Endogenous adenine mediates kidney injury in diabetic models and predicts diabetic kidney disease in patients

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Abstract

Diabetic kidney disease (DKD) can lead to end-stage kidney disease (ESKD) and mortality, however, few mechanistic biomarkers are available for high risk patients, especially those without macroalbuminuria. Urine from participants with diabetes from Chronic Renal Insufficiency Cohort (CRIC), Singapore Study of Macro-Angiopathy and Reactivity in Type 2 Diabetes (SMART2D), and the Pima Indian Study determined if urine adenine/creatinine ratio (UAdCR) could be a mechanistic biomarker for ESKD. ESKD and mortality were associated with the highest UAdCR tertile in CRIC (HR 1.57, 1.18, 2.10) and SMART2D (HR 1.77, 1.00, 3.12). ESKD was associated with the highest UAdCR tertile in patients without macroalbuminuria in CRIC (HR 2.36, 1.26, 4.39), SMART2D (HR 2.39, 1.08, 5.29), and Pima Indian study (HR 4.57, CI 1.37-13.34). Empagliflozin lowered UAdCR in non-macroalbuminuric participants. Spatial metabolomics localized adenine to kidney pathology and transcriptomics identified ribonucleoprotein biogenesis as a top pathway in proximal tubules of patients without macroalbuminuria, implicating mammalian target of rapamycin (mTOR). Adenine stimulated matrix in tubular cells via mTOR and stimulated mTOR in mouse kidneys. A specific inhibitor of adenine production was found to reduce kidney hypertrophy and kidney injury in diabetic mice. We propose that endogenous adenine may be a causative factor in DKD.

Introduction

Progression to organ failure is marked by fibrosis and loss of architecture in solid organs, such as the kidney. In almost all progressive chronic kidney diseases (CKD) the features that are most consistently associated with functional loss of the glomerular filtration rate (GFR) are the degree of glomerulosclerosis, tubulointerstitial fibrosis, vascular injury, and proteinuria (1-4). However, many patients who eventually develop end-stage kidney disease (ESKD) are non-proteinuric at the time impaired GFR is recognized. Non-proteinuria is defined as urine albumin-to-creatinine ratio (ACR) ≤ 300 mg/creatinine or urine albumin excretion ≤ 300mg/day (5). As non-proteinuric or non-macroalbuminuric DKD accounts for >40% of prevalent ESKD in patients with type 2 diabetes (5-7) and 75% of prevalent CKD (GFR <60 mL/min/1.73m²) (8) identifying the patients at risk for progression in early stages of disease is an important step.
to improve clinical outcomes. This is especially relevant as the armamentarium of therapies for DKD to mitigate kidney disease progression has rapidly expanded (9-11).

Establishing novel biomarkers that predict progression and represent biologically relevant pathways in DKD could improve the care of patients with diabetes. To identify novel biomarkers, we recently performed an untargeted urine metabolomics study in patients with type 2 diabetes (T2D) and impaired eGFR from the Chronic Renal Insufficiency Cohort (CRIC) study (12) and identified 15 candidate metabolites associated with ESKD. A targeted assay validated 13 of these metabolites, one of which was adenine. As exogenous adenine has been found to cause kidney failure in mice, rats, and dogs (13-15), we evaluated whether endogenous adenine could play a role in progression of kidney disease in patients with diabetes.

Results

Urine adenine/creatinine ratio predicts kidney failure and all cause mortality in the CRIC and SMART2D cohorts. The baseline clinical characteristics of the participants with diabetes from CRIC and SMART2D are shown in Table 1. Of the 904 subjects evaluated from CRIC, 558 had either normoalbuminuria or microalbuminuria, 341 had macroalbuminuria, and 5 had no data for 24h albumin. The mean eGFR was 40 mL/min/1.73m². The top tertile of baseline urine adenine/creatinine ratio (UAdCR) was found to identify the participants with diabetes who were at high risk for ESKD and all cause mortality (adjusted HR 1.57, 95% CI 1.18, 2.10, as compared to the lowest tertile) (Figure 1A) and a similar significant relationship was found using UAdCR as a continuous variable (Table 2). The value of the top tertile of UAdCR to identify patients with diabetes at high risk for ESKD and all cause mortality was confirmed in participants from SMART2D who had reduced eGFR and normoalbuminuria or microalbuminuria (adjusted HR 1.77, 95% CI 1.00, 3.12) (Figure 1B, Table 2, datasets combined in Supplementary Figure 2A).

UAdCR predicts kidney failure in the non-macroalbuminuric Pima, CRIC, and SMART2D cohorts and empagliflozin reduces UAdCR. The UAdCR was also evaluated in early-stage disease (measured GFR>90 mL/min/1.73m²) in a Pima Indian cohort with >20 year follow up (Table 1). As the majority of the participants in the Pima Indian cohort had non-macroalbuminuria (n=42 of the 54 participants), the association of UAdCR with longitudinal progression to ESKD is presented in this non-macroalbuminuric cohort. ESKD was associated with the top UAdCR tertile (HR 4.57, CI 1.37-13.34) (Supplementary Table 1). UAdCR was also measured from 2 untimed spot urine samples obtained 1 year apart and found to be consistent across the individual paired samples (r=0.665,
p<0.0001) (Supplementary Figure 3). Similar relationships to predict ESKD was found in the non-macroalbuminuric participants in CRIC (adjusted HR 2.36, 95% CI 1.26, 4.39) and SMART2D (adjusted HR 2.39, 95% CI 1.08, 5.29) (Figure 2A, 2B, Supplementary Table 2, combined datasets in Supplementary Figure 2B). Of note, there were no significant correlations of UAdCR with the UACR or eGFR in the non-macroalbuminuric subjects from CRIC or SMART2D (Supplementary Table 3). Of the CRIC subjects with macroalbuminuria, there were modest associations between the top tertile of UAdCR and ESKD (HR 1.10, CI 0.75, 1.60) and mortality (HR 1.33, CI 0.59, 3.01).

To determine if UAdCR could be modified in non-macroalbuminuric participants with normal or elevated measured GFR by glycemia or a therapeutic intervention with an SGLT2 inhibitor, the UAdCR was measured during euglycemia or hyperglycemia before and after empagliflozin in patients with T1D (clinical characteristics described in Supplementary Table 4). Acute hyperglycemia did not alter UAdCR levels (Supplementary Figure 4), however empagliflozin significantly lowered UAdCR by 36.4% (Figure 2C).

**Adenine is localized to regions of kidney fibrosis and is increased in patients with diabetes.** A spatial metabolomics platform was developed to annotate small molecules (<700 Da) and performed on kidney biopsies from healthy controls and in patients with diabetes (clinical characteristics in Supplementary Table 4). Adenine was present at low intensity in normal glomeruli and blood vessels in the healthy control kidney (Figure 3A) and enhanced in regions of arteriolosclerosis, tubulointerstitial fibrosis and early glomerulosclerosis in the diabetic kidney (Figure 3B). There was an overall increase in adenine in the whole section of kidney biopsies from participants with diabetes as compared to healthy controls (Figure 3C). The spatial adenine values in rat kidney sections were found to correlate well with the UAdCR in a ZDF diabetic model (r=0.73, p<0.001, Supplementary Table 5).

**Single cell transcriptomics identify ribonucleoprotein biogenesis as a dominant pathway in non-macroalbuminuric DKD.** As adenine was prominent in regions of tubular pathology in the diabetic kidney and empagliflozin treatment lowered the UAdCR in patients, the proximal tubular cells were considered to be a target cell type affected by adenine. Single cell transcriptomics from proximal tubular cells were studied in DKD patients from the KPMP study (n=28) and an unbiased pathway analysis was performed based on differentially regulated genes. The top pathway identified was the ribosomal nucleoprotein biogenesis pathway in patients without macroalbuminuria and low eGFR (Figure 4A, B). In addition, small and large ribosomal subunit organization pathways were also upregulated in these patients. Replication of these results from KPMP was found in the CROCODILE study in diabetic patients without macroalbuminuria and normal GFR (Figure 4C). As ribonucleoprotein biogenesis, and small and large
ribosomal subunit organization is closely linked to activity of mammalian target of rapamycin (mTOR) (16) and adenine has been found to stimulate mTOR (17), this pathway was evaluated to mediate adenine-induced effects on proximal tubular cells.

Mechanism of adenine induced matrix production is via the mTOR pathway and adenine increases KIM1 and sTNFR1 in mice. To determine whether adenine could be in the causative pathway for tissue fibrosis, adenine was added to mouse and human proximal tubular cells. There was a robust and early stimulation of fibronectin by adenine (Figure 4D, Supplementary Figure 5A). In addition, adenine stimulated mTOR activity as demonstrated by enhanced phosphorylation of S6 kinase (Figure 4E, Supplementary Figure 5B). Inhibition of mTORC1 with rapamycin blocked adenine-induced production of fibronectin (Figure 4F). Exposure of adenine to normal mice stimulated blood and kidney levels of soluble tumor necrosis factor receptor 1 (sTNFR1) and kidney injury molecule-1 (KIM1), kidney hypertrophy, kidney mTOR activity, and kidney matrix production (Figures 4 G-K; Supplementary Figure 6).

Endogenous adenine contributes to diabetic kidney disease in db/db mice. To determine whether endogenous adenine plays a role in progression of diabetic kidney disease, methylthio-DADMe-Immcillin-A (MTDIA) a small molecule specific inhibitor of methylthioadenosine phosphorylase (MTAP) was administered to db/db mice, a model of obese type 2 diabetes. MTAP converts methylthioadenosine to adenine and is responsible for approximately 80% of adenine production in mammalian cells (18). MTDIA was well tolerated and did not affect food intake, water intake, blood glucose levels or body weight (Supplementary Table 6). MTDIA significantly reduced kidney adenine in db/db mice (Figure 5A) but not other metabolites linked to progression of kidney disease (Supplementary Table 7) (19). MTDIA significantly reduced serum cystatin C, kidney hypertrophy, kidney KIM1, and partially reduced urine ACR, serum creatinine, urine KIM1, kidney matrix proteins and mTOR activity in db/db mice (Figure 5 B-I).

Discussion

The results from the present study demonstrate a role for endogenous adenine in kidney disease progression in the context of DKD. Urine levels of the AdCR identified patients with diabetes at high risk of kidney failure and all cause mortality at all levels of albuminuria in the CRIC study and verified in a cohort study from Singapore. The UAdCR can also identify patients who will develop ESKD even in the setting of normal or elevated GFR without macroalbuminuria across ethnicities. Spatial metabolomics localized adenine to regions of vascular, tubular, and glomerular pathology in patients with diabetes who have normoalbuminuria and GFR. Adenine appears to be in the
causal pathway of kidney fibrosis as adenine was demonstrated to stimulate matrix molecules in proximal tubular cells via mTOR, was causative of kidney matrix production in mice and inhibiting adenine production was protective in diabetic mice.

Biomarkers in the causal pathway have not previously been identified for kidney disease progression in non-macroalbuminuric patients with diabetes. Microalbuminuria is clearly a risk factor for kidney disease progression, however as microalbuminuria can revert to normoalbuminuria (20) the dependence upon microalbuminuria alone may not provide reliable prognostication for event rates of GFR decline or kidney failure. Non-invasive omics approaches using plasma and urine have identified promising candidate biomarkers (21-23), however, demonstration of a contributory role of these biomarkers to the disease process has been difficult to establish (24). In the present study, integration of spatial metabolomics and single cell transcriptomics of human kidney biopsies converged on adenine and the mTOR pathway as highly relevant to DKD progression. The link with adenine and pathologic features was suggested by spatial metabolomics as adenine could be localized adjacent to atrophic tubules, in regions of arteriosclerotic blood vessels and glomerulosclerosis. The spatial localization implicated adenine to potentially be an endogenous pro-fibrotic factor.

Adenine is known to cause kidney pathology as an exogenous toxin in mouse (25) and rat models (14) of CKD, and possibly as an endogenous toxin in humans (26). The pathology of adenine-induced kidney disease includes glomerulosclerosis, tubular atrophy, interstitial fibrosis, and inflammatory cell infiltration (27, 28). The mechanism of adenine induced kidney disease has not been established although it has been postulated that conversion of adenine to 2-8 dihydroxyadenine (26) is a driver of CKD in patients with mutations of adenine phosphoribosyltransferase (APRT), the major enzyme that metabolizes adenine to AMP. However, CKD patients with APRT mutations are rare. Adenine itself is likely an endogenous tubular toxin based on the spatial metabolomic analysis and our finding that high urine adenine identifies patients at high risk of ESKD. Adenine exposure enhances tubular cell matrix production via the mTOR pathway and a prior study found that adenine is a potent stimulus for mTOR (17). Several published studies in mice and rats have also found that inhibiting mTOR protects against adenine-induced kidney disease (29-31). The mTOR pathway is likely relevant to human DKD as a recent study found stimulation of mTOR activity in kidney biopsies from patients with DKD (32) and our study with kidney biopsies from KPMP and CROCODILE demonstrate that a number of outputs of mTOR are elevated in DKD. This includes pathways involved in bioenergetics and pathways related to stimulation of extracellular matrix molecules. Further, adenine can increase the levels of
KIM1 and sTNFR1 demonstrating that adenine is likely an initiator of downstream injury and inflammatory markers.

Endogenous adenine production was blocked with a specific small molecule inhibitor of MTAP (MTDIA) and found to protect against diabetic renal hypertrophy, elevation of kidney KIM1 and was protective of decline in kidney function, as measured by serum cystatin C. It is possible that chronic MTAP inhibition with MTDIA could be developed as a safe therapeutic as a prior study found that MTDIA extended lifespan in mice with colon cancer and was provided for 294 days without evidence of toxicity (33). The role of adenine to accentuate mortality is not clear although it is possible that adenine could be directly toxic to vascular cells.

The measure of UAdCR was closely associated with DKD progression in the non-macroalbuminuric diabetic Pima Indian, CRIC, and SMART2D cohorts. As non-macroalbuminuric DKD leads to ESKD in many patients with CKD and diabetes (7, 8, 34), the new UAdCR biomarker could be of clinical value to identify those patients likely to progress. Further, the benefit of SGLT2 inhibitors may be due in part to reduce adenine levels as our study documented that short term use of empagliflozin significantly attenuated the UAdCR.

Strengths of our study included multiple analysis of several independent cohorts across different ages, ethnicities and stages of DKD. Additional strengths include application of spatial metabolomics and single cell transcriptomics to identify a pathway linking adenine to mTOR in human kidney disease pathology and progression. Limitations of our study was that the role of adenine was not demonstrated in type 1 DKD and other causes of CKD.

In conclusion, urine samples from independent well characterized cohorts of patients with diabetes identified the UAdCR as a robust predictor of ESKD and mortality independent of albuminuria and baseline eGFR, and spatial metabolomic and single cell transcriptomic studies from human kidney biopsies identified a potential role for endogenous adenine and the mTOR pathway in DKD. Studies in cells and mice identified a causative role for adenine and a small molecule therapeutic was found to block adenine production and was nephroprotective in a mouse model of type 2 diabetes. Our results thus demonstrate that endogenous adenine could contribute to progressive kidney disease in the context of type 2 diabetes.

Methods

Clinical Cohorts. Chronic Renal Insufficiency Cohort (CRIC): The parent CRIC Study recruited a racially diverse group aged 21 to 74 years, ~50% with diabetes, with a broad range of kidney function (35). Informed consent was obtained from participants; protocols were approved by Scientific and Data Coordinating Center). The current study
analyzed the urine sample at study entry (from baseline 24h urine samples) of 904 CRIC participants with diabetes and eGFR between 20-70 mL/min/1.73 m² who had samples and outcomes data available. Singapore Study of Macro-Angiopathy and microvascular Reactivity in Type 2 Diabetes cohort (SMART2D): SMART2D is an ongoing prospective cohort study of Southeast Asian T2D participants recruited between 2011-2014 (36). Fasting spot urine samples were collected at baseline and stored at -80°C. To validate findings from the CRIC cohort, 309 participants with baseline eGFR 20-70 mL/min/1.73 m² and urine ACR < 300 µg/mg were evaluated. All participants gave written informed consent, and the study was approved by the Singapore National Healthcare Group. Pima Indians with early DKD were enrolled in a randomized clinical trial (37) ([ClinicalTrials.gov number, NCT00340678]). GFR was measured annually throughout the trial by the urinary clearance of iothalamate. Stored spot urine samples collected for two consecutive years were available from 54 participants and included for analysis. Additionally, urine samples were obtained under controlled euglycemic and hyperglycemic clamp conditions from a previously published clinical study in patients with type 1 diabetes (T1D) without macroalbuminuria (n=42) to evaluate the effects of Empagliflozin (Adjunctive-to-insulin and Renal Mechanistic (ATIRMA), NCT01392560) (38). Euglycemic clamp (4-6 mM glucose) conditions were maintained for approximately 4 hours prior to urine collection. The following day hyperglycemia (9-11 mM glucose) was maintained for 4 hours. Urine samples for adenine measurements were performed from samples obtained at the 4-hour time point following euglycemia or hyperglycemia before and after empagliflozin (25 mg/d) treatment for 8 weeks.

**Urine metabolomics (Zip-Chip Analysis).** Urine samples from Pima Indians, CRIC, SMART2D cohorts, and ATIRMA urines were all analyzed using ZipChip (908 Devices, Boston, MA) coupled with mass spectrometry (39). A rapid throughput urine adenine/creatinine assay was developed that showed excellent correlation with the gold standard assay using LC-MS/MS (Supplementary Figure 1). The reportable linear range for urine adenine assay was 100 nM to 100 uM, with a limit of detection at 10 nM and coefficient of variation (CV)<10% across the reportable linear range. Metabolite separation was achieved with a microfluidic chip which integrates capillary electrophoresis (CE) with nano-electrospray ionization through a ZipChip interface. Data acquisition was performed with Q-Exactive mass spectrometer (Thermo, San Jose, CA) and Thermo Scientific’s software Xcalibur-Quan Browser for data processing. Detailed procedures were previously published (39).

**Human kidney biopsies.** Human kidney samples were obtained via the Kidney Precision Medicine Project (KPMP; The trial registration number from ClinicalTrials.gov is NCT04334707) and the Control of Renal Oxygen
Consumption, Mitochondrial Dysfunction, and Insulin Resistance (CROCODILE) studies (40-42). The KPMP and CROCODILE studies were approved by the Institutional Review Board at Washington University, St. Louis, MO and the University of Colorado, respectively, and written consent was obtained from all patients. Samples were frozen in liquid nitrogen and stored at -80 °C until analysis. Snap frozen sample preparation and sectioning procedures for MALDI-MSI were published at dx.doi.org/10.17504/protocols.io.bcrav2e.

Animal studies. Zucker Diabetic Fatty (ZDF) rat kidney and urine samples were provided by Epigen, Inc. to verify that kidney spatial adenine correlated with the targeted urine adenine assay. C57Bl6J mice, db/m and db/db mice were obtained from Jackson Labs. C57Bl6J mice were administered adenine for 4 weeks in drinking water before sacrificing mice and harvesting tissues and blood samples after IACUC approval at UTHSA. db/m and db/db mice were administered vehicle or methylthio-DADMe-Immmucillin-A (MTDIA) MTAP inhibitor for a period of 8 weeks from week 10 to week 18. Albumin ELISA kit (Cat. #, E101 and E90-134, Bethyl Laboratories Inc.) and creatinine colorimetric kit (catalog ADI-907-030A, Enzo Life SciencesInc.) were used for the urinary ACR. Serum cystatin C was measured by Quantikine ELISA kit (Cat. #, MSCTC0, R&D systems). Plasma creatinine and metabolites in kidney tissue were measured by the ZipChip-mass spectrometry as previously described (19). Urine and kidney KIM-1 was measured by ELISA (Cat. #DY1817, R&D System).

Mass Spectrometry Imaging (MSI) and Optical Imaging of Kidney Biopsies. A multimodal imaging approach was developed to investigate regional localization of metabolites in kidney sections. Bright-field (BF) and autofluorescence (AF) microscopy outlined glomeruli and tubules, and PAS-H staining revealed regions of pathology in serial sections. Matrix assisted laser desorption/ionization (MALDI)-MSI was performed with a Thermo Scientific Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific) in combination with a novel elevated pressure MALDI/ESI interface (Spectroglyph LLC, Kennewick, WA) (43). Metabolite annotation was performed on METASPACE (44) and the optical image was uploaded to METASPACE and SCiLS Lab for visual overlay of metabolites with optical images to provide an assessment of metabolites associated with normal-appearing and pathologic features. Detailed procedures for MALDI-MSI are available at dx.doi.org/10.17504/protocols.io.bctfiwjn.

Cell culture. Human kidney proximal tubular (HK-2) cells were purchased from American Type Culture Collection (Manassas, VA) and cultured as previously described (45). Murine kidney proximal tubular epithelial (MCT) cells were cultured as previously described (46). Cells were treated with 20 µM of adenine for the indicated
time points with and without rapamycin (Fisher Scientific). Phosphorylation of S6 kinase, ribosomal protein S6 and expression of fibronectin and type 1 collagen α2 were analyzed by immunoblotting using antibodies against phosphor-Thr389-S6 kinase (Cat. # 9205, Cell Signaling Technology), phosphor-Ser240/244 ribosomal protein S6 (Cat. #, 2215, Cell Signaling Technology), ribosomal protein S6 (Cat. #, 2217, Cell Signaling Technology), MTAP (Cat. #, 62765, Cell Signaling Technology), fibronectin (Cat. #, ab2413, Abcam Plc), type 1 collagen α2 (Cat. #, 14695-1-AP, Proteintech Group Inc), and beta-actin (Cat. #, A2066, Millipore Sigma).

**Statistical Analysis.** A composite kidney endpoint was defined as sustained kidney replacement therapy, progression to GFR or eGFR < 15 mL/min/1.73m², or >50% GFR or eGFR decline from baseline level. All-cause mortality included death from any cause before reaching ESKD endpoint. Urine adenine was normalized to urine creatinine concentrations and log2 transformed. In the CRIC and SMART2D cohorts, the association of urine adenine levels (tertile) with clinical endpoints was studied by multivariable Cox proportional hazard regression models with adjustment for age, gender, ethnicity/race, body-mass index (BMI), hemoglobin A1c (HbA1c), mean arterial pressure (MAP), baseline eGFR, and urine ACR (natural log-transformed) as covariates. The group with a urine adenine/creatinine ratio in the lowest tertile was used as reference. Due to the limited number of cases in the Pima Indian cohort we only reported univariate Cox proportional hazards analysis for this cohort. To evaluate the pre-treatment and post-treatment effect of empagliflozin on urine adenine in the ATIRMA cohort, we performed a linear regression analysis for repeated measures. Student’s t test (2-tailed) was used for comparisons of features between two groups. A P value less than 0.05 was considered significant.

**Bioinformatic and systems medicine analysis.** Single cell transcriptomics and spatial metabolomics datasets generated from healthy kidney tissue, unaffected tissue in kidney nephrectomy and biopsy samples (KPMP and CROCODILE) were analyzed as recently described (40-42). The top pathway genes and proteins from the top 600 significant genes or proteins from proximal tubular cells were mapped onto pathways for subcellular processes using the MBCO ontology (47).

**Study Approval.** For the CRIC study, Informed consent was obtained from participants; protocols were approved by IRBs and Scientific and Data Coordinating Center. All participants gave written informed consent, and the study was approved by the Singapore National Healthcare Group. The KPMP and CROCODILE studies were approved by the Institutional Review Board at Washington University, St. Louis, MO and the University of Colorado, respectively, and written consent was obtained from all patients.
Data Availability. Data are available in the ‘Supporting data values’ XLS file. Raw human subject data used in Tables and Supplementary Tables are confidential and they are available from the corresponding author upon request.

Author Contributions

The first 9 coauthors each made unique and critical contributions to this manuscript, and authorship order was determined after discussion among writing group members. KS, PB, SCL, JJL, RI, RGN, MK, and GZ designed the study. KS, PB, SCL, DC and RGN acquired funding for the study. GZ, JH, HJL, RM, PB, JYL, HL, LH, PB, LK, VSS acquired or generated the data. GZ, JH, HJL, HL, BC, LK and JG analyzed the data. KS, GZ, PB, MK, RGN and RI wrote the manuscript. KS, PB, MK, SCL, LN, JZ, VS, BK, SW, JH, KT, BK, TF, HF, IB, JS, HDL, JS, RM, EO, CA, TA, SCL, RGN, JG and RI provided scientific guidance and insights. All authors reviewed, edited, and approved the manuscript.

Conflicts of Interest

K.S. reports serving as consultant for Visterra, Bayer, Sanofi, and receiving research support from Boehringer-Ingelheim. K.S. also reports having equity in a startup company, SygnaMap. P.B. reports serving as a consultant for AstraZeneca, Bayer, Bristol-Myers Squibb, Boehringer Ingelheim, Eli-Lilly, LG Chemistry, Sanofi, Novo Nordisk, and Horizon Pharma. P.B. also serves on the advisory boards of AstraZeneca, Bayer, Boehringer Ingelheim, Novo Nordisk, and XORTX. K.R.T. reports other support from Eli Lilly; personal fees and other support from Boehringer Ingelheim; personal fees and other support from AstraZeneca; grants, personal fees and other support from Bayer AG; grants, personal fees and other support from Novo Nordisk; grants and other support from Goldfinch Bio; other support from Gilead; and grants from Traver outside the submitted work. R.G.N. and H.C.L. report no conflicts. J.H. reports serving as a consultant for Maze Therapeutics, Chinook Therapeutics, Renalytix AI, and Seattle Genetics. D.C. has received honoraria from Boehringer Ingelheim-Lilly, Merck, AstraZeneca, Sanofi, Mitsubishi-Tanabe, AbbVie, Janssen, Bayer, Prometric, BMS, Maze, CSL Behring, and Novo Nordisk. H.L.H. has received honoraria for participation in steering committees from AstraZeneca, Janssen, Eli-Lilly, Gilead, Bayer, Chinook, Novartis, and CSL Behring; honoraria for participation in advisory boards from AstraZeneca, Vifor, Novartis, NovoNordisk, and Idorsia; fees for consultancy from AstraZeneca, Traver Pharmaceuticals, Boehringer Ingelheim, and Novo Nordisk; and
research grant support from AstraZeneca, Janssen, Boehringer Ingelheim and NovoNordisk. Honoraria are paid to his institution [University Medical Center Groningen].

Acknowledgments

G.Z., L.H., H.J.L, A.F, and K.S. receives salary and research support from NIH (UH3DK114920, 5U2CDK114886, RO1DK110541). L.N., J.Z., B.K. were supported by NIDDK 5R01DK110541. J.J.L. receives research support from Alexandra Health Fund (STAR grant 18203 and 20201). S.C.L. receives research support from Singapore National Medical Research Councile (MOH-000066, 0000714 and OFLCG/001/2017). P.B. receives salary and research support from NIDDK (R01 DK129211, R21 DK129720, K23 DK116720, UC DK114886, and P30 DK116073), JDRF (2-SRA-2019-845-S-B, 3-SRA-2017-424-M-B, 3-SRA-2022-1097-M-B), Boettcher Foundation, American Heart Association (20IPA35260142), Ludeman Family Center for Women’s Health Research at the University of Colorado, the Department of Pediatrics, Section of Endocrinology and Barbara Davis Center for Diabetes at University of Colorado School of Medicine. K.R.T. receives salary and research support from the NIDDK, NIMHD, NCATS, and NHLBI (R01MD014712, U2CDK114886, UL1TR002319, U54DK083912, U01DK100846, OT2HL161847, UM1AI109568) and the CDC (75D301-21-P-12254). R.G.N. was supported by the American Diabetes Association (Clinical Science Award 1-08-CR-42) and R.G.N. and H.C.L. were supported by the Intramural Research Program of NIDDK. J.H. and R.I. received salary support from U3CDK114886, R01GM137056, P01HL134605. Funding for the CRIC Study was obtained under a cooperative agreement from National Institute of Diabetes and Digestive and Kidney Diseases (U01DK060990, U01DK060984, U01DK061022, U01DK061021, U01DK061028, U01DK060980, U01DK060963, U01DK060902 and U24DK060990). In addition, this work was supported in part by: the Perelman School of Medicine at the University of Pennsylvania Clinical and Translational Science Award NIH/NCATS UL1TR000003, Johns Hopkins University UL1 TR-000424, University of Maryland GCRC M01 RR-16500, Clinical and Translational Science Collaborative of Cleveland, UL1TR000439 from the National Center for Advancing Translational Sciences (NCATS) component of the National Institutes of Health and NIH roadmap for Medical Research, Michigan Institute for Clinical and Health Research (MICHR) UL1TR000433, University of Illinois at Chicago CTSA UL1RR029879, Tulane COBRE for Clinical and Translational Research in Cardiometabolic Diseases P20 GM109036, Kaiser Permanente NIH/NCRR UCSF-CTSI UL1 RR-024131, Department of Internal Medicine, University of New Mexico School of Medicine Albuquerque, NM R01DK119199. We acknowledge technical help
for animal studies from Richard Montellano, transcriptomics data organization from Fadhl AlAkwaa and Philip McCown, method development from Annapurna Pamreddy and data analysis by Rabiul Islam. D.C. has received operational funding for clinical trials from Boehringer Ingelheim-Lilly, Merck, Janssen, Sanofi, AstraZeneca, and Novo Nordisk. We acknowledge Afaf Saliba’s help in creating the graphical abstract using BioRender. The Kidney Precision Medicine Project (KPMP) is funded by the following grants from the NIDDK: U01DK133081, U01DK133091, U01DK133092, U01DK133093, U01DK133095, U01DK133097, U01DK114866, U01DK114908, U01DK133090, U01DK133113, U01DK133766, U01DK133768, U01DK114907, U01DK114920, U01DK114923, U01DK114933, U24DK114886. Please see Supplemental Acknowledgments for KPMP consortium details.

Appendix

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References


Table 1. Baseline characteristics of patients with diabetes in the Pima American Indians, CRIC and SMART2D studies.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Pima cohort (N=54)</th>
<th>CRIC cohort (N=904)</th>
<th>SMART2D cohort (N=309)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD/N (%)</td>
<td>Mean ± SD/N (%)</td>
<td>Mean ± SD/N (%)</td>
</tr>
<tr>
<td>Index age (years)</td>
<td>45.1 ± 9.6</td>
<td>60 ± 9.4</td>
<td>64.5 ± 9.6</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13 (24%)</td>
<td>515 (57%)</td>
<td>176 (57%)</td>
</tr>
<tr>
<td>Female</td>
<td>41 (76%)</td>
<td>390 (43%)</td>
<td>133 (43%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Black: 376 (41%)</td>
<td></td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>American Indian: 54 (100%)</td>
<td></td>
<td>Hispanic: 141 (16%)</td>
<td>Chinese: 163 (53%)</td>
</tr>
<tr>
<td>Hispanic: 141 (16%)</td>
<td></td>
<td>Other: 37 (4%)</td>
<td>Malay: 71 (23%)</td>
</tr>
<tr>
<td>Other: 37 (4%)</td>
<td></td>
<td>White: 350 (39%)</td>
<td>Asian Indian: 75 (24%)</td>
</tr>
<tr>
<td>White: 350 (39%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>..</td>
<td>510 (56%)</td>
<td>20 (7%)</td>
</tr>
<tr>
<td>No</td>
<td>..</td>
<td>391 (43%)</td>
<td>289 (93%)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>35.4 ± 7.1</td>
<td>34 ± 7.8</td>
<td>27.7 ± 5.3</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>9.6 ± 2</td>
<td>7.6 ± 1.6</td>
<td>7.6 ± 1.3</td>
</tr>
<tr>
<td>Mean artery pressure (mmHg)</td>
<td>93.2 ± 10</td>
<td>90 ± 13</td>
<td>98 ± 11</td>
</tr>
<tr>
<td>eGFR* (mL/min/1.73m²)</td>
<td>139 ± 49</td>
<td>40 ± 12</td>
<td>53 ± 13</td>
</tr>
<tr>
<td>ACR** (median (IQR), mg/g)</td>
<td>40 (15-164)</td>
<td>116 (16-756)</td>
<td>27 (11-87)</td>
</tr>
<tr>
<td>ACR category (N, %)***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 30 mg/g</td>
<td>26 (48%)</td>
<td>298 (33%)</td>
<td>162 (52%)</td>
</tr>
<tr>
<td>30-300 mg/g</td>
<td>16 (30%)</td>
<td>260 (28%)</td>
<td>147 (48%)</td>
</tr>
<tr>
<td>&gt;300 mg/g</td>
<td>12 (22%)</td>
<td>341 (39%)</td>
<td>..</td>
</tr>
</tbody>
</table>

BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; UACR, urine albumin-to-creatinine ratio; *data for Pima cohort is measured GFR in ml/min; **Continuous ACR is summarized using median (IQR, interquartile range) because of its skewed distribution. ***Data for CRIC cohort is based on 24h urine albumin or albumin/creatinine values. For ACR category and all other continuous variables are summarized using mean ± SD.
Table 2. Association of baseline urine adenine/creatinine ratio (UAdCR) with risk for progression to ESKD and all-cause mortality in CRIC and SMART2D participants with type 2 diabetes with 7 years follow up.

<table>
<thead>
<tr>
<th>Adenine/Creatinine ratio</th>
<th>CRIC cohort (N=889)</th>
<th>SMART2D cohort (N=309)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td>1-SD increment</td>
<td>1.15 (1.03-1.28)</td>
<td>0.010</td>
</tr>
<tr>
<td>Tertile 2 vs tertile 1</td>
<td>1.59 (1.21-2.09)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tertile 3 vs tertile 1</td>
<td>1.57 (1.18-2.10)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Multivariate Cox proportional hazard regression models were adjusted for baseline age, sex, ethnicity, body mass index, mean arterial pressure, hemoglobin A1c, eGFR and natural-log transformed urine albumin/creatinine ratio. UAdCR was modelled as both continuous variable (1-SD increment in log2-transformed adenine/creatinine ratio) and categorical variable (low tertile as reference). There were 15 subjects in CRIC with missing values for the clinical covariates.
Figure 1. High urine adenine/creatinine (UAdCR) levels identify patients with diabetes who are at high risk of end stage kidney disease (ESKD) and mortality. Participants with diabetes in the Chronic Renal Insufficiency Cohort (CRIC) cohort (n=904) had UAdCR measured within 1 year of enrollment and followed for 10 years. The participants in the top tertile had the highest risk of ESKD and all cause mortality (A). Participants from the Singapore Study of Macro-Angiopathy and microvascular Reactivity in Type 2 Diabetes (SMART2D) study (n=309) had UAdCR measurements at the time of enrollment and were followed for 7 years. The participants in the top tertile for UAdCR had the highest risk for ESKD and all-cause mortality.
Figure 2. High urine adenine/creatinine (UAdCR) tertile identifies end stage kidney disease (ESKD) outcome in non-macroalbuminuric patients with diabetes and empagliflozin reduced urine adenine/creatinine ratio. The participants with the top UAdCR tertile had a significant increase in risk of ESKD from CRIC (n=551) (A) and SMART2D (n=309) (B) studies. Patients with T1 diabetes underwent treatment with empagliflozin for 8 weeks which reduced UAdCR levels (n=40 patients) (C).
Figure 3. Spatial metabolomics identifies adenine in regions of pathology in non-macroalbuminuric patients with diabetes. Adenine was localized to regions of normal glomeruli and vessels in the normal kidney (A). In a diabetic kidney, adenine is diffusely increased across the tissue section and prominent in regions of sclerotic blood vessels, glomeruli with mild sclerosis and regions of atrophic tubules and interstitial inflammation (B). Quantitative assessment across healthy controls (n=5 from Control of Renal Oxygen Consumption, Mitochondrial Dysfunction and Insulin Resistance (CROCODILE) study) and diabetic samples (n=8 T1D from CROCODILE and n=8 T2D (2 from CROCODILE and 6 from Kidney Precision Medicine Project (KPMP)) demonstrates a statistically significant increase of adenine in kidney tissue sections. Two-tailed Student’s t test was used for the comparison. Data represent mean ± SEM (C).
Figure 4. Molecular pathways and events implicating ribonucleoprotein biogenesis and mammalian target of rapamycin (mTOR) pathway with adenine in diabetic kidney disease (DKD): Protein synthesis (Ribonucleoprotein (RNP) biogenesis) pathway increased in proximal tubule cells of DKD patients without proteinuria (A) Single cell- transcriptomic data obtained from DKD kidney biopsies for the Kidney Precision Medicine Project (KPMP) was analyzed for differentially expressed genes in proximal tubule (PT) of each DKD patient versus healthy reference tissue. Upregulated genes with an adjusted p-value ≤ 0.01 and ranked among the top 600 significant DEGs were subjected to pathway enrichment analysis using the Molecular Biology of the Cell Ontology (MBCO). Ranking for the RNP biogenesis pathway (a Level 2 pathway canonically regulated by the mTOR pathway) is shown for 28 individual patients. Vertical dashed line indicates p value ≤ 0.01 for pathway ranking (B) Up to top five, five, ten and five level-1 (dark red), level-2 (red), level-3 (blue) and level-4 (green) pathways using MBCO are shown for patient #1 (p-value ≤ 0.01). See blue lines from A to B. Single cell transcriptomic data from T2D patients (N=6) with low albuminuria compared to cohort specific healthy samples was analyzed to identify upregulated pathways in PT cells (C). Note that the RNP biogenesis pathway is the top ranked Level-2 pathway in both independent studies. Cell culture studies in mouse proximal tubular cells demonstrated an increase in fibronectin (D), phospho-S6 kinase and
mediation of fibronectin (FN) upregulation is blocked by rapamycin (E, F), indicating mTOR mediates adenine effect. Adenine administration to mice increases serum soluble tumor necrosis factor-1 (sTNFR1) (G), plasma kidney injury marker-1 (KIM-1) (H), stimulates kidney (I) and matrix molecules in the kidney (J, K) (n=12 in control group and n=7 in adenine treated group, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Two-tailed Student’s t test was used for two group comparisons. One-way ANOVA was used for multiple group comparisons. Data represent mean ± SD.
Figure 5. Methylthioadenosine phosphorylase (MTAP) inhibitor ameliorates kidney injury in db/db mice with type 2 diabetes: Methylthio-DADMe-Immucillin-A (MTDIA) significantly reduced kidney adenine levels (A), kidney hypertrophy (B), kidney KIM-1 levels (C) in diabetic mice. MTDIA significantly reduced diabetes-increased serum cystatin C (E), and partially reduced plasma creatinine (F), and albuminuria (G) in diabetic mice. Diabetes induced kidney matrix protein levels were partially reduced by MTDIA (H, I). Ribosomal S6 phosphorylation was partially reduced by MTDIA in the kidney of db/db mice (J) (n=6 per group, *p<0.05, **p<0.01, ***p<0.001). Two-tailed Student’s t test was used for two group comparisons. Data represent mean ± SD.