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Targeted metabolomics as a tool for the diagnosis of kidney disease in Type II diabetes mellitus

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ABSTRACT

Background: Diabetic kidney disease (DKD) is an increasing health problem and an extra burden to health services. The study of characteristic metabolic alterations of DKD is crucial for a better understanding of pathogenesis to identify new potential biomarkers and drug targets. We hypothesized that metabolic profiling of amino acids, acylcarnitines, and organic acids are useful new biomarkers for the diagnosis of the early stages of DKD

Methods: The hypothesis was testing in a case-control study of 232 patients with type 2 diabetes mellitus and 150 healthy controls. Patients were classified according to urinary albumin and estimated glomerular filtration rate (eGFR) into 100 with normoalbuminuria and 132 with microalbuminuria group. Eighteen AcylCNs and 17 amino acids were measured in the blood by tandem mass spectrometry while 17 urinary organic acids were quantitatively measured by gas chromatography – mass spectrometry.

Results: Regression analysis found that dodecanoylcarnitines C12 (effect size 2.03 [95%CI 1.73–2.32]), triglylcarnitine C5:1 (2.01 [1.70–2.30]), and isovalerylcarnitine C5 (1.78 [1.48–2.07]) were stronger predictors of albumin/creatinine ratio than HbA1c (1.50 [1.20–1.78]) and hence they could serve as potential biomarkers for the diagnosis of the early stages of DKD.

Conclusions: Targeted metabolic profiling offers a new, non-invasive approach for detecting biomarkers for the early diagnosis of DKD suggesting new pathogenetic phases that might be new targets for treatment.

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Acylcarnitines; albuminuria; diabetes mellitus type 2; diabetic kidney disease; metabolomics; urinary organic acids

Introduction

Diabetic Kidney disease (DKD) is an important cause of end-stage renal disease (ESRD) with expected global increase in its prevalence reaching 44% by 2030 [1]. It is characterized by increased urinary albumin excretion with an enhanced rise in proteinuria and a drop in estimated glomerular filtration rate (eGFR) in the absence of other renal diseases [2,3]. Renal function may also be determined by the albumin to creatinine ratio (ACR), although affected by the muscle mass and the physical activity due to the variable creatinine excretion in male and female [4], and the equations such as the Modification of Diet in Renal Disease (MDRD) used for the estimation of GFR. However, the MDRD is suitable only for patients with a GFR level less than 60 mL/min/1.73 m^2 [5], and there are several drawbacks to the use of urine albumin [5–7], leading to the need for better markers [8].

DKD is a consequence of a complicated interaction among metabolic, inflammatory and hemodynamic changes with the involvement of energy pathway-related metabolites such as the fatty acids and Krebs cycle intermediates [9]. As the early detection and management of DKD leads to a reduction in the risk of kidney damage by as much as 50%, the ability to detect asymptomatic renal dysfunction is crucial in minimising DKD progression [10].

Acylcarnitines (AcylCNs) consist of an acyl group esterified to carnitine, allowing crossing of long-chain fatty acids through the mitochondria membrane for β-oxidation [11]. They also participate during the branched-chain amino acids catabolism, with acyl-CoA intermediate status through carnitine acyltransferases [12]. Organic acids are intermediate metabolites in certain critical metabolic pathways involved in carbohydrate, lipid, and protein metabolism, with the Krebs cycle and fatty acid β-oxidation gaining increasing interest in assessing the health status, pathogenesis and development of many diseases, including DKD [13].

The use of a single biomarker such as HbA1C or eGFR alone, or in combination, may not be adequate in recognising the subtle pathogenic pathways underlying complex diseases such as DKD [14]. With advances such as mass spectrometer dependent metabolomics, it is possible to identify and quantify *in vivo* metabolites with molecular mass <1.5 KDa and so develop a profile of biomarkers for certain disease or

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diseases [15]. Additionally, if metabolites are pathognomic, they might also be new targets for treatment [16]. However, although metabolomics have discovered pathways possibly related to DKD development and progression, targeted blood and urine metabolomics studies are limited [17].

We hypothesised that the metabolic profiling of amino acids, AcylCNs and organic acids can provide superior definition of T2DM patients with normo-albuminuria and micro-albuminuria compared to HbA1C.

Patients and methods

The institutional research board of Menoufia University school of medicine approved the study (11/2018 INTM) and written informed consent obtained from all participants. This study took place from December 2018 to December 2019. It enrolled 232 patients with early DKD (urinary albumin-to-creatinine ratio [ACR] <300 mg/g and eGFR ≥ 60 ml/min/1.73 m2) [18] classified into two groups; 100 with normo-albuminuria (<30 mg/g) and 132 with microalbuminuria (30 to 300 mg/g). The study also enrolled 150 non-diabetic healthy subjects as a control group. The MDRD was used to estimate the GFR (eGFR) [19]. Exclusion criteria were macro-albuminuria ≥ 300 mg/g (overt DKD) and renal diseases such as chronic glomerulonephritis or insulin-dependent type one DM.

Sample collection: Five millilitres of fasting venous blood collected from all participants and divided into two samples; 3 ml was collected in a plain vacationer, centrifuged, and the resulting sera were used for biochemical investigations. Two ml was collected into EDTA and centrifuged as soon as possible; plasma was separated within 10 min of collection and used immediately for HbA1c (Sysmex XT-1800i, Japan). Fasting blood glucose (FBG), 2 hours postprandial blood sugar (2HPPBS), lipid profile [total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C); high-density lipoprotein cholesterol (HDL-C)] and creatinine in blood were measured by the Beckman Coulter (Synchron CX 9 ALX) Clinical Auto analyser (Beckman Instruments, Fullerton, California, USA). Blood spots obtained by a sterile puncture in the thumb and spotted on the filter paper (Guthrie card, GE Healthcare, NJ, USA), left to dry on a clean surface, then stored at −80 C°. Urine samples were collected in a sterile plastic container for measuring urine creatinine and albumin (Beckman Instruments, Synchron CX 9 ALX, Fullerton, CA, USA) for albumin to creatine ratio (ACR) [20]. Some was stored immediately at −80°C till analysis of organic acids using GC/MS.

Chemicals and reagents: Component of MassChrom® Amino acids and Acylcarnitines were from Chromsystems Instruments & Chemicals GmbH, München, Germany. Pentadecanoic acid (PDA) was obtained from across organics (New Jersy, USA). N, O-bis-(trimethylsilyl)–trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS) was from SUPELCO, Bellefonte Pennsylvania, USA, and used as derivatizing reagents. The solvent was of HPLC Grade; methanol was purchased from Fisher Scientific (Loughborough, U.K.). All other chemicals and standards were purchased from Sigma-Aldrich (Fluka, St.Louis, Mo, USA).

AcylCNs and amino acids assay by MS/MS: Previously reported method [21] was used with modifications. Briefly, 3 mm of the dried blood spot disk punched into a well of the v-bottomed plate, containing 100 µl of lyophilized internal standard reconstituted with 25 ml Extraction Buffer. The plate was sealed with a protective sheet and agitated at 600 rpm for 20 min at room temperature. The supernatant moved to a new v-bottomed well plate and covered by aluminiuman foil sheet. Ten µl of the elute injected into the MS/MS system (Acquity UPLC H-Class. Water Corporation, MA, USA) at a two-min interval in a flowing stream of 80% acetonitrile at a flow rate of 200 µl/min and reduced to 20 µl/min in 0.25 min. The flow rate increased to (600 µl/min in 1.25 min) then decreased again to (200 µl/min). The scan time of the MS/MS system was 1.25 min. The obtained spectra of all analytes analysed with multiple reactions monitoring (MRM) mode. Quantitative analysis (expressed as µmol/l) achieved using Neolynx software (Neolynx Inc., Glendale, CA, USA) by comparing the signal intensity of an analyte against the corresponding internal standard.

Quantitative urinary organic acid assay by GC/MS: Frozen urine samples incubated at 37°C for 15 min, vortexed for 15 sec and urine creatinine levels adjusted to 1 µMol creatinine [22]. Extraction and derivatization of the urine samples performed according to the previously reported method [23] with some quantitative modifications. All standards were prepared in stock solutions after obtaining the molecular mass of each organic acid. The internal standard (PDA) stock solution was prepared by dissolving 48.8 mg in 100 ml of absolute methanol. One µl aliquot of derived sample injected in spitless mode into the Agilent 7890 GC system supported by a 30.0 m × 0.25 mm i.d. fused-silica capillary column with 0.25 µm HP-5 MS stationary phase (Agilent, USA), with the injector temperature at adjusted at 250°C. Helium used as a carrier gas at a flow rate of

1 ml/min through the column. The column temperature primarily kept at 80°C for 2 min and then increased to 280°C by 4°C/min, then hold for 3 min, and the run time for 55 min. The column effluent introduced into the ion source of an Agilent 5975 mass selective detector (Agilent Technologies). The MS quadruple temperature fixed at 150°C and the ion source temperature at 230°C. Turning on the acceleration voltage after a solvent delay of 3 minutes Followed by attaining masses from 50 to 550 m/z. Calibration linear graphs were created by plotting the linear regression of the peak area ratio of the analytes to the internal standard (IS) at seven concentration levels. For quantitation purposes, selective ion mentoring (SIM) mode was applied. Auto-acquisition of GC total ion chromatograms (TICs) and fragmentation patterns were done by GC/MSD ChemStation Software (Agilent, USA). Data were expressed as μM/mM creatinine.

Sample size: Using G Power 3.1, sample size was calculated for linear regression analysis with power = (1-β) = 0.95 and CI95% and it was estimated to be 220 patients. Accounting for a drop-out of 10%, the sample size was increased to 244 patients. From 244 patients, 232 completed the study with a response rate of 95%. Healthy controls were 150 subjects recruited from the hospital and relatives of the patients matched for age, sex, residence and socio-economic standard as controls. For correlation analysis, the sample size was estimated at power 80%, and 90% to be 84 and 112 respectively. We calculated the sample size at a level of 95% based on regression analysis (n = 232) which at the same time cover and exceeds the sample required for correlation (n = 112 at 90%) [24,25].

Statistical analysis: Results were statistically analysed by SPSS version 22(SPSS Inc., Chicago, IL, USA). Tests of normality were performed. An independent sample t-test was used for parametric data. Mann-Whitney test was used for non-parametric data. Chi-Squared ($\chi 2$) test was used for qualitative variables. Linear trend analysis using the Jonckheere-Terpstra Test was applied to detect whether there was an increasing or decreasing trend across the ordered groups. Spearman correlation determined the strength and direction of the association between variables. Correlation was considered significant if r >0.30 [26]. Effect Size for Multivariate linear regression analysis determined the independent predictors of various amino acids, acylcarnitines and organic acids in patients with (normo and micro) albuminuria. Multiple comparisons were tested using Holm-Bonferroni Sequential Correction. The p values <0.05 are statistically significant after this correction.

Results

Demographic and laboratory characteristics of the patients and controls are shown in Table 1. The two groups were matched for age and sex, and all other indices (except triglycerides) were, as expected, significantly different. One hundred and four patients were on dyslipidemic drugs, 33 on blood pressure lowering drugs. Mean (SD) creatinine in those with normal- and microalbuminuria were 76 [5] and 115 [14] respectively. Similarly, eGFR was 92 [10] and 64 [11], and ACR (median/IQR) was 16 [14–24] and 145 (99–180). These three sets of data all give a linear trend estimate with levels in the healthy controls of $p < 0.001$.

Table 2 shows data of the 17 amino acids. In linear trend estimation, most were highly significant, the most significant were (in order) arginine, valine, phenylalanine:tyrosine and citrulline. Table 3 shows data of 18 AcylCNs, and again, almost all showed a significant trend, the most significant being (in order) decanoylcarnitine (C10), propionylcarnitine (C3), propionylcarnitine: acetylcarnitine (C3:C2) and decenoylcarnitine (C10:1). Table 4 shows data on the urinary organic acids with many showing very significant linear trends, the most significant being lactic acid, 2- and 3-hydroxybutric acid and 5-hydroxyhexanoic acid.

On univariate analysis between Albumin/creatinine ratio (ACR) and HbA1c, eGFR, Blood amino acid, acylcarnitines, and urinary organic acids levels in the diseased groups, the highest correlations were reported with 2hydroxy butyric acid (r = 0.90), Sebacic (r = 0.81), Adipic (r = 0.78), Suberic (r = 0.78) and L-pyroglutamic acid (0.78) while lower values were reported with HbA1c (r = 0.57) and eGFR (r = −0.68). The effect size (95% CI) of key analytes in predicting DKD by multivariate regression analysis were C12 2.03 (1.73-2.32), C5:1 2.01 (1.70-2.30), C5 1.78 (1.48-2.07), HbA1c 1.50 (1.20-1.78), C5DC 1.12 (0.41-1.72), suberic acid 1.0 (0.70-1.29), methionine 0.78 (0.48-1.07), tiglyl-glycine 0.59 (0.29-0.89), c0 0.52-0.22-0.82), adipic acid 0.37 (0.07-0.66), L-pyroglutamic 0.35 (0.05-0.64), arginine 0.33 (0.03-0.62), 2-hydroxybutric acid 0.23 (0.06-0.52), lactic acid 0.22 (0.07-0.52) and citric acid 0.15 (0.14-0.44).

Discussion

Changes in amino acids, acylcarnitines and organic acid metabolites have been detected in some genetic disorders such as aminoacidopathies, fatty acid oxidation disorders and organic acidurias by using MS/MS of dried blood spots and GC/MS of urine samples [27]. We performed targeted, quantitative metabolic profiling of a few sets of these metabolites diabetic patients with normo and micro albuminuria for identifying more specific and sensitive markers for the early diagnosis of diabetic kidney disease.

Table 1. Demographic and laboratory characteristics of the patients and healthy controls.

	Groups	
	Controls (n = 150)	Patients (n = 232)
	Mean ± SD	Mean ± SD
Age (Years)	56.8 ± 3.9	56.1 ± 3.9
Sex: Male/Female	72/78	115/117
BMI (kg/m^2)	21.4 ± 0.9	30.0 ± 4.1
SBP (mm Hg)	119 ± 9	130 ± 8
DBP (mm Hg)	74 ± 6	80 ± 6
DM Duration (y)	-	6.1 ± 2.2
HbA1 c%	4.7 ± 0.3	8.0 ± 0.8
Glucose (mmol/l)	4.5 ± 0.3	8.6 ± 0.5
2hPPBG (mmol/l)	6.0 ± 0.7	12.7 ± 2.2
Cholesterol (mmol/l)	3.7 ± 0.3	4.7 ± 0.4
Triglycerides (mmol/l)	1.32 ± 0.06	1.30 ± 0.17
HDL-C (mmol/l)	1.55 ± 0.19	1.35 ± 0.14
LDL-C (mmol/l)	1.7 ± 0.4	2.7 ± 0.3
Creatinine (µmol/l)	64 ± 6	98 ± 10
eGFR (mL/min/1.73 m^2)	119 ± 10	80 ± 10
ACR (mg/g)	9 (6–11)	80 (56 – 102)

BMI: Body Mass Index, (ACR): albumin to creatinine ratio, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, 2hPPBG: 2-hour post-prandial blood glucose. Data is mean/SD or median/IQR. All differences p < 0.001 except triglycerides p = 0.08.

Sustained hyperglycaemia in diabetes encourages fatty acid synthesis and triglycerides build-up causing lipid accumulation in ectopic non-adipose tissues playing a role in the pathogenesis of DKD [28,29]. Altered blood and muscle amino acid profile in DKD may be a cause of chronic inflammation and oxidative stress, leading to progressive renal disease so they have been proposed as nutritional markers for early renal dysfunction in diabetic patients [30]. In early DKD groups, the blood concentrations of the amino acids arginine, citrulline, and ornithine showed an increasing trend, most so by arginine. We speculate these may be related to increased levels of blood urea nitrogen in patients with early stages of renal insufficiency and thus enhanced urea cycle activity might be beneficial in cases of diabetic kidney disease [12]. Additionally, the current study revealed that the blood levels of valine and leucine:alanine significantly increased in linear trend over the three groups. This was in line with the study of Chuang et al [13] due to reduced take-up of branched chain amino acids, especially valine, into the muscles due to insulin resistance and decreased expression levels of adipose-tissue enzymes [31]. Regarding aromatic amino acids, phenylalanine and tyrosine showed a moderately increasing trend, as increased phenylalanine levels are related to higher macrovascular risk and mortality [32]. Raised concentrations of branched chain and aromatic amino acids were detected as predictors of diabetic kidney disease in a longitudinal liquid chromatography mass spectrometry (LC-MS)-based plasma metabolomic study [15].

Numerous studies have documented altered plasma AcylCNs in T2DM patients with various stages of albuminuria [12,33]. The present study showed that the short-chain acylcarnitines (C2, C4) showed a moderately increasing trend, while medium-chain acylcarnitines (C8 and C10) had a highly decreasing trend especially C10, additionally, the long-chain acylCNs (C18:1) showed a moderately decreasing trend as there is a mismatch between energy substrate flux and its consumption due to impaired mitochondrial function and initiation of alternative ω- FAO as it was reported in Liu et al [18] playing a role in the development and progression of DKD [34].

Table 2. Levels of blood amino acids in controls and patients.

	Groups				
Blood amino acids (µmol/l)	Controls (n = 150)	Normoalbuminuria (n = 100)	Microalbuminuria (n = 132)	Linear trend analysis test	*p value*
Arginine	5.7 ± 2.6	10.9 ± 2.8	18.2 ± 5.2	18.35	<0.001
Valine	58.4 ± 14.5	72.5 ± 15.1	83.9 ± 17.7	11.60	<0.001
Phe:Tyr	0.9 ± 0.2	1.2 ± 0.1	1.2 ± 0.3	10.39	<0.001
Citruline	19.7 ± 7.3	22.5 ± 5.6	28.9 ± 9.1	8.89	<0.001
Leu: Ala	0.34 ± 0.1	0.40 ± 0.1	0.48 ± 0.2	8.85	<0.001
Glu:cit	2.7 ± 1.6	2.4 ± 1.3	1.7 ± 0.7	7.19	<0.001
Aspartate	51.2 ± 17.2	63.8 ± 19.6	67.6 ± 24.4	7.07	<0.001
Cit: phe	0.35 ± 0.09	0.43 ± 0.12	0.48 ± 0.16	6.89	<0.001
Ornithine	95 ± 25	110 ± 18	119 ± 29	6.85	<0.001
Tyrosine	41.9 ± 11.6	45.1 ± 11.8	52.2 ± 14.6	6.77	<0.001
Phenylalanine	33.8 ± 8.5	37.5 ± 4.0	41.5 ± 9.6	6.31	<0.001
Glutamate	102 ± 33	166 ± 54	132 ± 36	6.14	<0.001
Leu: Ile	85.7 ± 16.8	63.5 ± 16.5	75.3 ± 14.9	4.61	<0.001
Proline	90 ± 26	84 ± 18	106 ± 29	4.06	<0.001
Alanine	210 ± 66	178 ± 21	184 ± 42	3.84	<0.001
Gly:Ala	0.66 ± 0.18	0.71 ± 0.21	0.73 ± 0.22	2.97	0.003
Met:ph	0.11 ± 0.03	0.11 ± 0.03	0.10 ± 0.02	2.32	0.020
Leu:Phe	1.6 ± 0.3	1.6 ± 0.3	1.7 ± 0.4	2.14	0.032
Methionine	5.3 ± 1.5	4.1 ± 1.1	6.1 ± 2.1	1.70	0.087
Glycine	103 ± 32	128 ± 28	99 ± 20	0.67	0.500

Leu, leucine; Ile, Isoleucine; . Met, Methionine; Tyr, Tyrosine; Cit, Citrulline; Ala, Alanine. Linear trend analysis using Jonckheere-Terpstra Test was applied to detect whether there was an increasing or decreasing trend across the ordered groups. The Mann-Kendall (M-K) test is used to detect the presence of linear or non-linear trends (steadily increasing/decreasing or unchanging) in a series data by estimating the effect size following Jonckheere-Terpstra (J-T) Test. Data mean with SD

Table 3. Levels of AcylCNs in controls and patients.

AcylCNs (μmol/l)	Groups			Linear Trend analysis test	*p value*
	Controls (n = 150)	Normoalbuminuria (n = 100)	Microalbuminuria (n = 132)		
C10	0.14 ± 0.08	0.08 ± 0.03	0.06 ± 0.04	11.17	<0.001
C3	0.5 ± 0.3	0.9 ± 0.4	0.8 ± 0.4	8.12	<0.001
C3:C2	0.10 ± 0.02	0.14 ± 0.05	0.16 ± 0.07	8.09	<0.001
C10:1	0.06 ± 0.01	0.07 ± 0.03	0.08 ± 0.03	7.77	<0.001
C8	0.07 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	7.26	<0.001
C6	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	6.91	<0.001
C18:1	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	6.90	<0.001
C2	5.8 ± 2.6	9.3 ± 2.9	8.3 ± 3.3	6.80	<0.001
C16	0.45 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	5.96	<0.001
C14	0.03 ± 0.02	0.05 ± 0.01	0.03 ± 0.01	5.07	<0.001
C12	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.02	4.74	<0.001
C3DC	0.03 ± 0.01	0.05 ± 0.01	0.04 ± 0.02	4.50	<0.001
C4	0.07 ± 0.02	0.07 ± 0.01	0.08 ± 0.02	4.42	<0.001
C5DC	0.11 ± 0.03	0.15 ± 0.02	0.13 ± 0.04	2.78	0.005
C0	13.8 ± 5.2	7.8 ± 2.2	15.7 ± 5.8	2.24	0.025
C5:1	0.07 ± 0.01	0.02 ± 0.01	0.08 ± 0.05	1.89	0.058
C5	0.15 ± 0.03	0.08 ± 0.02	0.14 ± 0.03	1.49	0.136

C0, free carnitine; C2, Acetylcarnitine; C3, Propionylcarnitine; C3-DC, Malonylcarntine; C4, Isobutyrylcarnitine;; C5, Isovalerylcarnitine, C5:1, Tiglylcarnitine;; C5-DC, Glutarylcarnitine; C6, Hexanoylcarnitine; C8, Octanoylcarnitine; C10, Decanoylcarnitine; C10:1,Decenoylcarnitine; C12, Dodecanoylcarnitines; C14, Tetradecanoylcarnitine; C16, Hexadecanoylcarnitine;; C18, Octadecanoylcarnitine; C18:1, Octadecenoylcarnitine. Data: mean with SD.

Table 4. Distribution of urinary organic acids levels in controls and patients.

Urinary organic acids(μmol/l)	Groups			Linear Trend analysis test
	Controls (n = 150)	Normoalbuminurea (n = 100)	Microalbuminuria (n = 132)	
Lactic acid	3.2 ± 0.9	25.6 ± 4.3	89.9 ± 13.6	20.56
2hydroxy butyric acid	1.6 ± 0.3	15.5 ± 3.3	73.9 ± 17.0	20.55
3hydroxy butyric acid	2.4 ± 0.4	71.9 ± 4.4	88.7 ± 6.9	20.38
5hydroxy hexanoic acid	2.1 ± 0.6	1.4 ± 0.1	1.1 ± 0.1	19.49
Hydroxy propionic acid	3.9 ± 1.6	6.4 ± 1.3	14.8 ± 4.3	18.90
Sebacic acid	0.9 ± 0.2	3.6 ± 2.2	11.7 ± 0.9	18.54
Adipic acid	2.2 ± 0.7	3.4 ± 0.9	13.12 ± 4.4	18.04
Methyl malonic acid	3.8 ± 0.9	5.4 ± 1.6	12.9 ± 1.5	17.98
L-pyroglutamic acid	19.8 ± 3.5	24.7 ± 2.9	46.4 ± 11.8	17.88
Suberic acid	1.6 ± 0.1	1.9 ± 0.5	7.1 ± 1.9	16.34
Citric acid	3.7 ± 1.6	13.7 ± 1.6	21.4 ± 5.8	14.74
Succinic acid	5.6 ± 1.5	5.5 ± 1.5	24.7 ± 7.5	14.13
Ethyl malonic acid	4.6 ± 1.6	1.8 ± 0.6	1.7 ± 0.6	13.92
Azelaic acid	1.5 ± 0.3	1.0 ± 0.2	1.01 ± 0.1	13.82
Tiglyl glycine	0.9 ± 0.2	3.9 ± 0.8	2.5 ± 1.7	10.23
Glutaric acid	4.1 ± 17	4.0 ± 1.7	4.1 ± 1.4	1.31
Pimelic acid	1.7 ± 0.2	1.7 ± 0.2	1.7 ± 0.3	0.27

Linear trend analysis using Jonckheere-Terpstra Test was applied to detect whether there was an increasing or decreasing trend across the ordered groups. All trends strongly significant except glutamic acid (p = 0.188) and pimelic acid (p = 0.785). Data: mean with SD.

Compared with blood metabolomics, urine metabolomics may offer a straight understanding of biological pathways related to kidney dysfunction as urine metabolites are directly emitted by the kidney [35]. Organic anion transporters (OAT), which were involved in the elimination of these organic anions via the kidney, are affected in DKD. This hypothesis was supported, as there was a greater than twofold reduction in the gene expression levels of OAT1 and OAT3 in kidney biopsy samples from patients with diabetic nephropathy compared with that of the non-diseased kidney [36]. Analysis of the organic acids in the current study revealed that the tricarboxylic cycle metabolites had a significantly highly increasing linear trend over the three groups that could be an indicator of kidney function [37]. Remarkably, the main bulk of the 17 organic acids or the enzymes generating metabolites is oxidized in mitochondria, therefore implicating mitochondrial dysfunction and reduced mitochondrial biogenesis as the main feature associated with early DKD [15]. The metabolite 5-hydroxyhexanoic acid is produced from fatty acids degradation with medium-chain lengths (particularly hexanoic acid) [35]. Its level showed a highly significant decreasing trend in the groups of early DKD, this was in line with the study of Tang et al, that may increase the risk of ESRD progression in T2DM patients with microalbuminuria [35]. A novel view of this metabolism could present a probable new medical theory for the avoidance of renal function decline by increasing their levels, such as supplementation which necessitates further confirmation [35]. In addition, the diseased groups showed a highly decreasing trend of

azelaic acid in urine. Azelaic acid increases the amounts of enzymatic and nonenzymatic antioxidants that was related to the development of DKD [35]. Furthermore, L-pyroglutamic acid showed an increasing trend in the diseased groups, in accordance with the study of Kim et al. [38] due to impaired fasting glucose of the diabetic subjects [38]. These results provide new pathogenetic phases that may be new targets for treatment.

Interestingly, on multivariate analysis; C12, C5:1 and C5 served as significantly stronger predictors of DKD than others, and also HbA1c, that may be due to the mismatch of fatty acid delivery and the tricarboxylic acid cycle capacity [39]. Acyl-carnitines may interact with NF-Kβ, that stimulates inflammation and insulin resistance affecting the development and progression of DKD [39]. Hence, acylcarnitines, especially C12, C5:1 and C5, may be more sensitive biomarkers for the diagnosis of early DKD in T2DM patients with normoalbuminria and microalbuminuria than HbA1c that might offer additional therapeutic goals for limiting DKD progression. The limitations to metabolome coverage in this study result from multiple steps of metabolite extraction, and the chemical derivatization of the urine samples analysed via GC-MS [40].

This work represents an advance in biomedical science because it shows an innovative, non-invasive approach for detecting specific and sensitive biomarkers for the diagnosis of early DKD in T2DM patients with normoalbuminuria and microalbuminuria that might offer additional therapeutic goals for limiting DKD progression.

Summary table

What is known about this subject?

- Diabetic Kidney disease (DKD) is an important cause of the end-stage renal disease (ESRD).
- Determination of the urinary microalbuminuria is the standard non-invasive test for the detection and assessment of DKD.

What this study adds:

- Acylcarnitines are stronger predictors of the ACR compared to HbA1c, and so may be of value in diagnosing early DKD (normoalbuminuria to microalbuminuria)

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