



Urine Complement Proteins and the Risk of Kidney Disease Progression and Mortality in Type 2 Diabetes

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OBJECTIVE

We examined the association of urine complement proteins with progression to end-stage renal disease (ESRD) or death in people with type 2 diabetes and proteinuric diabetic kidney disease (DKD).

RESEARCH DESIGN AND METHODS

Using targeted mass spectrometry, we quantified urinary abundance of 12 complement proteins in a predominantly Mexican American cohort with type 2 diabetes and proteinuric DKD ($n = 141$). The association of urine complement proteins with progression to ESRD or death was evaluated using time-to-event analyses.

RESULTS

At baseline, median estimated glomerular filtration rate (eGFR) was 54 mL/min/1.73 m² and urine protein-to-creatinine ratio 2.6 g/g. Sixty-seven participants developed ESRD or died, of whom 39 progressed to ESRD over a median of 3.1 years and 40 died over a median 3.6 years. Higher urine CD59, an inhibitor of terminal complement complex formation, was associated with a lower risk of ESRD (hazard ratio [HR] [95% CI per doubling] 0.50 [0.29–0.87]) and death (HR [95% CI] 0.56 [0.34–0.93]), after adjustment for demographic and clinical covariates, including baseline eGFR and proteinuria. Higher urine complement components 4 and 8 were associated with lower risk of death (HR [95% CI] 0.57 [0.41–0.79] and 0.66 [0.44–0.97], respectively); higher urine factor H-related protein 2, a positive regulator of the alternative complement pathway, was associated with greater risk of death (HR [95% CI] 1.61 [1.05–2.48]) in fully adjusted models.

CONCLUSIONS

In a largely Mexican American cohort with type 2 diabetes and proteinuric DKD, urine abundance of several complement and complement regulatory proteins was strongly associated with progression to ESRD and death.

Diabetic kidney disease (DKD) is the leading cause of chronic kidney disease (CKD) and end-stage renal disease (ESRD) (1). Our diagnostic and therapeutic tools have not kept pace with the rising incidence of diabetes and DKD (2), significantly curtailing our ability to diagnose DKD, particularly early in its course, and to modify its natural history beyond what is possible under current standards of care. Ethnic minorities, in particular, bear a large and growing burden of diabetes and DKD (3).

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As such, there is an urgent need to identify new pathogenic mechanisms and evaluate the potential of these mechanisms as diagnostic and therapeutic targets.

Substantial evidence, largely derived from rodent models, links complement and complement regulatory proteins to the DKD pathogenesis (4). Notably, complement activation has been implicated in mediating the tubulointerstitial injury by proteinuria in CKD (5). However, a comprehensive assessment of the role of the complement cascade in human DKD has not been conducted to date. Such an evaluation has been impeded in part due to absence of methods to quantify the ensemble of complement proteins in parallel.

We developed a novel targeted proteomics method to quantify the abundance of 12 complement and complement regulatory proteins, which were detectable in urine, and used this method to quantify these proteins in a cohort of predominantly Hispanic participants with type 2 diabetes and proteinuric DKD. Urine was selected as the biofluid of choice because it is readily and noninvasively available, is already extensively used in clinical diagnostics, and is of increasing interest for biomarker discovery. In addition, mounting evidence suggests that urine protein composition is substantially modified by proteins reabsorbed or elaborated from the cells lining the nephron (6–9), suggesting that urine protein composition may inform on the intrarenal pathogenic mechanisms and as such has prognostic value for disease progression. In this study, we report the association of the urine abundance of several complement proteins with progression to ESRD and all-cause mortality.

RESEARCH DESIGN AND METHODS

Study Populations

This study used samples from two populations: for the discovery analysis using untargeted proteomics, we used urine samples and clinical data from a cross-sectional subset of participants from the University of Washington (UW) DKD repository. The validation step using targeted proteomics used samples from the longitudinal Golden State cohort. For the UW DKD repository, as previously described (10), clinic patients with type 1 diabetes were recruited from 2011 to

2016. DKD was defined as: 1) an estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m² and a urine albumin-to-creatinine ratio (ACR) ≥ 30 mg/g or 2) ACR ≥ 300 mg/g. Control participants had ≥ 30 years of type 1 diabetes, eGFR ≥ 90 mL/min/1.73 m², and ACR <300 mg/g. This study was approved by the UW Institutional Review Board (IRB).

The Golden State cohort, a prospective study of vascular calcification in patients with type 2 diabetes and DKD, has also been previously described (11). From 2004 to 2008, participants with type 2 diabetes, defined as diabetes diagnosed at ≥ 30 years of age and treated with diet or oral hypoglycemic agents for ≥ 6 months, were recruited. DKD was defined as a urine protein-to-creatinine ratio ≥ 0.5 g/g at enrollment or in the preceding 12 months and one or more of the following: 1) typical histologic changes on kidney biopsy, 2) diabetes duration ≥ 5 years and diabetic retinopathy, or 3) diabetes duration ≥ 10 years without diabetic retinopathy. Patients with a kidney transplant or on dialysis were excluded. All participants provided written informed consent. The study was approved by the IRB at the Los Angeles Biomedical Research Institute. Links to participant identification were destroyed after the last study contact (2011). Use of de-identified samples from this study was approved by the UW IRB.

Covariates

Demographic data and past medical history were obtained from medical records and self-report and medications from pill bottles. Vital signs were average of duplicate measurements. Hypertension was defined as systolic blood pressure/diastolic blood pressure (SBP/DBP) $>140/90$, use of antihypertensive medications, or a clinical history of hypertension. Hemoglobin A_{1c} (HbA_{1c}) was measured using high-performance liquid chromatography (Toshio Medics, Inc., Foster City, CA). Plasma lipids were measured using conventional enzymatic methods with LDL calculated from Friedewald formula. Serum creatinine, urine albumin, and urine creatinine were measured using the enzymatic reaction (12), immunoturbidometric assays (13), and the modified Jaffé reaction (14), respectively. eGFR was estimated using the

Modification of Diet in Renal Disease (MDRD) Study equation (15).

Outcomes

Following the baseline visit, the Golden State cohort participants were reassessed at 12 and 24 months or within 3 months of the first dialysis treatment, whichever occurred first. Progression to ESRD and occurrence of death was verified by the study staff who contacted the study participants or next of kin every 6 months via phone, letters, or home visits, as well as linkage to the U.S. Renal Data System (USRDS; through 31 December 2008) and National Death Index (through 31 December 2007). ESRD was defined as a requirement for maintenance dialysis or kidney transplantation. The time to ESRD was ascertained from the last study contact or the date of the linkage with the USRDS, whichever occurred later. Time to death was ascertained from the last study contact or the date of the linkage with the National Death Index or USRDS, whichever occurred later.

Urine Sample Collection and Processing for Proteomic Analysis

Random urine samples were aliquoted and stored at -80°C . The samples used for this study had not undergone prior freeze-thaw and showed no evidence of volume loss on inspection. A total of 100–300 μL of stored samples were thawed, precipitated with 4 \times volume of ice-cold 1:1 mixture of acidified methanol/acetone (1 mmol/L HCl in methanol added to acetone in 1:1 ratio), and incubated overnight at -20°C . Precipitated proteins were reconstituted in 0.2% RapiGest/100 mmol/L ammonium bicarbonate with vigorous mixing overnight at 4°C . Protein concentration was measured by Bradford assay (Pierce). α -Enolase (*Aspergillus oryzae*) was spiked into samples (1 μg enolase/10 μg protein) as a protein internal standard for digestion and liquid chromatography–mass spectrometry (LC-MS). A total of 10 μg urine protein per sample was reduced with dithiothreitol, alkylated with iodoacetamide, and digested with two additions of trypsin (Promega, Madison, WI) at a 1:20 weight-for-weight ratio for 3 h, and overnight, at 37°C with mixing. After digestion, RapiGest was hydrolyzed by addition of 1% trifluoroacetic acid and incubation at 37°C for 45 min.

Samples were spun (14,000g for 10 min), and the supernatant was dried down and stored at -80°C until LC-MS. Before analysis, samples were reconstituted with 0.1% formic acid (FA)/5% acetonitrile.

Untargeted Proteomics

Peptides (0.375 μg) were injected on a trap column (40 \times 0.1 mm, XBridge BEHC18, 5 μm ; Waters, Milford, MA), desalted for 5 min at a flow of 4 $\mu\text{L}/\text{min}$, separated on a pulled-tip analytical column (150 \times 0.075 mm, XBridge BEHC18, 3.5 μm ; Waters), and heated to 50°C with a three-segment linear gradient of acetonitrile, 0.1% FA (B) in water, and 0.1% FA (A) as follows: 0–3 min 1–7% B, 3–53 min 7–25% B, 53–60 min 25–35% B, and then 80% B and re-equilibration at 0.4 $\mu\text{L}/\text{min}$ (nanoACQUITY UPLC; Waters). Tandem mass spectrometry (MS/MS) spectra were acquired on Orbitrap Fusion (Thermo Fisher Scientific, Waltham, MA) operated in data-dependent mode with higher-energy collisional dissociation fragmentation (normalized collisional energy 25%) and MS/MS acquisition. MS spectra were acquired at resolution 60,000 and MS/MS spectra (MS/MS selection window 2 Da) at resolution 15,000. Peptides and proteins were identified using the Comet search engine, with PeptideProphet and ProteinProphet validation (20 ppm tolerance window for precursors and products, Cys alkylation and methionine oxidation as fixed and variable modifications, respectively).

Targeted Proteomics

Proteins of interest were quantified using parallel reaction-monitoring (PRM) LC-MS on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) connected to a nanoACQUITY UPLC (Waters). Peptides were selected from untargeted proteomics and prioritized based on proteotypic properties using public resources, prediction models, and the human SRMatlas (<http://www.srmatlas.org/>). Best peptides by chromatographic data were selected for PRM LC-MS (Supplementary Table 1). Selected peptides were monitored by targeting precursor ions in the quadrupole analyzer (selection window 2.0 Da) and full-scan MS/MS after higher-energy collisional dissociation fragmentation (normalized collisional energy

29%) in the Orbitrap analyzer with high resolution (15,000). Scheduled acquisition with 5-min acquisition windows was set up for each peptide precursor using Skyline (<https://skyline.ms/project/home/software/skyline/begin.view>), allowing a maximum of 20 concurrent PRM experiments. Acquired data were processed in Skyline, and automated integration was manually checked. The identity of the chromatographic peaks was ascertained by matching the PRM MS/MS spectra to those from the untargeted dataset (dot product >0.9 and mass precision <5 ppm). For quantification, peptides were selected from those measured in PRM analyses based on correlation ≥ 0.9 (Supplementary Fig. 1). Of the two peptides in the most highly correlated pair, one was selected for quantification based on the abundance and the smallest number of samples with missing or undetectable peptides (Supplementary Table 2).

Statistical Methods

Continuous variables were summarized by mean, SD, median, and interquartile range (IQR) and categorical variables by number and percent. Peptide and protein abundance data (targeted proteomics) were \log_2 -transformed and converted to z scores. The correlation between the abundance of each protein and the eGFR and urine protein was calculated using the Pearson correlation. The time to the first occurrence of death or ESRD was modeled by abundance of each complement protein and other variables using Cox proportional hazards models. Three single-protein models were fitted per protein. Model 1 included the abundance of the given protein in urine and the sample LC-MS batch. For complement factor H-related protein 2 (FHR2), model 1 also included a second variable indicating whether the abundance was below the limit of detection. This variable was added to prevent undue influence by the individuals with undetectable urine FHR2 (outliers) on the hazard ratio (HR). Model 2 included covariates from model 1 plus age, sex, race, smoking status, renin-angiotensin system (RAS) inhibitor use, and hypertension. Model 3 included the covariates in model 2 plus $\log_2(\text{eGFR})$ and $\log_2(\text{total urine protein concentration})$. Prevalent cardiovascular disease (CVD), HbA_{1c} , and LDL were excluded from all models

because they changed the HRs for complement proteins $<5\%$. Diabetes duration was excluded from the models for the outcomes of death and combined ESRD/death for the same reason. The Lunn-McNeil method was used to examine association between complement proteins and ESRD while accounting for the competing risk of death (16). The nominal significance for all analyses was a two-sided P value of <0.05 . Analyses were conducted using R, version 3.3.3 (R Core Team, 2017). The Cox proportional hazards analyses were conducted using the R package survival, version 2.41-3.

RESULTS

Participant Characteristics

The initial discovery study was conducted using the urine samples from a cross-sectional study of participants with type 1 diabetes with or without proteinuric DKD ($n = 61$) (Supplementary Table 3). Subsequent targeted proteomics studies used urine samples from participants with type 2 diabetes and proteinuric DKD from the longitudinal Golden State cohort ($n = 141$) (Table 1). The latter participants had a mean age of 57 years, were mostly male (57%) and Hispanic (73%), and had a high prevalence of current/past smoking (55%) with a median (IQR) diabetes duration of 14 (10, 19) years. At baseline, 94% had hypertension and 35% CVD. A majority (82%) were treated with RAS inhibitors. Median (IQR) SBP, DBP, and HbA_{1c} were 155 (136, 176), 77 (70, 85), and 8.0% (7.1%, 10.0%), respectively. Median LDL and HDL were 108 and 41 mg/dL, respectively. Median eGFR and urine protein-to-creatinine ratio (PCR) were 54 mL/min/ 1.73 m^2 and 2.6 g/g, respectively. Compared with participants who neither died nor developed ESRD in follow-up, those who did were more likely to be male (60% vs. 54%) and white (19% vs. 11%), had a higher prevalence of current/past smoking (58% vs. 52%) and hypertension (96% vs. 93%), had a lower prevalence of diagnosed CVD (31% vs. 39%) and RAS inhibitor use (79% vs. 84%), and had a higher median SBP (159 vs. 152) and urine PCR (3.6 vs. 2.0) and lower median eGFR (44 vs. 62). Other than baseline eGFR and proteinuria, none of the above differences between the groups reached statistical significance (Table 1). The two

Table 1—Baseline characteristics of the participants from the Golden State cohort

Variable	All	ESRD or death	Neither	<i>P</i> value for trend
<i>N</i>	141	67	74	
Age (years)	57 (8)	57 (7)	57 (8)	0.4
Male, <i>N</i> (%)	80 (57)	40 (60)	40 (54)	0.6
Race/ethnicity, <i>N</i> (%)				0.1
Hispanic	103 (73)	50 (75)	53 (72)	
Caucasian	21 (15)	13 (19)	8 (11)	
African American	12 (9)	4 (6)	8 (11)	
Other	5 (4)	0 (0)	5 (6)	
Smoking, <i>N</i> (%)				0.6
Current	21 (15)	9 (13)	12 (16)	
Past	57 (40)	30 (45)	27 (36)	
Never	63 (45)	28 (42)	35 (47)	
Diabetes duration (years)	14 (10, 19)	14 (10, 20)	14 (10, 18)	0.6
Hypertension, <i>N</i> (%)	133 (94)	64 (96)	69 (93)	1.0
CVD, <i>N</i> (%)	50 (35)	21 (31)	29 (39)	0.4
RAS inhibitor use, <i>N</i> (%)	115 (82)	53 (79)	62 (84)	0.6
Aspirin use, <i>N</i> (%)	63 (45)	27 (40)	36 (49)	0.3
HMG-CoA reductase use, <i>N</i> (%)	89 (63)	42 (63)	47 (64)	0.9
SBP (mmHg)	155 (136, 176)	159 (138, 179)	152 (135, 173)	0.6
DBP (mmHg)	77 (70, 85)	77 (69, 85)	77 (71, 85)	0.5
BMI (kg/m ²)	32 (9)	31 (8)	32 (9)	0.5
HbA _{1c} (%)	8.0 (7.1, 10.0)	8.0 (6.9, 9.9)	8.0 (7.2, 10.0)	0.8
LDL (mg/dL)	108 (81, 136)	108 (85, 140)	109 (77, 129)	0.5
HDL (mg/dL)	41 (35, 49)	41 (35, 49)	41 (33, 49)	0.9
eGFR (mL/min/1.73 m ²), mean	59 (23)	53 (25)	63 (21)	0.002
eGFR (mL/min/1.73 m ²), median	54 (40, 75)	44 (33, 74)	62 (45, 76)	
Urine protein (g/L), mean	3.1 (3.4)	3.8 (3.8)	2.5 (2.9)	0.03
Urine protein (g/L), median	1.8 (0.9, 4.3)	2.5 (0.9, 5.2)	1.5 (0.9, 3.1)	
Urine PCR (g/g), mean	3.7 (3.5)	4.6 (4.2)	2.9 (2.5)	
Urine PCR (g/g), median	2.6 (1.2, 5.3)	3.6 (1.4, 6.5)	2.0 (1.1, 3.6)	0.01

Data are displayed as mean (SD) and median (IQR) for continuous covariates and *N* (%) for categorical covariates where noted. To convert HDL and LDL to mmol/L, multiply by 0.0259. HMG, hydroxymethylglutaryl.

groups were similar in age, diabetes duration, median HbA_{1c}, LDL, HDL, and DBP.

Untargeted Proteomics

Abundance of Complement Proteins in Urine of People With Diabetes With or Without DKD

Untargeted proteomics was used to broadly compare the urine proteins in people with type 1 diabetes and proteinuric DKD (*n* = 32) versus those without DKD (control subjects; *n* = 29). Of the 469 proteins identified in a majority of samples, 95 were higher in people with DKD versus control subjects (*P* < 0.05). The complement pathway was one of the most highly represented pathways, with nine proteins (complement component 3 [C3], 4, 5, 6, 7, 8α, 8γ, 9, and complement factor H) for which median abundance per unit of urine protein was 1.8–7.8-fold higher in people with DKD than

control subjects (*P* < 0.05) (Supplementary Fig. 2).

Targeted Proteomics

Correlation Between Urine Complement Proteins and eGFR or Proteinuria

The abundance of most complement proteins per unit of total urine protein (i.e., the fraction of total urine protein that was composed of a given complement protein) was negatively correlated with eGFR (Pearson *r* = −0.16 to −0.49). The exception was CD59, which was not correlated with eGFR (*r* = 0.06) (Fig. 1A). In contrast, the abundance of most urine complement proteins (per unit of total urine protein) was positively correlated with total urine protein concentration, ranging from *r* = 0.15 (clusterin) to *r* = 0.42 (C3 and C4). Only CD59 showed a strong negative correlation with total urine protein (*r* = −0.53) (Fig. 1B).

Complement Proteins and the Risk for the Combined Outcome of ESRD and/or Death

Of the 141 participants at risk, 67 developed ESRD and/or died during a median (IQR) follow-up of 3.1 (1.7, 4.9) years for ESRD and 3.6 (2.1, 5.5) years for all-cause death. Urine abundance of each quantified complement protein was included individually in time-to-event analyses for the combined ESRD/death outcome (Table 2). Four complement proteins were associated with ESRD/death after adjustment for demographic and clinical parameters, including baseline eGFR and proteinuria (model 3). C4 and C8 were significantly associated with ESRD/death only in the fully adjusted model (model 3); CD59 and FHR2 were significantly associated with ESRD/death in all models. Higher urine C4, C8, and CD59 were associated with a lower risk (HRs [95% CIs per doubling] 0.70 [0.53–0.91],

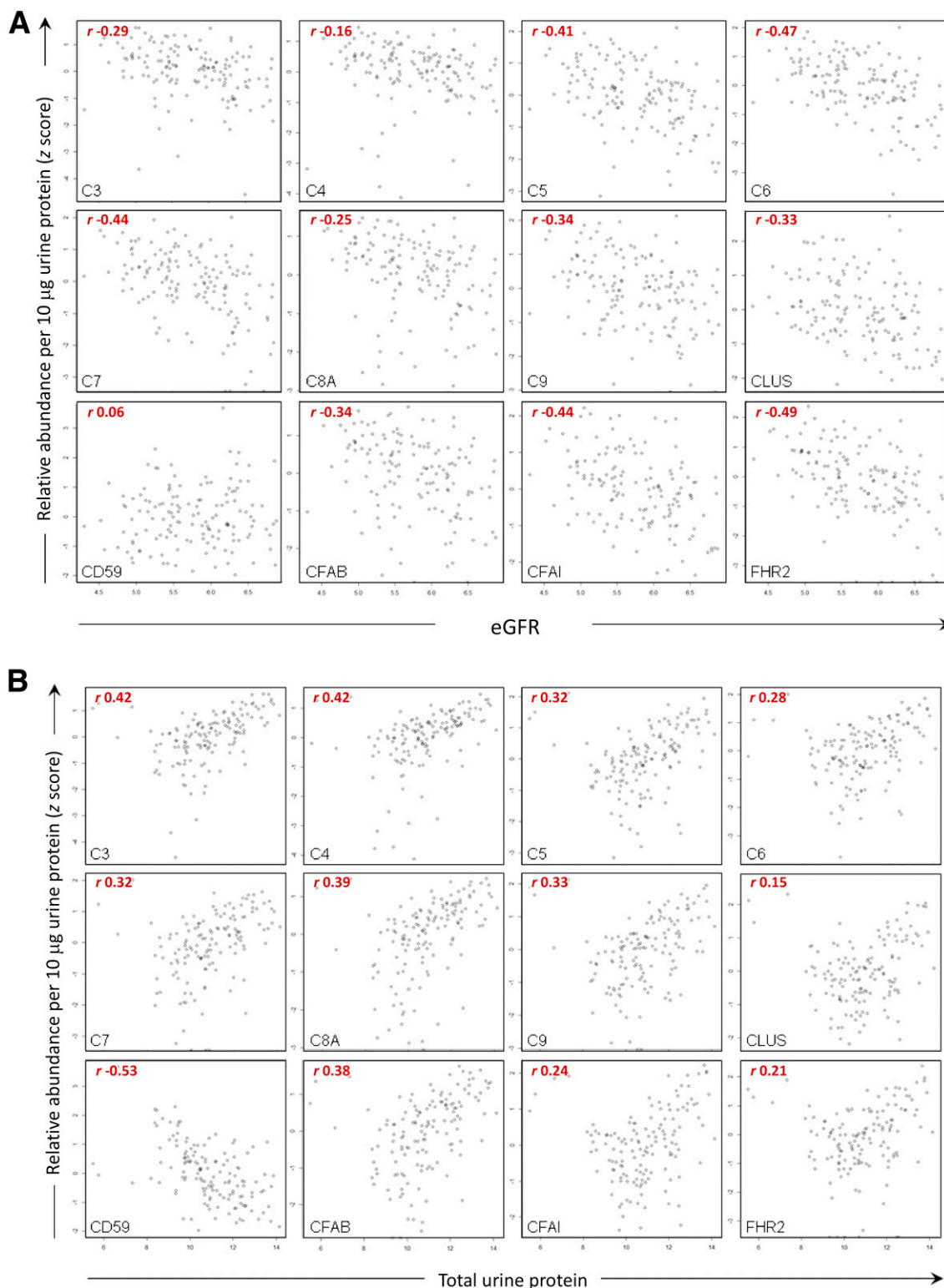


Figure 1—Correlations between the abundance of individual complement proteins and baseline eGFR (A) and total urine protein concentration (B). Correlations are represented by the Pearson correlation coefficient (*r*). CFAB, complement factor B; CFAI, complement factor I; CLUS, clusterin.

0.70 [0.51–0.95], and 0.60 [0.40–0.88], respectively), whereas higher urine FHR2 was associated with a higher risk (HR [95% CI] 1.77 [1.25–2.50]) for ESRD/death.

Replacing hypertension with SBP/DBP (Supplementary Table 4), inclusion of BMI (Supplementary Tables 5, 7, and 8), or aspirin and hydroxymethylglutaryl-

CoA reductase inhibitor use (Supplementary Table 6) did not alter these findings significantly. Furthermore, to limit model overfitting, LDL, HbA_{1c}, diabetes duration,

Table 2—Association of individual complement proteins with the combined outcome of ESRD and/or death

Protein	Model 1		Model 2		Model 3	
	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
C3	1.06 (0.79, 1.40)	0.71	1.07 (0.79, 1.45)	0.66	0.77 (0.57, 1.05)	0.10
C4	0.92 (0.71, 1.19)	0.52	0.88 (0.66, 1.16)	0.36	0.70 (0.53, 0.91)	0.008
C5	1.37 (1.03, 1.82)	0.03	1.47 (1.10, 1.95)	0.01	1.16 (0.85, 1.60)	0.35
C6	1.39 (1.04, 1.87)	0.03	1.45 (1.06, 1.98)	0.02	0.99 (0.69, 1.43)	0.97
C7	1.18 (0.89, 1.58)	0.25	1.21 (0.90, 1.61)	0.20	0.84 (0.59, 1.19)	0.33
C8A	0.96 (0.73, 1.27)	0.78	0.99 (0.74, 1.32)	0.92	0.70 (0.51, 0.95)	0.021
C9	1.19 (0.91, 1.55)	0.20	1.24 (0.93, 1.64)	0.14	0.97 (0.71, 1.32)	0.85
CLUS	1.45 (1.10, 1.91)	0.01	1.47 (1.11, 1.96)	0.01	1.24 (0.90, 1.69)	0.19
CD59	0.70 (0.53, 0.93)	0.01	0.64 (0.47, 0.87)	0.004	0.60 (0.40, 0.88)	0.009
CFAB	1.29 (0.97, 1.71)	0.08	1.33 (0.99, 1.79)	0.06	0.93 (0.67, 1.31)	0.69
CFAI	1.52 (1.14, 2.02)	<0.01	1.59 (1.18, 2.14)	<0.01	1.23 (0.88, 1.73)	0.23
FHR2	2.11 (1.55, 2.86)	<0.00001	2.19 (1.61, 2.97)	<0.00001	1.77 (1.25, 2.50)	0.0014

Model 1 was adjusted for experimental sample batch. Model 2 was additionally adjusted for age, sex, race/ethnicity (Hispanic, non-Hispanic), smoking, hypertension, and use of RAS inhibitors. Model 3 was further adjusted for \log_2 (baseline eGFR) and \log_2 (baseline urine protein concentration). Statistically significant associations appear in boldface type. LDL, HbA_{1c}, diabetes duration, and prevalent CVD were excluded from models because their inclusion changed HR <5%. CFAB, complement factor B; CFAI, complement factor I; CLUS, clusterin.

and prevalent CVD were excluded from models because their inclusion changed HR <5% (Supplementary Tables 7 and 8).

Association of C4, C8, CD59, and FHR2 with the Individual Outcomes of ESRD or Death

Of the 67 participants who sustained ESRD and/or death, 39 developed ESRD and 40 died, including 12 who developed ESRD and subsequently died (Supplementary Table 9). C4, C8, CD59, and FHR2 were individually included in the time-to-event analysis for each outcome of ESRD or death (Table 3). In a fully adjusted model (model 3), higher urine CD59 remained associated with lower risk of ESRD (HR [95% CI] 0.50 [0.29–0.87]), whereas FHR2 association with ESRD lost significance after adjustment for eGFR and proteinuria. Accounting

for the competing risk of death did not alter these findings (Supplementary Table 10). All four proteins were associated with death in the fully adjusted model. Higher urine C4, C8, and CD59 were associated with a lower risk (HRs [95% CI for doubling] 0.57 [0.41–0.79], 0.66 [0.44–0.97], and 0.56 [0.34–0.93], respectively), whereas higher urine FHR2 was associated with a greater risk (1.61 [1.05–2.48]) of death.

CONCLUSIONS

In a cohort of predominantly Mexican American participants with type 2 diabetes, proteinuric DKD, and high rates of ESRD and all-cause mortality, higher urine CD59, an inhibitor of the complement pathway, was associated with lower risk of ESRD and all-cause mortality. Higher

urine C4 and C8 were associated with lower, and higher FHR2 with higher, risk of all-cause mortality. These associations remained significant after adjustment for demographic and clinical covariates, including eGFR and proteinuria.

Several lines of evidence implicate complement pathway activation in DKD pathogenesis. Complement deposition in the kidneys has been noted in DKD animal models (17) and people with DKD (18,19). Complement activation products were increased in urine of people with DKD, but not those with minimal change disease with comparable proteinuria (20). Two more findings suggested that urine complement might reflect complement production and/or deposition in the kidneys in human DKD: transcription of complement genes was

Table 3—Association of individual complement proteins C4, C8, CD59, and FHR2 with ESRD or death

Protein	Model 1		Model 2		Model 3	
	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
ESRD						
C4	1.39 (0.86, 2.22)	0.18	1.25 (0.76, 2.06)	0.38	0.84 (0.55, 1.29)	0.43
C8A	1.25 (0.83, 1.89)	0.28	1.14 (0.73, 1.79)	0.56	0.68 (0.45, 1.06)	0.08
CD59	0.55 (0.37, 0.81)	0.002	0.56 (0.36, 0.87)	0.01	0.50 (0.29, 0.87)	0.01
FHR2	2.59 (1.69, 3.97)	<0.001	2.60 (1.67, 4.05)	<0.001	1.48 (0.90, 2.45)	0.13
Death						
C4	0.70 (0.53, 0.92)	0.01	0.66 (0.48, 0.90)	0.009	0.57 (0.41, 0.79)	0.0009
C8A	0.74 (0.54, 1.00)	0.05	0.81 (0.58, 1.13)	0.21	0.66 (0.44, 0.97)	0.03
CD59	0.91 (0.65, 1.27)	0.57	0.73 (0.49, 1.08)	0.12	0.56 (0.34, 0.93)	0.02
FHR2	1.45 (1.03, 2.04)	0.03	1.61 (1.13, 2.28)	0.008	1.61 (1.05, 2.48)	0.03

Models were adjusted as described in Table 2, except that models for ESRD also included diabetes duration. Statistically significant associations appear in boldface type.

increased in the tubulointerstitium of people with DKD versus control subjects (21). Furthermore, in people with proteinuric DKD, urine C3 excretion correlated with tubular C3 deposition (22) but not with plasma C3 (20). In addition, inhibition of complement activation in experimental DKD reduced renal complement deposition, mesangial expansion, and glomerular injury, supporting a causal role for complement activation in DKD progression (23,24).

The timing of any complement pathway involvement in human DKD is unknown. Elegant studies in experimental models of proteinuric CKD outline a chain of events beginning with activation of the complement pathway by proteinuria, leading to complement-mediated release of proinflammatory cytokines and growth factors from endothelial and tubular epithelial cells. This then promotes inflammatory cell infiltration into the renal interstitium, leading to tubulointerstitial inflammation and subsequent fibrosis (25–28). Consistently, human proximal tubular cells express a membrane-bound C3-convertase capable of activating the alternative complement pathway, culminating in insertion of the membrane attack complex (MAC). When exposed to human serum proteins, these cells activate complement, leading to induction of inflammatory cytokines and cell lysis (25,29). Furthermore, inhibiting complement activation in proteinuric rodents reduces tubulointerstitial injury without affecting the degree of proteinuria (30–32). As early as a decade ago (5), these observations led to the intriguing hypothesis that complement activation may be a potential mechanism by which proteinuria leads to tubulointerstitial fibrosis and progression of CKD. The current study is the first report of an association between urine complement proteins and progression of proteinuric kidney disease in people with diabetes.

A key finding in this study is that in people with proteinuric DKD, higher urine CD59 is associated with a lower ESRD risk. CD59, a major regulator of the complement activity, is a *trans*-membrane protein that binds to the C5–8 complex and inhibits assembly of C9 monomers into the terminal complement complex, thus protecting cells from complement-mediated injury (33). This protective function has been

implicated in CD59-mediated attenuation of kidney injury (34), as well as atherosclerotic endothelial injury in experimental diabetes (35,36). Our findings do not identify the source of urine CD59, which may be renal (i.e., shed into urine from apical membranes of renal cells) or systemic (i.e., serum CD59 filtered through the glomerulus). CD59 is expressed by most cells in the body, including those in the kidneys. Its expression is increased in kidney tubulointerstitium in people with DKD (21), so renal expression may contribute to the urine CD59 protein. If the higher urine CD59 is a consequence of higher renal CD59 expression, its association with a lower risk of ESRD may reflect the CD59-mediated protection from proteinuria-induced complement activation and subsequent tubulointerstitial injury and fibrosis, eventually leading to slower DKD progression and longer time to ESRD. On the other hand, CD59 is a small protein (14 kDa), and higher urine CD59 may well represent greater filtration of serum CD59. If so, its association with lower ESRD risk may be unrelated to complement activity in the kidneys, but instead reflect a systemic process that affects DKD progression. In a further twist, hyperglycemia glycosylates CD59 on a critical histidine residue (His44) within its active site, rendering CD59 incapable of inhibiting MAC assembly (37). In kidneys of people with diabetes, glycosylated CD59 and MAC codeposit in glomerular capillaries (34). Interestingly, His44 is absent in CD59 from all animal species sequenced to date and is proposed to contribute to the unique human susceptibility to DKD (37). The CD59 glycosylation motif includes a lysine (K), which, once glycosylated, cannot be cleaved by trypsin. As a result, the pattern of peptides generated from a trypsin digest of glycosylated CD59 will be distinct from that of unglycosylated CD59 (Supplementary Fig. 3). The pattern of CD59 peptides identified in urine, and used for this study, together with the near-complete correlation between them suggests that all peptides identified in this study were derived from unglycosylated CD59. Furthermore, the observed association between this unglycosylated urine CD59 and lower risks of ESRD and death in this study fits well with prior reports showing that the unglycosylated (but not the glycosylated) CD59 can inhibit complement activation. It would be additionally informative to

specifically quantify glycosylated CD59, in parallel with the unglycosylated CD59, and compare the association of each with renal outcomes. Specific quantification of glycosylated CD59 would require an immunoassay using an antibody specific to glycosylated CD59 or development of a targeted MS method to detect its specific peptide pattern (Supplementary Fig. 3C).

Higher urine CD59 was also associated with lower all-cause mortality. Lack of adjudicated causes of death precluded further dissection of this finding. However, CVD is a significant cause of death in people with DKD, and CD59 deficiency markedly exacerbates atherosclerosis in rodents with diabetes, suggesting that complement activation plays a role in progression of atherosclerosis and that complement inhibition by CD59 may reduce complement-mediated endothelial injury in CVD (36). As such, the observed association between higher urine CD59 and lower mortality may reflect CD59-mediated protection of endothelial cells from complement-mediated injury in CVD. This hypothesis would suggest that higher urine CD59 reflects higher serum CD59. In contrast, given the strong association between reduced eGFR and mortality, the association between urine CD59 and mortality may be confounded by CD59 association with DKD progression and ESRD. Adjustment for baseline eGFR did not affect the association between urine CD59 and mortality. However, despite adjustment for baseline eGFR, a greater rate of eGFR loss during the follow-up in people with lower baseline urine CD59 may still confound the association between urine CD59 and mortality.

In this study, higher urine C4 and C8 were associated with a lower risk and higher urine FHR2 with a greater risk of death. C4 is a nonenzymatic component of the C3- and C5-convertases, generated via the classical or lectin arms of the complement cascade. C8 is a component of the terminal complement complexes. FHR2 belongs to the family of the complement FHR proteins that block the inhibition of the alternative pathway by complement factor H. Taken at face value, these findings suggest that greater activation of the classical/lectin complement pathways may be associated with longer survival, whereas greater activation of the alternative pathway may be

associated with shorter survival in this population. However, the explanation is likely much more complex. A majority of deaths in the population with CKD/ESRD are due to CVD and cancer (~62% combined), with a much lower proportion (~4%) from infectious causes (38). Although complement activation is beneficial against inflammatory and infectious etiologies, it may promote cancer growth and metastasis (39). Also, the role of complement system in CVD is complex and incompletely understood, with some components inhibiting, and others promoting, progression of atherosclerosis (40). In addition, given the marked clinical differences in CVD between the CKD/ESRD and general population, it is unclear how much of what is known about the complement pathway in CVD in the general population can be extrapolated to people with CKD/ESRD. As such, deciphering the exact molecular mechanisms behind the association of C4, C8, and FHR2 with all-cause mortality requires further study. However, it is worth noting that whereas higher complement inhibitor CD59 is associated with lower ESRD, activation of different arms of the complement pathway may be associated with both higher and lower risk of mortality, perhaps dependent on the arm of the pathway that is engaged. As such, targeting the complement pathway for therapeutic intervention requires a more nuanced understanding of the effect of each of its components on DKD progression, as well as mortality.

The strengths of this study are the comprehensive quantification of urine complement proteins using a robust targeted MS method specifically developed for use in urine, the use of a clinically relevant population with high prevalence of DKD and its adverse outcomes, use of the hard outcomes of ESRD and death, and careful adjustment for potential confounders. The study limitations are the modest cohort size, absence of parallel quantification of complement proteins in serum and kidney, and lack of adjudicated causes of death. Furthermore, given the strong association between eGFR and both ESRD and death, adjustment with eGFR (instead of measured GFR) may leave residual confounding. In addition, study participants were not evaluated for occult urinary tract infections, which may increase complement proteins in urine.

However, as we are not aware of data suggesting that urinary tract infections are more common in people who progress to ESRD or die, undetected urinary tract infections are more likely to add to noise than false-positive findings. Finally, the findings of this study require validation in independent cohorts. Additionally, it would be informative to include control subjects without diabetes to assess the impact of diabetes without kidney disease on urine complement. Furthermore, the above studies should be repeated in timed urine collections to determine if the urinary excretions of these proteins are more strongly associated with the outcome than their abundance in random samples. It is worth noting that at least for urine albumin, its quantity in spot urine samples is more strongly predictive of renal events than its excretion in timed urine collections.

In summary, in a group of largely Hispanic patients with type 2 diabetes and proteinuric CKD, higher urine CD59 was strongly associated with a substantially lower risk of subsequent ESRD and death. Three other complement proteins were strongly associated with mortality. These findings suggest a role for complement pathway activation in DKD progression in people with proteinuria.

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