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TRANSLATIONAL RESEARCH

Profiling of Plasma Metabolites Suggests Altered Mitochondrial Fuel Usage and Remodeling of Sphingolipid Metabolism in Individuals With Type 2 Diabetes and Kidney Disease

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Introduction: Pathophysiology of diabetic kidney disease (DKD) is incompletely understood. We aim to elucidate metabolic abnormalities associated with DKD in type 2 diabetes mellitus (T2DM) by targeted plasma metabolomics.

Methods: A total of 126 T2DM participants with early DKD (urinary albumin-to-creatinine ratio [ACR] 30−299 mg/g and eGFR $\geq$ 60 ml/min/1.73 m^2), 154 overt DKD (ACR $\geq$ 300 mg/g or eGFR $<$ 60 ml/min/1.73 m^2), and 129 non-DKD T2DM controls (ACR $<$ 30 mg/g and eGFR $\geq$ 60 ml/min/1.73 m^2) were included in discovery study. Findings were subsequently validated in 149 T2DM with macroalbuminuria (ACR $\geq$ 300 mg/g) and 149 matched non-DKD T2DM controls. Plasma amino acid, acylcarnitine, Krebs cycle organic acid, and sphingolipids/ceramide levels were quantified by liquid chromatography−mass spectrometry and gas chromatography−mass spectrometry.

Results: Of 123 metabolites included in the data analysis, 24 differed significantly between DKD and controls in the same direction in both discovery and validation subpopulations. A number of short acylcarnitines including their dicarboxylic derivatives (C2−C6) were elevated in DKD, suggesting abnormalities in fatty acids and amino acids metabolic pathways. Five phosphatidylcholines were lower whereas 4 metabolites in the sphingomyelin−ceramide subfamily were higher in DKD. Principal component regression revealed that long-chain ceramides were independently associated with ACR but not eGFR. Conversely, essential amino acids catabolism and short dicarboxylacylcarnitine accumulation were associated with eGFR but not ACR.

Discussion: DKD is associated with altered fuel substrate use and remodeling of sphingolipid metabolism in T2DM with DKD. Associations of albuminuria and impaired filtration function with distinct metabolomic signatures suggest different pathophysiology underlying these 2 manifestations of DKD.

Kidney Int Rep (2017) **2,** 470–480; http://dx.doi.org/10.1016/j.ekir.2016.12.003
KEYWORDS: diabetic kidney disease; energetic fuel substrate; metabolomics; pathophysiology; type 2 diabetes mellitus

Diabetic kidney disease (DKD), defined as a decline in renal filtration function and/or albuminuria, affects more than 50% of patients with diabetes.[1] It is not only the leading cause of end stage renal disease (ESRD) but also a major risk factor for cardiovascular disease and mortality in patients with diabetes.[2] Although the management of DKD has improved, the number of patients with DKD is still increasing, and the ESRD risk attributable to diabetes has remained unabated in the past decade.[3] These unmet clinical needs may stem from the incomplete understanding in pathophysiology of DKD.[4]

Unlike other omics technologies such as genomics and epigenomics, metabolomics offers a window to elucidate intermediate and end products of metabolic

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Received 5 September 2016; revised 6 December 2016; accepted 8 December 2016; published online 16 December 2016

pathways. Therefore, it has emerged as a novel tool to gain insights into disease pathophysiology.[5] Encouragingly, a few large-scale metabolomics studies have identified metabolic pathways associated with chronic kidney disease (CKD) in the general population. For instance, Yu *et al*. found that elevated plasma levels of kynuerenine, the metabolite derived from tryptophan catabolism, was associated with incident CKD in African Americans, which was consistent with studies performed in the Framingham Heart Study and the KORA study.[6] Given that the pathogenesis of DKD is closely related to perturbed energy metabolism, metabolomics may provide insights into pathophysiological pathways associated with DKD and may help to identify novel interventional targets.[7,8] For instance. Sharma *et al*. have reported a urinary metabolomic signature that is suggestive of mitochondrial dysfunction in patients with DKD.[9]

Although recent metabolomics studies have revealed potential pathways associated with DKD development and its progression,[10] several important limitations are noteworthy: (i) The sample sizes of the early studies are generally small, and patient profiles are relatively heterogeneous; (ii) most of these studies lack a robust validation by an independent population[10,11]; (iii) the coverage of metabolites, especially for targeted plasma metabolomics studies, is relatively limited[10]; and (iv) most studies to date have focused on urinary metabolomics in patients with DKD.[9,12] To the best of our knowledge, no large-scale metabolomics study has characterized plasma metabolites systemically, especially in patients with type 2 diabetes mellitus (T2DM) and kidney disease. In the present work, we profiled plasma acylcarnitines, Krebs cycle intermediates, amino acids, and sphinogolipids/phospholipids by targeted metabolomics in participants with T2DM. *A priori* knowledge and literature review suggest that these metabolites/pathways may be associated with DKD development or progression.[9,11,13,14] We aim to systematically study metabolic pathways associated with DKD in an Asian T2DM population.

METHODS

Participant Recruitment

Participants were recruited consecutively in the diabetes center from a regional public hospital in Singapore between 2003 and 2013. Diagnosis of T2DM was based on American Diabetes Association criteria as follows: (i) fasting plasma glucose $\geq$ 7 mmol/l; (ii) random plasma glucose level $\geq$ 11.1 mmol/l, or (iii) self-reported T2DM on hypoglycemic medication. Exclusion criteria were age less than 21 years, pregnancy, manifest infectious diseases, active cancer, and autoimmune diseases. Volunteers were excluded when renal impairment was likely caused by other diseases such as polycystic kidney disease, presence of overt hematuria, or history of glomerulonephritis. Volunteers with an estimated GFR (eGFR) $\leq$ 15 ml/min/1.73 m^2, those undergoing dialysis, and those with renal transplants were also excluded from the study. The study was approved by the Singapore National Health Group domain-specific ethnical committee, and written consent was obtained from each participant.

Study Design

Participants with T2DM and a broad spectrum of albuminuria and eGFR levels were included in the discovery substudy (Table 1). We categorized participants as follows: (i) early DKD (urinary albumin-to-creatinine ratio [ACR] between 30 and 299 mg/g and eGFR $\geq$ 60 ml/min/1.73 m^2); (ii) overt DKD (ACR $\geq$ 300 mg/g or eGFR $<$ 60 ml/min/1.73 m^2); and (iii) non-DKD controls (T2DM with ACR $<$ 30 mg/g and eGFR $\geq$ 60 ml/min/1.73 m^2). Cognizant of the technical variability of metabolomics and that DKD is a heterogeneous disease in which many nondiabetic causes may have been involved in eGFR decline and mild albuminuria,[2] we subsequently validated findings from a discovery substudy in an independent subpopulation. A few lines of evidence suggest that macroalbuminuria is highly correlated with typical diabetic glomerulopathy on renal biopsy.[15,16] Therefore, we included 149 T2DM patients with macroalbuminuria (ACR $\geq$ 300 mg/g) as cases in the validation subpopulation and matched them 1-to-1 by age, sex, ethnicity, and diabetes duration with non-DKD controls to partially control for confounding. We expected that metabolites would differ between DKD and non-DKD in both substudies and might be more likely attributed to classical diabetic glomerulopathy.

Biochemical Variables

Estimated glomerular filtration rate (eGFR) was calculated by the Modified Diet in Renal Disease (MDRD) formula which has performed well in the diabetic population.[17] Urinary albumin was measured by solid-phase competitive chemiluminescent enzymatic immunoassay with a lower detection limit of 2.5 mg/L (Immulite, DPC, Gwynedd, UK). HbA_{1c} was measured using an immunoturbidimetric method (Cobas Integra 800 Chemistry Analyser, Roche, Basel, Switzerland). Triacylglycerol, creatinine, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol levels were quantified by enzymatic methods (Roche/Hitachi Cobas C System; Roche Diagnostic GmbH, Mannheim, Germany).

Table 1. Clinical and biochemical characteristics of T2DM participants in discovery study

	T2DM control (n = 129)	Early DKD (n = 126)	Overt DKD (n = 154)	*P* value[a]
Age (yr)	51.6 ± 10.9	55.4 ± 11.6	64.1 ± 10.2	<0.0001
Male sex (%)	63.6	63.5	48.7	0.013
Diabetes duration (yr)	8.3 ± 7.2	11.4 ± 7.3	14.1 ± 9.0	<0.0001
Ethnicity (%)				<0.0001
Chinese	68.2	62.7	64.3	
Malay	7.0	18.3	31.2	
South Asian	24.8	19.0	4.5	
Current smoker (%)	11.3	13.4	10.7	0.796
BMI (kg/m^2)	26.2 ± 4.4	26.9 ± 4.7	27.3 ± 5.1	0.156
HbA_{1c} (%)	8.0 ± 1.3	8.8 ± 2.2	8.0 ± 1.8	<0.0001
HbA_{1c} (mmol/mol)	64 ± 11	73 ± 18	64 ± 17	<0.0001
Systolic BP (mm Hg)	128 ± 15	135 ± 18	142 ± 22	<0.0001
Diastolic BP (mm Hg)	78 ± 9	77 ± 10	77 ± 12	0.703
HDL cholesterol (mmol/l)	1.33 ± 0.38	1.25 ± 0.37	1.25 ± 0.32	0.124
LDL cholesterol (mmol/l)	2.83 ± 0.70	2.80 ± 0.74	2.80 ± 0.84	0.940
Triacylglycerol (mmol/l, IQR)	1.24 (0.98–1.96)	1.46 (1.16–2.09)	1.64 (1.25–2.32)	0.001
eGFR (ml/min/1.73 m^2)	91 ± 22	90 ± 23	49 ± 19	<0.0001
Urinary ACR (mg/g, IQR)	9 (5–17)	59 (38–127)	249 (55–633)	<0.0001
Statin use (%)	66.4	72.2	72.7	0.454
RAS blocker use (%)	45.0	73.8	85.1	<0.0001
Insulin use (%)	20.2	37.3	44.8	<0.0001

ACR, albumin-to-creatinine ratio; BMI, body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; RAS, renin–angiotensin system; T2DM, type 2 diabetes mellitus.
[a]One-way analysis of variance or χ^2 test where appropriate.

Biosample Collection, Metabolite Extraction, and Quantification by Liquid Chromatography–Mass Spectrometry and Gas Chromatography–Mass Spectrometry

Participants fasted overnight. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA)–containing tubes by phlebotomy, and plasma was separated by centrifugation. Spot urine samples were collected in a sterile container. Biosamples were transferred to the laboratory within 30 minutes after collection, aliquoted, and stored at −80 °C. Samples used for this metabolomics study did not undergo repeated freeze-and-thaw cycles. Early studies have indicated that plasma acylcarnitines and amino acids were stable when stored at −80 °C.[11]

Methods for metabolite extraction and metabolic profiling for amino acids, acylcarnitines, and organic acids have been described (see Supplementary Methodology).[18–20] Briefly, plasma acylcarnitines, amino acids, and lipids were extracted by methanol and derivatized with 3 mol/l hydrochloric acid in methanol or butanol, respectively. Organic acid was extracted with ethylacetate and derivatized with N,O-Bis(trimethylsilyl) trifluoroacetamide with protection of the α-keto groups using ethoxyamine. Urine samples were normalized to 4 mmol/l (45.24 mg/dl) of creatinine concentration by deionized water before extraction. Amino acids, lipids, and acylcarnitines were quantified by tandem mass spectrometry (MS). Organic acids were measured by gas chromatography–mass spectrometry (GC-MS).

An aliquot of the same volume from each study sample was pooled as a quality control (QC) sample. The relative SD (RSD) of the QC samples may indicate stability of response of individual metabolites. The QC sample was injected after every eighth study sample. The average relative SD of the QC was 9.5% for acylcarnitines, 4.0% for organic acids, 9.5% for amino acids, 21.0% for ceramides, 16.7% for sphingomyelins and 21.6% for phosphatidylcholines. Due to the high relative SD for lipid metabolites, all the raw readings of ceramide, sphingomyelin, and phosphatidylcholine of the study samples were normalized against their respective QC readings before data analysis. To account for run-order effects, the samples were coded and mixed randomly in each substudy. The assay operators were blinded to the clinical profiles.

Statistical Analysis

Continuous data are presented as mean ± SD or as median (interquartile range [IQR]) if skewed. Categorical data are expressed as percentage. Urinary ACR was natural logarithmically transformed. Differences in means were compared by the Student *t* test or analysis of variance, whereas categorical variables were compared by χ^2 tests.

We quantified 169 plasma metabolites in total. Among them, 2 of 12 sphingomyelins, 18 of 33 ceramides, 12 of 18 glucosylceramides, and 14 of 16 lactosylceramides have > 10% missing values (below

the lower detection limit). These metabolites with high proportions of missing values usually have low plasma concentrations with unknown pathophysiological implications, based on literature search. Hence, we excluded these 46 sphingomyelin/ceramide metabolites from data analysis. For the remaining 123 metabolites, 17 amino acids, 45 acylcarnitines, 7 organic acids, 19 phosphatidylcholines, sphingosine, and sphinganine have $< 1\%$ missing values, whereas the missing values for the remaining 35 metabolites range between 2% and 9%. For the metabolites included in final analysis, missingness was replaced by the lowest detected value. Metabolite concentrations were presented as median (IQR) to visualize centrality and dispersion, as most of them were right-skewed. Data were log2 transformed before analysis for normalization of distribution and to partially reduce the variation across orders of magnitude.

We compared differences in metabolite concentrations among early DKD patients, overt DKD patients, and non-DKD controls by analysis of covariance after adjustment for age, sex, and ethnicity. The false discovery rate (FDR, q value) was calculated (Benjamini-Hochberg approach) to account for type I errors attributable to multiple comparisons. Differences with both P value and q value < 0.05 were considered as statistically significant and subjected to validation. Differences in metabolite concentrations between DKD and non-DKD controls in the validation substudy were then compared by the Student t test, and the q value threshold was set at the < 0.10 level. Furthermore, Jonckheere–Terpstra tests were applied to examine which metabolites exhibited a linear trend across the non-DKD control, early DKD, and overt DKD groups in the discovery substudy.

We used principal component analysis (PCA) to aggregate the individual metabolites based on their degree of correlation with each other under the assumption that highly correlated metabolites may be regulated by the same pathway. We focused PCA on the discovery subpopulation because the profile of these DKD individuals was closer to the clinical settings. Principal component (PC) factors were extracted (eigenvalue > 1) by orthogonal rotation procedure with the varimax method. Metabolites with loading values > 0.5 were considered as major loadings in each factor. The associations of PC factor scores with clinical variables and renal function were further studied by multivariable linear regression models. We first entered the score of each factor as dependent variable, respectively, and clinical variables as independent variables to study the association of each PC factor with clinical phenotype (5 models) (Table 2). Next, we studied which PC factors were independently associated with renal filtration function and albuminuria after adjustment for clinical risk factors and other PC factors. Both ACR (log-transformed) and eGFR were entered into the model as dependent variables, respectively. Clinical variables and scores of all 5 PC factors were entered as independent variables (Table 3). Data analysis was performed using SPSS 22.0 for Windows (IBM, Armonk, NY) and R software version 3.2.3 (Comprehensive R Archive Network, Wirtschaftsuniversität Wien, Austria).

RESULTS

Plasma Metabolites That Differed Significantly Between DKD and Non-DKD Controls

Participants with DKD in the discovery subpopulation were of older age and had higher HbA1c, systolic blood pressure (SBP), and triacylglycerol levels as compared with non-DKD controls. They were more likely on insulin and renin-angiotensin system (RAS) blockers. Consistent with the DKD prevalence in Singapore,[21] Malays were overrepresented whereas South Asian participants were underrepresented in DKD groups, especially in the overt DKD group, as compared to their proportions in the non-DKD control group (Table 1). Similar clinical and biochemical profile could be observed in the validation subpopulation (Table 4). There were no significant differences in body mass index (BMI), diastolic blood pressure, use of statin and proportion of current smokers between DKD and non-DKD controls in both subpopulations.

Of the 123 plasma metabolites included in data analysis, 33 differed significantly between DKD and non-DKD controls in discovery substudy (Table S1), and 24 of these 33 metabolites differed significantly in the same direction in the validation subpopulation (Table S2). Of these 24 plasma metabolites, C2, C4, C4-OH, C5-DC, Cer18:1/16:0, GlcCer18:1/18:0, and 5 phosphatidylcholine subspecies levels differed significantly between early DKD and non-DKD controls (Table S1); notably, 19 of them showed a linear trend across the non-DKD, early DKD, and overt DKD groups in the discovery subpopulation (Table S3). As shown in Table 5 and Figure 1, most acylcarnitines with significant differences between DKD and non-DKD had short and medium chains (2–8 carbons) and many of them belonged to the short dicarboxylacylcarnitine subfamily (C3-DC, C4-DC, C5-DC, and C6-DC).[22] Four sphingomyelin–ceramide metabolites (sphingomyelin 18:1/16:1, ceramide 18:1/16:0, glucosylceramide 18:1/18:0, and sphingosine) were elevated in DKD patients as compared to those in non-DKD controls.

Short plasma acylcarnitines can be filtered into urine. Hence, we measured urinary short- and medium-chain acylcarnitine concentrations to examine whether plasma accumulation of these acylcarnitine subspecies

Table 2. Association of metabolite principal component factor scores with clinical and biochemical variables in multivariable linear regression analysis in discovery subpopulation (N = 409)

	Factor 1		Factor 2		Factor 3		Factor 4		Factor 5	
% of Total variance	16.7		13.4		9.5		9.1		8.8	
Main loading	C2, C4-OH, C6, C14:1-OH, C14-OH/C12-DC, C18-OH/C16-DC		PC32:2, PC34:3, PC36:6, PC38:3; PC40:5		C4-DC, C5-DC, C8-OH/C6-DC		Cer18;1/16:0, GlcCer18:1/18:0, SM18:1/16:1		C3, C5, C5:1	
Clinical variables	β	*P* value	β	*P* value	β	*P* value	β	*P* value	β	*P* value
Age (yr)	0.002	0.773	0.007	0.207	0.011	0.035	–0.005	0.299	0.008	0.144
Male sex[a]	0.084	0.428	–0.171	0.100	0.347	0.001	–0.115	0.254	0.168	0.106
Malay ethnicity[b]	–0.243	0.079	–0.043	0.751	–0.131	0.325	0.409	0.002	–0.038	0.780
South Asian ethnicity[b]	–0.004	0.976	0.336	0.020	0.263	0.064	0.020	0.886	–0.236	0.101
BMI (kg/m^2)	0.014	0.222	0.007	0.740	–0.014	0.201	0.003	0.756	0.009	0.407
Diabetes duration (yr)	0.011	0.111	–0.012	0.072	–0.009	0.174	0.007	0.268	–0.008	0.229
HbA_{1c} (%)	–0.049	0.101	–0.057	0.053	0.045	0.124	0.037	0.195	0.062	0.038
HDL cholesterol (mmol/l)	–0.063	0.706	0.286	0.081	0.062	0.699	0.407	0.011	0.164	0.316
LDL cholesterol (mmol/l)	0.000	0.997	–0.001	0.988	–0.056	0.424	0.221	0.001	0.035	0.615
LnTG	0.247	0.031	0.358	0.001	0.035	0.751	0.050	0.643	0.333	0.003
Systolic BP (mm Hg)	0.000	0.903	0.000	0.865	–0.002	0.456	0.001	0.607	–0.004	0.139
eGFR (ml/min/1.73 m^2)	–0.001	0.586	–0.003	0.104	–0.008	<0.0001	–0.002	0.309	–0.004	0.059
LnACR	0.008	0.808	–0.052	0.100	0.006	0.848	0.087	0.005	–0.010	0.745
RAS blocker use (yes)	–0.029	0.799	–0.020	0.859	0.001	0.990	–0.071	0.518	0.167	0.139
Insulin use (yes)	–0.101	0.389	–0.003	0.980	–0.018	0.873	–0.121	0.280	–0.180	0.117

The score of each factor was entered as a dependent variable. All clinical variables were entered as independent variables. BMI, body mass index; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LnACR, natural logarithmically transformed albumin-to-creatinine ratio; LnTG, natural logarithmically transformed triacylglycerol; RAS, renin-angiotensin system.
[a]Female as reference.
[b]Chinese ethnicity as reference.

was due to their reduced renal filtration in participants with DKD. The majority of urinary short and medium-chain acylcarnitines did not differ significantly between DKD patients and non-DKD controls (Table S4). Spearman correlation analysis showed that plasma and urine short- and medium-chain acylcarnitine levels were only modestly to moderately correlated (Table S5). These data, together with the observation that plasma acylcarnitine levels were elevated even in those with preserved renal filtration function (early DKD), suggest that accumulation of short and medium-chain acylcarnitine subspecies in plasma may not be attributed to their reduced renal excretion in DKD.

Cognizant of the difference in designs of discovery and validation substudies, we performed a sensitivity analysis to compare the metabolite levels between non-DKD controls (n = 129) and those with macroalbuminuria (ACR > 300 μg/g regardless of eGFR, n = 67) in a discovery subpopulation after adjustment for age, sex, ethnicity, and diabetes duration. The clinical and biochemical profiles of cases and controls included in the sensitivity analysis (Table S6) were similar to those in the validation subpopulation (Table 4). In agreement with the primary analysis, essential amino acids and phosphatidylcholine levels were lower, whereas short-chain acycarnitine and sphingolipid levels were higher in macroalbuminuric DKD patients. Among 24 metabolites that differed significantly between DKD and controls in 2 subpopulations in the primary analysis, 16 of them also showed statistically significant differences between case patients and controls in the sensitivity analysis ($P < 0.05$ and $q < 0.05$), and another 3 metabolites trended in the same direction ($P < 0.1$ and $q < 0.1$) (Table S7). In another sensitivity analysis, we compared plasma metabolite levels between case patients and controls after adjustment for multiple potential confounders by analysis of covariance. As shown in Table S8, differences in short acylcarnitines and phosphatidylcholine levels between case patients and controls remained statistically significant in both substudies after adjustment for multiple covariates. Interestingly, the differences in ceramide 18:1/16:0 and sphingomyelin 18:1/16:1 levels between controls and macroalbuminuric DKD patients in a validation subpopulation were attenuated after adjustment for lipids profile (HDL cholesterol, LDL cholesterol, and triacylglycerol) in the multivariable model ($P = 0.034$ and $P = 0.025$, respectively, without adjustment for lipid profile).

PCA and Association of Metabolite Component Factors With Clinical Variables

Seven PC factors derived from 24 metabolites by PCA explained 70% of the total variances in the discovery subpopulation. As shown in Table S9, major loadings (loading value > 0.5) in factor 1 were even-number acylcarnitines (C2, C4-OH, C6, C14:1-OH,

Table 3. Association of eGFR and urinary ACR with metabolite component factor scores in discovery subpopulation (N = 409)

Covariate	eGFR		Urinary ACR[a]	
	β	*P* value	β	*P* value
Age (yr)	−0.836	<0.0001	0.020	0.015
Male sex[b]	2.127	0.411	−0.168	0.325
Malay ethnicity[c]	−7.206	0.031	0.401	0.068
South Asian ethnicity[c]	5.728	0.015	−0.547	0.019
BMI (kg/m^2)	−0.466	0.086	0.002	0.909
Diabetes duration (yr)	−0.369	0.027	0.023	0.034
HbA_{1c} (%)	2.708	<0.0001	0.093	0.049
HDL cholesterol (mmol/l)	6.448	0.108	−0.557	0.035
LDL cholesterol (mmol/l)	−1.836	0.289	0.009	0.940
Ln TG	−2.805	0.318	0.114	0.537
SBP (mm Hg)	−0.066	0.331	0.021	<0.0001
Insulin use (yes or no)	−5.176	0.064	0.476	0.010
RAS blocker (yes or no)	−5.786	0.033	0.682	<0.0001
Factor 1 (flux/oxidation imbalance and FAO)	−0.918	0.448	0.021	0.795
Factor 2 (phosphatidylcholine)	−1.819	0.138	−0.108	0.179
Factor 3 (short dicarboxylacylcarnitine)	−5.228	<0.0001	0.071	0.378
Factor 4 (sphingomyelin–ceramide)	−1.866	0.135	0.249	0.003
Factor 5 (amino acid catabolism)	−2.619	0.033	0.010	0.906

Both eGFR and ACR were entered as dependent variables. All clinical variables and scores of 5 principal component analysis factors were entered as independent variables. ACR, albumin-to-creatinine ratio; BMI, body mass index; eGFR, estimated glomerular filtration rate; FAO, fatty acid oxidation; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LnTG, natural logarithmically transformed plasma triacylglycerol; RAS, renin-angiotensin system.
[a]ACR is natural logarithmically transformed.
[b]Female as reference.
[c]Chinese as reference.

Table 4. Clinical and biochemical characteristics of T2DM participants in validation study

	T2DM Control (n = 149)	Macroalbuminuric DKD (n = 149)	*P* value[a]
Age (yr)	57.0 ± 10.2	57.0 ± 10.3	NA
Male sex (%)	62.4	62.4	NA
Diabetes duration (yr)	10.8 ± 7.1	10.9 ± 6.9	NA
Ethnicity (%)			NA
Chinese	71.1	71.1	
Malay	16.1	16.1	
South Asian	12.8	12.8	
Current smoker (%)	11.9	17.2	0.241
BMI (kg/m^2)	26.2 ± 4.4	27.4 ± 5.5	0.061
HbA_{1c} (%)	8.1 ± 1.8	8.8 ± 2.2	0.004
HbA_{1c} (mmol/mol)	65 ± 14	73 ± 18	0.004
Systolic BP (mm Hg)	131 ± 17	144 ± 21	<0.0001
Diastolic BP (mm Hg)	77 ± 10	79 ± 12	0.120
HDL cholesterol (mmol/l)	1.29 ± 0.37	1.19 ± 0.31	0.009
LDL cholesterol (mmol/l)	2.75 ± 0.83	3.09 ± 1.10	0.003
Triacylglycerol (mmol/l, IQR)	1.34 (0.98–1.93)	1.95 (1.28–2.95)	<0.0001
eGFR (ml/min/1.73 m^2)	94 ± 21	62 ± 32	<0.0001
Urinary ACR (mg/g, IQR)	9 (6–18)	861 (463–2098)	<0.0001
Statin use (%)	73.3	78.9	0.312
RAS blocker use (%)	53.1	83.9	<0.0001
Insulin use (%)	24.5	35.6	0.038

ACR, albumin-to-creatinine ratio; BMI, body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; NA, not applicable; RAS, renin-angiotensin system; T2DM, type 2 diabetes mellitus.
[a]Student *t* test or χ^2 test where appropriate.

C14-OH/C12-DC, C18-OH, and C16-DC) that were indicative of incomplete fatty acid oxidation (FAO), substrate flux−oxidation imbalance or increased alternative FAO pathway.[23–27] Main loadings in factor 2 were phosphatidylcholines including PC 32:2, PC 34:3, PC 36:6, PC 38:3, and PC 40:5, whereas the main loadings in factor 4 were metabolites in sphingomyelin−ceramide metabolism pathway, namely, sphingomyelin 18:1/16:1, ceramide 18:1/16:0, and glucosylceramide 18:1/18:0. The main loadings in factor 3 mainly consisted of short-chain dicarboxylacylcarninies that were derived from either ω-FAO or amino acid catabolism (C4-DC, C5-DC, C8-OH/C6-DC).[22,28] In factor 5, the main loadings were C3, C5, and C5:1, which were mainly derived from amino acid catabolism.[19,24,25] As factor 6 and 7 represented only a small proportion of total variances, we focused our further analysis on the first 5 factors.

Multiple linear regression models revealed that PC factor suggestive of substrate flux−utilization imbalance/incomplete FAO and factor for phosphatidylcholine reduction were independently associated with plasma triacylglycerol levels. Notably, only the factor for short dicarboxylacylcarnitine was independently associated with eGFR after adjustment for multiple clinical risk factors. The factor for sphingomyelin−ceramide metabolism was independently associated with urinary ACR as well as HDL and LDL cholesterol levels. The factor indicative of amino acid catabolism was independently associated with both HbA_{1c} and plasma triacylglycerol levels (Table 2).

Association of Renal Filtration Function and Albuminuria Levels With Distinct Metabolite Component Factors in Individuals With T2DM

Next, we examined the relationship of PC factor scores with renal filtration function and albuminuria levels in participants with T2DM. Interestingly, factors indicative of short dicarboxylacylcarnitine accumulation and amino acid catabolism were independently associated with eGFR but not urinary ACR after adjustment for multiple clinical and biochemical variables and mutual adjustment for other component factors. Conversely, the factor for sphingomyelin−ceramide metabolism was independently associated with urinary ACR but not eGFR (Table 3). In an exploratory analysis in a matched case−control validation subpopulation in which participants with "extreme phenotype" were included, metabolites suggestive of short dicarboxylacylcarnitine accumulation and amino acid catabolism (C3, C4, C5, C51:1, C4-DC, C5DC, C5-OH/C3-DC, C6, and C8-OH/C6-DC) were aggregated in the same PC factor (Table S10). Multivariable linear regression showed that it was the strongest variable that was independently associated with eGFR. In agreement with findings in discovery subpopulation, factors for ceramide metabolism

Table 5. Plasma metabolites that differed significantly in discovery and validation subpopulations in the same direction

	Discovery substudy					Validation substudy			
	T2DM Control (n = 129)	Early DKD (n = 126)	Overt DKD (n = 154)	*P* value	q Value	T2DM Control (n = 149)	Overt DKD (n = 149)	*P* value	q Value
Serine (μmol/l)	106 (91–123)	108 (91–123)	97 (85–114)	0.022	0.017	113 (101–132)	111 (89–128)	0.0018	0.00313
C2 (μmol/l)	3.2 (2.6–4.0)	3.7 (2.7–4.6)	3.8 (2.9–5.0)	0.001	0.007	2.8 (2.3–3.6)	3.5 (2.9–4.6)	4.57E-6	1.29E-5
C3 (nmol/l)	204 (144–243)	218 (165–276)	228 (175–279)	0.043	0.027	202 (157–253)	231 (183–289)	8.60E-4	1.58E-3
C4 (nmol/l)	100 (74–127)	122 (87–189)	143 (103–197)	0.001	0.007	106 (82–135)	142 (104–237)	1.62E-9	2.18E-8
C4-OH (nmol/l)	13 (9–18)	17 (12–25)	18 (12–24)	4.38E-4	0.007	12 (8–16)	16 (10–23)	2.07E-4	4.02E-4
C5 (nmol/l)	45 (33–54)	49 (37–60)	53 (42–69)	0.017	0.014	46 (34–54)	57 (45–75)	1.27E-09	6.99E-9
C4-DC (nmol/l)	14 (12–18)	16 (13–21)	20 (16–25)	1.03E-4	0.006	14 (11–16)	20 (15–28)	9.00E-18	1.49E-16
C5:1 (nmol/l)	10 (7.8–13)	10 (8.2–13)	13 (10–17)	0.001	0.007	9.3 (7.3–11.0)	11.4 (9.0–14.6)	2.32E-8	9.57E-8
C5-DC (nmol/l)	26 (21–30)	28 (22–33)	30 (24–37)	0.009	0.010	20 (17–24)	28 (22–39)	1.50E-15	1.65E-14
C5-OH/C3-DC (nmol/l)	18 (14–21)	20 (15–23)	22 (18–31)	0.001	0.007	13 (11–15)	20 (14–25)	1.50E-19	6.60E-18
C6 (nmol/l)	37 (31–48)	43 (32–57)	48 (38–65)	0.003	0.008	32 (25–39)	45 (31–60)	9.95E-11	6.57E-10
C8-OH/C6-DC (nmol/l)	24 (20–35)	30 (20–42)	36 (26–51)	0.001	0.007	23 (18–32)	38 (23–60)	1.01E-11	8.33E-11
C14:1-OH (nmol/l)	6.0 (5.0–7.7)	6.5 (5.1–8.9)	7.2 (5.4–9.4)	0.045	0.028	5.4 (4.2–7.1)	6.1 (4.6–7.8)	0.038	0.0522
C14-OH/C12-DC (nmol/l)	4.6 (3.5–5.3)	4.9 (3.9–6.7)	5.3 (3.9–6.7)	0.016	0.014	3.7 (2.8–4.8)	4.4 (3.2–5.6)	0.019	0.0285
C18-OH/C16-DC (nmol/l)	2.5 (1.9–2.9)	2.9 (2.1–3.6)	2.7 (2.0–3.5)	0.023	0.017	2.5 (1.9–3.4)	2.9 (2.2–3.8)	0.007	0.0116
Cer18:1/16:0 (nmol/l)	244 (198–297)	277 (229–330)	272 (224–334)	0.005	0.009	245 (202–288)	271 (233–342)	9.38E-5	2.06E-4
GlcCer18:1/18:0 (nmol/l)	61 (48–81)	80 (63–103)	75 (58–111)	1.48E-4	0.006	63 (47–79)	68 (51–103)	0.029	0.0416
SM 18:1/16:1 (μmol/l)	14 (12–16)	14 (12–17)	15 (13–18)	2.52E-4	0.006	9.2 (7.6–10.7)	9.8 (8.3–11.4)	1.02E-4	2.10E-4
Sphingosine (nmol/l)	23 (16–36)	26 (17–37)	26 (17–42)	0.041	0.026	27 (16–47)	28 (19–80)	0.009	0.0141
PC 32:2 (μmol/l)	2.5 (2.0–3.3)	2.2 (1.6–2.9)	2.2 (1.6–2.9)	0.005	0.009	1.0 (0.8–1.4)	0.8 (0.5–1.1)	7.58E-8	2.52E-7
PC 34:3 (μmol/l)	8.7 (7.0–10.5)	7.8 (6.1–9.8)	7.9 (6.2–10.3)	0.031	0.021	3.4 (2.4–4.7)	2.5 (2.0–3.4)	1.82E-6	5.46E-6
PC 36:6 (μmol/l)	0.5 (0.4–0.6)	0.4 (0.3–0.6)	0.5 (0.4–0.6)	0.007	0.010	0.4 (0.3–0.5)	0.3 (0.2–0.4)	3.82E-5	9.69E-5
PC 38:3 (μmol/l)	21 (16–27)	19 (13–24)	20 (16–26)	0.025	0.019	13 (10–17)	10 (7–13)	7.63E-8	2.52E-7
PC 40:5 (μmol/l)	5.0 (3.8–6.1)	4.4 (3.2–5.9)	4.8 (3.7–6.1)	0.040	0.024	3.9 (3.1–4.8)	3.3 (2.6–4.1)	8.27E-5	1.95E-4

P values in discovery study have been adjusted for age, sex, and ethnicity by analysis of covariance. GlcCer, glucosylceramide; SM, sphingomyelin; PC, phosphatidylcholine; T2DM, type 2 diabetes mellitus.

(ceramide 18:1/16:0, glucosylceramide 18:1/18:0, and sphingosine) were independently associated with urinary ACR but not eGFR (Table S11). Similarly, in the sensitivity analysis, component factors for short dicarboxylacylcarnitines were independently associated with eGFR ($P = 0.001$) but not ACR ($P = 0.847$), whereas component factors for sphingomyelin–ceramide metabolism were associated with ACR ($P = 0.019$) but not eGFR ($P = 0.202$) after adjustment for multiple clinical covariates and scores of other component factors (data not shown).

DISCUSSION

There are several novel findings in this targeted plasma metabolomics study. First, profiling of plasma acylcarnitines revealed evidence for incomplete FAO and intriguingly, accelerated catabolism of amino acids in individuals with T2DM and kidney disease. Second, a reduction in plasma phosphatidylcholine levels and elevation of long-chain sphingomyelin and ceramide levels suggested remodeling of sphingolipids in T2DM with DKD. Finally, we found that impaired renal filtration function and albuminuria levels were associated with different metabolite signatures.

Targeted metabolomics provides an excellent survey of energy-yield pathways and fuel substrate selection.[5] In addition to incomplete FAO as indicated by an elevation in β-oxidation–derived even-number acylcarnitines (Table 5 and Figure 1), our data point to an accelerated catabolism of amino acids, especially aromatic amino acids, in patients with DKD.[23–25] This is suggested by elevated levels of amino acid–derived acylcarnitines (C3, C5, C3-DC, C4-DC, C5-DC, and C6-DC) and lower plasma levels of tyrosine and tryptophan in participants with DKD ($P = 0.002$ and $P = 0.014$ in an unadjusted model, borderline significance after adjustment for age, sex, and ethnicity) (Table S1). The depletion of aromatic amino acids has been reported by other studies in both diabetic and non-diabetic populations with chronic kidney diseases.[6,11,29] It is worth noting that the flux of amino acids and its oxidation in mitochondria occur only under special circumstances such as aerobic physical exercise.[30,31] Therefore, the accelerated amino acid catabolism in DKD is intriguing, and the sensory signals that trigger amino acid flux and oxidation in these patients remain largely unknown. On the other hand, a large body of evidence has shown that excess intracellular and circulating acylcarnitines may exacerbate metabolic disturbance and lead to cytotoxicity.[24,32,33] Hence, it is conceivable that accumulation of FAO and amino acid–derived metabolic

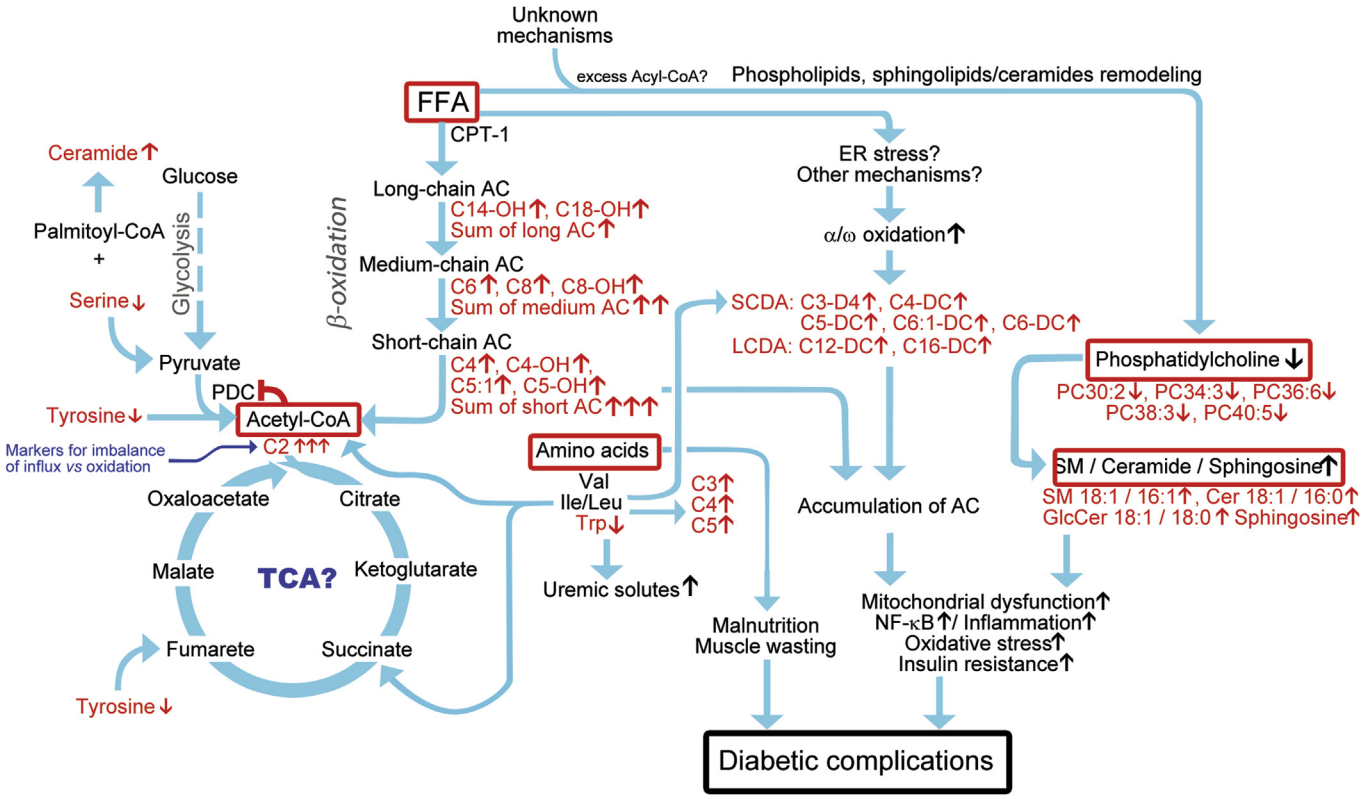

Figure 1. Plasma metabolites that differed significantly between diabetic kidney disease (DKD) patients and non-DKD controls in the same direction in both discovery and validation subpopulations (highlighted in red) and the potential metabolic pathways associcated with DKD. AC, acylcarnitine; CPT-1, carnitine palmitoyltransferase I; ER, endoplasmic reticulum; FFA, free fatty acid; LCDA, long-chain dicarboxylic acid; NF-κB, nuclear factor−κB; PDC, pyruvate dehydrogenase complex; SCDA, short-chain diacids; SM, sphingomyelin; TCA, tricarboxylic acid cycle.

intermediates in plasma may play a role in the progression of DKD (Figure 1).

Another metabolic feature of DKD observed in the current study is the elevation of a cluster of dicarboxyl and hydroxyl acylcarnitines in plasma (Table 5 and Figure 1). Increased formation of dicarboxyl and hydroxyl dicarboxylic acids via ω- FAO and ω-1 FAO and their conversion to the corresponding acylcarnitines may be attributed to acyl-CoA accumulation in DKD.[34] ω-Oxidation is a rescue pathway when mitochondrial fatty acid oxidation is impaired.[27,34] A recent study by Kraus *et al*. indicated that endoplasmic reticulum stress might elevate short-chain diacid levels.[22] Thus, accumulation of short- and long-chain dicarboxylacylcarnitines may indicate mitochondrial stress and/or endoplasmic reticulum stress that may lead to activation of alternative FAO pathways in DKD.

Accumulation of acetyl-CoA−derived C2 (acetylcarnitine), as observed in our study, points to a mismatch between energy substrate flux and its utilization in DKD. However, due to the observational nature of our study, we are unable to elucidate whether the imbalance is due to accelerated flux or a reduced downstream utilization, or both. A strong body of evidence supports that mitochondrial function is markedly impaired in patients with DKD. A decrease in electronic transport chain activities accompanied by an overall reduction in mitochondrial content and PGC-1α expression has been found in diabetic kidney.[9,35] Similar defects have been observed in diabetic islet, heart, and skeletal muscle.[36] Therefore, impaired mitochondrial function may at least partially explain the substrate flux/oxidation imbalance in DKD. On the other hand, insulin resistance and other related metabolic disturbance in DKD may impair mitochondria and restrict its ability to select glucose as preferred substrate for oxidation (metabolic inflexibility).[35,37] Therefore, the increased flux of fatty acids and amino acids into mitochondria may be a compensation for impaired glucose utilization.[38] Notably, fuel supply and oxidation may not necessarily be reduced in the presence of mitochondrial dysfunction. For instance, frank mitochondrial dysfunction in humans (e.g., myopathies with impaired electron transport chain activity) is accompanied by increased fuel flux and oxidation to compensate for the impaired ATP synthesis.[39] Taken together, we postulate that both impaired mitochondria function and energy substrate oversupply in individuals with T2DM may be involved in intramitochondrial metabolic intermediates accumulation and their extracellular overflow to circulation. A future *in vivo* tracing study by isotope-labeled

substrates may provide insights into substrate flux, selection, and oxidization in DKD.

We found that long-chain sphingomyelin and ceramides are elevated in participants with DKD, suggesting that the sphingomyelin–ceramide pathway may play a part in renal complications. The underlying pathological mechanisms may involve impairment of mitochondria function, enhanced generation of reactive oxygen species, activation of apoptotic pathway, elevation of inflammation tone, and endoplasmic reticulum stress.[40] The latter may contribute to the aforementioned overproduction of short-chain diacids and long-chain dicarboxylacylcarnitines in DKD.[22] The observed pattern of reduction in phosphatidylcholine and increment in sphingomyelin levels may suggest sphingolipid remodeling in DKD, as sphingomyelin can be synthesized from phosphatidylcholine by cholinephosphotransferase action.[41]

Another novel finding in our current study is the association of renal filtration function and albuminuria levels with distinct metabolite signatures. Early studies have found that both eGFR and ACR are independent predictors of CKD progression.[42] In addition, genetic studies have found little overlap between loci of eGFR and ACR.[43] Association of sphingomyelin with albuminuria has been reported in diabetic patients, and inhibiting conversion of sphingomyelin to ceramide might protect kidney function and reduce albumin excretion in animal models.[14,44,45] Interestingly, we found that metabolite signature for sphingomyelin–ceramide metabolism was independently associated only with albuminuria but not with renal filtration function in T2DM. These data echo findings from an early study showing that polymorphism of ceramide synthase 2 (CerS2) was associated only with changes in albuminuria but not eGFR in patients with diabetes.[46] Our data reinforce the notion that albuminuria and decline of filtration function may represent complementary, if overlapping, manifestations of kidney damage.[47]

An inverse association of acylcarnitine levels with eGFR has been reported in nondiabetic populations.[13] Our current study extended the early findings by showing that among many acylcarnitine subspecies that differed significantly between DKD versus non-DKD controls, only short-chain diacids and amino acid–derived acylcarnitines were strongly and independently associated with eGFR (Tables 2 and 3). This finding is principally agreeable with findings from an early, small study that reported that amino acid–derived acylcarnitines predicted progression to ESRD in T2DM.[11]

Our study has several strengths. Multiple uncontrolled factors may contribute to heterogeneity of metabolomic profile. We included 2 subpopulations with relatively large sample sizes to partially address the inherent shortcoming of a metabolomics study. In addition, the coverage of metabolites in our study is broader than that in early reports. These strengths have enabled us to identify several novel pathophysiological features associated with DKD. Nevertheless, several weaknesses should be mentioned, as follows: (i) This is a cross-sectional study in its design. Our data can show association but not causality. (ii) Some important clinical variables such as physical activities, diet, and comorbidities were not considered in our current study. Although the inclusion of 2 independent subpopulations may partially address this concern, validation of our findings by other independent studies is warranted. (iii) We measured urinary albumin level only once. It has been known that the day-to-day variation of albuminuria level is high.[2] (iv) Due to technical limitations, we are not able to separate the isomeric species including C8-OH/C6-DC, C14-OH/C12-DC, and C18-OH/C16-DC. An early study showed that the signals of these isomers come mainly from branched-chain dicarboxylacylcarnitines.[22] (v) Although targeted metabolomics provides accurate metabolite identification and quantification, it can cover only a subset of the human metabolome. Nontargeted metabolomics is needed to complement our findings. Finally, we would highlight that participants included in a validation substudy had "extreme phenotype" in terms of DKD phenotype. Therefore, data obtained from this subpopulation should be interpreted with caution. In addition, clinical characteristics of participants and the loadings of factors are different in discovery and validation subpopulations (Tables 1 and 4, Tables S9 and S10). Hence, direct comparisons of factors derived from these 2 subpopulations are not relevant.

In conclusion, our targeted plasma metabolomics study implicates altered energy substrate selection, imbalance of substrate flux and utilization, as well as remodeling of sphingolipids in T2DM with DKD. The association of distinct plasma metabolite signatures with eGFR and albuminuria suggest different pathophysiological processes underlying these 2 related manifestations of DKD in individuals with T2DM.

DISCLOSURE

SAS, a cofounder and consultant for Centaurus Therapeutics, receives grants from the company for research of drugs targeting sphingolipids pathway. All the other authors declared no competing interests.

ACKNOWLEDGMENTS

This study was supported by Singapore Stratified Medicine Programme Office grant (SMPO201401). We thank Mr Billy Aswin, Ms Babitha Jeevith, Mr Melvin Wong, Ms

Sylvia Liu, and Dr Aik Kia Khaw from Khoo Teck Puat Hospital Singapore for assistance with participant recruitment, data input, participant selection, and plasma/urine sample preparation. We thank Mr Melvin Wong for assistance with language editing for the manuscript. We also thank Dr Aik Kia Khaw for drawing the metabolic pathway illustration (Figure 1).

SUPPLEMENTARY MATERIALS

Supplementary Methodology. Metabolites Extraction and Quantification.

Table S1. Plasma metabolite levels in participants with type 2 diabetes mellitus (T2DM) alone (controls), early diabetic kidney disease (DKD), and overt DKD in discovery substudy.

Table S2. Plasma metabolite levels in participants with macroalbuminuria (cases) and those with type 2 diabetes mellitus (T2DM) alone (controls) in validation substudy.

Table S3. Jonckheere–Terpstra tests for linear trend of plasma metabolites across non–diabetic kidney disease (DKD) controls, early DKD patients, and overt DKD patients.

Table S4. Urinary short- and medium-chain acylcarnitine levels in type 2 diabetes mellitus (T2DM) participants.

Table S5. Correlation of plasma and urine short- and medium-chain acylcarnitines by Spearman correlation analysis.

Table S6. Clinical and biochemical characteristics of type 2 diabetes mellitus (T2DM) participants in sensitivity analysis (T2DM control versus macroalbuminuric T2DM in discovery subpopulation).

Table S7. Plasma metabolite levels that differed between case patients and controls in sensitivity analysis.

Table S8. Comparison of plasma metabolite levels between case patients and controls after adjustment for multiple covariates by analysis of covariance.

Table S9. Loading of principal component factors derived from discovery subpopulation.

Table S10. Loading of principal component factors derived from validation subpopulation.

Table S11. Association of estimated glomerular filtration ratio (eGFR) and urinary albumin-to-creatinine ratio (ACR) with principal component factor scores in validation subpopulation.

Supplementary material is linked to the online version of the paper at www.kireports.org.

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