

Supplementary information

Urinary Complement proteome strongly linked to diabetic kidney disease progression

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Supplementary Methods

Advanced DKD: two-cohort study - expanded description

Joslin Diabetes Center's large outpatient clinic provides clinical care for approximately twenty thousand patients with type 1 and type 2 diabetes, who are mainly residents of Eastern Massachusetts, United States. A large proportion of patients remains under Joslin care for a long time, frequently for life. Over the last 25 years, approximately 3,500 patients attending the Joslin Clinic between 1991 and 2009 were enrolled into the longitudinal Joslin Kidney Study (Supplementary Fig. 1). Enrolled individuals were examined during routine clinic visits. Those with less frequent clinic visits and those who stopped coming to the Joslin Clinic¹⁻⁴ were examined at their homes.

Design of the two cohorts with both types of diabetes and advanced diabetic kidney disease (DKD) has already been published^{5,6}. This study included the type 1 diabetes cohort, aged 18-64 years old, participating in the Joslin Kidney Study with urinary specimens available (189 subjects), whereas the type 2 diabetes cohort comprised 115 subjects with advanced DKD, aged 35-64 years old, from Joslin Kidney Study participants with urinary specimens available.

Classification of albuminuria and glomerular filtration rate (GFR) categories in this study was based on the nomenclature of The Kidney Disease: Improving Global Outcomes Clinical Practice Guidelines for diabetes management in chronic kidney disease and based on current guidelines of the American Diabetes Association^{7,8}.

Albuminuria categories were as follows: A1 – albumin-to-creatinine ratio <30 mg/g; A2 – 30-299 mg/g; A3 – $\geq$ 300 mg/g. The albumin-to-creatinine ratio from the same timepoint as Complement protein determinations was used in the analyses.

GFR categories at baseline were as follows: G1 – ≥ 90 ml/min/1.73m²; G2 – 60-89 ml/min/1.73m²; G3 – 30-59 ml/min/1.73m²; G4 – 15-29 ml/min/1.73m². The serum creatinine-based Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) 2021 formula⁹ was used to estimate kidney function to determine GFR at baseline, to define GFR categories and to formulate GFR slopes described below. Additional analyses in the Cox models exercised the adjustment by GFR based on the newly described creatinine-based formula without race¹⁰. Serum creatinine concentrations were determined using the Roche enzymatic assay (Catalog (Cat.) No. 11775685, Roche Diagnostics, Indianapolis, IN) on a Roche/Hitachi Modular P Chemistry Analyzer at the University of Minnesota. This method was standardized to be traceable to an isotope dilution mass spectrometry (IDMS) reference assay and was verified by measuring National Institutes of Standards and Technology Standard Reference Material (NIST SRM) No. 967. Throughout the manuscript, GFR indicates serum creatinine-based estimated GFR.

The Joslin Diabetes Center Committee on Human Studies approved the informed consent, recruitment and examination procedures for the Joslin Kidney Studies, as well as for the current project.

Early-to-moderate DKD: three-cohort study - expanded description

The early-to-moderate DKD cohort in type 1 diabetes included participants of the Preventing Early Renal Loss in Diabetes (PERL) trial, ClinicalTrials.gov identifier NCT02017171. This international, double blind, placebo-controlled, randomized clinical trial investigated the effect of allopurinol on DKD progression. Allopurinol is a xanthine oxidase inhibitor impacting the conversion of hypoxanthine to xanthine and of xanthine to uric acid.

The PERL trial included 530 subjects with type 1 diabetes, aged 18-70 years, who were at high risk of GFR loss on the basis of the presence of Macro- or Micro-albuminuria, or rapid GFR

decline (historical slopes), and a relatively high serum uric acid (≥ 4.5 mg/dL), but who still had only a mild or moderate decreased kidney function (GFR of 40.0 to 99.9 ml/min/1.73m²). The study design included a run-in period during which renin-angiotensin system inhibitors use was standardized and blood pressure was normalized. Visit 4 was scheduled 6 weeks into the run-in period and 3 weeks prior to randomization where a comprehensive medical history, examination and blood and urine specimen were collected. PERL participants were then randomized 1-to-1 at visit 5 to receive either allopurinol (n=267) or placebo (n=263), for 3 years (visit 16) followed by a 2-month washout period (visit 17). This clinical trial found no evidence of a clinical benefit of urate-lowering treatment in the population examined. Details of the PERL study design and results are published^{11,12}.

For the purpose of this study, the albuminuria in PERL participants was based on the timed urinary collection at visit 4. For 1 missing value at visit 4, visit 3 (2 weeks of run-in) value was used instead. Corresponding albuminuria categories were then as follows: A1 – albumin excretion rate < 20 $\mu\text{g}/\text{min}$; A2 – 20-200 $\mu\text{g}/\text{min}$; A3 – > 200 $\mu\text{g}/\text{min}$. Serum creatinine was measured by enzymatic methods on the Roche Cobas 6000 analyzer (Roche Diagnostics, Indianapolis, IN)¹¹. GFR was also based on the serum creatinine-based CKD-EPI formula⁹.

The current study included PERL subjects with determinate recruitment modality (qualified by albuminuria or historical GFR slope); albumin excretion rate ≥ 20 $\mu\text{g}/\text{min}$; seven or more GFR measurements including required GFR at baseline (visit 4 of the trial), and at the end of the follow-up (visit 16 and/or visit 17 of the trial); and had sufficient urine specimens for targeted protein immunoassay quantifications. Ninety-six (46%) of the identified subjects meeting these criteria were in the allopurinol arm, and 111 (54%) in the placebo arm.

Spot urines were used for the Complement protein determinations in this study, as well as in the other three cohorts. Urine specimens collected at baseline from the four cohorts were stored at -80°C until analyses.

The study protocols including informed consent forms were approved by all local institutional review boards at each study site and the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-appointed data and safety monitoring board. The Joslin Diabetes Center Committee on Human Studies approved the current project.

The early-to-moderate DKD cohort in type 2 diabetes included Joslin Kidney Study participants with early-to-moderate DKD¹³. Subjects, aged 35 to 64 years at study enrollment, were recruited from patients attending the Joslin Clinic between 2003 and 2009. Albuminuria and GFR were determined as in the other Joslin Kidney Study cohorts described above. None of the four cohorts included subjects with normoalbuminuria. The Joslin Diabetes Center Committee on Human Studies approved the informed consent, recruitment and examination procedures for the Joslin Kidney Studies, as well as for the current research.

The African American early-to-moderate DKD cohort in type 2 diabetes included participants of the Diabetes Heart Study. Urine samples from 123 African Americans were collected in the Wake Forest University School of Medicine African American-Diabetes Heart Study (AA-DHS), AA-DHS Longitudinal Follow-Up, and AA-DHS MIND (Memory IN Diabetes) studies. All participants self-identified as African American with clinically diagnosed and actively treated type 2 diabetes mellitus (insulin and/or hypoglycemic medications). Participants had relatively preserved kidney function, as those with a known serum creatinine concentration >2.0 mg/dL were not recruited. Fasting (morning) urine samples were collected, with multiple aliquots frozen at -80°C. Analyses of urine Complement concentration were performed in never thawed

samples. Serum creatinine, urine albumin, and urine creatinine concentrations were measured at each study visit. Full details of the study population and recruitment methods have previously been reported¹⁴⁻¹⁶.

Other study groups

The advanced DKD cohort subset for high-throughput determinations of the Complement proteome in the paired plasma and urine

Approximately half of the subjects with type 1 diabetes from the advanced DKD cohort (N=97), Joslin Kidney Study participants, having a representative range of baseline values of albumin-to-creatinine ratio and GFR (albumin-to-creatinine ratio: 1219±1371 mg/g; GFR: 40±11 ml/min/1.73m²), annual GFR slope of -5.1±5.7 ml/min/1.73m²/year, 48% of incident kidney failures and good specimen availability were included in this analysis. All subjects had ethylenediamine tetraacetic acid plasma specimens collected at baseline and stored at -80°C until analyses.

The case-control study group for high-throughput determinations of the Complement proteome in the kidney tissue

The clinical characteristics of this study group has been described elsewhere¹⁷. Briefly, tissue samples were obtained from surgical nephrectomies. The normal, non-neoplastic part of the tissue taken ≥ 2 cm from the malignant lesion was used. The DKD group comprised subjects with type 2 diabetes, persistent kidney function impairment, GFR in the range of 15 to < 60 ml/min/1.73m², and at least class II of the histological classification of the diabetic nephropathy (glomerulosclerosis and mesangial expansion) as per national guidelines^{7,8,18}. The control group comprised subjects without diabetes or hypertension and with normal kidney function. Kidney

tissue specimens linked to the clinical characteristics were collected through an honest broker and de-identified.

The case-control study group for single-cell/single-nucleus transcriptomics

Single-cell/single-nucleus RNA sequencing (sc/snRNA-seq) was conducted in 31 human kidney tissue samples obtained from 25 subjects. The DKD group comprised 8 subjects with diabetes and either GFR <60 ml/min/1.73m² or kidney fibrosis $>10\%$. The control group comprised 17 subjects with GFR ≥ 60 ml/min/1.73m² and fibrosis $<5\%$.

Urinary creatinine normalization

All Complement proteins measured in urine were normalized to urinary creatinine¹⁹. Subjects from the three Joslin cohorts had creatinine measurements performed by the Clinical Laboratory at Joslin Diabetes Center. Over the 18 calendar years of the recruitment, the Joslin Clinic has used four different Chemistry Analyzers (Beckman CX9, Ortho Diagnostics Vitros 5.1, Roche Integra 800 and Roche Cobas C501; in chronological order) for the measurement of urinary creatinine. Three out of four analyzers rely on the Jaffe method principle, in which creatinine reacts with picric acid in an alkaline environment to cause a color change, except for the Ortho Vitros Analyzer, which utilizes the enzymatic method. Eighty-seven percent of the samples were measured with the Jaffe method and 13% with the enzymatic method, respectively. As regulated by the Clinical Laboratory Improvement Amendments and followed by the Joslin Clinic laboratory, precision and linearity procedures were performed during transitions between the analyzers. No recalibration scheme was required.

Creatinine levels in urine samples from the early-to-moderate DKD cohort with type 1 diabetes were measured with the Jaffe-based method by MicroVue Creatinine assay (Cat. No. 8009, Quidel, San Diego, CA); dilution factor 1:40. We have also performed an orthogonal

comparison between creatinine measured at the Joslin clinic and by the MicroVue assay in 37 subjects with DKD of comparable phenotype to our study. The two measurements were strongly correlated (Pearson $r = 0.993$, $P < 10^{-36}$).

Kidney tissue pre-analytical processing for proteomics

Specimen acquisition and processing were previously described¹⁷. Briefly, each kidney tissue specimen was cryopulverized using a TissueLyser instrument (QIAGEN, Hilden, Germany) and subjected to the T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA). The total protein concentration was measured using bicinchoninic acid method with the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific). These specimens were stored at -80°C, until subjected to high-throughput aptamer proteomics described in methods.

Kidney tissue sc/snRNA-seq

For scRNA-seq, fresh kidney tissue was homogenized per the manufacturer's protocol for the dissociation of human kidney using the Multi Tissue Dissociation Kit 1 (Cat. No. 30-110-201, Miltenyi Biotec, Bergisch Gladbach, Germany) with red blood cell lysis. Cells were counted with the Countess AutoCounter (Cat. No. C10227, Invitrogen, Waltham, MA), and viability was determined to be >80%. For snRNA-seq, nuclei were isolated, counted and checked for quality. Gel beads, complementary DNA and libraries for both scRNA-seq and snRNA-seq materials were created per the manufacturer's protocol using the Chromium Next GEM Single Cell 3' GEM Kit v3.1 (Cat. No. PN-1000121, 10X Genomics, Pleasanton, CA) and Single Index Kit T Set A (Cat. No. PN-120262, 10X Genomics, Pleasanton, CA). Libraries were quality checked using the Agilent Bioanalyzer High Sensitivity DNA kit (Cat. No. 5067-4626, Agilent Technologies, Santa Clara, CA) and sequenced on the Illumina Novaseq 6000 system²⁰.

Analysis was performed using Seurat (version 4.0.3). The Seurat object was processed, annotated, and subset to control and DKD samples. The DotPlot function was used to visualize gene expression, and the AverageExpression function was used to quantify average gene expression.

Spatial RNA-seq data analysis

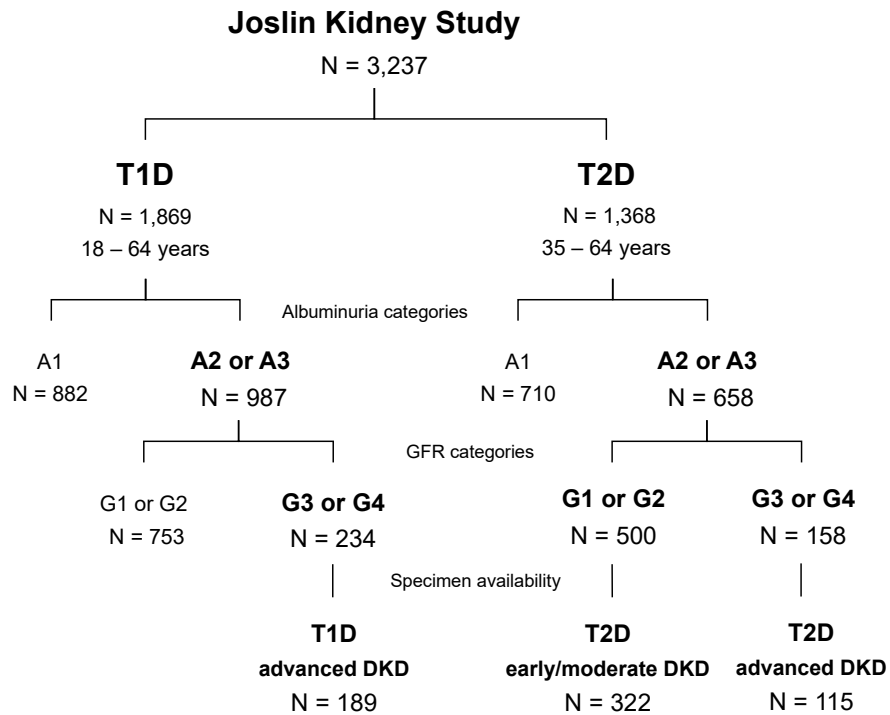
We re-analyzed spatial RNA-seq data from human kidney tissue to elucidate gene expression patterns. The original dataset, which involved Visium formalin-fixed paraffin-embedded sample preparation and quality control, is detailed in a previous study²¹. Our reanalysis employed the Samui Browser²², an interactive web-based tool for visualizing gene expression data, enabling a detailed exploration of spatial gene expression profiles. The spatial RNA-seq data were processed using Space Ranger (v1.0.0) with the GRCh38 reference genome and the Visium Human Transcriptome Probe Set v1.0 GRCh38. Data normalization was carried out using SCTransform. For analysis, we utilized Seurat for principal component analysis, integrated the dataset using Harmony, and created uniform manifold approximation and projection (UMAP) visualizations. Spot-specific markers were identified with Seurat's "FindAllMarkers," applying a log-fold-change > 0.25 and an FDR < 0.05 threshold.

Supplementary Fig. 1. Flowchart illustrating a structured overview of the subject selection for the current study.

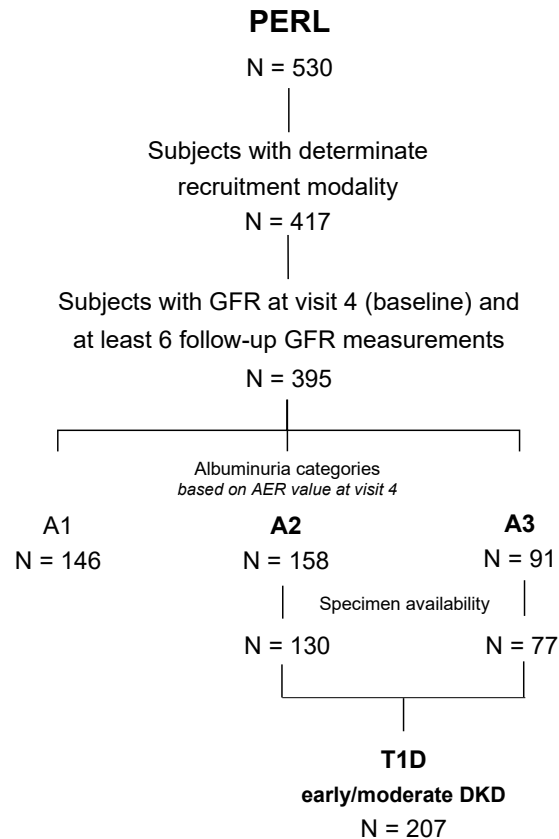
a, The Joslin Kidney Study enrolled approximately 3,500 patients with type 1 or type 2 diabetes who were receiving care at the Joslin Clinic between 1991 and 2009. The type 1 diabetes (T1D) cohort comprised 1,869 subjects, of whom 987 were classified as A2 or A3 based on albuminuria levels (albuminuria categories; A2: 30-299 or A3: ≥ 300 mg/g creatinine). Subsequent criteria for inclusion were based on glomerular filtration rate categories with 234 subjects were categorized as G3 or G4 (G3: 30-59 or G4: 15-29 ml/min/1.73m²). Among these G3/G4 subjects, urine specimens were available from 189 subjects who formed the advanced DKD T1D cohort. The type 2 diabetes (T2D) cohort included 1,368 subjects. Initial stratification was based on albuminuria categories with 658 subjects classified having A2 or A3 category. Among these, 500 were further categorized into G1 or G2 (G1 – ≥ 90 ml/min/1.73m²; G2 – 60-89 ml/min/1.73m²) of whom 322 had urine specimens available and constituted the early-to-moderate DKD T2D cohort. The remaining 158 subjects were categorized as G3 or G4, with urine specimens available from 115 subjects who formed the advanced DKD T2D cohort.

b, The early-to-moderate DKD T1D cohort included participants of the Preventing Early Renal Loss in Diabetes (PERL) trial. In total, 530 participants were enrolled in this trial and 207 subjects were selected for inclusion in the Early-to-moderate DKD T1D cohort based on (1) determinate recruitment modality (qualified by albuminuria or historical GFR slope, N=417) and (2) having seven or more GFR measurements including required GFR at baseline (N=395). Subjects further were stratified by (3) albuminuria status: 146 were categorized as A1 but were not included in the final study, 158 were categorized as A2 and 91 were categorized as A3. Urine specimens for targeted protein quantifications were available for 130 out of the 158 subjects in the A2 category and for 77 among the A3 subjects, totaling specimens from 207 subjects.

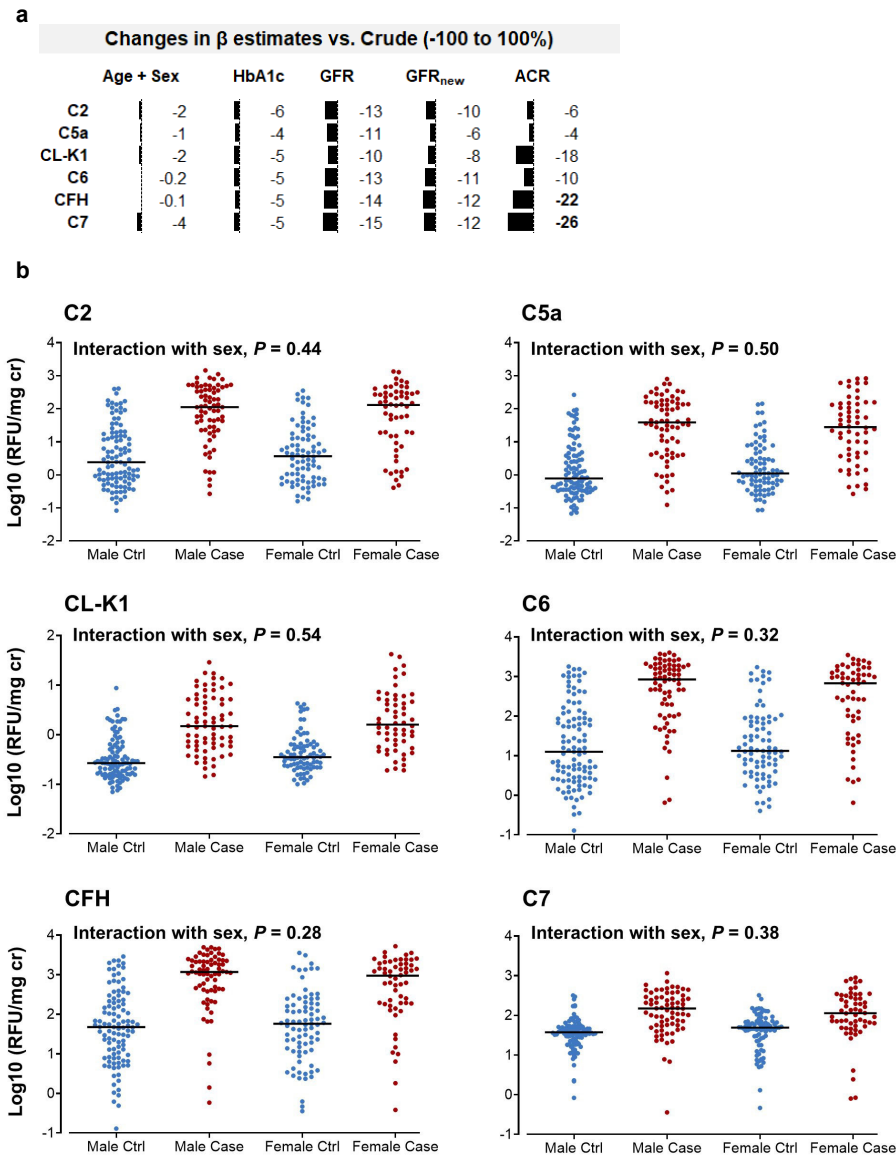
a



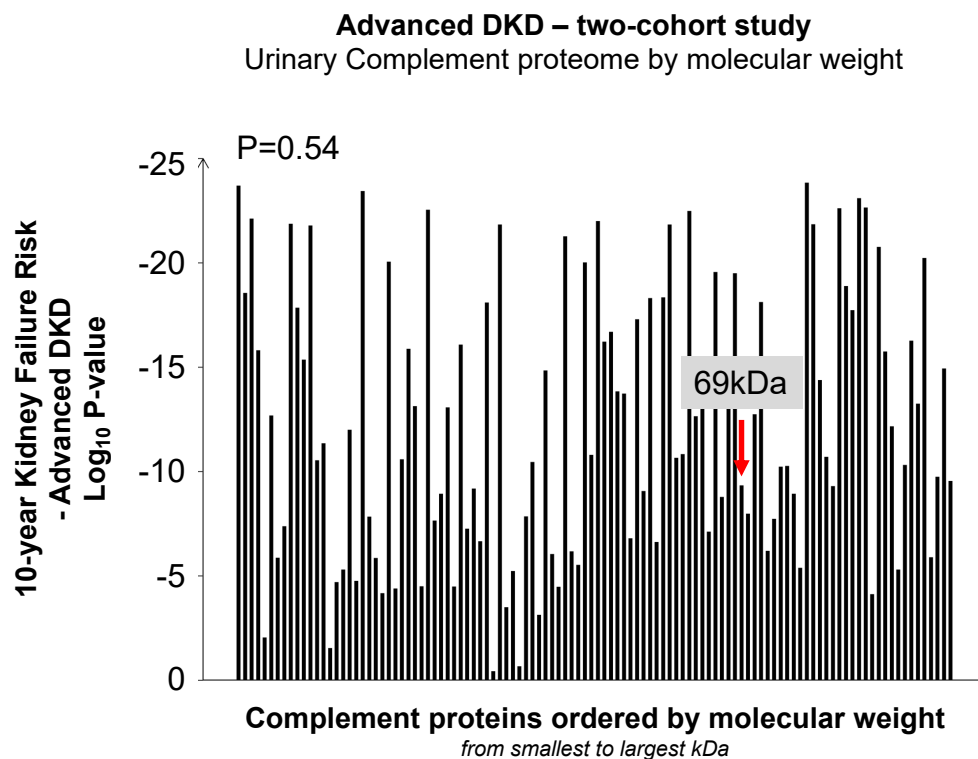
b



Supplementary Fig. 2. The assessment of potential confounding by clinical variables on top 5% Complement proteins and 10-year kidney failure risk in the advanced DKD two-cohort study (n=304). **a**, Confounding was evaluated by inspection of β estimate changes in partially adjusted models. A change in β effect estimate of 20% or higher was indicated in bold. GFR_{new} indicates estimated GFR calculated using the new creatinine-based formula without race. **b**, The distribution of top 5% Complement proteins stratified by sex and prospective caseness (male ctrl: n=99, male case: n=70, female ctrl: n=79, female ctrl: n=56). Cases represent subjects who developed kidney failure within 10 years of follow-up. The horizontal line within each dot plot indicates the median protein value. All P values are two-sided. HbA1c, glycated hemoglobin; GFR, estimated creatinine-based glomerular filtration rate; ACR, urinary albumin-to-creatinine ratio; Ctrl, control; RFU, relative fluorescence units.

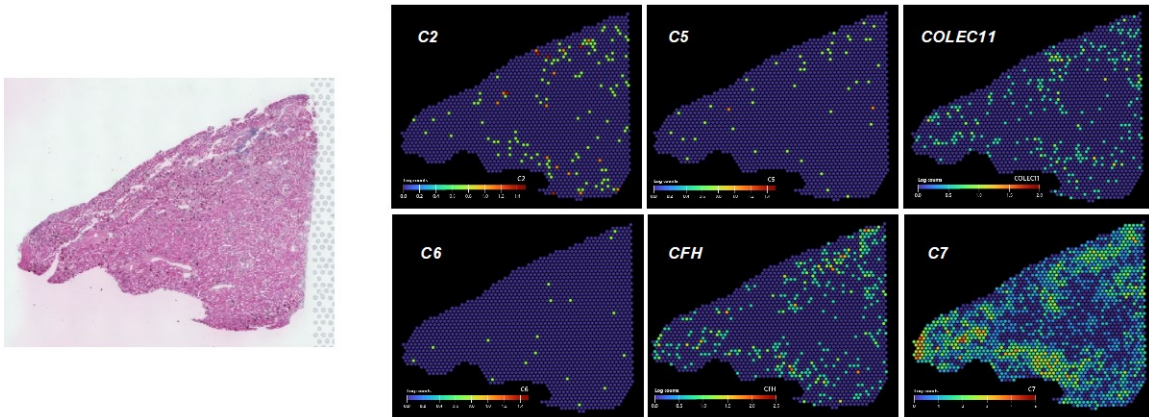


Supplementary Fig. 3. Association between Complement proteins with 10-year kidney failure risk by molecular weight in advanced DKD cohorts (n=304). Each needle plot illustrates a specific Complement protein ordered by its molecular weight from largest to smallest. One vertical needle represents a two-sided P value transformed to its base 10 logarithm from the diabetes type-adjusted model (base model) evaluating one creatinine-adjusted Complement proteins at a time. Sixty-nine kiloDaltons (kDa) indicates the size of albumin. DKD, diabetic kidney disease.

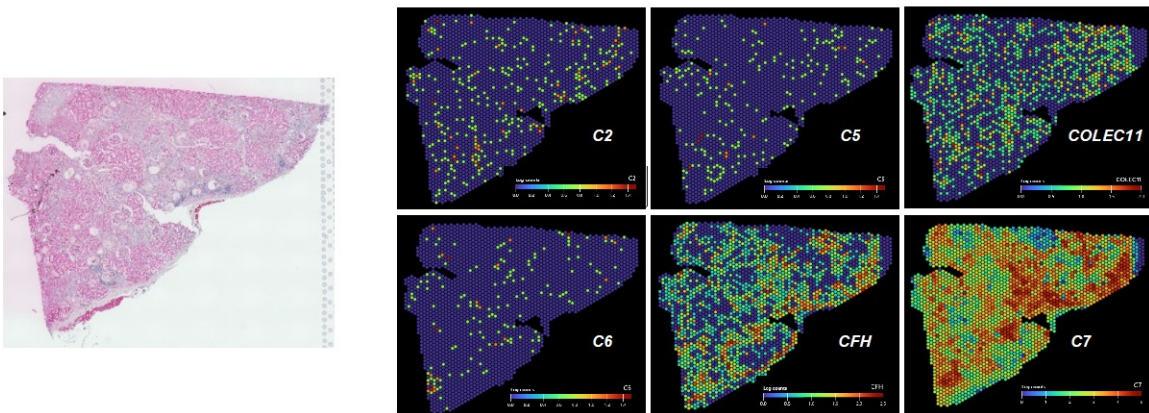


Supplementary Fig. 4. Spatial transcriptomics of genes corresponding to top 5% Complement proteins. This figure illustrates the hematoxylin-stained image and the spatial distribution of six genes (*C2*, *C5*, *COLEC11*, *C6*, *CFH* and *C7*) in human kidney tissues from (a) a control sample and (b) a DKD sample. The data were obtained using the Visium spatial transcriptomics platform. Each panel represents the log-transformed expression counts for one gene, visualized across the tissue sample. **a**, The top six panels show the control sample and **b**, the bottom six panels show the DKD sample. Color gradients indicate varying levels of gene expression, with blue representing lower expression and red indicating higher expression. Scale bar = 250 μ m. DKD, diabetic kidney disease.

a Control

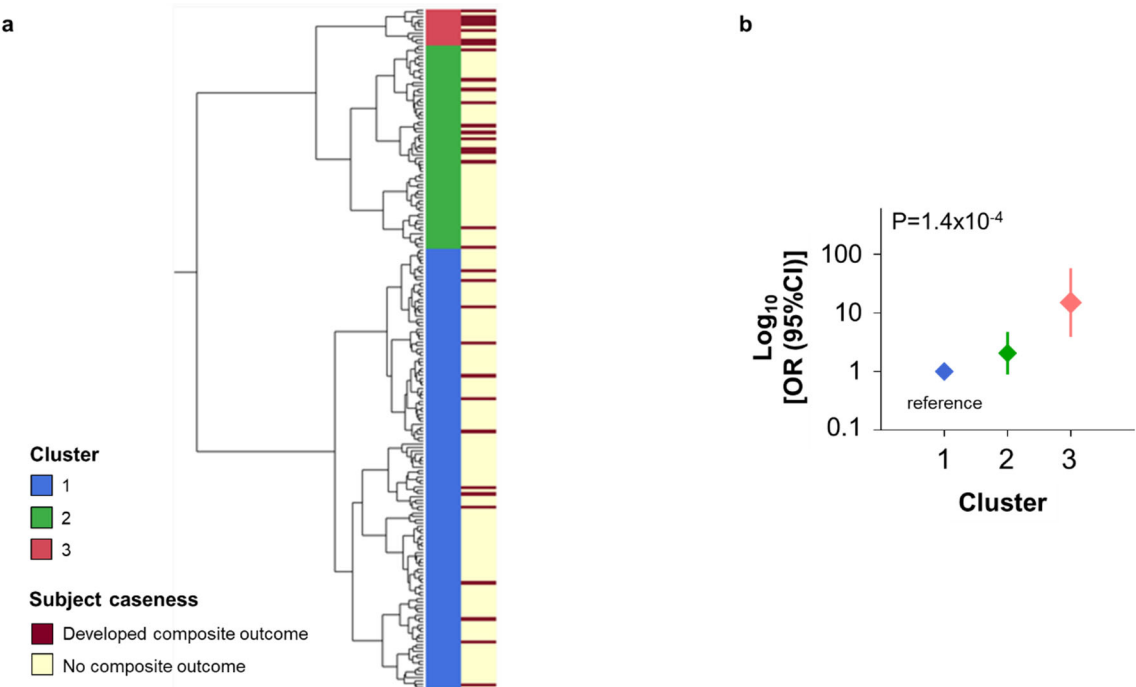


b DKD

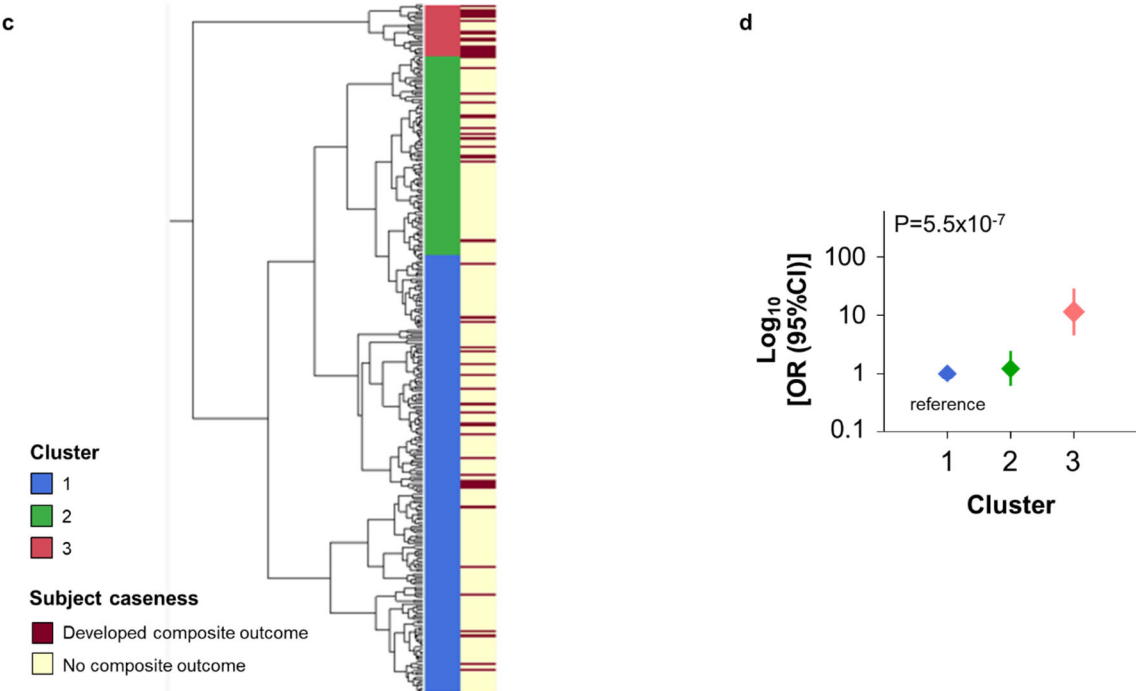


Supplementary Fig. 5. Complement proteins-informed clustering and prospective kidney outcome in the early-to-moderate DKD cohorts. **a**, An unsupervised approach performed in the form of hierarchical clustering built upon the top 5% Complement proteins in type 1 diabetes (n=207). Subjects were clustered based on Complement protein levels disregarding the outcome. Vertical colored bars show cluster groups and prospective subject caseness. **b**, The plot shows the association of the top 5% proteins with composite kidney outcome across clusters in type 1 diabetes. **c**, An unsupervised approach performed in the form of hierarchical clustering built upon the top 5% Complement proteins in type 2 diabetes (n=322). **d**, The plot shows the association of the top 5% proteins with composite kidney outcome across clusters in type 2 diabetes. Effect sizes in **(b)** and **(d)** are presented per one tertile change in urinary creatinine-adjusted Complement protein distribution with cluster 1 serving as the reference group. The two-sided Fisher-exact P-value is reported.

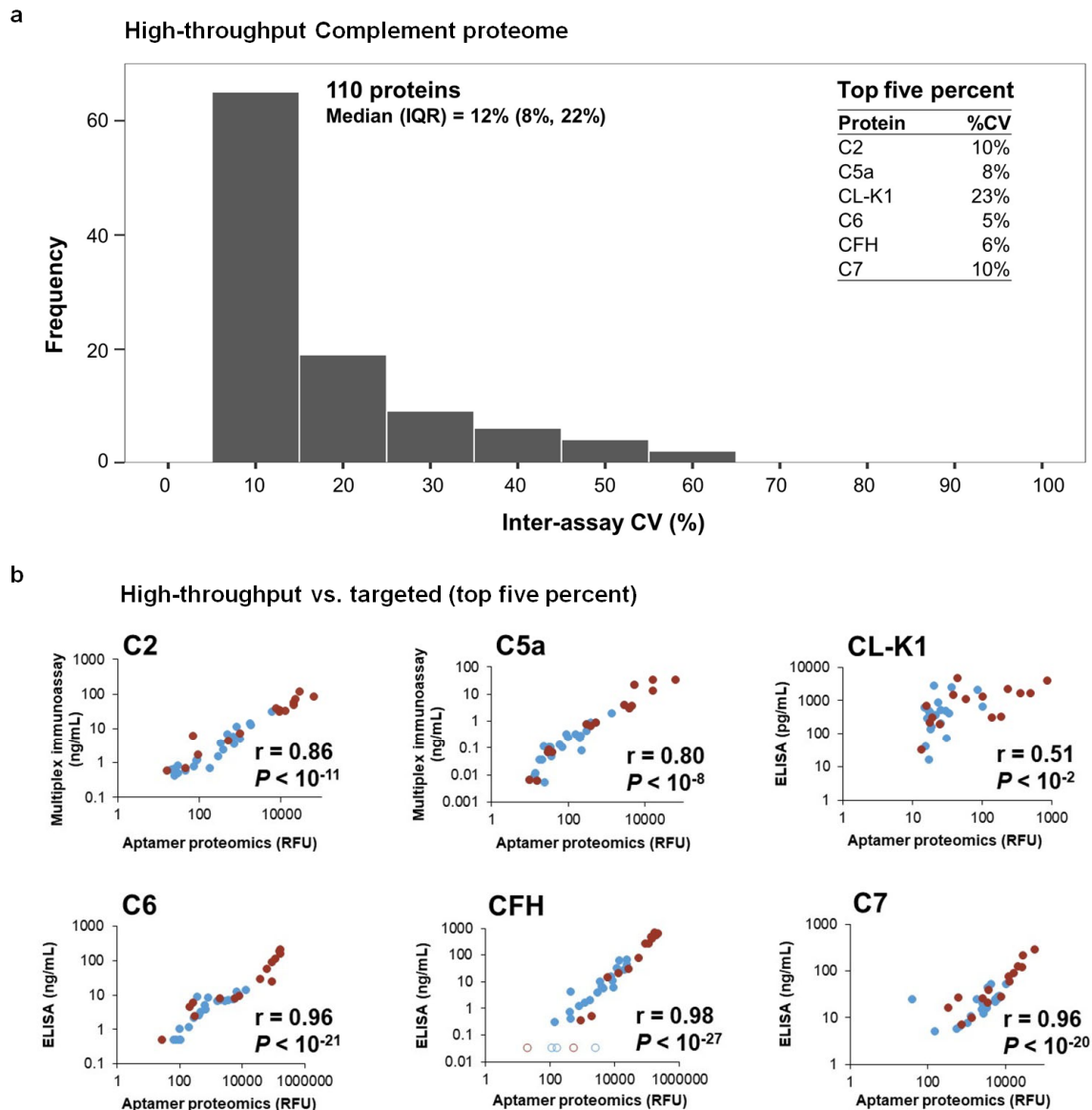
Early-to-moderate DKD cohort in Type 1 diabetes



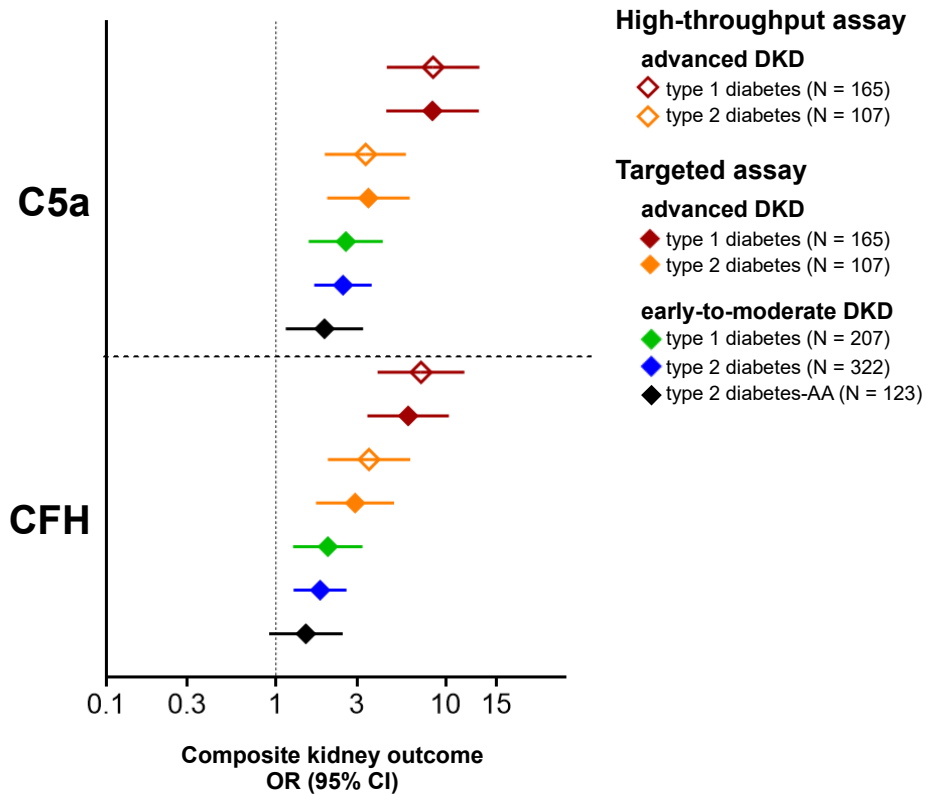
Early-to-moderate DKD cohort in Type 2 diabetes



Supplementary Fig. 6. Precision of the high-throughput assay and orthogonal method validation in the advanced DKD samples. **a**, Histogram shows the distribution of the inter-assay coefficients of variation (CV) of all 110 urinary Complement proteins. CVs of the top 5% Complement proteins were $\leq 10\%$, except for CL-K1, as listed in the top-right corner of the graph. **b**, Orthogonal method validation for the top Complement proteins between high-throughput and targeted assays. Pearson correlation coefficients (r) and corresponding two-sided P values are based on the non-urinary creatinine adjusted protein values. Cases ($n=15$) represent subjects who developed kidney failure within 10 years of follow-up, are marked with red dots and controls ($n=22$) are marked with blue dots. Open circles indicate non-quantifiable, extrapolated values by targeted means. IQR, interquartile range; RFU, relative fluorescence units.



Supplementary Fig. 7. Urinary Complement proteins and kidney disease progression across five cohorts - high-throughput and targeted protein quantifications. Association of Complement C5a and Complement factor H with prospective, composite kidney outcome by cohort. In the advanced DKD cohorts, 165 out of 189 subjects with type 1 diabetes had data available for both high-throughput (open diamond symbol) and targeted (closed diamond symbol) assays, while in type 2 diabetes, 107 out of 115 subjects were similarly assessed. Color-annotated and cohort-specific effect estimates (diamond symbols) and 95% confidence intervals (horizontal bars) represent changes in kidney function over time per one tertile increase in the distribution of a urinary creatinine-adjusted Complement protein. DKD, diabetic kidney disease; OR, odds ratio; CI, confidence intervals; AA, African American.



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