SHORT COMMUNICATION

Complement 7 Is Up-Regulated in Human Early Diabetic Kidney Disease

Monica Sircar,^{*} Ivy A. Rosales,^{*} Martin K. Selig,^{*} Dihua Xu,^{*} Zsuzsanna K. Zsengeller,^{†‡§} Isaac E. Stillman,^{†‡§} Towia A. Libermann,[¶] S. Ananth Karumanchi,^{†‡§} and Ravi I. Thadhani^{*}

From the Division of Nephrology, Massachusetts General Hospital and Partners Health Care, the Center for Vascular Biology Research,[†] the Departments of Medicine[‡] and Pathology,[§] and the Genomics, Proteomics, Bioinformatics, and Systems Biology Center,[¶] Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts*

Accepted for publication
June 21, 2018.

Address correspondence to
Monica Sircar, M.D., Division
of Nephrology, Massachusetts
General Hospital, 55 Fruit St.,
Boston, MA 02114. E-mail:
msircar@partners.org.

There is a temporal window from the time diabetes is diagnosed to the appearance of overt kidney disease during which time the disease progresses quietly without detection. Currently, there is no way to detect early diabetic nephropathy (EDN). Herein, we performed an unbiased assessment of gene-expression analysis of postmortem human kidneys to identify candidate genes that may contribute to EDN. We then studied one of the most promising differentially expressed genes in both kidney tissue and blood samples. Differential transcriptome analysis of EDN kidneys and matched nondiabetic controls showed alterations in five canonical pathways, and among them the complement pathway was the most significantly altered. One specific complement pathway gene, complement 7 (C7), was significantly elevated in EDN kidney. Real-time PCR confirmed more than a twofold increase of C7 expression in EDN kidneys compared with controls. Changes in C7 gene product level were confirmed by immunohistochemistry. C7 protein levels were elevated in proximal tubules of EDN kidneys. Serum C7 protein levels were also measured in EDN and control donors. C7 levels were significantly higher in EDN serum than control serum. This latter finding was independently confirmed in a second set of blood samples from a previously collected data set. Together, our data suggest that C7 is associated with EDN, and can be used as a molecular target for detection and/or treatment of EDN. (*Am J Pathol* 2018, 188: 2147–2154; <https://doi.org/10.1016/j.ajpath.2018.06.018>)

Over the past two decades, the incidence of end-stage renal disease in the United States has nearly doubled because of a steady increase in diabetic nephropathy (DN). In 1982, only 23% of new end-stage renal disease cases were attributed to diabetes, but by 1999, at 43%, DN became the leading cause of end-stage renal disease.¹ In 2015, the incidence of end-stage renal disease was >130,000, with prevalence >700,000 and an annual death rate of >100,000 individuals.² Despite progress made in delaying dialysis dependence, these trends are expected to increase, at least for the near future.

DN affects 15% to 25% of type 1 diabetics and 30% to 40% of type 2 diabetics.³ The detection of albumin in urine is often the first clinical indication of kidney disease. The natural progression of diabetic kidney disease has been closely examined in type 1 diabetic patients, in whom timing of

disease onset is generally known. Investigation into type 1 diabetes has shown progressive decline in renal function over time (>3.5 mL/minute per year).⁴ However, for many years, the decline was not detected by standard laboratory testing because patients were deemed to have normal renal function if the estimated glomerular filtration rate was >60 mL/

Supported by NIH-National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) T32 grant DK 5 7540 (principal investigator: R.I.T.; trainee: M.S.), Massachusetts General Hospital NIH/NIDDK grant K24 DK094872-04 (principal investigator: R.I.T.), and American Society of Nephrology Ben J. Lipps Research Fellowship (M.S.).

Disclosures: The authors have filed for a provisional patent application to use complement 7 for risk determination in diabetic nephropathy/chronic kidney disease.

Current address of S.A.K., Cedars-Sinai Medical Center, Los Angeles, CA.

minute. Later in the disease process, individual patients can have differing rates of renal decline. Progressive renal decline precedes the onset of microalbuminuria and, as it continues, it increases the risk of proteinuria.⁴

Evidence from autopsy studies indicates that diabetic nephropathy remains relatively underdiagnosed,^{5–7} and diabetic kidney damage,⁸ often with no overt clinical findings, is usually confirmed during postmortem pathologic examination. In 2010, the Renal Pathology Society developed a new classification system for diabetic kidney disease.⁹ According to this classification: class I kidneys show increased thickness of glomerular basement membrane (≥ 395 nm in females and ≥ 430 nm in males), class II kidneys have mild (class IIa) to severe (class IIb) mesangial expansion, class III kidneys show at least one convincing Kimmelstiel-Wilson lesion, and in class IV kidneys show 50% of glomeruli have global glomerular sclerosis. Pathologic staging of diabetic kidney disease appears to be unrelated to clinical staging of chronic kidney disease, such as blood creatinine level.

Mechanisms underlying early diabetic nephropathy remain unknown, and there is a paucity of literature on renal molecular targets in early DN (EDN).^{10–16} Most differential gene expression studies have been performed in rodent diabetic models, and although rodent experiments provide important insights into disease mechanisms, they are limited because of the inherent differences between murine and human diabetic kidney disease. In the few human studies that have been reported in the literature, all have looked at kidneys with advanced diabetic kidney disease. There appears to be no study in the literature that has investigated gene expression in postmortem human kidneys with early diabetic nephropathy, otherwise scheduled for the kidney donor pool. Herein, we have compared transcriptome profiles of human postmortem early diabetic kidneys with those of nondiabetic kidneys. We then selected one of the most significantly increased EDN genes for further examination in postmortem human kidney and blood.

Materials and Methods

This study was approved by the Massachusetts General Hospital Institutional Review Board and abided by the guidelines set forth by the Declaration of Helsinki. No donor organs were obtained from executed prisoners or other institutionalized individuals at any national transplant center. Organ procurement offices were coordinated to retrieve organs with appropriate informed consents. All patient information was deidentified and was thus Health Insurance Portability and Accountability Act compliant.

Study Population and Sample Collection

Postmortem biological samples (blood and kidney tissue) from nondiabetics and individuals with EDN were obtained

Table 1 Summary of Patient Demographics

Sample type	Nondiabetic	Diabetic
Harvested kidneys		
<i>n</i> (%)	18 (51)	17 (49)
Male sex, <i>n</i> (%)	8 (44)	6 (35)
Age in years, mean (range)	50 (17–75)	57 (34–78)
Hypertension, <i>n</i> (%)	9 (50)	17 (100)
Obese, <i>n</i> (%)	5 (28)	12 (71)
Tobacco use, <i>n</i> (%)	12 (67)	10 (59)
White, <i>n</i> (%)	14 (78)	12 (71)
Serum creatinine, mean (range), mg/dL*	1.4 (0.4–4.5)	1.3 (0.5–2.3)
Proteinuria (≥ 1 + by UA), <i>n</i> %	4 (22)	7 (41)
Hemoglobin A1c, mean % (range)	5.4 (4.7–5.9)	8.2 (6.5–11.3)
eGFR (per MDRD), mean mL/minute	69.1	51.6
Stage I to IIIA eGFR, <i>n</i> % [†]	11 (61)	9 (53)
Serum complement C3, mg/dL	112	137
Serum complement C4, mg/dL	25	29
Total complement CH50, U/mL	51	57
Alternative complement AH50, U/mL	97	127
Kidney samples for microarray[‡]		
<i>n</i>	4	4
Male sex, <i>n</i>	2	2
Age in years, mean (range)	52 (24–65)	64 (55–78)
Hypertension, <i>n</i> (%)	3 (75)	4 (100)
BMI in kg/m ² , mean (range)	27 (26–29)	35 (22–43)
Tobacco use, <i>n</i> (%)	2 (50)	2 (50)
White, <i>n</i> (%)	3 (75)	3 (75)
Terminal serum creatinine, mean, mg/dL	0.9	1.4
Proteinuria (≥ 1 + by UA), %	50	50
Hemoglobin A1c, mean, %	5.4	9.5
eGFR, mean mL/minute	>60	>60
PRIMO blood samples		
<i>n</i>	20	39
Male sex, <i>n</i> (%)	14 (70)	30 (77)
Age in years, mean (range)	66 (47–81)	66 (48–84)
Hypertension, <i>n</i> (%)	19 (95)	38 (97)
Obese, <i>n</i> (%)	6 (30)	24 (62)
Tobacco use, <i>n</i> (%)	11 (55)	23 (59)
White, <i>n</i> (%)	15 (75)	29 (74)
Serum creatinine, mean, mg/dL*	2.1	2.1
Proteinuria (≥ 1 + by UA), <i>n</i> (%)	10 (50)	24 (62)
eGFR (per MDRD), mean mL/minute	33	34
Class I to IIIA or better eGFR, <i>n</i> (%) [†]	2 (10)	5 (13)

*At time of admission.

[†]Based on MDRD equations.

[‡]Subset of harvested kidneys.

AH50, alternative pathway complement activity; BMI, body mass index; CH50, total complement activity; eGFR, estimated glomerular filtration rate; MDRD, Modification of Diet in Renal Disease; PRIMO, Paricalcitol Capsules Benefits Renal Failure Induced Cardiac Morbidity in Subjects with Chronic Kidney Disease Stage 3/4; UA, urinalysis.

from the National Disease Research Interchange (Philadelphia, PA). Patient demographics have been provided in Table 1. Except for obesity, there was no significant difference in any of the parameters between study groups; there were more obese individuals in the EDN group than among nondiabetic controls. All diabetic donors had type 2 diabetes. Diabetes status was defined as hemoglobin A1c $\geq 6.5\%$. Proteinuria was identified by urine dipstick test, performed during the terminal hospital admission. These diagnoses were confirmed by careful review of premortem and postmortem clinical health records. There were stringent inclusion and exclusion criteria. Inclusion criteria required that: i) donors be between 17 and 80 years of age, ii) diabetic status be confirmed as noted above, and iii) the postmortem time be within 24 hours from cross-clamping in the operating room. The only exception with respect to hemoglobin A1c testing was if a donor had been prescribed and/or using glycemic agent(s), including but not limited to metformin and insulin, in which case repeated hemoglobin A1c testing was not mandatory. Exclusion criteria included active infection; malignancy; severe glomerulosclerosis, as determined in frozen operating room biopsy material; history of renal replacement therapy (hemodialysis or peritoneal dialysis at any time); and any known genetic renal condition, such as polycystic kidney disease. Pathologic staging of diabetic tissues was performed using the 2010 Renal Pathology Society classification system for diabetic kidney disease.⁹ From each postmortem nephrectomy sample, >100 glomeruli were reviewed in tissue sections. Pathologic classes II and III diabetic kidney disease were considered as EDN; class III kidneys were included because the renal function in these donors was preserved.

Isolation of Total RNA

For all real-time PCR studies, mRNA was extracted exclusively from kidney cortical tissue. Total RNA from flash-frozen kidney tissue was subjected to Trizol (Invitrogen, Carlsbad, CA) extraction and purified using an RNeasy mini kit. Total RNA extracted was quantitated using a NanoDrop Spectrophotometer (ND-2000; Thermo Scientific, Waltham, MA), and stored at -80°C until use. mRNA quality was confirmed by gel electrophoresis. Good quantities of high-quality RNA were obtained from all postmortem kidney samples.

Affymetrix Chip Hybridization and Expression Profiling

mRNA preparations were processed by the Beth Israel Deaconess Medical Center Genomics, Proteomics, Bioinformatics, and Systems Biology Center, per the standard Affymetrix protocol using the Affymetrix Human Transcriptome 2.0 GeneChip (Affymetrix, Inc., Santa Clara, CA), as previously described.¹⁷ Gene expression data have been deposited with the National Center for Biotechnology

Information Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE111154).

Gene Ontology, Pathway, and Interactive Network Analysis

To assess for potential molecular pathways underlying the early diabetes-specific gene expression signature and to more precisely understand the complex interactions between the differentially expressed genes, the 285 differentially expressed genes were further analyzed using the Ingenuity Pathway Analysis software package version 8.0 (Qiagen Bioinformatics, Redwood City, CA). *P* values were calculated on the basis of gene enrichments in biological pathways or networks using the Fisher exact test ($P < 0.05$). Gene ontology, functional category, canonical pathway, upstream regulator, and interactive network analyses were performed on the 285 differentially expressed genes. Interactive network analysis identified the complex interactions between the differentially expressed EDN-specific genes and the key focus hub genes (genes with the largest number of connections and anticipated to be the ones that ensure the stability and integrity of the network). Ingenuity Pathway Analysis also helped identify the canonical pathways with the statistically highest enrichment of EDN-specific genes.

cDNA Synthesis and Real-Time PCR

Protocol, as described in the literature,¹⁸ was used to perform real-time PCR for complement 7 (C7). Briefly, 1 μg of total cortical RNA from each specimen was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA) with a Qiagen RNase inhibitor for 10 minutes at 25°C , 120 minutes at 37°C , and 5 minutes at 85°C in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems). The resulting cDNA was amplified using primers specific for the human C7 gene (UniGene Hs78065; chromosome 5: 40909497-40982939 on Build GRCh38; forward primer, 5'-TGTAACACGACGGCCAGT-3'; reverse primer, 5'-CAGGAAACAGCTATGACC-3'; Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) and the housekeeping β -actin gene (Applied Biosystems catalog number 4333762T; Thermo Fisher Scientific, Waltham, MA).

Enzyme-Linked Immunosorbent Assay

Serum component C7 protein levels from postmortem donors (Table 1) were measured using a commercially available quantitative enzyme-linked immunosorbent assay kit (catalog number 125964; Abcam, Cambridge, MA), as per manufacturer's instructions. The serum component C7 assay was specific for C7 and is not known to cross-react with any other complement component. The interassay CV was $<12\%$.

Immunohistochemistry and Histology

Immunohistochemistry of formalin-fixed, paraffin-embedded kidney tissue sections was performed using a rabbit polyclonal IgG antibody to human C7 (catalog number HPA001465; Sigma, Billerica, MA; 1:200 dilution) and a goat secondary antibody staining kit (catalog number MP-7405; Vector Laboratories, Burlingame, CA). Scoring of immunohistochemical staining was performed as follows: 0, no staining; 1, low staining; 2, medium staining; and 3, high staining.

For light microscopic histology, kidney tissues were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin by conventional techniques. Sections were stained with periodic acid-Schiff or hematoxylin-eosin. Ultrastructural images were captured using an FEI Morgagni Transmission Electron Microscope with an AMT 2K digital charge-coupled device camera (Advanced Microscopy Techniques, Woburn, MA). As per the protocol outlined in Dickersin’s Diagnostic Electron Microscopy Text/Atlas, glomerular basement membrane thickness was measured using a rectangular grid. Tangential measurements and examination of paramesangial areas were avoided.

Additional Outcome Measures

Serum levels of complement component 3 (C3) and complement 4 (C4), total complement activity, alternative pathway complement activity, and urine protein and urine creatinine were measured by the Massachusetts General Hospital Clinical Resource Center. Creatinine measurements were also obtained from the primary hospital caring for the donor before death. An estimated glomerular function was determined using an online calculator, as established by the National Kidney Foundation (New York, NY; https://www.kidney.org/professionals/kdoqi/gfr_calculator, last accessed November 8, 2017).

Statistical Analysis

Demographic characteristic differences between nondiabetic donors and donors with EDN were compared using the χ^2 test, the Fisher exact test, the *t*-test, or the *U*-test, as deemed appropriate. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). Differences between serum component C7 levels were analyzed using paired *t*-test. All reported *P* values are two sided, and *P* < 0.05 was regarded as statistically significant.

Results

Gene Expression Changes in EDN Kidney

Microarray analysis was performed on four EDN and four nondiabetic kidneys (patient demographics provided in Table 1). Ingenuity pathway analysis revealed top canonical

Figure 1 Hierarchical cluster analysis of the top 80 genes in kidneys from donors with EDN and matched controls. Red indicates up-regulated genes; blue, down-regulated genes. Rows are genes, and columns are individual kidneys. ID, identification.

pathways enriched among the EDN-specific differentially expressed genes. Compared with nondiabetic kidneys, EDN kidneys had 245 up-regulated genes and 44 down-regulated genes (Figure 1).

Gene Ontology and Pathway Enrichment Analysis

Compared with nondiabetic controls, EDN kidneys had overrepresentation of up-regulated genes in pathways linked to the complement system, hepatic fibrosis, phagosome formation, and acute phase response signaling. The complement system was identified as the canonical pathway with the statistically highest enrichment of EDN-specific genes, and was predicted to be activated because most of the genes in this pathway were increased in EDN kidneys. Quantitative PCR analysis of EDN kidneys showed a twofold to threefold higher level of C7 compared with kidneys from nondiabetic controls (Figure 2A).

Component C7 Protein in EDN Kidney

Component C7 immunostaining was predominantly noted in the tubular segments (both cortical and medullary), with proximal tubules showing greater staining. Vessels in controls showed patchy mild staining of the media, whereas diabetics showed a similar but more intense pattern in the tubules. In addition, areas of hyalinosis in both glomeruli and vessels were intensely positive (Figure 2B). Semi-quantification of immunohistochemical staining confirmed greater staining in EDN kidneys compared with nondiabetic controls (Figure 2C).

Component C7 Protein Level in EDN Donor Blood

Serum component C7 protein levels in EDN donors and nondiabetic controls were determined by enzyme-linked immunosorbent assay sandwich assay. C7 protein levels were significantly higher ($P < 0.05$) in the serum of donors with EDN ($n = 8$) than in nondiabetic controls ($n = 8$) (Figure 3A).

Validation of Increased Serum C7 Protein Level in an Independent Cohort

The validity of elevated C7 level was tested in EDN blood by examining additional blood samples collected from an earlier clinical trial^{19,20} (Table 1). Serum C7 protein levels were significantly elevated in patients with clinically identified diabetes compared with nondiabetic controls (Figure 3B); both groups had chronic kidney disease. To determine the specificity of C7 changes, levels of additional C3, C4, total complement activity, and alternative pathway complement activity were measured in diabetic and control sera. Levels of all these complements in nondiabetic and EDN samples were within normal limits (Table 1).

Discussion

Early diagnosis of EDN has the potential of improving the quality and duration of life in diabetic patients. Earlier, gene expression analysis studies were performed in animal models,^{21,22} and the few human studies reported in the literature investigated gene expression in kidneys with advanced diabetic kidney disease.^{10,23} This is the first study to perform differential gene expression analysis in well-characterized diabetic kidneys in the early stages of nephropathy when kidneys have undergone relatively little glomerulosclerosis or reduction in estimated glomerular filtration rate compared with matched nondiabetic kidneys. In the present study, pathologic class II and III kidney disease was considered as EDN; class III kidneys had at least one glomerulus with a nodular increase in mesangial matrix (Kimmelstiel-Wilson). The presence of unequivocal mesangial expansion, often with limited Kimmelstiel-Wilson nodules, was deemed the earliest time point when

Figure 2 **A:** Real-time PCR for C7: C7 expression was measured in postmortem normal kidneys (ND) and early diabetic (ED) kidneys. Real-time PCR analysis shows that kidneys with EDN (white bar) have significantly higher levels of C7 gene expression (quantified as fold change in mRNA expression compared with the housekeeping β -actin gene) compared with matched nondiabetic control kidneys (black bar). Significance shown compared with ND. **B:** C7 immunohistochemistry in normal kidney and kidney with EDN. C7 is expressed in both renal tubular cells and endothelial cells. **C:** C7 protein levels in normal and EDN kidneys. C7 immunohistochemistry data were semiquantitated. Levels are significantly higher in EDN kidneys than nondiabetic kidneys. Closed bar: nondiabetic kidneys. Open bar: EDN kidneys. $n = 4$ (A, ND); $n = 3$ (A, ED kidneys, and C, EDN and nondiabetic kidneys). *** $P < 0.005$ versus ND. Original magnification, $\times 20$ (B).

Figure 3 **A:** Serum C7 levels in postmortem blood samples from nondiabetic and diabetic donors. Postmortem serum C7 levels were measured using enzyme-linked immunosorbent assay sandwich assay, and levels are significantly increased ($P < 0.05$) in donors with diabetic nephropathy (DN) compared with nondiabetic (ND) controls. **B:** Serum levels of C7 in patients enrolled in the Paricalcitol Capsules Benefits Renal Failure Induced Cardiac Morbidity in Subjects with Chronic Kidney Disease Stage 3/4 (PRIMO) trial. Serum C7 levels are significantly higher ($P < 0.005$) in patients with clinically identified diabetes compared with nondiabetic matched chronic kidney disease (CKD) controls. Closed dots indicate nondiabetic; open dots, diabetic. $n = 8$ (**A**, donor with DN and ND controls); $n = 34$ (**B**, patients with clinically identified diabetes); $n = 18$ (**B**, nondiabetic matched CKD controls).

diabetic kidney disease could be identified.²⁴ Several families of genes were dysregulated in EDN kidneys, one of which was the complement system gene family. C7 expression was significantly increased in EDN kidneys. Compared with controls, component C7 protein levels were elevated in the blood of EDN donors. There was no alteration in the levels of any of the other complements tested (C3, C4, total complement activity, or alternative pathway complement activity) in the serum of EDN and control donors. Together, these data suggest that before any clinical manifestations of diabetic nephropathy, such as proteinuria or changes in glomerular filtration rate, C7 gene and gene product levels are elevated in postmortem human kidney and blood.

An association between complement activation and diabetic kidney disease has been shown in animals.^{25–27} In humans, complement activation has been noted in a variety of conditions, including Alzheimer disease,^{28,29} fibromuscular disease,³⁰ and preeclampsia.^{31,32} Complement activation has been hypothesized to be important in the initiation and development of peripheral vasculopathy,³³ diabetic neuropathy,³⁴ and proliferative diabetic retinopathy.^{35,36} In one human study,¹⁰ gene expression profiling was done in cadaveric donor kidneys from two individuals with normal renal function and histologic characteristics and in two individuals with type 2 diabetes (both diabetic kidneys showed extensive glomerular hypertrophy, significant glomerulosclerosis, and interstitial fibrosis). Of the 12,000 genes on the chip, 96 were up-regulated and 519 were down-regulated. Authors reported down-regulation of vascular endothelial growth factor, nephrin (*NPHS1*), and *TGFB1* genes in advanced diabetic kidney disease kidneys. It is unclear whether members of the complement family were investigated or not. Another human transcriptome analysis study performed in normal kidneys ($n = 13$) and

kidneys with advanced diabetic kidney disease ($n = 9$) reported that the complement system (*C3*, *CD55*, and *C1qA*) was differentially expressed in diabetic kidney disease kidney, with a threefold increase in *C3* expression.²³ Both these human studies used renal tissues from subjects with advanced diabetic kidney disease, where kidneys had already undergone extensive renal pathologic damage.

Three pathways are involved in the activation of the complement system.³⁷ The classic pathway, when activated by the complement 1 complex, cleaves complex components C4 and complement 2. The lectin pathway is activated by mannose-binding lectin and N-acetylglucosamine residue of ficolins. When this pathway is activated, it also cleaves C4 and complement 2. The alternate pathway is activated after activation of C3. Activation of all three pathways results in the formation of the membrane attack complex (MAC). MAC deposition has been reported in diabetic kidney.³⁸ C7 is a critical component of the terminal pathway of complement activation.³⁹ C7, along with complements 6, 8, and 9, is involved in MAC formation.⁴⁰ In this study, marked elevations were noted only in C7 levels in kidneys with EDN. Levels of C3 and C4 did not show any significant alterations in EDN kidneys, and total complement activity and alternative pathway complement activity levels were within normal limits; levels for complement 6, 8, and 9 were not examined. Future studies will investigate these latter complements in EDN kidneys. The membrane-bound C7 complex forms the transmembrane pore.⁴¹ C7 is expressed in endothelial cells as a trap for assembling MAC.^{42,43} Alterations in MAC formation have been reported in several pathologic conditions. Vasil and Magro³³ found MAC deposition in skin biopsy specimens from diabetics with vascular lesions. MAC deposition has also been seen in glomerular injury, dermatomyositis microvasculature,^{44,45} smooth muscle,⁴⁶ and mesangium of kidneys with more advanced diabetic disease.⁴⁷ In this

study, tubular C7 deposition was observed. It is possible that the proximal tubule is an early target of injury.⁴⁸ It is not clear if there is juxtaglomerular feedback involving tubular C7 and mesangial MAC formation. Further studies will be required to identify a causal relationship between the two. Most diabetic donors experienced a fatal cardiovascular condition, stroke, or anoxia from presumed cardiac causes, whereas causes for death in nondiabetic donors included blunt head trauma and suicide from hanging. Whether these conditions affect complement regulation needs investigation.

These findings show that in the early stages of diabetes, there is increased expression of C7 and its gene product in EDN kidney. C7 is released into circulation and is reflected as high levels of C7 in blood. These findings are supported by other studies where vascular deposition of complement proteins has been reported. Falk³⁸ reported complement deposition in kidney from diabetics but not in kidneys from membranoproliferative renal disease. This study suggests that elevated C7 gene, and gene product, can be a biomarker for early diabetic nephropathy. Therapeutics based on this terminal complement may be used to treat diabetic patients in a precise and timely manner to prevent the development of clinically significant diabetic nephropathy. Complement activation has been suggested as a therapeutic measure in the progression of other diseases, including sepsis, age-related macular degeneration, Alzheimer disease, and atypical hemolytic-uremic syndrome.^{37,49} Recently, complement inhibitors, such as the C5 antibody, have been approved by the US Food and Drug Administration for therapeutic use in paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome.^{50,51}

In conclusion, based on differential transcriptome microarray analysis of postmortem human EDN and nondiabetic kidney tissues, we report the identification of a novel target gene C7 with a strong signal in early diabetic nephropathy. C7 expression is significantly increased in early diabetic kidneys compared with nondiabetic kidneys. We further show a concurrent increase in C7 protein levels in the postmortem blood from EDN donors. Together, these data provide a novel approach to identify EDN before signs of the disease become clinically apparent. These findings may lead to the development of distinctive therapeutic strategy(s) in the early detection and/or treatment of EDN, and slow the progression of the disease. A noninvasive diagnostic method would allow for early detection of previously imperceptible renal damage, and may be more informative than hemoglobin A1c level, creatinine clearance, or urine protein quantification.

Acknowledgments

Z.K.Z., I.E.S., and I.A.R. evaluated histology/immunostaining; Z.K.Z. and I.E.S. scored stained samples and generated data for Figure 2, B and C; T.A.L. analyzed microarray data, deposited microarray data with the

National Center for Biotechnology Information Gene Expression Omnibus, and wrote the relevant portion of Results; M.K.S. prepared and processed tissue samples for electron microscopy; D.X. performed the final statistical analyses; M.S. collected all tissues and processed them, performed experiments, collected and analyzed data, including statistical analysis, and wrote the manuscript; R.I.T. and S.A.K. guided the project and provided inputs with manuscript preparation.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.06.018>.

References

1. Excerpts from the United States Renal Data System 2000 Annual Data Report: atlas of end-stage renal disease in the United States. *Am J Kidney Dis* 2000, 36:S1–S7
2. Wetmore JB, Collins AJ: Meeting the world's need for maintenance dialysis. *J Am Soc Nephrol* 2015, 26:2601–2603
3. Fineberg D, Jandeleit-Dahm KA, Cooper ME: Diabetic nephropathy: diagnosis and treatment. *Nat Rev Endocrinol* 2013, 9:713–723
4. Krolewski AS: Progressive renal decline: the new paradigm of diabetic nephropathy in type 1 diabetes. *Diabetes Care* 2015, 38:954–962
5. Klessens CQ, Woutman TD, Veraar KA, Zandbergen M, Valk EJ, Rotmans JJ, Wolterbeek R, Bruijn JA, Bajema IM: An autopsy study suggests that diabetic nephropathy is underdiagnosed. *Kidney Int* 2016, 90:149–156
6. Mazzucco G, Bertani T, Fortunato M, Bernardi M, Leutner M, Boldorini R, Monga G: Different patterns of renal damage in type 2 diabetes mellitus: a multicentric study on 393 biopsies. *Am J Kidney Dis* 2002, 39:713–720
7. White KE, Bilious RW: Type 2 diabetic patients with nephropathy. *J Am Soc Nephrol* 2000, 66:1596–1605
8. Osterby R, Gall MA, Schmitz A, Nielsen FS, Nyberg G, Parving HH: Glomerular structure and function in proteinuric type 2. *Diabetologia* 1993, 36:1064–1070
9. Tervaert TW, Mooyaart AL, Amann K, Cohen AH, Cook HT, Drachenberg CB, Ferrario F, Fogo AB, Haas M, de Heer E, Joh K, Noel LH, Radhakrishnan J, Seshan SV, Bajema IM, Bruijn JA: Pathologic classification of diabetic nephropathy. *J Am Soc Nephrol* 2010, 21:556–563
10. Baelde HJ, Eikmans M, Doran PP, Lappin DWP, de Heer E, Bruijn JA: Gene expression profiling in glomeruli from human kidneys with diabetic nephropathy. *Am J Kidney Dis* 2004, 43:636–650
11. Lim SC, Liu JJ, Low HQ, Morgenthaler NG, Li Y, Yeoh LY, Wu YS, Goh SK, Chionh CY, Tan SH, Kon YC, Soon PC, Bee YM, Subramaniam T, Sum CF, Chia KS: Microarray analysis of multiple candidate genes and associated plasma proteins for nephropathy secondary to type 2 diabetes among Chinese individuals. *Diabetologia* 2009, 52:1343–1351
12. Brennan EP, Morine MJ, Walsh DW, Roxburgh SA, Lindenmeyer MT, Brazil DP, Peadar OG, Roche HM, Sadlier DM, Cohen CD; GENIE Consortium, Godson C, Martin F: Next-generation sequencing identifies TGF-beta1-associated gene expression profiles in renal epithelial cells reiterated in human diabetic nephropathy. *Biochim Biophys Acta* 2012, 1822:589–599
13. Zheng S, Huang Y, Yang L, Chen T, Xu J, Epstein PN: Uninephrectomy of diabetic OVE26 mice greatly accelerates albuminuria,

- fibrosis, inflammatory cell infiltration and changes in gene expression. *Nephron Exp Nephrol* 2011, 119:e21–e32
14. Wu T, Li Q, Liu HY: Identification of biological targets of therapeutic intervention for diabetic nephropathy with bioinformatics approach. *Exp Clin Endocrinol Diabetes* 2014, 122:587–591
 15. Fu F, Wei X, Liu J, Mi N: Bioinformatic analysis of specific genes in diabetic nephropathy. *Ren Fail* 2015, 37:1219–1224
 16. Wang Z, Zhou Z, Ren Y: Crucial genes associated with diabetic nephropathy explored by microarray analysis. *BMC Nephrol* 2016, 17:128
 17. Jones J, Otu H, Spentzos D, Kolias S, Inan M, Beecken WD, Fellbaum C, Gu X, Joseph M, Pantuck AJ, Jonas D, Libermann TA: Gene signatures of progression and metastasis in renal cell cancer. *Clin Cancer Res* 2005, 11:5730–5739
 18. Reimold FR, Braun N, Zsengeller ZK, Stillman IE, Karumanchi SA, Toka HR, Latus J, Fritz P, Biegener D, Segerer S, Alscher MD, Bhasin MK, Alper SL: Transcriptional patterns in peritoneal tissue of encapsulating peritoneal sclerosis, a complication of chronic peritoneal dialysis. *PLoS One* 2013, 8:e56389
 19. Thadhani RI, Appelbaum E, Chang Y, Pritchett Y, Bhan I, Agarwal R, Zoccali C, Wanner C, Lloyd-Jones D, Cannata J, Thompson T, Audhya P, Andress D, Zhang W, Ye J, Packham D, Singh B, Zehnder D, Manning WJ, Pachika A, Solomon SD: Vitamin D receptor activation and left ventricular hypertrophy in advanced kidney disease. *Am J Nephrol* 2011, 33:139–149
 20. Thadhani RI, Appelbaum E, Pritchett Y, Chang Y, Wenger J, Tamez H, Bhan I, Agarwal R, Zoccali C, Wanner C, Lloyd-Jones D, Cannata J, Thompson BT, Andress D, Zhang W, Packham D, Singh B, Zehnder D, Shah A, Pachika A, Manning WJ, Solomon SD: Vitamin D therapy and cardiac structure and function in patients with chronic kidney disease: the PRIMO randomized controlled trial. *JAMA* 2012, 307:674–684
 21. Hsieh T-J, Chen R, Zhang SL, Liu M, Brezniceanu FL, Whiteside C, Fantus G, Ingelfinger JR, Hamet P, Chan JSD: Upregulation of osteopontin gene expression in diabetic rat proximal tubular cells revealed by microarray profiling. *Kidney Int* 2006, 69:1005–1015
 22. Yang L, Brozovic S, Xu J, Long Y, Kralik PM, Waigel S, Zacharias W, Zheng S, Epstein PN: Inflammatory gene expression in OVE26 diabetic kidney during the development of nephropathy. *Nephron Exp Nephrol* 2011, 119:e8–e20
 23. Woroniecka KI, Park AS, Mohtat D, Thomas DB, Pullman JM, Susztak K: Transcriptome analysis of human diabetic kidney disease. *Diabetes* 2011, 60:2354–2369
 24. Westberg NG, Michael AF: Immunohistopathology of diabetic glomerulosclerosis. *Diabetes* 1972, 21:163–174
 25. Burkholder PM: Immunohistopathologic study of localized plasma proteins and fixation of guinea pig complement in renal lesions of diabetic glomerulosclerosis. *Diabetes* 1965, 14:755–770
 26. Axelgaard E, Ostergaard JA, Thiel S, Hansen TK: Diabetes is associated with increased autoreactivity of mannan-binding lectin. *J Diabetes Res* 2017, 2017:1–12
 27. Fujita T, Ohi H, Komatsu K, Endo M, Ohsawa I, Kanmatsuse K: Complement activation accelerates glomerular injury in diabetic rats. *Nephron* 1999, 81:208–214
 28. Rogers J, Cooper NR, Webster S, Schultz J, McGeer PL, Styren SD, Civin WH, Brachova L, Bradt B, Ward P, Lieberburg I: Complement activation and P-amyloid-mediated neurotoxicity in Alzheimer's disease. *PNAS* 1992, 89:10016–10020
 29. Stoltzner SE, Grenfell TJ, Mori C, Wisniewski KE, Wisniewski TM, Selkoe DJ, Lemere CA: Temporal accrual of complement proteins in amyloid plaques in Down's syndrome with Alzheimer's disease. *Am J Pathol* 2000, 156:489–499
 30. Sewry CA, Dubowitz V, Abrahama A, Luzio JP, Campbell AK: Immunocytochemical localisation of complement components C8 and C9: the role of complement in muscle fibre damage. *J Neurol Sci* 1987, 81:141–153
 31. Rajakumar A, Cerdeira AS, Rana S, Zsengeller Z, Edmunds L, Jeyabalan A, Hubel CA, Stillman IE, Parikh SM, Karumanchi SA: Transcriptionally active syncytial aggregates in the maternal circulation may contribute to circulating soluble fms-like tyrosine kinase 1 in preeclampsia. *Hypertension* 2012, 59:256–264
 32. Penning M, Chua JS, van Kooten C, Zandbergen M, Buurma A, Schutte J, Bruijn JA, Khankin EV, Bloemenkamp K, Karumanchi SA, Baelde H: Classical complement pathway activation in the kidneys of women with preeclampsia. *Hypertension* 2015, 66:117–125
 33. Vasil KE, Magro CM: Cutaneous vascular deposition of C5b-9 and its role as a diagnostic adjunct in the setting of diabetes mellitus and porphyria cutanea tarda. *J Am Acad Dermatol* 2007, 56:96–104
 34. Rosoklija GB, Dwork AJ, Younger DS, Karlikaya G, Latov N, Hays AP: Local activation of the complement system in endoneurial microvessels of diabetic neuropathy. *Acta Neuropathol* 2000, 99:55–62
 35. Zhang J, Gerhardinger C, Lorenzi M: Early complement activation and decreased levels of glycosylphosphatidylinositol-anchored complement inhibitors in human and experimental diabetic retinopathy. *Diabetes* 2002, 51:3499–3504
 36. Gao BB, Chen X, Timothy N, Aielle LP, Feener EP: Characterization of the vitreous proteome in diabetes without diabetic retinopathy. *J Proteome Res* 2008, 7:2516–2525
 37. Flyvbjerg A: The role of the complement system in diabetic nephropathy. *Nat Rev Nephrol* 2017, 13:311–318
 38. Falk R: Ultrastructural localization of the membrane attack complex of complement in human renal tissues. *Am J Kidney Dis* 1987, 9:121–128
 39. DiScipio RG: The structure of human complement component 7 and the C5b-7 complex. *J Biol Chem* 1988, 263:549–560
 40. Podack ER, Kolb WP, Müller-Eberhard HJ: The SC5b-7 complex: formation, isolation, properties, and subunit composition. *J Immunol* 1977, 119:2024–2029
 41. Wang S, Gao Y, Shu C, Xu T: Characterization and evolutionary analysis of duplicated C7 in miuiy croaker. *Fish Shellfish Immunol* 2015, 45:672–679
 42. Bossi F, Rizzi L, Bulla R, Debeus A, Tripodo C, Picotti P, Betto E, Macor P, Pucillo C, Wurzner R, Tedesco F: C7 is expressed on endothelial cells as a trap for the assembling terminal complement complex and may exert anti-inflammatory function. *Blood* 2009, 113:3640–3648
 43. Serna M, Giles JL, Morgan BP, Bubeck D: Structural basis of complement membrane attack complex formation. *Nat Commun* 2016, 7:10587
 44. Kissel JT, Mendell JR, Rammohan KW: Microvascular deposition of complement membrane attack complex in dermatomyositis. *N Engl J Med* 1986, 314:329–334
 45. Lahoria R, Selcen D, Engel AG: Microvascular alterations and the role of complement in dermatomyositis. *Brain* 2016, 139:1891–1903
 46. Uesugi N, Sakata N, Nangaku M, Abe M, Horiuchi S, Hisano S, Iwasaki H: Possible mechanism for medial smooth muscle cell injury in diabetic nephropathy: glycoxidation-mediated local complement activation. *Am J Kidney Dis* 2004, 44:224–238
 47. Fearn A, Sheerin NS: Complement activation in progressive renal disease. *World J Nephrol* 2015, 4:31–40
 48. Chevalier RL: The proximal tubule is the primary target of injury and progression of kidney disease. *Am J Physiol Renal Physiol* 2016, 311:F145–F161
 49. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremiaux-Bacchi V, Roumenina LT: Complement system part II: role in immunity. *Front Immunol* 2015, 6:257
 50. Rother RP, Rollins SA, Mojcik CF, Brodsky RA, Bell L: Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. *Nat Biotechnol* 2007, 25:1256–1264
 51. Thurman JM: Many drugs for many targets: novel treatments for complement-mediated glomerular disease. *Nephrol Dial Transplant* 2017, 32:i57–i64