# Proteomics and Incident Kidney Failure in Individuals With CKD: The African American Study of Kidney Disease and Hypertension and the Boston Kidney Biopsy Cohort

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**Rationale & Objective:** Individuals with chronic kidney disease (CKD) are at increased risk of morbidity and mortality, particularly as they progress to kidney failure. Identifying circulating proteins that underlie kidney failure development may guide the discovery of new targets for intervention.

**Study Design:** Prospective cohort.

**Setting & Participants:** 703 African American Study of Kidney Disease and Hypertension (AASK) and 434 Boston Kidney Biopsy Cohort (BKBC) participants with baseline proteomics data.

**Exposures:** Circulating proteins measured using SomaScan.

**Outcomes:** Kidney failure, defined as dialysis initiation or kidney transplantation.

**Analytical Approach:** Using adjusted Cox models, we studied associations of 6,284 circulating proteins with kidney failure risk separately in AASK and BKBC and meta-analyzed results. We then performed gene set enrichment analyses to identify underlying perturbations in biological pathways. In separate data sets with kidney-tissue level gene expression, we ascertained dominant regions of expression and correlated kidney tubular gene expression with fibrosis and estimated glomerular filtration rate (eGFR).

**Results:** Over median follow-up periods of 8.8 and 3.1 years, 210 AASK (mean age: 55 years, 39% female, mean GFR: 46 mL/min/1.73 $m^2$) and 115 BKBC (mean age: 54 years, 47% female, mean eGFR: 51 mL/min/1.73 $m^2$) participants developed kidney failure, respectively. We identified 143 proteins that were associated with incident kidney failure, of which only 1 (Testican-2) had a lower risk. Notable proteins included those related to vascular permeability (endothelial cell-selective adhesion molecule), glomerulosclerosis (ephrin-A1), glomerular development (ephrin-B2), intracellular sorting/transport (vesicular integral-membrane protein VIP36), podocyte effacement (pigment epithelium-derived factor), complement activation (complement decay-accelerating factor), and fibrosis (ephrin-A1, ephrin-B2, and pigment epithelium-derived factor). Gene set enrichment analyses detected overrepresented pathways that could be related to CKD progression, such as ephrin signaling, cell-cell junctions, intracellular transport, immune response, cell proliferation, and apoptosis. At the kidney level, glomerular expression predominated for genes corresponding to circulating proteins of interest, and several gene expression levels were correlated with eGFR and/or fibrosis.

**Limitations:** Possible residual confounding.

**Conclusions:** Multimodal data identified proteins and pathways associated with the development of kidney failure.

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Chronic kidney disease (CKD) progression is associated with increased morbidity and mortality.[1] To date, general strategies to slow CKD progression include blood pressure (BP) and glycemic control, avoidance of nephrotoxins, and treatment of albuminuria with renin-angiotensin-aldosterone system and/or sodium-glucose cotransporter-2 inhibitors.[2] Despite these efforts, the number of registered incident kidney failure cases in the United States increased by 16% from 2009 to 2019.[1] The discovery of new targets for intervention is therefore of utmost importance to improve the outcomes of individuals with CKD.

Proteomics, the large-scale study of proteins, is one method by which novel protein targets might be identified. For example, among Joslin Kidney Study participants with type 1 and 2 diabetes mellitus, 194 circulating inflammatory proteins were evaluated and a Kidney Risk Inflammation Signature, based on 17 of these proteins, was developed and found to be associated with increased risk of kidney failure.[3] Recent advancements in this field now allow for the simultaneous measurement of thousands of proteins.[4,5] In the current study, we used an untargeted approach to investigate whether over 6,200 proteins were associated with kidney failure risk in 2 cohorts of adults primarily with nondiabetic CKD. We then performed gene set enrichment analyses (GSEA) and examined patterns of gene expression at the kidney-tissue level to expand our understanding of potential pathways involved in CKD progression.

## METHODS

### Study Populations

The African American Study of Kidney Disease and Hypertension (AASK) was a 3 x 2 factorial trial by which self-

**PLAIN-LANGUAGE SUMMARY**

Circulating proteins that underlie the development of kidney failure may be new targets for treatment. In the current study of adults with chronic kidney disease, we evaluated over 6,000 proteins detected in blood and found more than 100 proteins whose levels were associated with new-onset kidney failure. Further investigation using gene expression data showed that the genes encoding these proteins were expressed in the kidney and involved in pathways of immune responses as well as cell signaling, structure, transport, and survival.

identified African American adults with CKD attributed to hypertension were randomized to 1 of 3 BP drugs (ramipril, metoprolol, or amlodipine) and 1 of 2 BP goals (mean arterial pressure of 102-107 mm Hg or ≤92 mm Hg).[6] Inclusion criteria comprised an age of 18-70 years, a measured glomerular filtration rate (GFR) of 20-65 mL/min/1.73 $m^2$, and no other likely explanation for kidney disease. Individuals were excluded if they had a diastolic BP of <95 mm Hg, a history of diabetes mellitus, or a urine protein-creatinine ratio (PCR) of >2.5 g/g Cr. Trial enrollment occurred from February 1995 to September 1998. At the end of the trial phase, participants who did not develop kidney failure were invited to join the observational cohort phase, which began in April 2002.[7] AASK cohort participants were transitioned to ramipril therapy with a BP goal of <140/90 mm Hg until 2004, when this goal was further lowered to <130/80 mm Hg.

The Boston Kidney Biopsy Cohort (BKBC) is a prospective observational cohort of adults, aged ≥18 years, who underwent a clinically indicated native kidney biopsy between September 2006 and July 2019 at 1 of 3 tertiary care hospitals in Boston, Massachusetts (ie, Brigham and Women's Hospital, Massachusetts General Hospital, or Beth Israel Deaconess Medical Center).[8,9] Primary clinicopathologic diagnoses included proliferative glomerulonephritis, nonproliferative glomerulopathy, diabetic nephropathy, vascular disease, tubulointerstitial disease, paraprotein-related disease, or others. Individuals with severe anemia, pregnancy, or an inability to provide consent were excluded. On the date of kidney biopsy, each participant provided blood and urine samples.

Our study population consisted of 703 AASK and 434 BKBC participants with available proteomics and covariate data. For both cohorts, study protocols were approved by institutional review boards at each study center and participants provided written informed consent.[6-9]

## Proteomic Measurements

Proteins were quantified in relative fluorescence units using the SomaScan V4.1 platform (SomaLogic) from the serum collected at the baseline trial visit of AASK and the plasma collected on the date of kidney biopsy in BKBC. SomaScan assays use oligonucleotides that have been modified to bind to targeted proteins with slower off-rates compared with nonspecific proteins. Details regarding proteomics profiling have previously been published[10,11]; the SomaScan platform has previously been shown to perform well in both serum and plasma samples.[12] For both cohorts, we included human proteins that passed initial quality control, defined as having a Bland-Altman coefficient of variation of <50%. There were 18 blind duplicate pairs with a median coefficient of variation of 4% for AASK and 8 blind duplicate pairs with a median coefficient of variation of 3.7% for BKBC. In total, 6,284 proteins common across the 2 cohorts were evaluated in the current study. Proteomics measurements were performed in 2021 in AASK and in 2022 in BKBC.

## Outcomes and Other Measurements

The primary outcome of interest was kidney failure, defined as starting maintenance dialysis or receiving a kidney transplant. In AASK, GFR was measured by renal clearance of $I^{125}$ iothalamate and urine PCR was determined from 24-hour urine collections processed at a central laboratory.[6,7] In BKBC, the estimated GFR (eGFR) was determined using the creatinine-based 2021 Chronic Kidney Disease Epidemiology Collaboration equation.[13] Serum creatinine on the date of kidney biopsy and the most proximal albuminuria measure up to 3 months before biopsy were obtained from the electronic medical record.[14,15] If unavailable, then serum creatinine (by a Jaffe-based method) or albuminuria was measured from samples collected on the date of kidney biopsy. When necessary, urine PCR was harmonized to the urine albumin-to-creatinine (ACR) ratio.[16] Race was based on self-report.

## Statistical Analyses

Baseline characteristics for each cohort were presented as the mean ± standard deviation, number (percent), and median (Q1-Q3). We meta-analyzed data from both AASK and BKBC using Cox proportional hazards models in which each protein was an exposure and kidney failure was the outcome. Proteins were log-2 transformed to achieve a more normal distribution. Covariates included baseline age, sex, randomized treatment groups (AASK only), race (BKBC only), GFR (AASK) or eGFR (BKBC), and log-2-transformed albuminuria (urine PCR in AASK and urine ACR ratio in BKBC). To facilitate comparison across proteins, we scaled each protein to its standard deviation in AASK. To account for multiple comparisons, a Bonferroni-corrected P value of <0.05/6284 proteins or $7.96 \times 10^{-6}$ was considered statistically significant. Participants were followed until they developed kidney failure, died, were lost to follow-up, or underwent administrative censoring. Protein associations with kidney failure were visually depicted using volcano plots.

Next, we performed GSEA to identify pathways among the proteins associated with kidney failure. Details

regarding the GSEA method have previously been described.[17] Briefly, the goal of GSEA is to determine whether members of predefined gene sets (S) are randomly distributed or found at the top or bottom of a list (L) by which their locations on the list are sorted based on their correlations with a phenotype of interest (eg, kidney failure). An enrichment score is then determined as the maximum deviation from 0 after "walking down the list L" and calculating a running-sum statistic that increases when encountered by a gene that belongs to S and decreases when encountered by a gene not belonging to S.[17] Ontology packages used included those of Gene Ontology,[18,19] Kyoto Encyclopedia of Genes and Genomes,[20-22] and Reactome.[23,24] Pathways with a Benjamini-Hochberg (ie, a false discovery rate P value corrected for all pathways tested in a given package) P value of <0.05 were considered statistically significant.

We also used Netboost to cluster the protein hits into distinct modules. Netboost is an unsupervised 3-step dimension reduction technique that entails the following aspects: (1) use of a Spearman correlation–based filter and topological overlap measure to reduce a network to its essential edges (ie, eliminate spurious connections while preserving the interconnectedness of network structures), (2) sparse hierarchical clustering and dynamic tree cut procedure to identify modules from a dendrogram, and (3) robust principal components analysis to aggregate module information into a low-dimension description of the original data.[25] Netboost was applied to the residuals of the regression of the proteins on age, sex, GFR, and log-2–transformed urine ACR. Hub proteins for each module were identified as the top proteins most strongly associated with module assignment. We then examined Spearman correlations ($r_s$) of the following: (1) proteins with each other, sorted by module assignment, and (2) modules with each other.

Finally, we evaluated whether genes encoding the proteins of interest (ie, those with significant associations with kidney failure in meta-analysis) were detected in the following: (1) kidney tissue regional transcriptomics data from the Kidney Precision Medicine Project (www.kpmp.org) Atlas and (2) RNA-sequencing data in 431 kidney tubular samples from the Susztaklab Kidney Biobank (www.susztaklab.com). In the Kidney Precision Medicine Project, we used data from 36 participants, of whom 9 were healthy, 22 had CKD, and 5 had acute kidney injury. We defined predominance as having a beta value of >1.0 when comparing one cell type to all other cell types. Broad categories of cell types comprised glomerulus, proximal tubule, thick ascending limb, distal convoluted tubule, collecting duct, and interstitium. In the Susztaklab Kidney Biobank, we assessed correlations of genes that encoded the top 20 proteins with eGFR and fibrosis.

Data were analyzed using the Stata 17.0 SE (StataCorp LLC) and R Package, Netboost and fGSEA libraries (R Foundation for Statistical Computing). Unless otherwise specified, we considered a P value of <0.05 to be statistically significant.

## RESULTS

### Baseline Characteristics

At baseline, the mean age of AASK participants was 54.5 years and 39% were women (Table 1). Owing to the design of the original trial, all participants self-identified as African American, had hypertension, and did not have diabetes. The mean GFR was 46 mL/min/1.73 $m^2$ and the median urine PCR was 0.08 g/g Cr.

Among the BKBC participants included in the study, the mean age was 54.4 years, 47% were women, 20% self-identified as African American, 59% had hypertension, and 29% had diabetes. The mean eGFR was 51 mL/min/1.73 $m^2$ and the median urine ACR was 1.0 g/g Cr.

### Proteins Associated With Kidney Failure in AASK and BKBC

Over median follow-ups of 8.8 (Q1-Q3: 4.5-10.4) and 3.1 (Q1-Q3: 1.3-5.4) years, 210 (30%) AASK participants and 115 (26%) BKBC participants developed kidney

**Table 1.** Baseline Characteristics of AASK and BKBC Study Participants With Available Proteomics Data

| Characteristic | AASK (n = 703) | BKBC (n = 434) |
|---|---|---|
| Age (y) | 54.5 ± 10.7 | 54.4 ± 16.2 |
| Female | 271 (39%) | 206 (47%) |
| Self-reported Black race | 703 (100%) | 87 (20%) |
| Hypertension | 703 (100%) | 256 (59%) |
| Type 1 diabetes | 0 (0%) | 21 (5%) |
| Type 2 diabetes | 0 (0%) | 105 (24%) |
| History of chronic kidney disease | 703 (100%) | 157 (36%) |
| Glomerular filtration rate (mL/min/1.73 $m^2$) | 45.7 ± 13.0 | 50.9 ± 33.2[a] |
| Urine PCR or ACR[b] (g/g) | 0.08 (0.03-0.4) | 1.0 (0.2-3.0) |
| Randomized blood pressure drug and goal | | Not applicable[c] |
| Ramipril; MAP ≤ 92 mm Hg | 132 (19%) | |
| Ramipril; MAP 102-107 mm Hg | 142 (20%) | |
| Metoprolol; MAP ≤ 92 mm Hg | 141 (20%) | |
| Metoprolol; MAP 102-107 mm Hg | 147 (21%) | |
| Amlodipine; MAP ≤ 92 mm Hg | 70 (10%) | |
| Amlodipine; MAP 102-107 mm Hg | 71 (10%) | |

Data presented as mean ± standard deviation, number (percentage), or median (quartile 1-quartile 3).
There were no missing data unless otherwise specified.
Abbreviations: AASK, African American Study of Kidney Disease and Hypertension; ACR, albumin-to-creatinine ratio; BKBC, Boston Kidney Biopsy Cohort; MAP, mean arterial pressure; PCR, protein-to-creatinine ratio.
[a]Estimated using the 2021 Chronic Kidney Disease Epidemiology Collaboration equation based on creatinine levels.[13]
[b]PCR in AASK and ACR in BKBC.
[c]Thirty percent of the participants were on an angiotensin-converting enzyme inhibitor and 16% were on an angiotensin receptor blocker at the time of biopsy.

failure, respectively. In adjusted models, 143 out of 6,284 proteins (2.3%) were identified as associated with kidney failure (Fig 1; Tables S1-S3). Only one protein, Testican-2 (SPOCK2), was associated with a lower risk of kidney failure. Further details on the top proteins associated with kidney failure are provided in Table 2.[26-51] Endothelial cell-selective adhesion molecule (ESAM) was detected using 2 different aptamers (SeqId_2981_9 and SeqId_20536_11) with every 1 SD higher protein level associated with a 1.6-fold higher risk of developing kidney failure. Other top proteins with prior literature describing associations with kidney-related outcomes included ephrin-A1 (EFNA1), ephrin-B2 (EFNB2), vesicular integral-membrane protein VIP36 (LMAN2), and pigment epithelium-derived factor (SERPINF1), among others. Less-known proteins included CMRF35-like molecule 9, serine protease inhibitor Kazal-type 14, serine dehydratase-like (SDSL), and calcineurin B homologous protein 3.

## Pathways Enriched by Protein Hits

GSEA on the protein associations identified 25 distinct pathways enriched in CKD progression, with the majority (76%) being upregulated (Fig 2). In general, these pathways were involved in ephrin signaling, endoplasmic reticulum structure, cell-cell junctions, immune response, cell proliferation or apoptosis, and axon guidance and neural development. Other identified pathways included those related to valine, leucine, and isoleucine degradation, peptidyl-lysine acetylation, and serine-type endopeptidase inhibitor activity.

## Module Assignment and Correlations

Proteins significantly associated with kidney failure were clustered into 6 distinct modules. Hub proteins for each module are listed in Table 3. Module 2 was most distinct from the other modules (Fig 3 and Table 3). Correlations were strongest between Modules 1 (containing EFNB2, tumor necrosis factor receptor superfamily member 1A, and ESAM) and 6 (containing trefoil factor 3 and SDSL; $r_s = 0.84$), followed by Modules 1 and 3 (containing ecto-ADP-ribosyltransferase 3 and ephrin type-A receptor 1; $r_s = 0.76$) and Modules 1 and 5 (containing EFNB1; $r_s = 0.75$).

## Application to Kidney-Level Data

Of the 131 distinct proteins with significant associations with kidney failure (12 of the 143 significant associations represented different aptamers for the same protein), 126 were available in the Kidney Precision Medicine Project and 44 of these showed preferential cell type expression. The majority had primary expression in the glomerulus, followed by the proximal tubule, collecting duct, distal convoluted tubule, and thick ascending limb (Fig 4). The distribution favoring glomerular predominance was significantly different than the distribution of predominance among the other proteins from the Soma Platform ($P < 0.001$).

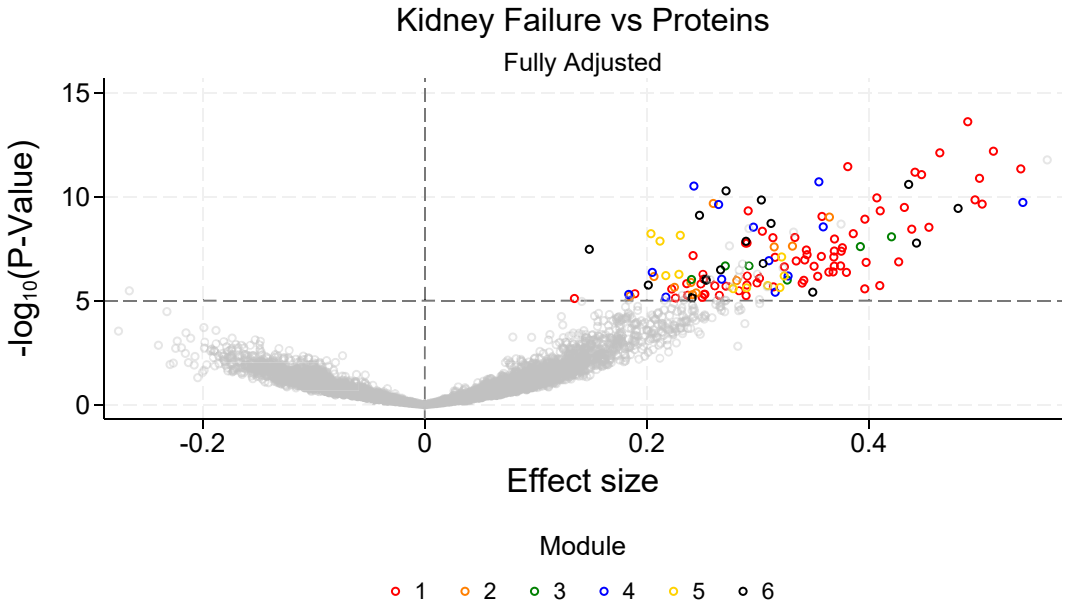


**Figure 1.** A volcano plot of proteins associated with kidney failure in AASK and BKBC. Models adjusted for age, sex, randomized treatment groups (AASK), race (BKBC), glomerular filtration rate (GFR in AASK; eGFR in BKBC), and log-transformed albuminuria (urine PCR in AASK; urine ACR in BKBC). The horizontal dashed line represents Bonferroni-corrected *P*-value threshold for significance. Colored dots represent the different modules. Abbreviations: AASK, African American Study of Kidney Disease and Hypertension; ACR, albumin-to-creatinine ratio; BKBC, Boston Kidney Biopsy Cohort; eGFR, estimated glomerular filtration rate; GFR, glomerular filtration rate; PCR, protein-to-creatinine ratio.

**Table 2.** Top Proteins Associated With Kidney Failure in AASK and BKBC

| Entrez Gene Symbol/ Sequence ID | Protein Name | Hazard Ratio per SD[a] (95% CI) | *P* Value | Known Associations With Kidney-Related Outcomes |
|---|---|---|---|---|
| ESAM[b] SeqId_2981_9 | Endothelial cell-selective adhesion molecule | 1.63 (1.44-1.85) | 2.44E-14 | • Present in glomerular endothelial cells; thought to contribute to integrity of tight junctions and regulate endothelial function within the kidney[26]<br>• Hyperglycemia reduces ESAM expression in vascular endothelial cells, which increases vascular permeability; ESAM-knockout mice exposed to high-glucose conditions have an increase in albuminuria[26]<br>• Higher levels associated with higher odds of having eGFR < 60 and albuminuria and greater increases in albuminuria[27] |
| EFNA1 SeqId_20091_138 | Ephrin-A1 | 1.67 (1.45-1.92) | 6.41E-13 | • Local overexpression of EphA1 in diabetic mouse kidneys reduces glomerulosclerosis[28] |
| LMAN2 SeqId_9468_8 | Vesicular integral-membrane protein VIP36 | 1.75 (1.50-2.05) | 1.68E-12 | • Associated with increased risk of 50% decline in eGFR or kidney failure in a community-based general population cohort (aged 54-74 and 66-90 y)[10]<br>• PWAS showed association of LMAN2 with eGFR, suggesting a causal role in CKD progression[10]<br>• Associated with increased risk of 50% decline in eGFR or kidney failure in a CKD cohort[29] |
| CPLX1 SeqId_18332_17 | Complexin-1 | 1.46 (1.31-1.63) | 3.54E-12 | • Associated with increased risk of 50% decline in eGFR or kidney failure in a community-based general population cohort (aged 66-90 y)[10] |
| GM2A SeqId_15441_6 | Ganglioside GM2 activator | 1.71 (1.47-1.99) | 4.55E-12 | • Localized to intercalated cells in the collecting duct[30]<br>• Associated with increased risk of 50% decline in eGFR or kidney failure in a community-based general population cohort (aged 66-90 y)[10] |
| CD300LG SeqId_20585_5 | CMRF35-like molecule 9 | 1.55 (1.37-1.76) | 6.57E-12 | • Not available |
| CD55 SeqId_5069_9 | Complement decay-accelerating factor | 1.56 (1.38-1.78) | 8.57E-12 | • Complement regulatory protein that has been localized to the juxtaglomerular apparatus of normal kidneys[31,32]<br>• Produced in diseased kidneys, with higher levels of DAF mRNA expression in mesangial cells among patients with IgA nephropathy and in glomerular epithelial cells among patients with membranous nephropathy[31]<br>• Downregulation of DAF in podocytes results in development of albuminuria and glomerulosclerosis in mice, by local activation of complement pathways[33] |
| FSTL3 SeqId_3438_10 | Follistatin-related protein 3 | 1.65 (1.43-1.90) | 1.29E-11 | • Associated with increased risk of 50% decline in eGFR or kidney failure in a community-based general population cohort (aged 54-74 and 66-90 y)[10]<br>• Associated with increased risk of 50% decline in eGFR or kidney failure in a CKD cohort[29]<br>• Higher levels associated with faster eGFR decline in 2 community-based cohorts[34] |

*(Continued)*

**Table 2 (Cont'd).** Top Proteins Associated With Kidney Failure in AASK and BKBC

| Entrez Gene Symbol/ Sequence ID | Protein Name | Hazard Ratio per SD[a] (95% CI) | *P* Value | Known Associations With Kidney-Related Outcomes |
|---|---|---|---|---|
| SERPINF1 SeqId_7735_17 | Pigment epithelium-derived factor | 1.43 (1.29-1.58) | 1.91E-11 | • In mouse models of unilateral ureteral obstruction, PEDF-knockout mice had more fibrosis, inflammation, and oxidative stress compared with wild-type mice[35]<br>• Intravascular injection of PEDF in mice results in foot process effacement and increased albuminuria[36]<br>• Patients with type 2 diabetes and albuminuria have a decrease in urine PEDF levels after being treated with irbesartan; diabetic rats treated with irbesartan have a decrease in kidney mRNA and protein expression of PEDF[37]<br>• Higher levels associated with more severe IFTA[38] and increased risks of albuminuria,[39,40] incident CKD,[39] CKD progression,[40] and kidney failure[38] |
| TFF3 SeqId_4721_54 | Trefoil factor 3 | 1.55 (1.36-1.76) | 2.55E-11 | • Expression localized to renal tubular epithelial cells in patients with CKD[41]<br>• Associated with increased risk of 50% decline in eGFR or kidney failure in a community-based general population cohort (aged 54-74 and 66-90 y)[10]<br>• Higher levels associated with faster eGFR decline in 2 community-based cohorts[34]<br>• Associated with long-term eGFR levels following hospitalization with COVID-AKI[42] |
| SPINK14 SeqId_8587_21 | Serine protease inhibitor Kazal-type 14 | 1.27 (1.19-1.37) | 3.05E-11 | • Not available |
| SDSL SeqId_17777_31 | Serine dehydratase-like | 1.31 (1.21-1.42) | 5.20E-11 | • Not available |
| CD46 SeqId_17682_1 | Membrane cofactor protein | 1.50 (1.33-1.70) | 1.13E-10 | • Mutations in CD46 associated atypical hemolytic uremic syndrome[43-45] |
| DSC2 SeqId_13126_52 | Desmocollin-2 | 1.64 (1.41-1.91) | 1.41E-10 | • DSC2 was strongly associated with a polygenic risk score for kidney function in a community-based general population cohort[46]<br>• Associated with long-term eGFR levels following hospitalization with COVID-AKI[42]<br>• Associated with increased risk of 50% decline in eGFR or kidney failure in a community-based general population cohort (aged 54-74 and 66-90 y)[10]<br>• Higher levels associated with faster eGFR decline in 2 community-based cohorts[34] |
| SLAMF1 SeqId_7953_20 | Signaling lymphocytic activation molecule | 1.35 (1.23-1.49) | 1.43E-10 | • Not available |
| AMBP SeqId_15453_3 | Alpha-1-microglobulin | 1.71 (1.45-2.02) | 1.87E-10 | • Urinary levels associated with increased risk of CKD progression in children[47]<br>• Urinary levels associated with future allograft failure among stable kidney transplant recipients[48]<br>• Associated with increased risk of 50% decline in eGFR or kidney failure in a CKD cohort[29] |

*(Continued)*

**Table 2 (Cont'd).** Top Proteins Associated With Kidney Failure in AASK and BKBC

| Entrez Gene Symbol/ Sequence ID | Protein Name | Hazard Ratio per SD[a] (95% CI) | *P* Value | Known Associations With Kidney-Related Outcomes |
|---|---|---|---|---|
| TESC Seqld_12831_21 | Calcineurin B homologous protein 3 | 1.30 (1.20-1.40) | 2.10E-10 | • Not available |
| EFNB2 Seqld_14131_37 | Ephrin-B2 | 1.65 (1.41-1.93) | 2.25E-10 | • Protects against capillary rarefaction and fibrosis following kidney injury[49]<br>• Plays a role in glomerular development[50]<br>• Higher levels associated with faster eGFR decline in 2 community-based cohorts[34]<br>• GWAS suggested EFNB2 as an all-cause kidney failure locus in African American individuals[51]<br>• Associated with increased risk of 50% decline in eGFR or kidney failure in a community-based general population cohort (aged 54-74 y)[10]<br>• Associated with increased risk of 50% decline in eGFR or kidney failure in a CKD cohort[29] |
| TRAPPC3 Seqld_14337_1 | Trafficking protein particle complex subunit 3 | 1.30 (1.20-1.41) | 2.36E-10 | • Associated with increased risk of 50% decline in eGFR or kidney failure in a community-based general population cohort (aged 54-74 y)[10] |

Bonferroni-corrected *P* value = 0.05/6284 common proteins = 7.96E-06.

Abbreviations: AASK, African American Study of Kidney Disease and Hypertension; ACR, albumin-to-creatinine ratio; AKI, acute kidney injury; BKBC, Boston Kidney Biopsy Cohort; CI, confidence interval; CKD, chronic kidney disease; COVID-19, coronavirus disease 2019; DAF, complement decay-accelerating factor; DSC2, desmocollin-2; EFNB2, ephrin-B2; eGFR, estimated glomerular filtration rate; ESAM, endothelial cell-selective adhesion molecule; GWAS, genome-wide association study; IFTA, interstitial fibrosis and tubular atrophy; IgA, immunoglobulin A; LMAN2, vesicular integral-membrane protein VIP36; mRNA, messenger RNA; PCR, protein-to-creatinine ratio; PEDF, pigment epithelium-derived factor; PWAS, protein-wide association study; SD, standard deviation.

[a]Models adjusted for age, sex, randomized treatment groups (AASK), race (BKBC), GFR (GFR in AASK; eGFR in BKBC), and log-transformed albuminuria (urine PCR in AASK; urine ACR in BKBC).

[b]ESAM was represented by 2 aptamers (Seqld_2981_9 and Seqld_20536_11) that were among the top 20 proteins. The aptamer with the stronger association is presented here.

Using RNA-sequencing data in 431 kidney tubular samples from the Susztaklab Kidney Biobank cohort, several genes encoding the top proteins associated with kidney failure in AASK and BKBC were found to be positively (ie, *EFNA1*, *CD300LG*, *SDSL*, and *CD46*) or negatively (*CPLX1*, *GM2A*, *CD55*, *SERPINF1*, *TFF3*, *SLAMF1*, and *TESC*) correlated with eGFR (Table S4). The following correlations for fibrosis were also observed: genes with positive correlations included *CPLX1*, *GM2A*, *CD55*, *FSTL3*, *SERPINF1*, *TFF3*, *SLAMF1*, and *TESC*, whereas genes with negative correlations included *EFNA1*, *CD300LG*, *SDSL*, *CD46*, *AMBP*, and *TRAPPC3*.

## DISCUSSION

In the current study, we identified 131 distinct circulating proteins associated with kidney failure risk among adults with moderate to severe CKD; top proteins were overrepresented in pathways of ephrin signaling, cell junction integrity, intracellular structure and transport, immune response, and cell survival. In addition, the predominant site of cognate gene expression within the kidney for many of the proteins was the glomerulus or a tubular segment. Given that the disease course of individuals with CKD can be quite variable, with some progressing to kidney failure more rapidly than others, we demonstrated that proteomic profiling can be used to detect novel proteins that contribute to CKD progression or herald future risk to kidney failure. Taken together, our results may provide insights into the underlying biology of CKD progression.

Of the top proteins that we identified, several have been described in the literature as having associations with kidney-related outcomes (Table 2) and some have potential therapeutics in development (Table S1).[52] SPOCK2, a podocyte-derived protein also known as testican-2, was the only protein associated with a lower risk of kidney failure. This finding is consistent with prior work demonstrating associations between higher testican-2 levels and reduced risk of subsequent eGFR decline in community-based cohorts, reduced risk of kidney failure among older adults and individuals with CKD, and less glomerulosclerosis among individuals who have undergone kidney biopsy.[34,53,54] Testican-2 has also been shown to increase from the artery to the renal vein in adults undergoing cardiac catheterization, suggesting that elevated levels are not due to reduced glomerular clearance.[34,53]

ESAM, the top protein, is selectively expressed in vascular endothelial cells.[55] Hara et al[26] previously reported that compared with wild-type mice, knock-out ESAM mice had the following: (1) more albuminuria; (2) fewer fenestrations of glomerular capillary endothelial cells; and (3) wider endothelial cell tight junctions. In contrast, there were no major structural differences in podocytes or the glomerular basement membrane. Among Heart and Soul Study participants, those in the highest versus lowest quartiles of serum ESAM experienced greater

**Table 3.** Correlations of Modules 1-6 in AASK and BKBC

| Hub Proteins (Entrez Gene Symbol) | EFNB2<br>FZD2<br>FZD7<br>UNC5B<br>EFNA5<br>TNFRSF1A | TIPRL<br>PDCD5<br>STMN2<br>CRK<br>NECAP2<br>NAXE | ART3[a]<br>CTSZ<br>EPHA1 | AMBP<br>ERP29<br>PSMB3<br>MCTS1<br>SUMF1<br>SPINK14 | REG1A<br>REG1B<br>REG3A<br>EFNB1<br>NECTIN2<br>GUCA2B | TFF3<br>IGFBP6<br>EPOR<br>CANT1<br>SLAMF1<br>DLL1 |
|---|---|---|---|---|---|---|
| Biological process[b] | Angiogenesis, differentiation, host-virus interaction, neurogenesis, Wnt signaling pathway, apoptosis | Apoptosis, endocytosis, protein and lipid transport, neurogenesis | Proteolysis, angiogenesis, cell adhesion | Host-virus interaction, cell-cycle, DNA damage response, growth regulation, protein biosynthesis, transcription regulation | Acute phase, inflammatory response, differentiation, neurogenesis, cell adhesion, host-virus interaction | Adaptive and innate immunity, cell adhesion, host-virus interaction, phagocytosis, differentiation, Notch signaling pathway |
| | Module 1 | Module 2 | Module 3 | Module 4 | Module 5 | Module 6 |
| Module 1 | 1.00 | 0.24 | 0.76 | 0.56 | 0.75 | 0.84 |
| Module 2 | | 1.00 | 0.18 | 0.18 | 0.30 | 0.27 |
| Module 3 | | | 1.00 | 0.51 | 0.58 | 0.67 |
| Module 4 | | | | 1.00 | 0.46 | 0.72 |
| Module 5 | | | | | 1.00 | 0.72 |
| Module 6 | | | | | | 1.00 |

Abbreviations: AASK, African American Study of Kidney Disease and Hypertension; AMBP, alpha-1-microglobulin; ART3, ecto-ADP-ribosyltransferase 3; BKBC, Boston Kidney Biopsy Cohort; CANT1, soluble calcium-activated nucleotidase 1; CRK, adapter molecule crk; CTSZ, cathepsin Z; DLL1, delta-like protein 1; EFNA5, ephrin-A5; EFNB1, ephrin-B1; EFNB2, ephrin-B2; EPHA1, ephrin type-A receptor 1; EPOR, erythropoietin receptor; ERP29, endoplasmic reticulum resident protein 29; FZD2, frizzled-2; FZD7, frizzled-7; GUCA2B, guanylate cyclase activator 2B; IGFBP6, insulin-like growth factor-binding protein 6; MCTS1, malignant T-cell-amplified sequence 1; NAXE, NAD(P)H-hydrate epimerase; NECAP2, adaptin ear-binding coat-associated protein 2; NECTIN2, nectin-2; PDCD5, programmed cell death protein 5; PSMB3, proteasome subunit beta type-3; REG1A, lithostathine-1-alpha; REG3A, regenerating islet-derived protein 3-alpha; REG1B, lithostathine-1-beta; SLAMF1, signaling lymphocytic activation molecule; SPINK14, serine protease inhibitor Kazal-type 14; STMN2, stathmin-2; SUMF1, sulfatase-modifying factor 1; TFF3, trefoil factor 3; TIPRL, TIP41-like protein; TNFRSF1A, tumor necrosis factor receptor superfamily member 1A; UNC5B, UNC5 netrin receptor B.
[a]ART was represented by 2 aptamers SeqID_7970_315 and SeqID_10970_3.
[b]From UniProt database (www.uniprot.org).

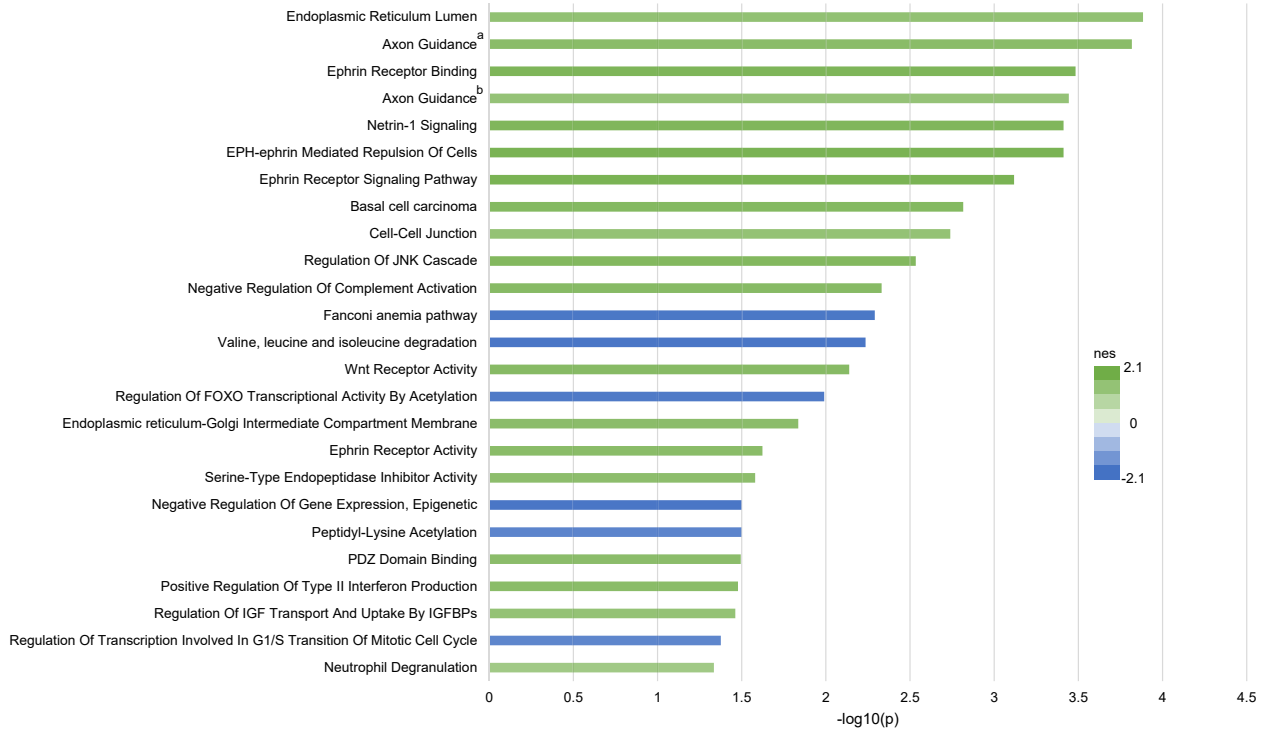


**Figure 2.** Pathways enriched in CKD progression using protein-kidney failure associations from AASK and BKBC. Abbreviations: AASK, African American Study of Kidney Disease and Hypertension; BKBC, Boston Kidney Biopsy Cohort; CKD, chronic kidney disease; NES, normalized enrichment score. Positive and negative scores indicate upregulation and downregulation of pathways, respectively. [a]From Kyoto Encyclopedia of Genes and Genomes 2021 human package. [b]From Reactome 2022 package.

mean increases in albuminuria over 5 years.[27] Faster rates of eGFR decline were also noted for the highest quartile; however, these findings did not meet the Bonferroni threshold for significance in fully adjusted models. In the current study, we also found that higher circulating levels of ESAM were associated with heightened risk of kidney failure and that, within the kidney, expression predominated in the glomerulus.

Recent studies suggest that members of the ephrin family may also be involved in kidney disease progression. EFNA1 has been shown to reduce glomerulosclerosis in vivo. More specifically, Li et al[28] injected adeno-associated virus overexpressing either EFNA1 or a negative control into the kidneys of mice with streptozotocin-induced diabetic nephropathy. Examination of kidney biopsy specimens showed that mice with intrarenal overexpression of EFNA1 had significantly less glomerulosclerosis and fewer cells staining for mesangial cell proliferation or fibrosis markers compared with their counterparts that had received the negative control. Further investigation suggested that this might be mediated by decreased phosphorylation of the ERK1/2, JNK, and myosin phosphatase target subunit1 pathways. EFNB2 is thought to play an important role in glomerular development. Takahashi et al[50] reported in mice that EFNB2 expression begins in glomerular epithelial cells next to the vascular cleft (ie, podocyte progenitors), which in turn recruit endothelial progenitor cells. EFNB2 expression was also described in mesangial cells, vascular smooth muscle, and tubular cells. The sequential expression of EFNB2 across specific cell lineages is believed to guide microvascular assembly in the developing glomerulus. Kida et al[49] reported an increase in EFNB2 kidney protein levels in mouse models of kidney injury. Moreover, the loss of EFNB2 PDZ-dependent signaling appeared to hamper response to kidney injury, with impaired angiogenesis and more differentiation of kidney pericytes to myofibroblasts, ultimately leading to fibrosis. Ngo et al[34] reported that higher baseline levels of EFNB2 were associated with faster rates of eGFR decline in both the Jackson Heart Study and the Framingham Heart Study. Finally, in a genome-wide association study, Guan et al[51] identified *EFNB2* as a novel locus (lead variant: rs77113398) associated with all-cause kidney failure among Black participants of 6 large cohorts (n = ~12,000).

LMAN2 has been localized to the early secretory pathway (eg, Golgi apparatus) and plasma membrane of Madin-Darby Canine Kidney cells.[56] LMAN2 is believed to play a role in the apical sorting and intracellular transport of mannose-rich glycoproteins. We previously reported in the Chronic Renal Insufficiency Cohort that higher baseline levels of LMAN2 were associated with CKD progression.[10,29] It is possible that circulating LMAN2 is a biomarker for other proteins expressed elsewhere; for example, LMAN2 and SLC34A1 share blood protein quantitative trait loci and kidney glomeruli and tubule

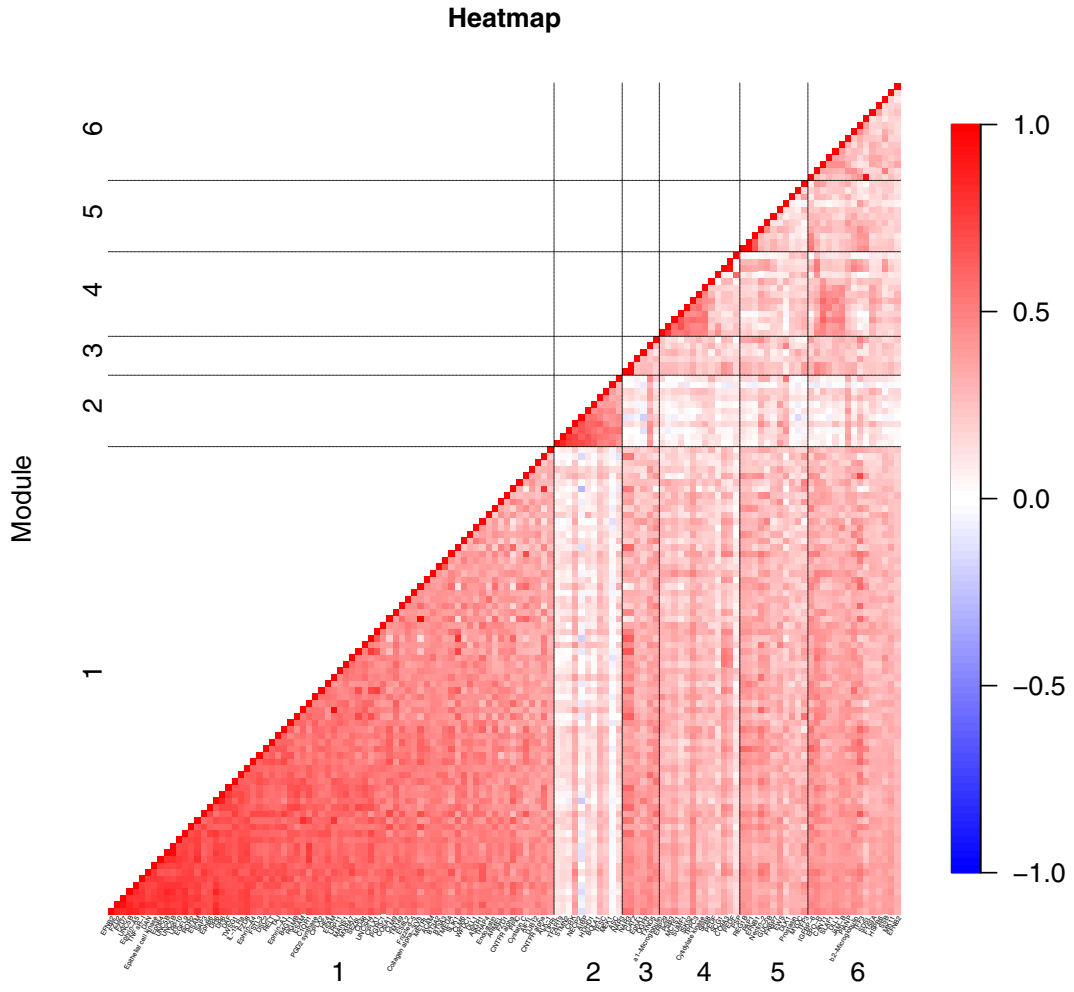


**Figure 3.** Heatmap of the square of correlations ($r^2$) between proteins, based on module assignment. The sign (+ or -) reflects correlation.

expression quantitative trait loci (eg, rs10051765 and rs11135015).[57] Future studies are needed to determine whether targeting these 2 proteins improves outcomes of individuals with CKD.

SERPINF1 is a 50-kDa glycoprotein and member of the serine protease inhibitor superfamily.[36] Huang et al[36] reported that in mice, the administration of exogenous SERPINF1 led to more albuminuria, increases in serum creatinine levels, glomerular atrophy, and podocyte foot process effacement. Interestingly, these findings were more pronounced in diabetic mice compared with normal controls. To further elucidate potential mechanistic pathways, the investigators then treated podocytes with SERPINF1 and found that this promoted actin filament rearrangement and apoptosis, likely by activation of the RhoA/ROCK1 signaling pathway. In humans, higher blood levels of SERPINF1 have been associated with increased risks of incident CKD[39] and albuminuria, and CKD progression,[40] among individuals with type 2 diabetes.

We also identified less-known proteins to be associated with kidney failure.[58,59] CMRF35-like molecule 9 is a receptor expressed by myeloid cells and has an immunoregulatory role. Serine protease inhibitor Kazal-type 14 may be a serine protease inhibitor, whereas SDSL may be involved in lipid metabolism, threonine and L-serine catabolism, and isoleucine biosynthesis. Calcineurin B homologous protein 3 is thought to play a role in maturation and transport of the $Na^+/H^+$ exchanger to the plasma membrane, which in turn regulates intracellular pH and cell volume.[60,61] Although replication of our findings in other cohorts is necessary, these proteins may represent exciting, future areas of research that provide insights into the progression of CKD to kidney failure.

On further investigation, we found that the top proteins were enriched in several pathways that could plausibly be related to CKD progression. These included ephrin signaling, cell-cell junctions, intracellular transport, immune response, cell proliferation, and apoptosis. In addition, most of these pathways appeared to be upregulated. The identification of pathways related to axon guidance and neural development was surprising, though Satake et al[62] previously reported an association between axon

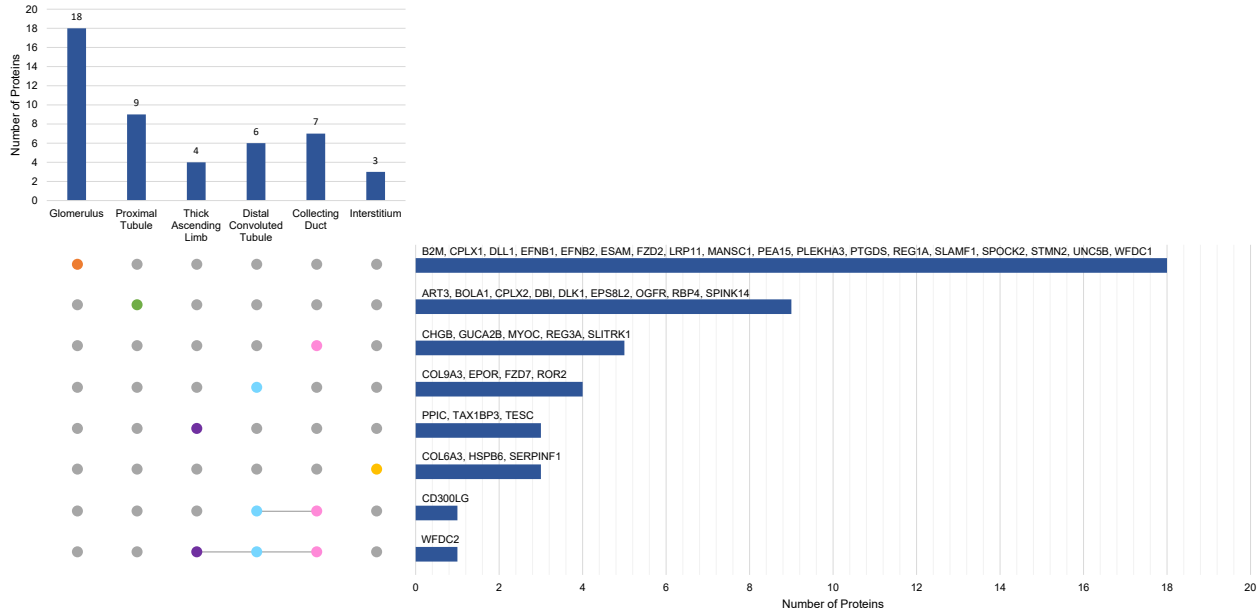


**Figure 4.** Predominant site of cognate gene expression in KPMP for proteins associated with kidney failure in meta-analysis of AASK and BKBC. Data presented are among 36 KPMP (9 healthy, 22 with chronic kidney disease, and 5 with acute kidney injury) participants with regional transcriptomics data. Predominance was determined as a beta of >1.0 when comparing one cell type to all other cell types. Abbreviations: AASK, African American Study of Kidney Disease and Hypertension; BKBC, Boston Kidney Biopsy Cohort; KPMP, Kidney Precision Medicine Project.

guidance pathway proteins and the risk of kidney failure among individuals with type 1 and 2 diabetes. Through an unbiased global analysis of circulating microRNAs, they identified 17 microRNAs that were associated with kidney failure. Further investigation of the genes predicted to be targets of these microRNAs led to the identification of axon guidance as the top candidate pathway, and by a targeted proteomics approach, 6 proteins (5 involved in ephrin signaling: EFNA4, EFNA5, ephrin type-A receptor 2, ephrin type-B receptor 2, and ephrin type-B receptor 6; 1 netrin receptor: UNC5C) were then found to be associated with 3.4- to 7.5-fold higher risks of developing kidney failure (comparing the fourth vs the first quartile). Among 105 Pima Indian Kidney Study participants who underwent a kidney biopsy, circulating levels of these proteins appeared to positively correlate ($r_s = 0.21$-$0.52$) with mesangial fractional volume, which is considered an early sign of diabetic kidney disease.[62] Given that 0% of AASK participants and only 29% of BKBC participants had baseline diabetes, our findings suggest that the potential role of axon guidance pathways in kidney failure development may extend beyond diabetes.

Our study has several strengths. First, through proteomic profiling, we were able to perform a comprehensive assessment of over 6,200 proteins. Second, we included participants from 2 different prospective cohorts of CKD. In particular, the primary clinicopathologic diagnoses in BKBC were very heterogenous.[8,9] Therefore, our results likely highlight shared pathways across multiple causes of CKD. Third, we adjusted for measured rather than estimated baseline GFR in AASK to account for reduced kidney clearance of proteins in CKD. Fourth, we used advanced analytical approaches to identify potential pathways involved in the progression of CKD to kidney failure. We also acknowledge some limitations. Despite adjusting for several baseline covariates, including GFR and eGFR, the potential for residual confounding remains and observed associations for proteins may be due to reduced glomerular clearance. In addition, only 11% of our study population had a history of diabetes. Our findings may therefore not be generalizable to patients with diabetic kidney disease. Still, many of the top proteins have been described in prior literature to have associations with kidney outcomes in the context of diabetes. Finally, the focus of the current study was on circulating proteins, which may not originate in the kidney; however, we chose this biofluid because it may be one of the easiest to target.

In conclusion, we used a proteomics approach to discover proteins associated with kidney failure in 2 cohorts of adults with CKD. Further investigation is needed to evaluate whether any of these proteins represent novel targets for therapeutic intervention.

## SUPPLEMENTARY MATERIALS

### Supplementary File 1 (xlsx)

**Table S1.** Proteins associated with kidney failure in AASK and BKBC.

### Supplementary File 2 (xlsx)

**Table S2.** Proteins associated with kidney failure in AASK.

**Supplementary File 3 (xlsx)**

**Table S3.** Proteins associated with kidney failure in BKBC.

**Supplementary File 4 (xlsx)**

**Table S4.** Correlations of top proteins with eGFR and fibrosis on RNA-sequencing data in 431 kidney tubular samples from Suszta-klab Kidney Biobank.

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