Original Article

Serum and urine metabolomics reveal potential biomarkers of T2DM patients with nephropathy

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Background: Diabetes is a metabolic disease and is often accompanied by severe microvascular and macrovascular complications. A comprehensive understanding of its complex mechanisms can help prevent type 2 diabetes mellitus (T2DM) complications, such as diabetic nephropathy (DN).

Methods: To reveal the systemic metabolic changes related to renal injury, clinical information of T2DM patients with or without nephropathy was collected, and it was found that serum urea levels of DN patients were significantly higher in T2DM patients without nephropathy. Further along the disease progression, the serum urea levels also gradually increased. We used gas chromatograph coupled with time-of-flight mass spectrometry (GC-TOFMS) metabolomics to analyze the serum and urine metabolites of T2DM patients with or without nephropathy to study the metabolic changes associated with the disease.

Results: Finally, we identified 61 serum metabolites and 46 urine metabolites as potential biomarkers related to DN ($P<0.05$, VIP >1). In order to determine which metabolic pathways were major altered in DN, we summarized pathway analysis based on P values from their impact values and enrichment. There were 9 serum metabolic pathways and 12 urine metabolic pathways with significant differences in serum and urine metabolism, respectively.

Conclusions: This study emphasizes that GC-TOFMS-based metabolomics provides insight into the potential pathways in the pathogenesis and progression of DN.

Keywords: Type 2 diabetes mellitus (T2DM); diabetic nephropathy; gas chromatograph coupled with time-of-flight mass spectrometry (GC-TOFMS); metabolomics

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Introduction

According to the International Diabetes Federation in 2017, 114 million (10.9%) Chinese have been diagnosed with diabetes in the last 20 years, and by 2045, the number is expected to be 119 million (11.6%) (1). The incidence and prevalence of diabetes have increased markedly, especially for type 2 diabetes mellitus (T2DM). A vast number of people with T2DM have progressed toward different complications in recent decades. Among them, the microvascular complications include diabetic nephropathy (DN), diabetic peripheral neuropathy (DPN) and diabetic retinopathy (DR), while the macrovascular complications include cardiovascular diseases (CVDs), stroke and peripheral vascular diseases, such as diabetic foot disease (DFD) (2). Chronic kidney disease (CKD) is a worldwide public health challenge and was first described as a advancing disease that begins with a small amount of albumin in the urine, accompanied with a decrease in kidney function, renal injury, and finally end-stage renal disease (ESRD) (3). CKD is diagnosed by the persistently increased urinary albumin excretion, low estimated glomerular filtration rate (eGFR), or additional characteristics of renal injury (4,5). It has been reported that DN has become the main cause of CKD in China (6). The rapidly elevating prevalence of diabetes global actually assures that the proportion of CKD due to diabetes, will continue to increase, so called diabetic kidney disease (DKD), also known as DN (7). According to World Health Organization data, about 25–40% of T2DM patients develop renal injury and CKD, which significantly worsens the quality of life of patients (8). Once disease progresses, about 20% to 40% of patients irreversibly would develop ESRD (9).

DKD is one of the usual complications of T2DM and is the major cause of ESRD, CVDs and death, but it is difficult to determine which T2DM patients have a higher risk of DKD based solely on glomerular filtration rate and proteinuria. Therefore, there is an urgent need to find biomarkers that can identify high-risk populations of DKD early in diabetes, through which we can predict the disease and prevent the progression of the disease at the early stage.

Metabolomics, a rapidly developing field in systems biology, measures metabolic changes in response to the development and progress of the disease (10,11). It has been widely used to detect metabolic profiles of diabetes-related diseases and to identify potential biomarkers (12). Rossi *et al.* (13) used liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to measure whole blood from streptozotocin-induced DN with tissue inhibitors of metalloproteinase 3-deficient mice, and reported that the abnormal fatty acid metabolism and the related elevations in serum acyl-carnitines (ACs) were associated with the pathogenic process of DN. Li *et al.* (14) used gas chromatograph coupled with time-of-flight mass spectrometry (GC-TOFMS) to determine the urinary metabolites acquired from DN patients and showed that reduced level of mitochondrial metabolites were closely related to the occurrence of albuminuria. Our former study showed that serum differential metabolites, such as carbohydrates, fatty acids, amino acids, bile acids, lipids, steroids, and tricarboxylic acids, were involved in the formation of T2DM complications according to a 292-patient cohort study using untargeted metabolomics (15). We also recently summarized many different metabolomics studies on diabetic complications (16). As far as we known, no large-scale metabolomics research has characterized serum and urine metabolites systemically, especially in DN.

In this research, we performed serum and urine metabolic profiling between T2DM patients with or without nephropathy using GC-TOFMS. We also analyzed the characteristics of serum and urine metabolites in patients at different stages of DN to investigate the association between serum and urine metabolites and during the development of DN. The purpose of this research was to probe the metabolite changes at different stages of DN, to define the metabolites that are potentially predictive markers for DN.

Methods

Chemicals and reagents

Methoxyamine HCl, fatty acid methyl ester (C7-C30, FAMEs) standards, pyridine and anhydrous sodium sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) with 1% (vol/vol) trimethylchlorosilane (TMCS), acetonitrile, methanol, dichloromethane, hexane, chloroform and acetone were obtained from Thermo-Fisher Scientific (FairLawn, NJ, USA). Ultrapure water was produced by a Mill-Q Reference system equipped with an LCMS Pak filter (Millipore, Billerica, MA).

Study population

From March 2015 to March 2016, T2DM patients were collected from the endocrinology clinics of Shuguang

Hospital, Putuo Hospital and Shanghai Traditional Chinese Medicine Hospital affiliated to Shanghai University of Traditional Chinese Medicine. The 2010 diagnostic criteria from the American Diabetes Association (ADA) were used: (I) glycosylated hemoglobin (HbA1c) ≥6.5%, (II) fasting plasma glucose (FPG) ≥7.0 mmol/L (fasting is defined as at least 8 hours without calorie); (III) 2 hours of blood glucose during oral glucose tolerance analysis ≥11.1 mmol/L, and (IV) in a typical hyperglycemia or hyperglycemia crisis patients, random blood glucose ≥11.1 mmol/L (17). DN was diagnosed as diabetes with proteinuria (the value ratio of urinary albumin to creatinine ≥30 mg/g), damaged glomerular filtration rate (GFR) [<60 mL/min/1.73 m^2 evaluated using the chronic kidney disease epidemiology collaboration (CKD-EPI) equation], or both (18). For women with serum creatinine ≤0.7, GFR was calculated as 144× (serum creatinine/0.7)$^{-0.329}$×(0.993)^{age}; for those who >0.7, GFR was computed as 144× (serum creatinine/0.7)$^{-1.209}$ ×(0.993)^{age}; for men with serum creatinine ≤0.9, GFR was computed as 141× (serum creatinine/0.9)$^{-0.411}$×(0.993)^{age}; and for those who >0.9, GFR was computed as 141× (serum creatinine/0.9)$^{-1.209}$×(0.993)^{age} (19). Renal biopsy is the gold standard for distinguishing DN and nondiabetic nephropathy, but renal biopsy is an invasive test and the patient's acceptance is low (20,21). The exclusion criteria were as follows: (I) primary kidney disease, this experiment is based on the diagnosis of diabetes, proteinuria, diabetic retinopathy, hematuria, and systolic blood pressure. (II) urinary tract infection, (III) recent acute disease, (IV) malignant tumor, (V) taking drugs affecting bone metabolism in the past six months, and (VI) primary osteoporosis and other endocrine diseases, autoimmune diseases, or similar conditions. Those who were difficult to accurately identify kidney disease was also excluded in this study. Additionally, hypertensive patients were excluded based on their medical histories and measured blood pressure. The information about weight, height, waist-to-hip ratio (WHR) and body mass index (BMI) was collected. All patients who participated in the study offered a signed informed consent form. The ethical approval for the present study was provided by the Ethics Committee of Shanghai University of Traditional Chinese Medicine.

GC-TOFMS analysis

All serum and urine samples were dissolved on ice and centrifuged at 4 ℃ and 3,000 g for 5 min (Microfuge 20R, Beckman Coulter, Inc., Indianapolis, IN, USA) to separate debris or a lipid layer. Each 50 μL of serum sample aliquot was mixed with 10 μL of the internal standard, to which 175 μL of prechilled methanol/chloroform (v/v =3/1) was added. After mixed at −20 ℃ for 20 min and centrifuged at 14,000 g and 4 ℃ for 20 min, the supernatant was transferred to an autosampler vial (Agilent Technologies, Foster City, CA, USA) carefully. In order to remove chloroform, all samples in autosampler vials were evaporated using a CentriVap vacuum concentrator (Labconco, Kansas City, MO, USA). Each urine sample aliquot of 75 μL was mixed with 10 μL of internal standard and was further lyophilized with a FreeZone dryer equipped with a stopping tray dryer (Labconco, Kansas City, MO, USA).

The samples were further analyzed by a GC-TOFMS system (Pegasus HT, Leco Corp., St. Joseph, MO, USA) coupled to an Agilent 7890B gas chromatograph and a Gerstel multipurpose MPS2 sampler with dual heads (Gerstel, Muehlheim, Germany). An Rxi-5 ms capillary column was used for separation (30 m × 250 μm, 0.25-μm film thickness; Restek Corporation, Bellefonte, PA, USA). The carrier gas was set at a constant flow rate of 1.0 mL/min using helium. Injection and transfer interface temperatures were both set to 270 ℃, and source temperature was 220 ℃. The analysis was made using electron impact ionization (70 eV) in the full scan mode (m/z 50-500).

Statistical analysis

SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) was used for analysis, and P<0.05 was considered statistically significant. Data are presented as the medians (interquartile ranges, IQRs). T-test, Wilcoxon test and Analysis of Variance (ANOVA) test were performed between two groups or among multiple groups. Chi-square tests were used to compare categorical variables. Partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were performed using SIMCA-P + 12.0 (Umetrics AB).

Results

Clinical information of T2DM patients with or without nephropathy

The clinical characteristics of the 88 subjects, including 44 DN and 44 T2DM subjects, are summarized in *Table 1*. According to clinical and pathological processes, the

Table 1 Clinical Information of T2DM patients with and without nephropathy and among DN-III, DN-IV and DN-V groups

Characteristics	T2DM without nephropathy (n=44)	T2DM with nephropathy (n=44)				P value			
		Overall	DN-III	DN-IV	DN-V	DN *vs.* T2DM	III *vs.* IV	III *vs.* V	IV *vs.* V
Gender (M/F)	31/13	26/18	7/6	10/7	9/5	0.697	1	1	1
Age (years)	71 (55.75–79.5)	62 (55.75–71.25)	71 [56–81]	62 [58–70]	61 (55.25–67)	0.176	0.519	0.519	0.72
BMI (kg/m^2)	24.515 (22.81–25.978)	23.83 (21.14–27.085)	23.14 (21.63–24.98)	26.79 (25.3–28.37)*	21.2 (20.55–23.31)$^{\#\#}$	0.932	0.034	0.521	0.008
2hPG (mmol/L)	11 (7.125–15.175)	14 (10.45–16.875)	15.3 (13.3–16.35)	16.1 (13.3–19.8)	9.05 (6.575–12.95)**$^{\#\#}$	0.06	0.716	0.008	0.008
2hC-peptide (pmol/mL)	2.005 (1.1–3.785)	3.01 (2.1–4.7)	2.29 (1.782–2.87)	3.19 (1.965–5.0425)	4.3 (2.35–4.85)	0.03	0.398	0.124	0.511
Insulin (mmol/L)	69.43 (42.985–105.31)	11.79 (7.48–20.93)	9.2 (7.112–12.903)	13.385 (11.175–23.033)	8.48 (5.56–18.18)	<0.001	0.143	0.828	0.143
Mon (%)	8 (7.175–9.85)	6.5 (5.5–8.025)	5.7 (5.3–7)	6 (5.5–7.7)	7.95 (6.05–9.1)	0.001	0.721	0.081	0.143
HGB-Mass (pg)	29 (27.8–30.45)	29.7 (28.8–31)	30.5 (29.8–31.8)	29.6 (28.8–31)	29.2 (28.65–29.9)*	0.042	0.147	0.023	0.35
PLT (*10^9/L)	246 (194.5–302.5)	193 (153.5–224)	184 [154–197]	199 [157–233]	194 (152.5–258.75)	0.003	0.497	0.497	0.889
MPV (fL)	10.5 (9.7–11.125)	11.1 (10.2–12.15)	12.3 (11.8–13.1)	10.5 (9.8–11.4)*	10.9 (10.125–11.9)*	0.01	0.011	0.019	0.462
hs-CRP (mg/L)	7 (4–34.5)	2 (1–9.5)	1.5 (1–6.25)	1 [1–5]	5 [1–17]	<0.001	0.556	0.556	0.451
GLB (g/L)	30.2 (27.35–34.825)	29 (25–31.5)	29 (27–30.5)	28 [25–31]	28 (24–31.5)	0.047	0.811	0.811	0.811
TBIL (μmol/L)	7.9 (6.025–8.625)	10 [7–13]	11.5 (8–13.5)	11 [8–13]	7 [7–8]	0.004	0.688	0.087	0.087
DBIL (μmol/L)	3.1 (2.1–3.8)	1.6 (1.2–2)	2.1 (1.45–2.75)	1.7 (1.5–1.9)	1.2 (0.925–1.45)*$^{\#}$	<0.001	0.351	0.017	0.019
Cr (μmol/L)	72 (58.5–94.25)	119 (69–431.75)	66 [58–88]	107 [70–243]	714.5 (431.75–854)***$^{\#\#\#}$	0.002	0.063	<0.001	<0.001
Urea (mmol/L)	6.85 (4.525–8.15)	9.15 (6.275–20.65)	6.4 (5.1–6.8)	9.1 (8–12.8)*	21.45 (16.725–24.05)***$^{\#\#\#}$	0.001	0.013	<0.001	<0.001
UA (μmol/L)	262.5 (210.75–327.75)	382 (309–432.25)	311 [284–403]	380 [330–425]	437.5 [384–459]*	<0.001	0.108	0.013	0.153
Ca (mmol/L)	2.24 (2.16–2.395)	2.165 (2.087–2.273)	2.165 (2.095–2.278)	2.165 (2.04–2.313)	2.16 (2.09–2.188)	0.02	0.921	0.921	0.921
Na (mmol/L)	139 (137–140)	141 (138.5–142)	140 (137.5–141)	142 [140–142]	140 (137.5–141.75)	0.028	0.26	0.938	0.403
TG (mmol/L)	1.03 (0.847–1.378)	1.61 (1.305–2.41)	1.945 (1.235–2.503)	2.11 (1.49–2.42)	1.355 (1.267–1.663)	<0.001	0.565	0.565	0.129
TC (mmol/L)	3.945 (3.522–4.873)	4.95 (3.83–5.57)	5.225 (3.917–5.645)	5.01 (4.43–5.76)	4.38 (3.607–5.198)	0.019	0.965	0.473	0.473
LDL (mmol/L)	2.545 (2.015–3.26)	3.15 (2.42–3.74)	3.55 (2.5075–3.84)	3.07 (2.55–3.71)	3 (2.12–3.425)	0.033	0.626	0.626	0.626
ApoB (g/L)	0.785 (0.635–0.873)	0.95 (0.747–1.093)	0.95 (0.76–1.13)	0.97 (0.852–1.108)	0.94 (0.682–1.048)	0.01	0.66	0.66	0.66
eGFR (mL/min/1.73 m^2)	8.775 (6.627–10.693)	6.405 (2.93–8.71)	7.91 (6.17–10.02)	4.72 (2.55–8.57)	6.46 (1.887–8.593)	0.028	0.054	0.285	0.451

Data were expressed as the medians (IQRs) at normal distribution. The Wilcoxon test was used to compare these variables between T2DM patients with and without nephropathy, and P<0.05 was considered significant. The ANOVA test was used to compare these variables among the three stages of DN. *P<0.05; **P<0.01; **P<0.001 when compared to DN-III, and #P<0.05; ##P<0.01; ###P<0.001 when compared to DN-IV. M, male; F, female; BMI, body mass index; 2hPG, two hours post-load plasma glucose; Mon, monocyte ratio; HGB-Mass, RBC average HGB content; PLT, platelet; MPV, mean platelet volume; hs-CRP, hypersensitive C-reactive protein; GLB, globulin; TBIL, total bilirubin; DBIL, direct bilirubin; TBA, total bile acid; Cr, creatinine; UA, uric acid; TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; ApoB, apolipoprotein b; eGFR, estimated glomerular filtration rate.

Mogensen staging method divides DN into 5 phases: glomerular hyperfiltration and renal hypertrophy (DN-I), normal albuminuria (DN-II), early diabetic nephropathy (DN-III), clinical diabetic nephropathy (DN-IV), and end-stage renal failure (DN-V) (22). At present, the clinical diagnosis of DN is mainly based on microalbuminuria, while patients with stage I and II DN have no good diagnostic signs, making the condition difficult to diagnose and intervene early. In the present study, according to the Mogensen staging criteria, patients with DN were further divided into three stages: DN-III (n=13), DN-IV (n=17) and DN-V (n=14).

This study showed that the average T2DM and DN patients gender, age and BMI had no significant differences between the T2DM and DN patients (P>0.05) (*Table 1*). Compared with the T2DM group, the serum insulin, monocyte ratio, platelet (PLT), total bilirubin (TBIL), direct bilirubin (DBIL), hypersensitive C-reactive protein (hs-CRP), creatinine (Cr), uric acid (UA) and triglyceride (TG) levels of DN patients showed significant differences (P<0.01). Accompanied with the progression of the disease, the serum urea level increased gradually (P<0.05), indicating that the serum urea level may be a potential clinical indicator for the diagnosis of whether T2DM progresses into DN or the severity of DN. The clinical features of other T2DM patients with or without nephropathy are detailed in *Table S1*.

GC-TOF-MS data analysis of serum and urine in T2DM patients with or without nephropathy

We used GC-TOFMS metabolomics to analyze the serum and urine metabolites of T2DM patients with or without nephropathy. A total of 177 serum metabolites and 159 urine metabolites were determined including amino acids, carbohydrates, fatty acids and organic acids. In order to determine if serum and urine metabolites differed between T2DM patients with or without nephropathy, we constructed OPLS-DA models, which have been broadly used in metabolomics researches (23,24). Typically, R2Y provides the fitting degree of the model to the Y data, while Q2Y is used to estimate the fitting degree of the model to Y. To obtain high predictive ability, Q2Y and R2Y values should be close to 1. As shown in *Figure 1A*,*B*, there is a distinct difference in clustering of between DN and T2DM groups. The R2Y and Q2Y were 0.87, 0.822 and 0.797, 0.71 in serum and urine models, respectively, indicating that the models had fine prediction features. The 20-time permutation test showed that the model was steady and reliable (25), with cumulative Q2 at –0.441/–0.265 (*Figure S1*). As shown in *Figure 1C*,*D*, we also obtained differential metabolites by Student's T-test for serum and urine. In *Figure 1C*, the P values and log 1.5 (fold change, FC) are shown, with cut-off values of 0.05, 0.01 for P value and 1.5 for log.1.5 FC, respectively. The highlighted serum metabolites from the top right corner are positively correlated with subjects on the right side of the OPLS-DA score plot (*Figure 1A*). The highlighted serum metabolites from the top left corner are negatively correlated with subjects on the right side of the OPLS-DA score plot (*Figure 1A*), indicating that these metabolites are decreased in these subjects compared to those on the left side of the OPLS-DA score plot. In *Figure 1D*, the P values and log 1.5 FC are introduced with cut-off values of 0.05 for P value and 1.5 for log.1.5 FC, respectively. The highlighted urinary metabolites from the top right corner are positively correlated with subjects on the right side of the OPLS-DA score plot (*Figure 1B*). The highlighted urine metabolites from the top left corner are negatively correlated with subjects located on the right side of the OPLS-DA score plot, indicating that these metabolites are decreased in these subjects compared to those on the left side of the OPLS-DA score plot (*Figure 1B*).

Metabolic features in serum and urine of T2DM patients with or without nephropathy

In order to select potential biomarkers associated with DN, independent sample T-test was performed for the variables between DN and T2DM group, and the differences were statistically significant. Significant differences in metabolites between the two groups (P<0.05) were recognized as potential biomarkers. Then a variable importance plot (VIP) was applied to screen out the important metabolites in the model. In the present research, metabolites with VIP >1 were recognized as candidate biomarkers. According to the above criteria, 61 serum metabolites and 46 urine metabolites were filtered as candidate biomarkers for DN. The summary of the serum and urine biomarker identified in DN is shown in *Tables 2* and *3*, respectively. More complete information is shown in Supplementary *Tables S2* and *S3*. Among them, the nine most significant changes in serum metabolites are shown in *Figure 2A* (P<5E-06 and VIP ≥1.5). Compared with T2DM patients, benzoic acid, fumaric acid, erythrose, and L-Arabitol in the DN group were significantly increased. In contrast,

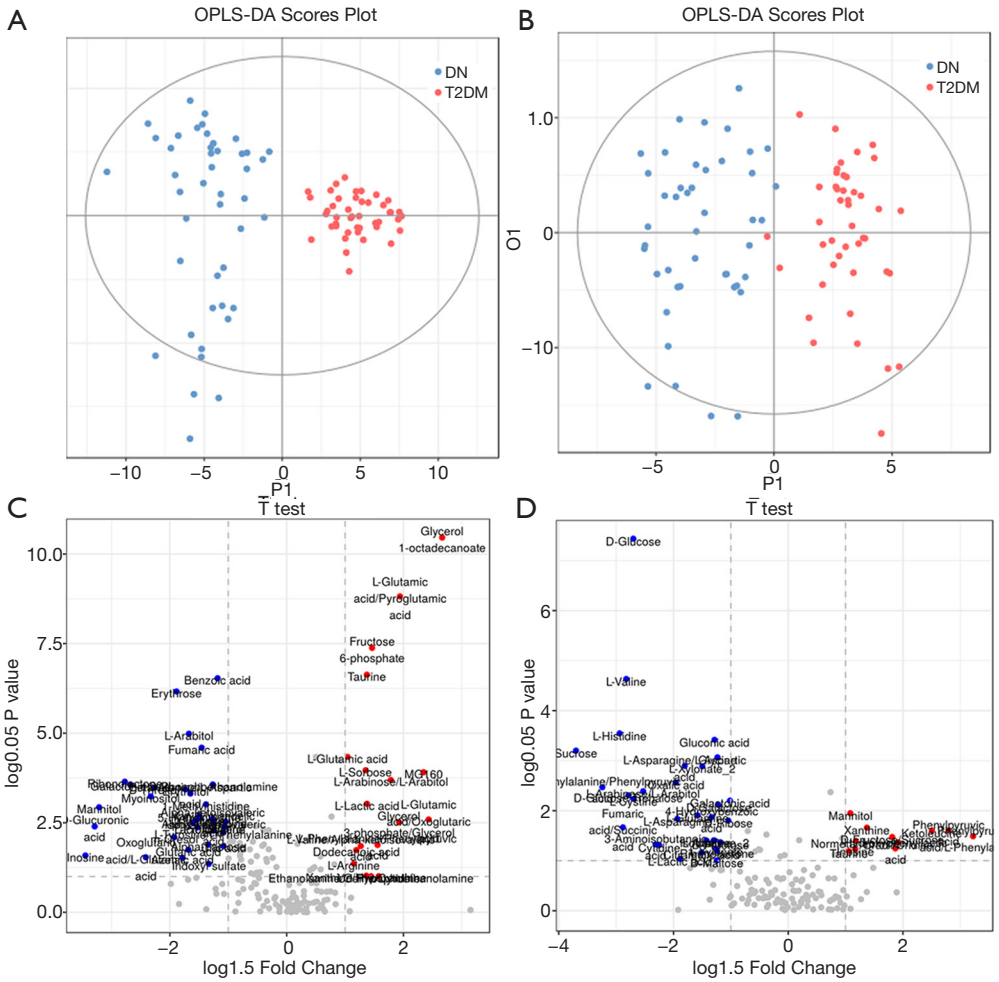

Figure 1 Visualization of overall serum and urinary metabolite profile differences between the two groups. (A) OPLS-DA score plot of serum. (B) OPLS-DA score plot of urine. T2DM with nephropathy group (blue), T2DM without nephropathy group (red). (C) Volcano plots of differentially expressed serum metabolites in T2DM patients with or without nephropathy. X-axis, log1.5 (fold change); Y-axis, log0.05 (P value). (D) Volcano plots of differentially expressed urinary metabolites in T2DM patients with or without nephropathy. X-axis, log1.5 (fold change); Y-axis, log0.05 (P value). The red highlighted points from the right (P<0.05, FC >1) indicated that these metabolites are significantly increased. The blue highlighted points from the left (P<0.05, FC <1) indicate that these metabolites are significantly decreased. The noncolored points indicate that these metabolites are not significant.

glycerol 1-octadecanoate, L-glutamic acid/pyroglutamic acid, fructose 6-phosphate, taurine and L-glutamine in DN patients were significantly decreased. The 9 most significant changes in urinary metabolites are shown in *Figure 2B* (P<5E-04 and VIP ≥1.5). Compared with T2DM patients, urine levels of D-glucose, L-valine, L-histidine, sucrose, gluconic acid, glycine, L-asparagine/L-aspartic acid, L-xylonate-2, and oxalic acid in the DN group were significantly increased.

In order to determine which metabolic pathways were major influenced by DN, we summarized pathways analysis based on P values from their impact values and enrichment. As shown in *Figure S2*, the "metabolome view", on the based of enrichment analysis (Y-axis) and topology analysis (X-axis), the arrangement shows all metabolic pathways, in which red presents the most significant P values, while yellow and white represent the lowest P values. In order to know the biological significance of the metabolic changes, we conducted a functional enrichment analysis of the experimental data using MetaboAnalyst (26), which provides metabolite set enrichment analysis (MSEA) for metabolites of serum and urine. Then, the metabolic pathways with

Table 2 Differential serum metabolites betweenT2DM with and without nephropathy

Class	Name	DN	T2DM	*T* test. P	VIP	Pathway
Alcohols	Glycerol	598,034.5 (388,745.25, 1,166,327)	387,146 (312,706.75, 526,477)	8.09E-04	1.4	Galactose metabolism, glycerolipid metabolism
	Myoinositol	438,968.5 (215,182.25, 1,198,811.75)	170,756.5 (114,897.5, 289,559.5)	6.18E-05	1.4	Galactose metabolism, inositol metabolism, inositol phosphate metabolism, phosphatidylinositol phosphate metabolism
Amino Acid	1-Methylhistidine	15,612 (7,125.5, 39,741)	8,910.5 (6,143, 12,853.75)	1.21E-04	1.3	Histidine metabolism
	5-Hydroxylysine	2,092.5 (1,624.5, 4,172.25)	1,141.5 (727, 1,643.75)	3.11E-04	1.6	Lysine degradation; metabolic pathways
	Alpha-ketoisovaleric acid	3,640 (1,992.5, 6,487)	2,190.5 (1,425.25, 3,217.5)	1.00E-03	1.4	Valine, leucine and isoleucine degradation
	Beta-Alanine	10,038 (8,363.25, 15,552.5)	7,151.5 (6,114, 8,661)	1.50E-04	1.4	Aspartate metabolism, beta-alanine metabolism, propanoate metabolism, pyrimidine metabolism
	Creatinine	338,468 (224,928.75, 1,273,947.25)	254,810.5 (179,753.5, 344,497.5)	6.36E-04	1.2	Arginine and proline metabolism; metabolic pathways
	L-Cysteine	60,307.5 (43,832, 82,471.5)	44,044 (34,087.25, 56,694.75)	5.15E-04	1.4	Cysteine metabolism, glutathione metabolism, glycine and serine metabolism, methionine metabolism, pantothenate and CoA biosynthesis, taurine and hypotaurine metabolism, transcription/translation
	L-Cystine	175,895 (120,707.75, 238,916)	117,730.5 (87,831.5, 169,729.25)	2.00E-03	1.3	Cysteine and methionine metabolism; metabolic pathways; ABC transporters; ferroptosis; protein digestion and absorption
	L-Glutamic acid	279,299.5 (167,237.25, 397,382)	426,439.5 (312,734.5, 578,261)	2.28E-06	1.5	Alanine metabolism, amino sugar metabolism, ammonia recycling, arginine and proline metabolism, cysteine metabolism, folate metabolism, glucose-alanine cycle, glutamate metabolism, glutathione metabolism, glycine and serine metabolism, histidine metabolism, malate-aspartate shuttle, transcription/translation, urea cycle
	L-Glutamine	2,323,035 (1,899,199, 2,747,632.5)	2,845,450 (2,567,941.25, 3,055,334.25)	2.28E-06	1.8	Amino sugar metabolism, ammonia recycling, glutamate metabolism, phenylacetate metabolism, purine metabolism, pyrimidine metabolism, transcription/translation, urea cycle
	L-Isoleucine	321,465 (283,167.75, 441,564.5)	271,347.5 (220,161.75, 359,676)	4.00E-03	1.2	Transcription/Translation, Valine, Leucine and Isoleucine Degradation
	L-Lysine	640,215 (549,289.25, 689,639.5)	515,362.5 (424,460.5, 599,079.5)	7.57E-04	1.5	Biotin metabolism, carnitine synthesis, lysine degradation, transcription/translation
	L-Phenylalanine	433,113 (323,081.25, 575,814)	620,982.5 (530,573.75, 707,172.25)	3.02E-06	1.6	Phenylalanine and tyrosine metabolism, transcription/translation
	L-Proline	961,090.5 (58,2251.5, 1,158,145)	516,571 (459,014.75, 757,644.75)	8.32E-04	1.2	Arginine and proline metabolism, transcription/translation
	L-Threonine	280,966 (212,282.5, 433,769.75)	248,005.5 (192,284.5, 298,326)	5.00E-03	1.2	Glycine and serine metabolism, threonine and 2-oxobutanoate degradation, transcription/translation

Table 2 *(continued)*

Table 2 *(continued)*

Class	Name	DN	T2DM	*T* test. P	VIP	Pathway
	N-Acetyl-L-aspartic acid	27,759 (10,844, 179,723.5)	29,926.5 (15,873.25, 46,000.5)	3.00E-03	1.1	Aspartate metabolism
	Pyroglutamic acid	1,992,442.5 (1415175, 2640418.75)	1,439,163 (1,185,021.5, 1,782,831.75)	3.00E-03	1.1	Glutathione metabolism
	Alpha-ketoisovaleric acid/ L-Valine	0.01 (0.003, 0.01)	0 (0.002, 0.005)	4.08E-04	1.3	Valine, leucine and isoleucine degradation; pantothenate and CoA biosynthesis
	Beta-Alanine/L-Aspartic acid	0.09 (0.061, 0.144)	0.05 (0.037, 0.076)	4.86E-05	1.5	Pantothenate and CoA biosynthesis; beta-Alanine metabolism
	L-Glutamic acid/ Oxoglutaric acid	25.75 (20.325, 42.947)	69.01 (40.097, 153.817)	4.25E-04	1.4	Arginine biosynthesis
	L-Glutamic acid/ Pyroglutamic acid	0.14 (0.092, 0.174)	0.3 (0.222, 0.384)	3.38E-12	2.1	Glutathione metabolism
	L-Tyrosine/L-Phenylalanine	1.87 (1.318, 2.323)	1.21 (0.861, 1.49)	1.00E-03	1.5	Phenylalanine metabolism; phenylalanine, tyrosine and tryptophan biosynthesis
	Sarcosine/Dimethylglycine	0.03 (0.018, 0.036)	0.02 (0.017, 0.026)	9.00E-03	1.4	Glycine, serine and threonine metabolism
	Sarcosine	9,147 (8,219.75, 10,872.75)	8,519.5 (7,869.5, 8,961.25)	2.30E-02	1.3	Glycine and serine metabolism
Carbohydrates	D-Glucuronic acid	90,337.5 (16,006.25, 165,173.25)	23,810.5 (12,463, 53,869.25)	7.63E-04	1.1	Inositol metabolism, starch and sucrose metabolism
	D-Maltose	29,752 (11,850.25, 74833)	16,010.5 (10,526.75, 24,970.5)	4.20E-04	1.1	Starch and sucrose metabolism
	D-Mannose	1,056,861 (494,489.5, 1326627.5)	1,442,658.5 (905,777.75, 2,018,569.5)	3.36E-04	1.3	Fructose and mannose degradation, galactose metabolism
	D-Threitol	13,649.5 (8,991, 51,131.5)	5,438.5 (3,241.5, 7,278.5)	3.04E-05	1.5	NA
	Erythritol	66,013 (49,723, 173,745.75)	32,681 (25,459.25, 42,582.5)	3.46E-05	1.4	NA
	Erythrose	535,478.5 (354,430.5, 92,3590)	249,182.5 (52,987, 324,922.75)	9.57E-09	1.8	NA
	Fructose 6-phosphate	516,665.5 (371,837.5, 627,208)	934,897 (717,232, 1,136,517.25)	2.50E-10	2	Amino sugar metabolism, fructose and mannose degradation, gluconeogenesis, glycolysis, pentose phosphate pathway
	Galactonic acid	44,632 (17,963, 138,125.5)	15,075 (11,043, 31,052)	2.29E-05	1.4	Galactose metabolism; metabolic pathways; microbial metabolism in diverse environments
	Isomaltose	8,272.5 (2,733.75, 19,707.75)	4,314 (2,844, 6,996.25)	5.60E-04	1.1	NA
	L-Arabitol	110,028 (65,100.75, 240,871)	55,786 (43,840.25, 67,209)	3.28E-07	1.7	Pentose and glucuronate interconversions; metabolic pathways
	L-Sorbose	214,365.5 (172,933, 306,600.5)	370,879 (293,727, 451,143.5)	6.97E-06	1.7	NA
	Mannitol	429,528.5 (98,217.25, 1,399,185.25)	116,745 (50,414.5, 306,783.75)	1.54E-04	1.3	Fructose and mannose metabolism; ABC transporters; phosphotransferase system (PTS)
	L-Arabinose/L-Arabitol	0.22 (0.143, 0.436)	0.46 (0.393, 0.6)	1.56E-05	1.5	Pentose and glucuronate interconversions
	L-Glutamine/L-Glutamic acid	9.28 (6.079, 14.034)	6.66 (4.532, 8.929)	1.00E-03	1.1	Glyoxylate and dicarboxylate metabolism; alanine, aspartate and glutamate metabolism

Table 2 *(continued)*

Table 2 *(continued)*

Class	Name	DN	T2DM	T test. P	VIP	Pathway
	Ribonolactone	27,147.5 (15,144, 7,7587.5)	8,821.5 (4,725.5, 14,265.25)	1.81E-05	1.4	NA
	Threonic acid	51,359.5 (34,386, 201,621.75)	45,318.5 (30,874.25, 56,184)	1.00E-03	1.2	NA
Esters	Glycerol 1-octadecanoate	3,563.5 (26,68.5, 4,804.75)	10,494.5 (8,536, 12,954)	2.45E-14	2.3	NA
Fatty Acids	Linoleic acid	162,340.5 (109,351.5, 223,310)	131,850 (91,854.25, 183,544.5)	3.60E-02	1.2	Alpha linolenic acid and linoleic acid metabolism
	Tetracosanoic acid	6,202.5 (5,134.75, 8,127.75)	5,272 (4,122, 6,202)	1.00E-03	1.2	Beta oxidation of very long chain fatty acids
Hormone	Normetanephrine	3,813.5 (2,690.25, 5,572.75)	2,953 (2,047.5, 3,518.5)	5.00E-03	1.4	Tyrosine metabolism
Lipids	MG160	3,829 (2,292.75, 5,679.5)	9,908 (7,105.75, 14,048)	8.20E-06	1.3	NA
	O-Phosphoethanolamine	24,043 (19,236, 30,112)	14,389.5 (10,347.75, 21,184)	2.29E-05	1.5	Sphingolipid metabolism
	Glycerol 3-phosphate/ Glycerol	0.06 (0.019, 0.122)	0.13 (0.089, 0.153)	5.41E-04	1.2	Glycerolipid metabolism
Nucleotide	Cytidine	761.5 (400.75, 2396.25)	498 (398, 615.5)	5.05E-04	1.2	Pyrimidine metabolism
	Hypoxanthine	44,123.5 (28,191, 77,664.75)	29,734.5 (22,674.75, 39,268.75)	3.00E-03	1.2	Purine metabolism
Organic Acids	Benzoic acid	24,087 (19,048.5, 29,166.5)	14,903 (13,031.25, 17,855.5)	3.16E-09	2	NA
	Fumaric acid	8,265 (6,316, 10,364.25)	4,577.5 (3,857.5, 5,716)	1.05E-06	2	Arginine and proline metabolism, aspartate metabolism, citric acid cycle, mitochondrial electron transport chain, phenylalanine and tyrosine metabolism, tyrosine metabolism, urea cycle
	Glyceric acid	47,358 (37,608.75, 59,286.25)	35,250 (29,520.75, 44,127.5)	6.86E-04	1.5	Glycerolipid metabolism, glycine and serine metabolism
	L-Lactic acid	1,806,745 (1,201,846, 2,235,344.25)	3,155,801 (1,616,732, 4,879,156.25)	1.15E-04	1.3	Gluconeogenesis, pyruvate metabolism
	Maleic acid	13,293.5 (11,825, 16,123)	11,344 (10,363.5, 13,526)	3.00E-03	1.4	NA
	Petroselinic acid	513,132 (322,569.25, 801,835.25)	416,994 (267,109.5, 580,415.25)	9.00E-03	1.3	NA
	Taurine	177245.5 (93433.75, 227747.5)	309,364.5 (206,290.25, 385,725.5)	2.35E-09	1.9	Bile acid biosynthesis, taurine and hypotaurine metabolism
	Uric acid	292,716.5 (207,345, 341,138.5)	188,751.5 (146,691.25, 270,719.75)	4.00E-03	1.3	Purine metabolism
	Vanillylmandelic acid	530 (327.75, 2513.5)	318 (220.25, 445.75)	5.37E-04	1.2	Tyrosine metabolism
Phenols	5-Hydroxydopamine	6,983.5 (4,712.75, 9,486)	5,617.5 (3,889, 7,842.5)	4.20E-02	1.1	NA
	m-Cresol	30,252 (8,040.5, 101,712.75)	13,858 (5,703.75, 26,689.25)	2.00E-03	1.2	Toluene degradation; microbial metabolism in diverse environments; degradation of aromatic compounds

Differential metabolites were selected according to VIP >1 and P<0.05. Values are expressed as medians (IQRs). P values were calculated from *t*-test for continuous variables and adjusted by FDR method; VIP, variable influence on projection.

Table 3 Differential urinary metabolites between T2DM with and without nephropathy

Class	Name	DN	T2DM	T test. P	VIP	Pathway
Amino acid	L-Valine	1,4824.5 (9,025.25, 31585.5)	4,716 (3,303.5, 7020.5)	9.40E-07	2.9	Propanoate metabolism, transcription/translation, valine, leucine and isoleucine degradation
	L-Histidine	10,364 (4,639.75, 18,147.25)	3,146 (899.25, 4,270.5)	2.44E-05	2.4	Ammonia recycling, histidine metabolism, transcription/translation
	Glycine	65,575 (39,493.75, 105,077)	39,768 (20,924, 53,651.75)	1.03E-04	2.2	Alanine metabolism, ammonia recycling, bile acid biosynthesis, carnitine synthesis, glutathione metabolism, glycine and serine metabolism, methionine metabolism, porphyrin metabolism
	Galactonic acid	54,048 (37,692.25, 93,398.25)	35,796.5 (19,514.75, 57,736)	1.00E-03	1.6	Galactose metabolism; metabolic pathways; microbial metabolism in diverse environments
	L-Glutamine	251,416 (123,474, 461,131.5)	132,467.5 (73,631.75, 241,030.25)	3.00E-03	1.9	Amino sugar metabolism, ammonia recycling, glutamate metabolism, phenylacetate metabolism, purine metabolism, pyrimidine metabolism, transcription/translation, urea cycle
	4-Hydroxybenzoic acid	40,798.5 (21,313.75, 115,454.25)	23,748.5 (14,890.5, 54,047)	4.00E-03	1.7	Ammonia recycling, aspartate metabolism, transcription/translation
	Ketoleucine	8,137 (4,966, 16,225.25)	22,470.5 (4,557.75, 49,945.5)	8.00E-03	1.3	Valine, leucine and isoleucine degradation
	L-Serine	92,666 (71,705.5, 163,200)	79,442.5 (36,488, 107,665.25)	1.00E-02	1.7	Ammonia recycling, glycine and serine metabolism, homocysteine degradation, methionine metabolism, sphingolipid metabolism
	L-Alanine	58,922.5 (29,373.75, 95,954.75)	32,996 (19,712.25, 52,954)	1.50E-02	1.9	Alanine metabolism, glucose-alanine cycle, glycine and serine metabolism, selenoamino acid metabolism, transcription/translation, urea cycle
	L-Threonine	61,327 (28,033.75, 150,853)	66,284 (28,502, 105,775)	1.50E-02	1.7	Glycine and serine metabolism, threonine and 2-oxobutanoate degradation, transcription/translation
	L-Glutamic acid	29,932.5 (18,307, 60,635)	29,173.5 (15,021.5, 48,765.75)	3.60E-02	1.4	Alanine metabolism, amino sugar metabolism, ammonia recycling, arginine and proline metabolism, cysteine metabolism, folate metabolism, glucose-alanine cycle, glutamate metabolism, glutathione metabolism, glycine and serine metabolism, histidine metabolism, malate-aspartate shuttle, transcription/translation, urea cycle

Table 3 *(continued)*

Table 3 *(continued)*

Class	Name	DN	T2DM	T test. P	VIP	Pathway
Amino acid metabolism	L-Asparagine/L-Aspartic acid	9.68 (5.958, 20.118)	4.66 (2.934, 7.522)	1.71E-04	2	Alanine, aspartate and glutamate metabolism
	L-Phenylalanine/ Phenylpyruvic acid	30.1 (11.584, 61.208)	8.09 (4.657, 15.047)	6.13E-04	1.7	Phenylalanine, tyrosine and tryptophan biosynthesis
	Phenylpyruvic acid/ L-Phenylalanine	0.03 (0.016, 0.088)	0.12 (0.066, 0.215)	1.20E-02	1.6	Phenylalanine metabolism
	Ornithine/L-Arginine	33.3 (9.814, 65.809)	23.33 (14.106, 29.792)	2.20E-02	1.5	Arginine biosynthesis; arginine and proline metabolism
	L-Arabinose/L-Arabitol	3.27 (1.428, 6.406)	1.17 (0.668, 2.598)	7.82E-04	1.8	Pentose and glucuronate interconversions
	D-Glucose/Trehalose	4.64 (2.208, 8.466)	1.5 (0.677, 2.537)	9.97E-04	1.6	Starch and sucrose metabolism
	Fumaric acid/Succinic acid	1.87 (0.783, 3.783)	0.58 (0.233, 1.478)	7.00E-03	1.5	Citrate cycle (TCA cycle)
	D-Fructose/Sucrose	0.11 (0.059, 0.259)	0.23 (0.071, 0.763)	1.20E-02	1.3	Starch and sucrose metabolism
Carbohydrates	D-Glucose	267,788.5 (181,641.25, 363,846.25)	89,577 (41,878.25, 157,795.5)	2.12E-10	3	Galactose metabolism, gluconeogenesis, glucose-alanine cycle, glycolysis, lactose degradation, lactose synthesis, transfer of acetyl groups into mitochondria
	Gluconic acid	382,496.5 (230,424.25, 531,628.25)	227,164.5 (124,367.75, 323,271.75)	3.60E-05	2	Pentose phosphate pathway; metabolic pathways; biosynthesis of secondary metabolites; microbial metabolism in diverse environments; biosynthesis of antibiotics; carbon metabolism
	Sucrose	1,200,070 (410,986.25, 1,750,933.5)	267,627.5 (108,851.5, 665,185.75)	6.89E-05	2.3	Galactose metabolism, starch and sucrose metabolism
	L-Cystine	133,144.5 (72,901, 283,660.5)	44,629 (16,423, 104,929.5)	1.00E-03	2	Cysteine and methionine metabolism; metabolic pathways; ABC transporters; ferroptosis; protein digestion and absorption
	D-Galactose	247,989.5 (167,010.5, 353,676.75)	151,497 (88,382, 237,218.75)	2.00E-03	1.6	Galactose Metabolism, Lactose Degradation, Nucleotide Sugars Metabolism
	Mannitol	257,485.5 (177,979, 411,349.25)	399,367 (266,932.25, 641,879.75)	3.00E-03	1.4	Fructose and mannose metabolism; ABC transporters; phosphotransferase system (PTS)
	D-Ribose	304,110.5 (148,998.75, 452,514.75)	199,112 (160,690.25, 289,500.5)	5.00E-03	1.4	Pentose phosphate pathway
	D-Threitol	45,409.5 (31,440, 66,992.5)	34,121 (19,136, 47,399.5)	8.00E-03	1.3	NA

Table 3 *(continued)*

Table 3 *(continued)*

Class	Name	DN	T2DM	T test. P	VIP	Pathway
	D-Fructose	104,449.5 (49,230.25, 185,290)	64,712.5 (30,731.75, 106,221)	1.70E-02	1.2	Amino sugar metabolism, fructose and mannose degradation, galactose metabolism, starch and sucrose metabolism
	L-Arabinose	442,924 (337,962, 666,296.25)	350,838.5 (184,538.25, 468,567.25)	2.90E-02	1.2	Pentose and glucuronate interconversions; ascorbate and aldarate metabolism; amino sugar and nucleotide sugar metabolism; metabolic pathways; ABC transporters
Hormone	Normetanephrine	5,526 (2,798.5, 10,694.25)	8,911.5 (3,673.25, 19,318.5)	1.50E-02	1.4	Tyrosine metabolism
Nucleotide	Xanthine	8,609.5 (5,751.25, 12,169.5)	15,018 (7,303.5, 26,031.75)	7.00E-03	1.6	Purine metabolism
	Uridine	3,777 (2,104.75, 6,605.5)	2,230.5 (1,601.25, 4,315.25)	1.50E-02	1.4	Pyrimidine metabolism
	Cytidine	434.5 (148.5, 720)	174.5 (84.75, 439.25)	2.00E-02	1.4	Pyrimidine metabolism
	Inosine	2,835.5 (1,850, 5,864.75)	4,552.5 (2,281, 14,824.75)	2.50E-02	1.1	Purine metabolism
Nucleotide metabolism	Inosine/Adenosine	0.35 (0.19, 0.599)	0.51 (0.301, 1.445)	1.30E-02	1.1	Purine metabolism
	Uracil/Uridine	2.01 (0.726, 3.555)	4.38 (1.92, 9.464)	1.70E-02	1.2	Pyrimidine metabolism
Organic acids	Oxalic acid	15,214.5 (7,031.5, 29,221.25)	6,881.5 (4,141.25, 9,998)	4.63E-04	1.4	Purine metabolism; chloroalkane and chloroalkene degradation; glyoxylate and dicarboxylate metabolism; metabolic pathways; microbial metabolism in diverse environments
	p-Hydroxyphenylacetic acid	194,089.5 (116,303, 313,946.25)	261,616.5 (167,321.75, 786,099.25)	8.24E-04	1.7	Tyrosine metabolism
	L-Asparagine	148,956 (71,175.5, 246,210.25)	67,998 (39,736.5, 132,908.75)	4.00E-03	1.9	Ubiquinone biosynthesis
	Phenylpyruvic acid	6,991 (3,393.5, 19,416.25)	21,715 (12,861, 39,259.75)	8.00E-03	1.5	Phenylalanine and tyrosine metabolism
	Hydroxyphenyllactic acid	83,610 (39,609.25, 132,933.5)	178,418.5 (75,759, 275,474.25)	2.40E-02	1.2	NA
	Hippuric acid	1331,521 (545,393.5, 2,606,043)	928,768 (330,140.25, 1,618,823.25)	2.50E-02	1.4	Phenylalanine metabolism
	L-Xylonate	319,783.5 (226,142.25, 471,741.5)	192,075.5 (124,227.5, 342,688)	2.50E-02	1.4	Pentose and glucuronate interconversions; ascorbate and aldarate metabolism
	Citramalic acid	20,538.5 (9,175.25, 43,236.75)	11,130.5 (6,810.25, 25,437.25)	3.00E-02	1.2	NA
	L-Lactic acid	430,096.5 (201,968.75, 737,092.5)	200,346.5 (91,188.75, 407,080)	4.50E-02	1.5	Gluconeogenesis, pyruvate metabolism
Phenols	1,2,3-Trihydroxybenzene	23,996 (6,720, 48,837)	33,284.5 (8,821.25, 85,409.75)	3.50E-02	1.3	Aminobenzoate degradation; microbial metabolism in diverse environments; catecholamine transferase inhibitors

Differential metabolites were selected according to VIP ≥1.5 and P<0.05. Values are expressed as medians (IQRs). P values were calculated from t test for continuous variables and adjusted by FDR method; VIP, variable influence on projection.

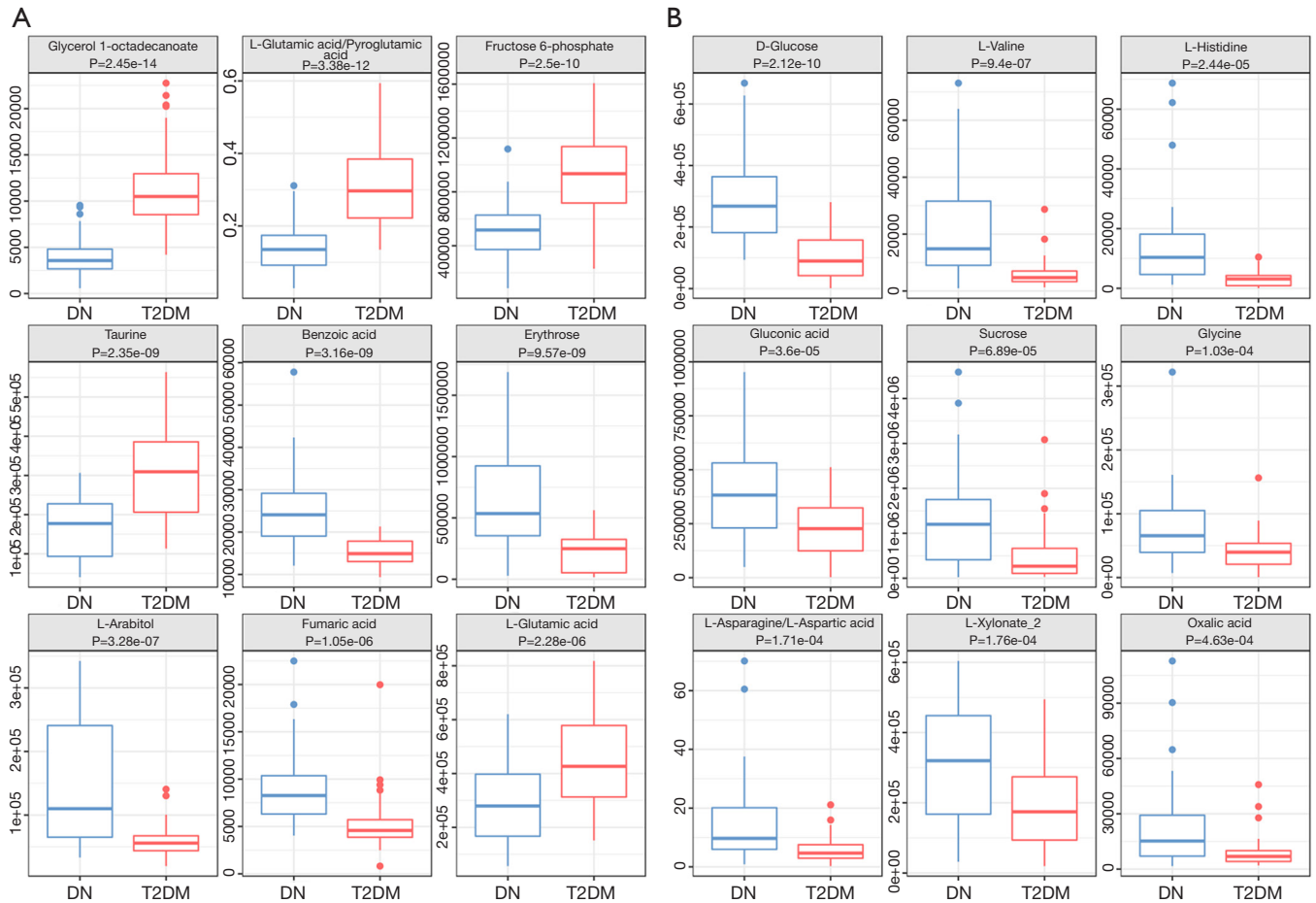

Figure 2 Top ranked differential serum and urinary metabolites between the two groups. (A) The peak height comparison of representative serum metabolites in two groups, T2DM with nephropathy group (blue), T2DM without nephropathy group (red), whose VIP value >1.5, P<5E-06. The statistical analysis of the significance between T2DM with nephropathy and without nephropathy group. Glycerol 1-octadecanoate, P=2.45E-14. L-glutamic acid/pyroglutamic acid, P=3.38E-12. Fructose 6-phophate, P=2.5E-10. Taurine, P=2.35E-09. Benzoic acid, P=3.16E-09. Erythrose, P=9.57E-09. L-Arabitol, P=3.28E-07. Fumaric acid, P=1.05E-06. L-Glutamine, P=2.28E-06. (B) The peak height comparison of representative urinary metabolites in two groups, T2DM with nephropathy group (blue) = T2DM without nephropathy group (red) = whose VIP value >1.5, P<5E-04. The statistical analysis of the significance between T2DM with nephropathy group and T2DM without nephropathy group. D-Glucose, P=2.12E-10. L-Valine, P=9.4E-07. L-Histidine, P=2.44E-05. Gluconic acid, P=3.6E-05. Sucrose, P=6.89E-05. Glycine, P=1.03E-04. L-Asparagine/L-aspartic acid, P=1.71E-04. L-Xylonate-2, P=1.76E-04. Oxalic acid, P=4.63E-04.

significant P values <0.05 were screened out (*Table 4*). Finally, 9 and 12 metabolic pathways were identified with significant differences in serum and urine metabolism, respectively. As the *Table 4* shows, 9 metabolic pathways are closely related to the occurrence of serum metabolism of DN, including arginine biosynthesis, galactose metabolism, valine, leucine and isoleucine biosynthesis, starch and sucrose metabolism, alanine, aspartate and glutamate metabolism, glutathione metabolism, D-glutamine and D-glutamate metabolism, glycine, serine and threonine metabolism, and nitrogen metabolism. Additionally, 12 metabolic pathways are closely related to the occurrence of urinary metabolism of DN, including starch and sucrose metabolism, valine, leucine, isoleucine biosynthesis, alanine, aspartate and glutamate metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, glyoxylate and dicarboxylate metabolism, phenylalanine metabolism; glycine, serine and threonine metabolism, D-glutamine and D-glutamate metabolism, arginine biosynthesis, nitrogen metabolism, galactose metabolism, and histidine metabolism. The

Table 4 Top-ranked altered serum and urine metabolic pathways

Sample	Pathway	P	Impact	Up	Down
Serum	Arginine biosynthesis	8.81E-04	0.44	L-Glutamic acid; Ornithine; L-Arginine; L-Glutamine	Fumaric acid
	Galactose metabolism	4.07E-03	0.16	D-Mannose; Sucrose	Glycerol; D-Galactose; Alpha-Lactose; Myoinositol
	Valine, leucine and isoleucine biosynthesis	9.24E-03	0.5		Alpha-ketoisovaleric acid; L-Threonine; L-Isoleucine
	Starch and sucrose metabolism	1.90E-02	0.16	Fructose 6-phosphate; Sucrose	D-Maltose; Isomaltose
	Alanine, aspartate and glutamate metabolism	2.23E-02	0.39	L-Glutamic acid; L-Glutamine; N-Acetyl-L-aspartic acid	Fumaric acid; L-Alanine
	Glutathione metabolism	2.23E-02	0.14	L-Glutamic acid; Ornithine	Glycine; Pyroglutamic acid; L-Cysteine
	D-Glutamine and D-glutamate metabolism	3.31E-02	0.67	L-Glutamic acid; L-Glutamine	
	Glycine, serine and threonine metabolism	4.25E-02	0.31		Glycine; Glyceric acid; L-Threonine; Sarcosine; L-Cysteine
	Nitrogen metabolism	4.51E-02	0.25	L-Glutamic acid; L-Glutamine	
Urine	Starch and sucrose metabolism	1.26E-03	0.31		D-Glucose; D-Maltose; Sucrose; Trehalose_2; Isomaltose_2
	Valine, leucine and isoleucine biosynthesis	5.24E-03	0	L-Threonine; Ketoleucine	L-Valine
	Alanine, aspartate and glutamate metabolism	9.83E-03	0.23		L-Glutamic acid; L-Alanine; L-Asparagine; Pyruvic acid; L-Glutamine
	Phenylalanine, tyrosine and tryptophan biosynthesis	1.32E-02	0.5	Phenylpyruvic acid	L-Phenylalanine
	Glyoxylate and dicarboxylate metabolism	1.52E-02	0.18		Glycine; L-Glutamic acid; L-Serine; Pyruvic acid; L-Glutamine
	Phenylalanine metabolism	1.79E-02	0.46	Phenylpyruvic acid	L-Phenylalanine; Hippuric acid
	Glycine, serine and threonine metabolism	1.96E-02	0.51	L-Threonine	Dimethylglycine; Glycine; L-Serine; Pyruvic acid
	D-Glutamine and D-glutamate metabolism	2.14E-02	1		L-Glutamic acid; L-Glutamine
	Arginine biosynthesis	2.75E-02	0.1		L-Glutamic acid; Ornithine; L-Glutamine
	Nitrogen metabolism	3.10E-02	0		L-Glutamic acid; L-Glutamine
	Galactose metabolism	3.91E-02	0.22		D-Glucose; D-Galactose; Myoinositol; Sucrose
	Histidine metabolism	3.95E-02	0.23		1-Methylhistidine; L-Glutamic acid; L-Histidine

significant serum and urinary metabolites observed in this study are shown in *Figure 3*.

Serum and urinary metabolic profiles in T2DM patients with nephropathy at different stages

We further compared the serum metabolites in three groups of patients with different stages of DN, and the score plots of PLS-DA are shown in *Figure 4A*. According to the model validation results, the PLS-DA (R2X =0.21; R2Y =0.326; Q2Y =0.29) showed distinct separation among all three groups. A 999-time permutation test was used to validate the reliability of the model, as illustrated in *Figure 4B*, with R2 =0.132 and Q2 =−0.105. Based on the VIP value of the PLS-DA (VIP >1), combined with ANOVA, 28 potential biomarkers were ultimately identified (P<0.05), including two species of organic acids (vanillylmandelic acid and taurine), ten carbohydrates, six amino acids and other metabolites (*Table 5*).

We also compared the urinary metabolites of the three groups of patients in different DN stages, and the PLS-DA score plot is shown in *Figure 4C*. PLS-DA model [R2X (cum) =0.09; R2Y (cum) =0.297; Q2Y =0.118] indicated good separation among all three groups. A 999-time permutation test was used to validate the reliability of the model, as illustrated in *Figure 4D*, with R2 =0.172 and Q2 =−0.079. Based on the VIP value of the PLS-DA (VIP >1), combined with ANOVA, 5 potential biomarkers were ultimately identified (P<0.05), including one organic acid, two types of carbohydrates (galactonic acid and L-arabinose), and their metabolites (*Table 5*).

Discussion

DN is a serious complication of T2DM. Patients with DN are at a significantly high risk for ESRD, and further the risks for cardiovascular morbidity and mortality increase persistently (3,9). Thus, the early diagnosis of DN can decrease morbidity by allowing for potential therapeutic interventions. However, there is no single diagnostic marker for the detection of DN. Over the last three decades, a large amount of scientific evidence has shown that many inflammatory markers are related to DN, for instance interleukin-1 (IL-1), IL-8, IL-6, tumor necrosis factor-alpha, transforming growth factor beta-1, cytokines, and the neutrophil-lymphocyte ratio in the complete blood count (27,28). Although there have been many studies on the occurrence and development of DN, the overall metabolic changes of T2DM that progress to kidney injury still lack clear characteristics. In this study, we used GC-TOFMS to identify distinct metabolic changes and related pathways during the occurrence and the development of DN in serum and urine samples from patients with DN. Dramatic changes in disease development and progression of DN metabolites could be a potential target for diagnosis or treatment.

Urea is a nitrogen-containing organic end product in protein metabolism, which can remove 80–90% of nitrogen from the human body (29). Recent studies suggest that it is probable that UA and Cr are involved in the pathogenesis of DN (30,31). Compared with T2DM patients, we found that serum UA and Cr levels of DN patients observably increased (P<0.05). Furthermore, when we compared the clinical features of the three stages of DN, we found that serum urea levels increased as the disease progressed (P<0.05). There is a obvious relationship between serum urea levels and disease stage, suggesting that DN progression may be associated with abnormal serum urea. However, we found that three different metabolites among the differential metabolites in serum and urine were involved in the metabolic process of the urea cycle. Those metabolites were fumaric acid, L-glutamine, and L-glutamic acid, which were present at significantly higher levels in the serum of DN patients than in T2DM patients, and L-glutamine, L-alanine and L-glutamate, which were present at significantly lower levels in the urine of DN patients, than in T2DM patients, as shown in *Table 2*. These changes may be a key warning sign, and if ignored, lead to irreversible kidney damage. However, the detailed mechanisms of serum and urine urea metabolism in T2DM DN need further research.

This research used GC-TOFMS metabolomics approach to identify metabolic changes associated with DN patients (32-34). Our data revealed a variety of organic acid metabolism disorders in DN patients, including lower serum taurine levels and higher serum benzoic acid and fumaric acid levels than in T2DM patients without nephropathy.

Taurine is a lesser-known organic acid in mammalian tissue and has multiple effects, such as bile acid conjugation, osmoregulation, viability and prevention of oxidant-induced tissue injury, and supplements that can stimulate prolactin and insulin release (35,36). Lin *et al.* (37) detected that taurine administration in diabetic rats conspicuously inhibited any further increase in urinary protein excretion. Winiarska *et al.* (38) found that taurine had the

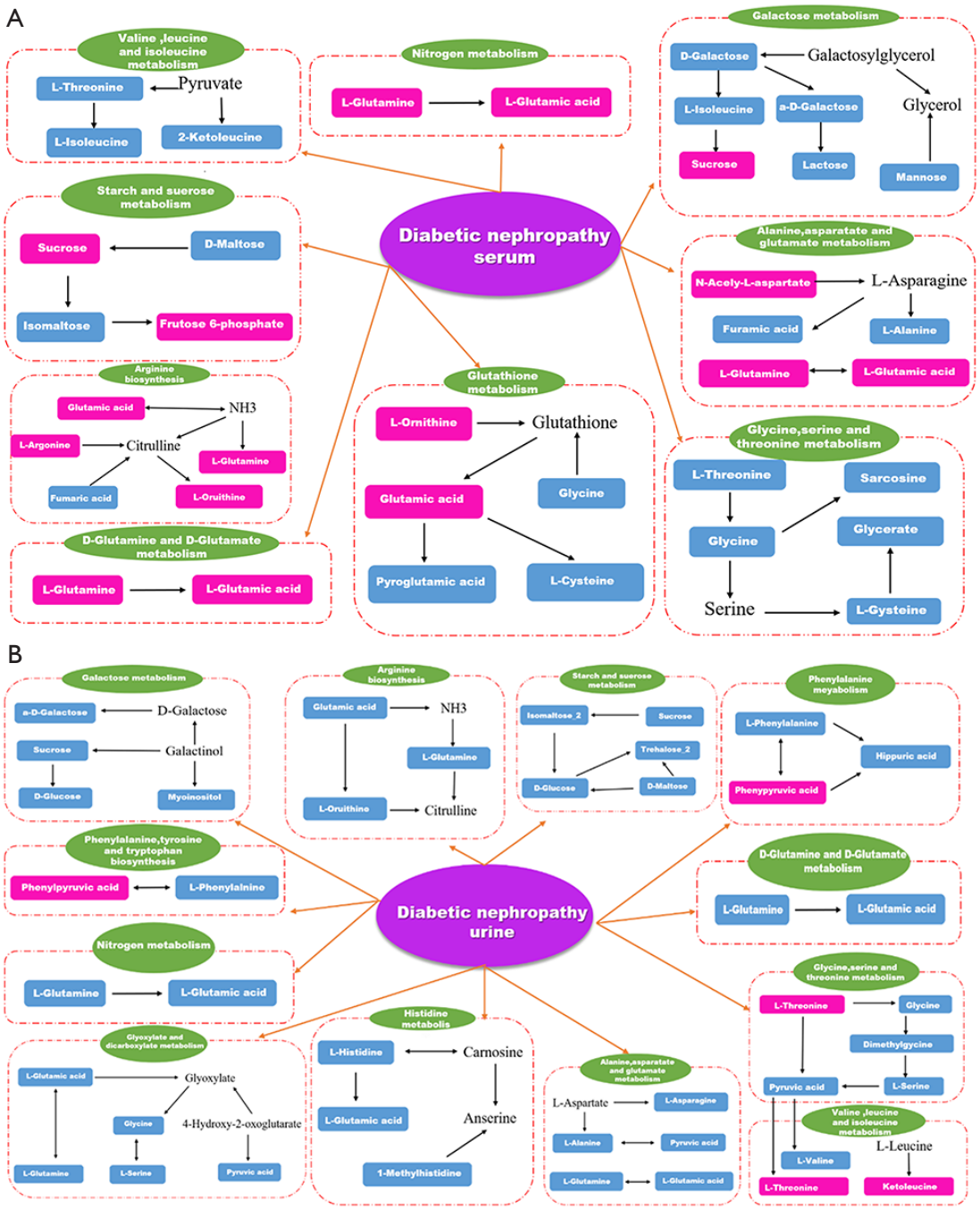

Figure 3 Serum and urinary top-ranked metabolic pathways. (A) For T2DM with nephropathy versus T2DM without nephropathy, the primary altered serum metabolism pathways are arginine biosynthesis; galactose metabolism; valine, leucine and isoleucine biosynthesis; starch and sucrose metabolism; alanine, aspartate and glutamate metabolism; glutathione metabolism; D-glutamine and D-glutamate metabolism; glycine, serine and threonine metabolism; and nitrogen metabolism ($P<0.05$). (B) For T2DM with nephropathy versus T2DM without nephropathy, the primary altered urinary metabolism pathways are starch and sucrose metabolism; valine, leucine and isoleucine biosynthesis; alanine, aspartate and glutamate metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; glyoxylate and dicarboxylate metabolism; phenylalanine metabolism; glycine, serine and threonine metabolism; D-glutamine and D-glutamate metabolism; arginine biosynthesis; nitrogen metabolism; galactose metabolism; and histidine metabolism ($P<0.05$). Red indicates elevated metabolites, blue indicates reduced metabolites.

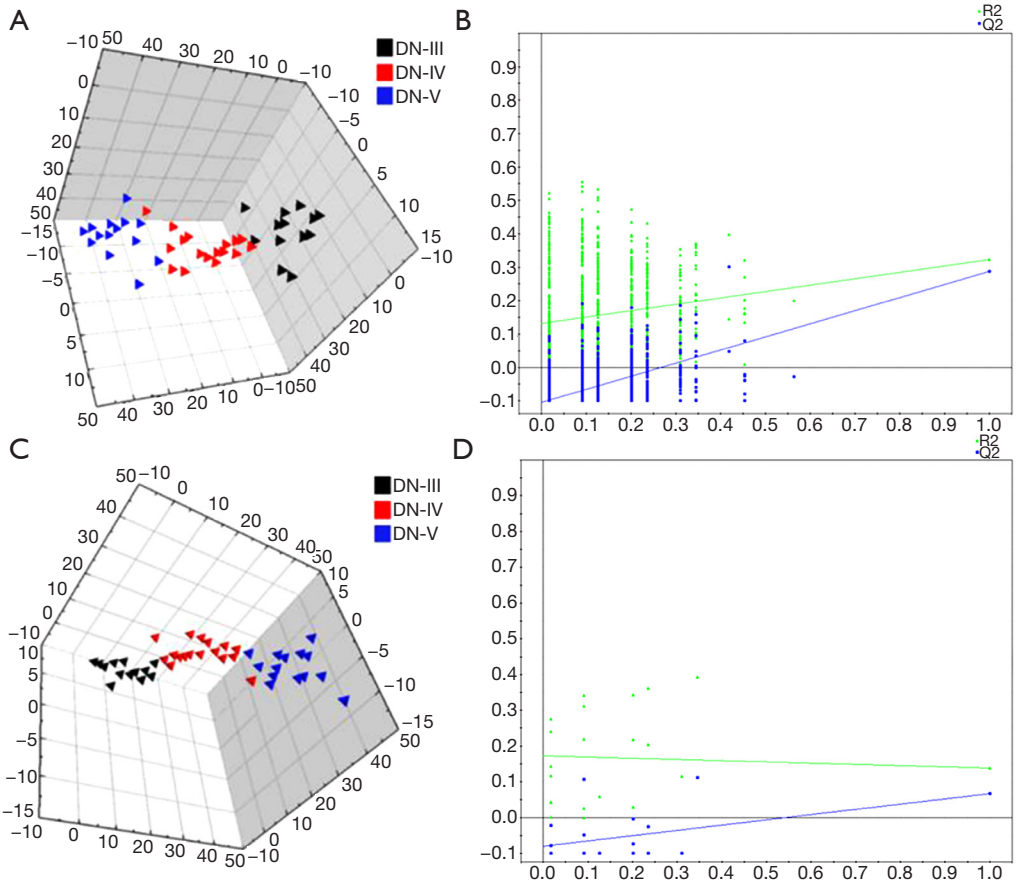

Figure 4 3D PLS-DA scores and validation plots for metabolic profiling of serum and urine in different stages of DN. (A) PLS-DA score plot of serum. (B) Validation plot of serum. (C) PLS-DA score plot of urine. (D) Validation plot of urine. Black triangles = DN-III, red triangles = DN-IV, blue triangles = DN-V.

characteristics of reducing proteinuria and glomerular disease in diabetes. In this study, GC-TOFMS-based metabolomics was used to detect DN-related metabolic changes in serum. Compared with T2DM patients, we observed a significantly decreased serum taurine levels in patients with DN, which suggests that taurine metabolic disorders are associated with diabetic kidney damage. In addition, in order to further assess kidney damage in patients with DN, we also compared the serum taurine levels in different stages of DN. The serum level of taurine decreased stepwise with the aggravation of kidney disease. This result also proves that taurine may be protective against DN (39).

We observed that the serum level of fumaric acid increased significantly in the pathways of arginine biosynthesis and alanine, aspartate, and glutamate metabolism of DN patients compared with the T2DM group. These findings indicate that disordered fumaric acid metabolism may be associated with diabetic renal impairment. Fumaric acid, a dicarboxylic acid, is a precursor to L-malate during the Krebs tricarboxylic acid (TCA) cycle. Zheng *et al.* (40) showed that the accumulated fumaric acid due to the inactivation of mutations enzyme fumarate hydratase (FH) in the TCA cycle led to oxidative stress. You *et al.* (41) found that Nox4 inhibited the regulation of fumarate levels, indicating that the TCA cyclic enzyme FH was the downstream target of Nox4. These results indicate that continuous oxidative stress cause diabetic renal injury, while fumaric acid may serve as a possible biomarker for evaluating the progression of DN.

Benzoic acid is a preservative in foods widely and is detoxified by glycine conjugation in human body. After binding to glycine, benzoic acid is excreted as hippuric acid. In this study, serum benzoic acid was significantly elevated

Table 5 Serum and urine metabolites that significantly changed among DN-III, DN-IV and DN-V groups

Sample	Class	Name	DN-III	DN-IV	DN-V	III *vs.* IV P value	III *vs.* V P value	IV *vs.* V P value	VIP	Pathway
Serum	Alcohols	Glycerol	554,871 (405,622–718,374)	530,712 (378,659–783,090)	1,356,328 (692,536–1,598,110.75)$^{*\#}$	0.8835416	0.0197352	0.048111	1.22482	Galactose metabolism, glycerolipid metabolism
	Amino acid	N-Acetyl-L-aspartic acid	7,770 (5,369–12,691)	23,028 (16,692–60,100)***	357,297 (179,723.5–535,292)$^{***\#\#\#}$	0.0006474	4.71E-05	0.0003343	1.52243	Aspartate metabolism
		Alpha-ketoisovaleric acid	6,586 (4,220–8,518)	3,687 (2,928–5,151)**	1,770 (1,290–2,491.75)$^{***\#\#}$	0.0061593	0.0003789	0.0061593	1.48702	Valine, Leucine and Isoleucine Degradation
		L-Glutamic acid	398,915 (323,825–470,549)	256,626 (199,139–396,871)*	139,187 (90,730.25–23,7134.5)$^{**\#\#}$	0.0225532	0.0024392	0.0072421	1.62093	Alanine metabolism, amino sugar metabolism, ammonia recycling, arginine and proline metabolism, cysteine metabolism, folate metabolism, glucose-alanine cycle, glutamate metabolism, glutathione metabolism, glycine and serine metabolism, histidine metabolism, malate-aspartate shuttle, transcription/translation, urea cycle
		1-Methylhistidine	6,977 (5,963–7,954)	9,821 (8,354–21,290)*	43,756.5 (38,094.25–57,711)$^{***\#\#\#}$	0.0251514	3.77E-05	0.0003343	1.98778	Histidine metabolism
		L-Proline	593,489 (374,549–952,896)	831,689 (525,705–1,032,912)	1,219,261 (1,048,785.5–1,424,675.75)$^{**\#\#}$	0.2673994	0.0038324	0.0038324	1.54998	Arginine and proline metabolism, transcription/translation
		Creatinine	242,432 (224,664–286,246)	312,233 (190,620–447,288)	158,3951.5 (1,255,862.5–2,448,715.75)$^{***\#\#\#}$	0.3909141	4.71E-05	9.14E-05	1.95035	Arginine and proline metabolism; Metabolic pathways
		Alpha-ketoisovaleric acid/L-Valine	0.0096 (0.0063–0.0113)	0.0061 (0.0037–0.0067)	0.0034 (0.0027–0.005)**	0.1165453	0.0024392	0.1017904	1.15383	Valine, leucine and isoleucine degradation; pantothenate and CoA biosynthesis
	Carbohydrate metabolism	L-Arabinose/L-Arabitol	0.4219 (0.2254–0.5234)	0.295 (0.1474–0.4964)	0.1359 (0.0498–0.1827)$^{**\#\#}$	0.4387786	0.004084	0.0081892	1.41058	Pentose and glucuronate interconversions
		L-Glutamine/L-Glutamic acid	5.9833 (4.2901–7.1692)	8.0008 (7.1466–11.0582)*	15.5129 (12.5254–27.2526)$^{***\#\#}$	0.0201914	0.0006781	0.0033596	1.78315	Glyoxylate and dicarboxylate metabolism; alanine, aspartate and glutamate metabolism
		L-Arabitol	62,868 (53,664–78,121)	99,356 (73,143–184,132)**	255,076 (232,777.25–322,292.5)$^{***\#\#}$	0.0069457	9.01E-05	0.0014783	2.01299	Pentose and glucuronate interconversions; metabolic pathways
		D-Threitol	8,982 (5,350–9,827)	13,297 (10,258–18,161)**	56,686 (48,846.5–74,620.5)$^{***\#\#\#}$	0.0069457	3.02E-05	0.0003905	1.71417	NA
		Threonic acid	30,265 (26,836–44,175)	45,008 (34,548–78,503)*	255,433.5 (183,747.75–337,353)$^{***\#\#\#}$	0.0113404	3.02E-05	0.0002858	1.56505	NA
		D-Maltose	11,284 (7,260–19,087)	20,425 (12,039–60,104)	138,927 (71,495.5–205,625.5)$^{***\#\#\#}$	0.0983015	9.01E-05	0.0002858	1.86072	Starch and sucrose metabolism
		Erythritol	52,721 (26,677–63,597)	57,960 (45,451–93,530)	178,694 (159,213.75–316,899.5)$^{***\#\#}$	0.1873931	3.77E-05	0.0012827	1.56967	NA
		Galactonic acid	18,020 (13,996–22,177)	25,906 (10,121–66,028)	164,484 (138,925.5–195,967.5)$^{***\#\#\#}$	0.2169695	9.01E-05	9.14E-05	2.10123	Galactose metabolism; metabolic pathways; microbial metabolism in diverse environments
		Isomaltose	3,297 (1,953–7,873)	6,234 (2,356–15,530)	48,503.5 (17,205.25–61,323)$^{***\#\#\#}$	0.3253535	0.0002542	0.0005304	1.86904	NA
		D-Glucuronic acid	25,651 (14,313–74,686)	58,577 (15,389–98,087)	256,148 (163,050.5–440,611)$^{***\#\#\#}$	0.4144377	0.0001769	0.0001769	1.77011	Inositol metabolism, starch and sucrose metabolism
		Ribonolactone	23,058 (20,454–32,604)	19,330 (9,341–35,691)	92,405.5 (60,316.5–141,768.5)$^{**\#\#}$	0.6303067	0.0085125	0.0085125	1.51333	NA
		Mannitol	117,196 (75,382–313,193)	203,909 (45,601–1,326,186)	14,67,111.5 (989,205.5–2,226,217.75)$^{***\#\#}$	0.753606	0.0009885	0.0038324	1.60645	Fructose and mannose metabolism; ABC transporters; phosphotransferase system (PTS)
	Hormone	Normetanephrine	4,808 (3,706–6,685)	4,142 (3,088–6,334)	2,886 (2,356.25–3,741.5)*	0.5165309	0.0348718	0.0530947	1.06807	Tyrosine metabolism
	Lipid metabolism	Glycerol 3-phosphate/Glycerol	0.1187 (0.0618–0.1576)	0.0769 (0.0461–0.1311)	0.0166 (0.0155–0.0419)$^{***\#\#}$	0.2017864	0.0006781	0.0072421	1.42454	Glycerolipid metabolism
	Metabolism of other amino acids	Beta-Alanine/L-Aspartic acid	0.0685 (0.0478–0.0878)	0.0826 (0.0493–0.104)	0.1561 (0.1266–0.1976)$^{**\#\#\#}$	0.7220354	0.0017235	0.0009109	1.49771	Pantothenate and CoA biosynthesis; beta-alanine metabolism
		L-Glutamic acid/Pyroglutamic acid	0.1722 (0.1365–0.2208)	0.1541 (0.1187–0.1799)	0.085 (0.0541–0.1064)$^{***\#\#}$	0.2017864	0.0004611	0.0019551	1.53127	Glutathione metabolism
	Nucleotide	Cytidine	497 (310–616)	596 (380–956)	3,504.5 (1,921.5–4,671.25)$^{***\#\#\#}$	0.3463645	0.0006781	0.0009613	1.86751	Pyrimidine metabolism
	Organic Acids	Vanillylmandelic acid	315 (223–394)	545 (332–991)*	3,943.5 (2,513.5–6,400)$^{***\#\#}$	0.0345568	0.000152	0.0011112	1.7413	Tyrosine metabolism
		Taurine	189,162 (163,813–268,372)	177,816 (83,163–225,459)	95,250.5 (59,044.5–196,355)*	0.2497638	0.0303438	0.1210744	1.1412	Bile acid biosynthesis, taurine and hypotaurine metabolism
	Phenols	m-Cresol	107,93 (5,845–32,654)	14,797 (6,190–35,789)	117,373 (63,152.5–169,724.25)$^{**\#\#}$	0.600872	0.0028477	0.0028477	1.51486	Toluene degradation; microbial metabolism in diverse environments; degradation of aromatic compounds
Urine	Organic acids	4-Hydroxybenzoic acid	21,514 (13,167–26,186)	56,709 (21,956–75,355)	116,479.5 (50,411–577,073.5)$^{*\#}$	0.051642	0.0125878	0.0287743	2.30879	Ubiquinone biosynthesis
	Carbohydrates	L-Arabinose	353,611 (253,073–675,129)	490,355 (408,902–728,241)	417,832.5 (340,508.5–460,281)$^{\#}$	0.206023	0.6623032	0.0415604	1.48153	Pentose and glucuronate interconversions; ascorbate and aldarate metabolism; amino sugar and nucleotide sugar metabolism; metabolic pathways; ABC transporters
		Galactonic acid	89,112 (50,457–104,671)	58,110 (48,030–94,308)	36,133 (23,138.75–43,421.75)$^{**\#}$	0.3463645	0.0067036	0.0148147	1.89233	Galactose metabolism; metabolic pathways; microbial metabolism in diverse environments
	Amino acid metabolism	L-Asparagine/L-Aspartic acid	20.8357 (11.5539–28.9061)	9.155 (6.7247–18.4256)	6.4576 (4.2255–9.2497)**	0.0574455	0.0024392	0.0878498	1.65339	Alanine, aspartate and glutamate metabolism
	Nucleotide metabolism	Inosine/Adenosine	0.1917 (0.1556–0.3425)	0.3472 (0.2507–0.5857)	0.5713 (0.3275–1.2048)*	0.107116	0.0107885	0.107116	1.9016	Purine metabolism

Differential metabolites were selected according to VIP >1 and P<0.05. Values are expressed as medians (IQRs). ANOVA test was conducted in the comparison among DN stages. T-test was performed in the comparison between two groups. *P<0.05; **P<0.01; ***P<0.001 when compared to DN-III, and $^{\#}$P<0.05; $^{\#\#}$P<0.01; $^{\#\#\#}$P<0.001 when compared to DN-IV.

in DN patients, probably because of the consumption of serum glycine in DN patients, resulting in a decrease in the binding of benzoic acid to glycine, resulting in the accumulation of benzoic acid and causing renal toxicity (42,43). Whether benzoic acid is a potential biomarker affecting DN progression requires further investigation.

Compared with T2DM patients without nephropathy, the levels of L-glutamic acid and pyroglutamic acid detected in serum samples from DN patients were significantly reduced. L-glutamic acid is a signaling molecule that regulates β-cells and secretes insulin to maintain blood sugar balance in the body (44). Insulin deficiency and increased gluconeogenesis through impaired cellular function can lead to increased L-glutamic acid catabolism, resulting in a significant decrease in L-glutamic acid contents (45). Our research found that serum L-glutamic acid levels differ in T2DM and DN patients, and from DN-III to DN-V, the serum L-glutamic acid content continues to decrease with the severity of kidney disease. Therefore, L-glutamic acid is considered to be a potential biomarker for kidney injury in DN.

We also found changes in carbohydrates in the serum of DN patients, including fructose 6-phosphate, erythrose, and L-Arabitol. The abnormality of these carbohydrates suggested that saccharides plays a vital role in the process of DN. Fructose 6-phosphate, one of the class of organic compounds, is known as hexose phosphates , and is a carbohydrate derivative with a hexose replaced by one or more phosphate groups (46). Elbein (47) reported that glutamine-fructose-6-phosphate aminotransferase is the major rate-limiting enzyme of this pathway, which increases the expression of the *glutamine-fructose-6-phosphate transaminase 1 (GFPT1)* gene, thereby increasing the susceptibility to diabetes and diabetic nephropathy. We observed a significant decreased serum level of fructose 6-phosphate in DN patients compared with T2DM patients, which suggests that diabetic renal impairment may affect glucose-6-phosphate metabolism. Gluconic acid occurs naturally in fruit. Normally, glucose is provided by glycolysis and cyclic decomposition. In T2DM, there is an obstacle to the catabolism of glucose (48).

Glycerol 1-octadecanoate, a monoacylglyceride or a monoacylglycerol, is a type of glycerol that is covalently bonded to a glycerol molecule by an ester bond through a fatty acid chain (49). Monoacylglycerols are the main end-products in animals during the intestinal digestion of dietary fats by the enzyme pancreatic lipase. They are absorbed directly by intestinal cells and are transformed into triacylglycerols through the monoacylglycerol pathway before being transitted in lymph to the liver (50). Our data show that DN patients had lower serum levels of glycerol 1-octadecanoate than patients with T2DM without nephropathy, which suggests that glycerol 1-octadecanoate metabolism disorder may be related to diabetic kidney damage. Whether glycerol 1-octadecanoate can be a biomarker for the early diagnosis of DN needs further study.

In addition, metabolic disorders in the urine are also traceable. In the present study, T2DM patients with nephropathy excreted daily averages of more urinary l-xylonate-2 and oxalic acid than those without nephropathy. Hyperoxaluria is a metabolic disorder with oxalate crystal deposition in various organs, including the kidney (51). The mechanism leading to hyperoxaluria is an increased load of free fatty acids in the intestine, resulting in reduced binding of calcium to free fatty acids and reduced synthesis of calcium oxalate. Then, an increasing amount of oxalic acid remains free and is absorbed in the intestine, leading to hyperoxaluria (52). Therefore, inhibition of oxalic acid may be a promising treatment strategy for patients with DN.

The levels of L-valine, L-histidine, glycine, L-asparagine, and L-aspartic acid found in urine samples from DN patients were observably elevated, indicating that urine amino acid metabolism associated with the development of DN.

The kidney is an important site of amino acid metabolism. Therefore, abnormal amino acid metabolism may be predicted as kidney damage. L-glycine has been shown the protection roles against several complications of diabetes in recent years (53,54). L-glycine protects the kidneys, but the precise mechanisms by which L-glycine ameliorates DN remain unclear (45). In our population, we observed a significant increase in urine L-glycine concentration in DN patients compared with T2DM patients. Therefore, L-glycine can not only be a drug for the treatment of diabetic complications but also a potential diagnostic biomarker for DN.

The levels of urine glucose, such as D-glucose, sucrose and gluconic acid, found in urine samples of patients with DN were significantly increased. Wei *et al.* (55) showed that increasing urine levels of TCA cycle intermediates in DN mice, including citrate, cis-aconitate, and fumarate, which might reflect the system pressure caused by hyperglycemia or local influences on kidney impaired tubule transport and mitochondrial function (56). In the ESRD group, the urine contents of galactonic acid and allose were significantly lower than those in the early DN and clinical DN groups. Thus, urine saccharides can be recognized as potential biomarkers for evaluating the progress of DN (57-59).

There are still several limitations in our study. First, the number of patients was relatively small in each group. Second, although we found that serum taurine concentrations were lower in DN patients than in those without nephropathy and decreased along with the progress of nephropathy, direct evidence is lacking to support the association between serum taurine concentration and deterioration of renal function. Third, the study lacks a robust validation in an independent population. In the near future, we plan to select several metabolites that have increased or decreased the most from the GC-TOFMS metabolomics analysis and use targeted metabolomics to quantitatively evaluate the serum or urine levels of those metabolites. In addition, we will combine molecular biology approaches and other methods to further explore factors and the relevant molecular mechanisms that influence the changes of these metabolites and to explain the reasons for the changes of these metabolites.

In addition, we also collected clinical serum and urine samples in other diabetic complications, such as patients with diabetic peripheral neuropathy, and used methods similar to GC-TOFMS-based metabolomics to reveal the metabolic differences between T2DM patients with or without peripheral neuropathy. Some interesting and novel results were found between the two groups, which we will show in the near future.

Conclusions

In the present study, we integrated information regarding serum and urine metabolites using GC-TOFMS-based metabolomics to reveal the differences between T2DM patients with or without nephropathy and metabolic changes in the development of DN. Our results showed that several metabolic disorders occur, including altered organic acid metabolism and disordered glucose metabolism, during the onset and pathogenesis of DN. These results show that GC-TOFMS-based metabolomics is a promising way to reveal potential metabolic pathways to assess the risk of DN complications and to evaluate therapeutic effects.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All patients who participated in the study offered a signed informed consent form. The ethical approval for the present study was provided by the Ethics Committee of Shanghai University of Traditional Chinese Medicine.

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Supplementary

Table S1 Clinical information of T2DM patients with and without nephropathy and among DN-III, DN-IV and DN-V groups

Characteristics	T2DM without nephropathy (n=44)	T2DM with nephropathy (n=44)				P value			
		Overall	DN-III	DN-IV	DN-V	DN *vs*. T2DM	III *vs*. IV	III *vs*. V	IV *vs*. V
Gender (M/F)	31/13	26/18	7/6	10/7	9/5	0.697	1	1	1
Age (years)	71 (55.75–79.5)	62 (55.75–71.25)	71 [56–81]	62 [58–70]	61 (55.25–67)	0.176	0.519	0.519	0.72
BMI (kg/m^2)	24.515 (22.81–25.978)	23.83 (21.14–27.085)	23.14 (21.63–24.98)	26.79 (25.3–28.37)*	21.2 (20.55–23.31)$^{\#\#}$	0.932	0.034	0.521	0.008
Glucose (mmol/L)	7.45 (4.95–9.7)	7.8 (5.475–9.525)	8.2 (6.7–11.9)	7.5 (4.9–9.6)	7.25 (4.95–8.775)	0.826	0.232	0.185	0.487
2hPG (mmol/L)	11 (7.125–15.175)	14 (10.45–16.875)	15.3 (13.3–16.35)	16.1 (13.3–19.8)	9.05 (6.575–12.95)**$^{\#\#}$	0.06	0.716	0.008	0.008
C-peptide (pmol/mL)	1.39 (0.85–1.98)	1.63 (0.89–2.54)	1.195 (0.79–1.57)	1.575 (0.8225–2.405)	3.44 (1.95–4.46)**$^{\#}$	0.17	0.202	0.004	0.021
2hC-peptide (pmol/mL)	2.005 (1.1–3.785)	3.01 (2.1–4.7)	2.29 (1.782–2.87)	3.19 (1.965–5.0425)	4.3 (2.35–4.85)	0.03	0.398	0.124	0.511
Insulin (mmol/L)	69.43 (42.985–105.31)	11.79 (7.48–20.93)	9.2 (7.112–12.903)	13.385 (11.175–23.033)	8.48 (5.56–18.18)	<0.001	0.143	0.828	0.143
HbAlC (mg/dL, %)	8.3 (7.05–9.5)	7.8 (6.6–9.7)	9.9 (9.1–11.4)	7.7 (7–9.375)*	6.65 (6.15–7.625)**$^{\#}$	0.618	0.043	0.005	0.043
WBC (*10^9/L)	7.27 (6.212–8.748)	6.1 (5.1–8)	5.9 (5.6–7.4)	6.1 (5.1–8)	7.25 (4.725–8.4)	0.065	0.802	0.802	0.802
Neu (%)	63.1 (58.025–71.35)	65 (58.6–73.75)	62.3 (53.6–74.9)	67.6 (57.2–70.4)	64.4 (62.4–79.2)	0.714	0.967	0.522	0.622
Lym (%)	23.05 (17.5–29.4)	23.1 (16.1–31.5)	29.5 (15.8–38.9)	23.3 (20.5–32.3)	19.4 (14.875–24)	0.89	0.544	0.168	0.168
Mon (%)	8 (7.175–9.85)	6.5 (5.5–8.025)	5.7 (5.3–7)	6 (5.5–7.7)	7.95 (6.05–9.1)	0.001	0.721	0.081	0.143
Eso (%)	2.3 (1.675–3.575)	2.3 (1.25–3.55)	2 (1.2–2.4)	2.4 (1.8–4.1)	2.3 (1.4–4.1)	0.538	0.475	0.475	0.9
Bas (%)	0.3 (0.175–0.5)	0.4 (0.2–0.5)	0.4 (0.2–0.5)	0.3 (0.275–0.5)	0.4 (0.2–0.4)	0.174	0.751	0.751	0.751
RBC (*10^{12}/L)	4.04 (3.725–4.403)	3.825 (3.17–4.288)	4.02 (3.52–4.44)	3.86 (3.14–4.54)	3.44 (2.95–4.083)	0.197	0.477	0.268	0.374
HGB (g/L)	116.5 (101.75–126)	110.5 (92.5–130.5)	128 [104–135]	110 [91–136]	98.5 (88.75–118.5)*	0.534	0.302	0.04	0.302
HCT (%)	34.25 (31.025–36.3)	32.1 (27.7–38.65)	36.7 (30–42.2)	32 (26.7–38.8)	30.15 (27.7–35.75)	0.751	0.19	0.132	0.62
MCV (%)	86.35 (82.725–89.75)	87.8 (85.2–90.575)	88.1 (85.2–89.8)	86.1 (84.4–89.2)	90.75 (86.75–93.65)	0.131	0.295	0.198	0.052
HGB-Mass (pg)	29 (27.8–30.45)	29.7 (28.8–31)	30.5 (29.8–31.8)	29.6 (28.8–31)	29.2 (28.65–29.9)*	0.042	0.147	0.023	0.35
HGB-Conc (g/L)	335 (328.5–344.25)	336.5 (321–351.25)	351 [347–358]	340 [329–354]	322 [318–333]**$^{\#}$	0.655	0.116	0.002	0.034
RDW-CV (%)	13.55 (12.85–14.525)	13.05 (12.575–13.9)	12.5 (12.3–12.9)	13 (12.6–13.8)	13.7 (13.4–14.5)**$^{\#}$	0.306	0.126	0.003	0.037
PLT (*10^9/L)	246 (194.5–302.5)	193 (153.5–224)	184 [154–197]	199 [157–233]	194 (152.5–258.75)	0.003	0.497	0.497	0.889
MPV (fL)	10.5 (9.7–11.125)	11.1 (10.2–12.15)	12.3 (11.8–13.1)	10.5 (9.8–11.4)*	10.9 (10.125–11.9)*	0.01	0.011	0.019	0.462
hs-CRP (mg/L)	7 (4–34.5)	2 (1–9.5)	1.5 (1–6.25)	1 [1–5]	5 [1–17]	<0.001	0.556	0.556	0.451
TP (g/L)	65.55 (60.5–68.825)	64 [56–69]	66.5 (63.75–70)	65 [55–72]	58 (53.5–64.75)*	0.364	0.412	0.017	0.398
ALB (g/L)	35.7 (32.25–37.225)	35 (29.5–38)	38 (35.75–39.5)	37 [30–40]	30.5 (28.25–33.75)***	0.69	0.258	0.001	0.142
GLB (g/L)	30.2 (27.35–34.825)	29 (25–31.5)	29 (27–30.5)	28 [25–31]	28 (24–31.5)	0.047	0.811	0.811	0.811
A/G	1.15 (1–1.3)	1.2 (1.08–1.365)	1.27 (1.197–1.418)	1.12 (1.06–1.39)	1.095 (0.967–1.21)	0.239	0.138	0.085	0.499
AST (U/L)	14.5 (11–23.25)	16 (11.5–22)	15 (12.75–18.25)	17 [13–27]	16 (11–22.25)	0.538	0.572	0.959	0.572
ALT (U/L)	17 (13.75–20.25)	19 (14.5–24)	16 [14–19]	21 [20–35]*	15.5 (12.5–22.5)$^{\#}$	0.186	0.011	0.857	0.011
AST_ALT	0.92 (0.71–1.285)	0.88 (0.605–1.11)	0.905 (0.725–1.103)	0.77 (0.58–0.95)	0.96 (0.555–1.2825)	0.259	0.426	0.959	0.426
r-GT (U/L)	23.5 (16.75–34)	25 [17–39]	25.5 (20.5–30.75)	23 [18–42]	24.5 (15–43.75)	0.884	0.965	0.965	0.965
AKP (U/L)	97 (77.25–118.25)	85 [75–91]	84.5 (63.75–89.75)	85 [75–89]	86.5 (75.75–100.5)	0.079	0.859	0.640	0.64
TBIL (μmol/L)	7.9 (6.025–8.625)	10 [7–13]	11.5 (8–13.5)	11 [8–13]	7 [7–8]	0.004	0.688	0.087	0.087
DBIL (μmol/L)	3.1 (2.1–3.8)	1.6 (1.2–2)	2.1 (1.45–2.75)	1.7 (1.5–1.9)	1.2 (0.925–1.45)*$^{\#}$	<0.001	0.351	0.017	0.019
TBA (μmol/L)	3.15 (2.475–3.925)	4 (2.5–7)	6 (6–9.25)	4 [2–8]	3 (2–3.75)**	0.244	0.066	0.002	0.132
Cr (μmol/L)	72 (58.5–94.25)	119 (69–431.75)	66 [58–88]	107 [70–243]	714.5 (431.75–854)***$^{\#\#\#}$	0.002	0.063	<0.001	<0.001
Urea (mmol/L)	6.85 (4.525–8.15)	9.15 (6.275–20.65)	6.4 (5.1–6.8)	9.1 (8–12.8)*	21.45 (16.725–24.05)***$^{\#\#\#}$	0.001	0.013	<0.001	<0.001
UA (μmol/L)	262.5 (210.75–327.75)	382 (309–432.25)	311 [284–403]	380 [330–425]	437.5 [384–459]*	<0.001	0.108	0.013	0.153
Ca (mmol/L)	2.24 (2.16–2.395)	2.165 (2.087–2.273)	2.165 (2.095–2.278)	2.165 (2.04–2.313)	2.16 (2.09–2.188)	0.02	0.921	0.921	0.921
P (mmol/L)	1.22 (1.1–1.3725)	1.225 (1.07–1.565)	1.08 (1.04–1.108)	1.265 (1.135–1.41)	1.54 (1.19–1.875)	0.65	0.111	0.105	0.111
K (mmol/L)	4 (3.75–4.2)	4.2 (3.8–4.45)	4.3 (4.025–4.325)	4.1 (3.9–4.5)	4.1 (3.575–4.975)	0.223	0.979	0.979	0.979
Na (mmol/L)	139 (137–140)	141 (138.5–142)	140 (137.5–141)	142 [140–142]	140 (137.5–141.75)	0.028	0.26	0.938	0.403
Cl (mmol/L)	102 (100.5–104)	104 (101–104.5)	103 (101–104.25)	104 [101–107]	103.5 (97.25–104)	0.443	0.461	0.461	0.322
TG (mmol/L)	1.03 (0.847–1.378)	1.61 (1.305–2.41)	1.945 (1.235–2.503)	2.11 (1.49–2.42)	1.355 (1.267–1.663)	<0.001	0.565	0.565	0.129
TC (mmol/L)	3.945 (3.522–4.873)	4.95 (3.83–5.57)	5.225 (3.917–5.645)	5.01 (4.43–5.76)	4.38 (3.607–5.198)	0.019	0.965	0.473	0.473
HDL (mmol/L)	1 (0.84–1.14)	1.05 (0.925–1.215)	1.13 (1.012–1.333)	1.11 (0.85–1.35)	1 (0.95–1.073)	0.573	0.690	0.227	0.425
LDL (mmol/L)	2.545 (2.015–3.26)	3.15 (2.42–3.74)	3.55 (2.5075–3.84)	3.07 (2.55–3.71)	3 (2.12–3.425)	0.033	0.626	0.626	0.626
ApoA (g/L)	1.06 (0.885–1.253)	1.12 (1.01–1.223)	1.17 (1.12–1.393)	1.12 (1.002–1.313)	1.04 (0.99–1.15)	0.59	0.544	0.284	0.353
ApoB (g/L)	0.785 (0.635–0.873)	0.95 (0.747–1.093)	0.95 (0.76–1.13)	0.97 (0.852–1.108)	0.94 (0.682–1.048)	0.01	0.66	0.66	0.66
Lp-A (mmol/L)	269 (111–415.25)	149.5 (35.75–364)	69 (35.25–151.75)	173 (30.5–364)	200.5 (50–386.25)	0.071	0.589	0.589	0.589
eGFR (mL/min/1.73 m^2)	8.775 (6.627–10.693)	6.405 (2.93–8.71)	7.91 (6.17–10.02)	4.72 (2.55–8.57)	6.46 (1.887–8.593)	0.028	0.054	0.285	0.451

Data were expressed as the medians (IQRs) at normal distribution. The Wilcoxon test was used to compare these variables between T2DM patients with and without nephropathy, and P<0.05 was considered significant. The ANOVA test was used to compare these variables among the three stages of DN. *P<0.05; **P<0.01; **P<0.001 when compared to DN-III, and #P<0.05; ##P<0.01; ###P<0.001 when compared to DN-IV. M, male; F, female; BMI, body mass index; 2hPG, two hours post-load plasma glucose; HbAlC, glycosylated hemoglobin or glycated hemoglobin; WBC, white blood cell; Neu, neutrophil ratio; Lym, lymphocyte ratio; Mon, monocyte ratio; Eso, eosinophil ratio; Bas, basophil ratio; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; HGB-Mass, RBC average HGB content; HGB-Conc, RBC average HGB concentration; RDW-CV, red blood cell distribution width coefficient of variation; PLT, platelet; MPV, mean platelet volume; hs-CRP, hypersensitive C-reactive protein; TP, total protein; ALB, albumin; GLB, globulin; A-G, albumin/globulin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AST-ALT, aspartate aminotransferase/alanine aminotransferase; r-GT, Glutamyl transpeptidase; AKP, alkaline phosphatase; TBIL, total bilirubin; DBIL, direct bilirubin; TBA, total bile acid; Cr, creatinine; UA, uric acid; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ApoA, apolipoprotein a; ApoB, apolipoprotein b; Lp-A, lipoprotein a; eGFR, estimated glomerular filtration rate.

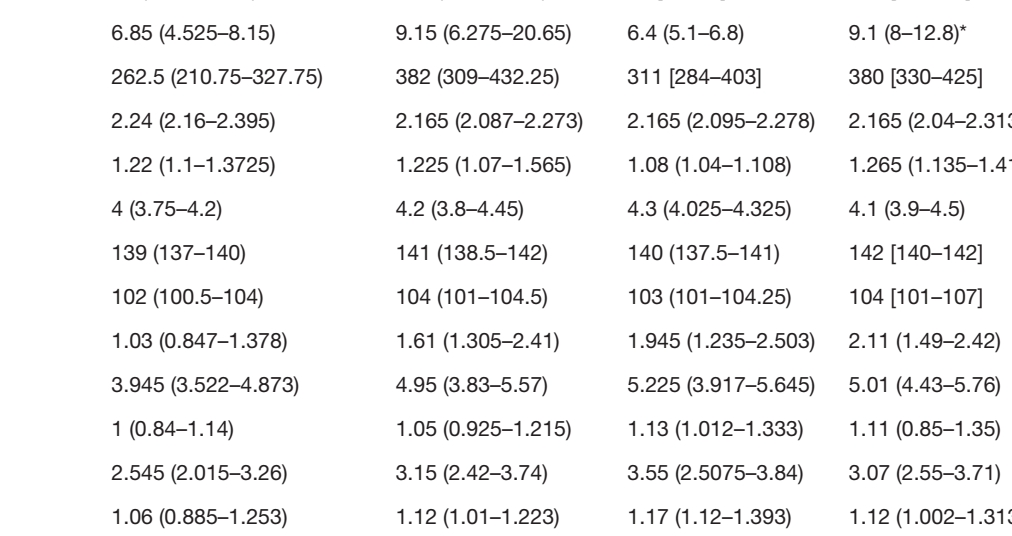

Figure S1 The permutation testing (20 times) result of the OPLS-DA model. (A) The permutation testing result of the serum OPLS-DA model. (B) The permutation testing result of the urine OPLS-DA model.

Table S2 Differential serum metabolites betweenT2DM with and without nephropathy

Name	Class	T test. P	VIP	FC	DN	T2DM	HMDBID	Kegg ID	Pathway
1-Methylhistidine	Amino acid	1.21E-04	1.3	0.6	15,612 (7,125.5, 39,741)	8,910.5 (6,143, 12,853.75)	HMDB00001	C01152	Histidine metabolism
Alpha-ketoisovaleric acid	Amino acid	1.00E-03	1.4	0.6	3,640 (1,992.5, 6,487)	2,190.5 (1,425.25, 3,217.5)	HMDB00019	C00141	Valine, leucine and isoleucine degradation
Alpha-ketoisovaleric acid/L-Valine	Amino acid metabolism; metabolism of cofactors and vitamins	4.08E-04	1.3	0.6	0.01 (0.003, 0.01)	0 (0.002, 0.005)	HMDB00019/HMDB00883	C00141/C00183	Valine, leucine and isoleucine degradation; pantothenate and CoA biosynthesis
Beta-Alanine	Amino acid	1.50E-04	1.4	0.7	10,038 (8,363.25, 15,552.5)	7,151.5 (6,114, 8,661)	HMDB00056	C00099	Aspartate metabolism, beta-alanine metabolism, propanoate metabolism, pyrimidine metabolism
Beta-Alanine/L-Aspartic acid	Metabolism of cofactors and vitamins; Metabolism of other amino acids	4.86E-05	1.5	0.5	0.09 (0.061, 0.144)	0.05 (0.037, 0.076)	HMDB00056/HMDB00191	C00099/C00049	Pantothenate and CoA biosynthesis; beta-alanine metabolism
Cytidine	Nucleotide	5.05E-04	1.2	0.7	761.5 (400.75, 2396.25)	498 (398, 615.5)	HMDB00089	C00475	Pyrimidine metabolism
Fructose 6-phosphate	Carbohydrates	2.50E-10	2	1.8	516,665.5 (371,837.5, 627,208)	934,897 (717,232, 1,136,517.25)	HMDB00124	C00085	Amino sugar metabolism, fructose and mannose degradation, gluconeogenesis, glycolysis, pentose phosphate pathway
Glycerol 3-phosphate/Glycerol	Lipid metabolism	5.41E-04	1.2	2.2	0.06 (0.019, 0.122)	0.13 (0.089, 0.153)	HMDB00126/HMDB00131	C00093/C00116	Glycerolipid metabolism
D-Glucuronic acid	Carbohydrates	7.63E-04	1.1	0.3	90,337.5 (16,006.25, 165,173.25)	23,810.5 (12,463, 53,869.25)	HMDB00127	C00191	Inositol Metabolism, starch and sucrose metabolism
Glycerol	Alcohols	8.09E-04	1.4	0.6	598,034.5 (388,745.25, 1,166,327)	387,146 (312,706.75, 526,477)	HMDB00131	C00116	Galactose metabolism, glycerolipid metabolism
Fumaric acid	Organic acids	1.05E-06	2	0.6	8,265 (6,316, 10,364.25)	4,577.5 (3,857.5, 5,716)	HMDB00134	C00122	Arginine and proline metabolism, aspartate metabolism, citric acid cycle, mitochondrial electron transport chain, phenylalanine and tyrosine metabolism, tyrosine metabolism, urea cycle
Glyceric acid	Organic acids	6.86E-04	1.5	0.7	47,358 (37,608.75, 59,286.25)	35,250 (29,520.75, 44,127.5)	HMDB00139	C00258	Glycerolipid Metabolism, Glycine and Serine Metabolism
L-Glutamic acid	Amino acid	2.28E-06	1.5	1.5	279,299.5 (167,237.25, 397,382)	426,439.5 (312,734.5, 578,261)	HMDB00148	C00025	Alanine metabolism, amino sugar metabolism, ammonia recycling, Arginine and proline metabolism, cysteine metabolism, folate metabolism, glucose-alanine cycle, glutamate metabolism, glutathione metabolism, glycine and serine metabolism, histidine metabolism, malate-aspartate shuttle, transcription/translation, urea cycle
L-Glutamic acid/Oxoglutaric acid	Amino acid metabolism	4.25E-04	1.4	2.7	25.75 (20.325, 42.947)	69.01 (40.097, 153.817)	HMDB00148/HMDB00208	C00025/C00026	Arginine biosynthesis
L-Glutamic acid/Pyroglutamic acid	Metabolism of other amino acids	3.38E-12	2.1	2.2	0.14 (0.092, 0.174)	0.3 (0.222, 0.384)	HMDB00148/HMDB00267	C00025/C01879	Glutathione metabolism
Hypoxanthine	Nucleotide	3.00E-03	1.2	0.7	44,123.5 (28,191, 77,664.75)	29,734.5 (22,674.75, 39,268.75)	HMDB00157	C00262	Purine Metabolism
L-Tyrosine/L-Phenylalanine	Amino acid metabolism	1.00E-03	1.5	0.6	1.87 (1.318, 2.323)	1.21 (0.861, 1.49)	HMDB00158/HMDB00159	C00082/C00079	Phenylalanine metabolism; Phenylalanine, tyrosine and tryptophan biosynthesis
L-Phenylalanine	Amino acid	3.02E-06	1.6	1.4	433,113 (323,081.25, 575,814)	620,982.5 (530,573.75, 707,172.25)	HMDB00159	C00079	Phenylalanine and tyrosine metabolism, transcription/translation
L-Proline	Amino acid	8.32E-04	1.2	0.5	961,090.5 (582,251.5, 1,158,145)	516,571 (459,014.75, 757,644.75)	HMDB00162	C00148	Arginine and proline metabolism, transcription/translation
D-Maltose	Carbohydrates	4.20E-04	1.1	0.5	29,752 (11,850.25, 74,833)	16,010.5 (10,526.75, 24,970.5)	HMDB00163	C00208	Starch and sucrose metabolism
L-Threonine	Amino acid	5.00E-03	1.2	0.9	280,966 (212,282.5, 433,769.75)	248,005.5 (192,284.5, 298,326)	HMDB00167	C00188	Glycine and serine metabolism, threonine and 2-oxobutanoate degradation, transcription/translation
D-Mannose	Carbohydrates	3.36E-04	1.3	1.4	1,056,861 (494,489.5, 1,326,627.5)	1,442,658.5 (905,777.75, 2,018,569.5)	HMDB00169	C00159	Fructose and mannose degradation, galactose metabolism
L-Isoleucine	Amino acid	4.00E-03	1.2	0.8	321,465 (283,167.75, 441,564.5)	271,347.5 (220,161.75, 359,676)	HMDB00172	C00407	Transcription/translation, valine, leucine and isoleucine degradation
Maleic acid	Organic acids	3.00E-03	1.4	0.9	13,293.5 (11,825, 16,123)	11,344 (10,363.5, 13,526)	HMDB00176	C01384	NA
L-Lysine	Amino acid	7.57E-04	1.5	0.8	640,215 (549,289.25, 689,639.5)	515,362.5 (424,460.5, 599,079.5)	HMDB00182	C00047	Biotin metabolism, carnitine synthesis, lysine degradation, transcription/translation
L-Lactic acid	Organic acids	1.15E-04	1.3	1.7	1,806,745 (1,201,846, 2,235,344.25)	3,155,801 (1,616,732, 4,879,156.25)	HMDB00190	C00186	Gluconeogenesis; pyruvate metabolism
L-Cystine	Amino acid	2.00E-03	1.3	0.7	175,895 (120,707.75, 238,916)	117,730.5 (87,831.5, 169,729.25)	HMDB00192	C00491	Cysteine and methionine metabolism; metabolic pathways; ABC transporters; ferroptosis; protein digestion and absorption
Myoinositol	Alcohols	6.18E-05	1.4	0.4	438,968.5 (215,182.25, 1,198,811.75)	170,756.5 (114,897.5, 289,559.5)	HMDB00211	C00137	Galactose metabolism, inositol metabolism, inositol phosphate metabolism, phosphatidylinositol phosphate metabolism
O-Phosphoethanolamine	Lipids	2.29E-05	1.5	0.6	24,043 (19,236, 30,112)	14,389.5 (10,347.75, 21,184)	HMDB00224	C00346	Sphingolipid metabolism
Taurine	Organic acids	2.35E-09	1.9	1.7	177,245.5 (93,433.75, 227,747.5)	309,364.5 (206,290.25, 385,725.5)	HMDB00251	C00245	Bile acid biosynthesis, taurine and hypotaurine metabolism
Pyroglutamic acid	Amino acid	3.00E-03	1.1	0.7	1,992,442.5 (1,415,175, 2,640,418.75)	1,439,163 (1,185,021.5, 1,782,831.75)	HMDB00267	C01879	Glutathione metabolism
Sarcosine	Amino acid	2.30E-02	1.3	0.9	9,147 (8,219.75, 10,872.75)	8519.5 (7,869.5, 8,961.25)	HMDB00271	C00213	Glycine and serine metabolism
Sarcosine/Dimethylglycine	Amino acid metabolism	9.00E-03	1.4	0.7	0.03 (0.018, 0.036)	0.02 (0.017, 0.026)	HMDB00271/HMDB00092	C00213/C01026	Glycine, serine and threonine metabolism
Uric acid	Organic acids	4.00E-03	1.3	0.6	292,716.5 (207,345, 341,138.5)	188,751.5 (146,691.25, 270,719.75)	HMDB00289	C00366	Purine metabolism
Vanillylmandelic acid	Organic acids	5.37E-04	1.2	0.6	530 (327.75, 2,513.5)	318 (220.25, 445.75)	HMDB00291	C05584	Tyrosine metabolism
5-Hydroxylysine	Amino acid	3.11E-04	1.6	0.5	2,092.5 (1,624.5, 4,172.25)	1,141.5 (727, 1,643.75)	HMDB00450	C16741	Lysine degradation; metabolic pathways
Creatinine	Amino acid	6.36E-04	1.2	0.8	338,468 (224,928.75, 1,273,947.25)	254,810.5 (179,753.5, 344,497.5)	HMDB00562	C00791	Arginine and proline metabolism; Metabolic pathways
Galactonic acid	Carbohydrates	2.29E-05	1.4	0.3	44,632 (17,963, 138,125.5)	15,075 (11,043, 31,052)	HMDB00565	C00880	Galactose metabolism; metabolic pathways; microbial metabolism in diverse environments
L-Cysteine	Amino acid	5.15E-04	1.4	0.7	60,307.5 (43,832, 82,471.5)	44,044 (34,087.25, 56,694.75)	HMDB00574	NA	Cysteine metabolism, glutathione metabolism, glycine and serine metabolism, methionine metabolism, pantothenate and CoA biosynthesis, taurine and hypotaurine metabolism, transcription/translation
L-Glutamine	Amino acid	2.28E-06	1.8	1.2	2,323,035 (1,899,199, 2,747,632.5)	2,845,450 (2,567,941.25, 3,055,334.25)	HMDB00641	C00064	Amino sugar metabolism, ammonia recycling, glutamate metabolism, phenylacetate metabolism, purine metabolism, pyrimidine metabolism, transcription/translation, urea cycle
L-Glutamine/L-Glutamic acid	Carbohydrate metabolism; Amino acid metabolism	1.00E-03	1.1	0.7	9.28 (6.079, 14.034)	6.66 (4.532, 8.929)	HMDB00641/HMDB00148	C00064/C00025	Glyoxylate and dicarboxylate metabolism; alanine, aspartate and glutamate metabolism
L-Arabinose/L-Arabitol	Carbohydrate metabolism	1.56E-05	1.5	2.1	0.22 (0.143, 0.436)	0.46 (0.393, 0.6)	HMDB00646/HMDB01851	C00259/C00532	Pentose and glucuronate interconversions
Linoleic acid	Fatty acids	3.60E-02	1.2	0.8	162,340.5 (109,351.5, 223,310)	131,850 (91,854.25, 183,544.5)	HMDB00673	C01595	Alpha Linolenic Acid and Linoleic Acid Metabolism
Mannitol	Carbohydrates	1.54E-04	1.3	0.3	429,528.5 (98,217.25, 1,399,185.25)	116,745 (50,414.5, 306,783.75)	HMDB00765	C00392	Fructose and mannose metabolism; ABC transporters; phosphotransferase system (PTS)
N-Acetyl-L-aspartic acid	Amino acid	3.00E-03	1.1	1.1	27,759 (10,844, 179,723.5)	29,926.5 (15,873.25, 46,000.5)	HMDB00812	C01042	Aspartate metabolism
Normetanephrine	Hormone	5.00E-03	1.4	0.8	3,813.5 (2,690.25, 5,572.75)	2,953 (2,047.5, 3,518.5)	HMDB00819	C05589	Tyrosine metabolism
Threonic acid	Carbohydrates	1.00E-03	1.2	0.9	51,359.5 (34,386, 201,621.75)	45,318.5 (30,874.25, 56,184)	HMDB00943	C01620	NA
L-Sorbose	Carbohydrates	6.97E-06	1.7	1.7	214,365.5 (172,933, 306,600.5)	370,879 (293,727, 451,143.5)	HMDB01266	C08356	NA
L-Arabitol	Carbohydrates	3.28E-07	1.7	0.5	110,028 (65,100.75, 240,871)	55,786 (43,840.25, 67,209)	HMDB01851	C00532	Pentose and glucuronate interconversions; metabolic pathways
Benzoic acid	Organic acids	3.16E-09	2	0.6	24,087 (19,048.5, 29,166.5)	14,903 (13,031.25, 17,855.5)	HMDB01870	C00180	NA
Ribonolactone	Carbohydrates	1.81E-05	1.4	0.3	27,147.5 (15,144, 77,587.5)	8,821.5 (4,725.5, 14,265.25)	HMDB01900	C02674	NA
Tetracosanoic acid	Fatty acids	1.00E-03	1.2	0.9	6,202.5 (5,134.75, 8,127.75)	5,272 (4,122, 6,202)	HMDB02003	C08320	Beta oxidation of very long chain fatty acids
m-Cresol	Phenols	2.00E-03	1.2	0.5	30,252 (80,40.5, 101,712.75)	13,858 (5,703.75, 26,689.25)	HMDB02048	C01467	Toluene degradation; microbial metabolism in diverse environments; degradation of aromatic compounds
Petroselinic acid	Organic acids	9.00E-03	1.3	0.8	513,132 (322,569.25, 801,835.25)	416,994 (267,109.5, 580,415.25)	HMDB02080	C08363	NA
Erythrose	Carbohydrates	9.57E-09	1.8	0.5	535,478.5 (354,430.5, 923,590)	249,182.5 (52,987, 324,922.75)	HMDB02649	C01796	NA
Isomaltose	Carbohydrates	5.60E-04	1.1	0.5	8,272.5 (2,733.75, 19,707.75)	4,314 (2,844, 6,996.25)	HMDB02923	C00252	NA
Erythritol	Carbohydrates	3.46E-05	1.4	0.5	66013 (49,723, 173,745.75)	32,681 (25,459.25, 42,582.5)	HMDB02994	C00503	NA
D-Threitol	Carbohydrates	3.04E-05	1.5	0.4	13,649.5 (8,991, 51,131.5)	5,438.5 (3,241.5, 7,278.5)	HMDB04136	C16884	NA
5-Hydroxydopamine	Phenols	4.20E-02	1.1	0.8	6,983.5 (4,712.75, 9,486)	5617.5 (3,889, 7,842.5)	HMDB04817	NA	NA
MG160	Lipids	8.20E-06	1.3	2.6	3,829 (2,292.75, 5,679.5)	9,908 (7,105.75, 14,048)	HMDB11564	NA	NA
Glycerol 1-octadecanoate	Esters	2.45E-14	2.3	2.9	3,563.5 (2,668.5, 4,804.75)	10,494.5 (8,536, 12,954)	HMDB31075	NA	NA

Differential metabolites were selected according to VIP >1 and P<0.05. Values are expressed as medians (IQRs). P values were calculated from t test for continuous variables and adjusted by FDR method; FC, fold change; VIP, variable influence on projection.

Table S3 Differential urinary metabolites betweenT2DM with and without nephropathy.

Name	Class	T test. P	VIP	FC	DN	T2DM	HMDBID	Kegg ID	Pathway
D-Glucose	Carbohydrates	2.12E-10	3.0	0.3	267,788.5 (181,641.25, 363,846.25)	89,577 (41,878.25, 157,795.5)	HMDB00122	C00031	Galactose metabolism, gluconeogenesis, glucose-alanine cycle, glycolysis, lactose degradation, Lactose synthesis, transfer of acetyl groups into mitochondria
L-Valine	Amino Acid	9.40E-07	2.9	0.3	14,824.5 (9,025.25, 31,585.5)	4,716 (3,303.5, 7,020.5)	HMDB00883	C00183	Propanoate metabolism, transcription/translation, valine, leucine and isoleucine degradation
L-Histidine	Amino Acid	2.44E-05	2.4	0.3	10,364 (4,639.75, 18,147.25)	3,146 (899.25, 4,270.5)	HMDB00177	C00135	Ammonia recycling, histidine metabolism, transcription/translation
Sucrose	Carbohydrates	6.89E-05	2.3	0.2	1,200,070 (410,986.25, 1,750,933.5)	267,627.5 (108,851.5, 665,185.75)	HMDB00258	C00089	Galactose metabolism, starch and sucrose metabolism
Glycine	Amino Acid	1.03E-04	2.2	0.6	65,575 (39,493.75, 105,077)	39,768 (20,924, 53,651.75)	HMDB00123	C00037	Alanine metabolism, ammonia recycling, bile acid biosynthesis, carnitine synthesis, glutathione metabolism, glycine and serine metabolism, methionine metabolism, porphyrin metabolism
L-Asparagine/L-Aspartic acid	Amino acid metabolism	1.71E-04	2.0	0.5	9.68 (5.958, 20.118)	4.66 (2.934, 7.522)	HMDB00168/HMDB00191	C00152/C00049	Alanine, aspartate and glutamate metabolism
Gluconic acid	Carbohydrates	3.60E-05	2.0	0.6	382,496.5 (230,424.25, 531,628.25)	227,164.5 (124,367.75, 323,271.75)	HMDB00625	C00257	Pentose phosphate pathway; metabolic pathways; biosynthesis of secondary metabolites; microbial metabolism in diverse environments; biosynthesis of antibiotics; carbon metabolism
L-Cystine	Amino Acid	1.00E-03	2.0	0.3	133,144.5 (72,901, 283,660.5)	44,629 (16,423, 104,929.5)	HMDB00192	C00491	Cysteine and methionine metabolism; metabolic pathways; ABC transporters; ferroptosis; protein digestion and absorption
L-Alanine	Amino Acid	1.50E-02	1.9	0.6	58,922.5 (29,373.75, 95,954.75)	32,996 (19,712.25, 52,954)	HMDB00161	C00041	Alanine metabolism, glucose-alanine cycle, glycine and serine metabolism, selenoamino acid metabolism, transcription/translation, urea cycle
L-Asparagine	Amino Acid	4.00E-03	1.9	0.5	148,956 (71,175.5, 246,210.25)	67,998 (39,736.5, 132,908.75)	HMDB00168	C00152	Ammonia recycling, aspartate metabolism, transcription/translation
L-Glutamine	Amino Acid	3.00E-03	1.9	0.5	251,416 (123,474, 461,131.5)	132,467.5 (73,631.75, 241,030.25)	HMDB00641	C00064	Amino sugar metabolism, ammonia recycling, glutamate metabolism, phenylacetate metabolism, purine metabolism, pyrimidine metabolism, transcription/translation, urea cycle
L-Arabinose/L-Arabitol	Carbohydrate metabolism	7.82E-04	1.8	0.4	3.27 (1.428, 6.406)	1.17 (0.668, 2.598)	HMDB00646/HMDB01851	C00259/C00532	Pentose and glucuronate interconversions
4-Hydroxybenzoic acid	Organic Acids	4.00E-03	1.7	0.6	40,798.5 (21,313.75, 115,454.25)	23,748.5 (14,890.5, 54,047)	HMDB00500	C00156	Ubiquinone biosynthesis
p-Hydroxyphenylacetic acid	Organic Acids	8.24E-04	1.7	1.3	194,089.5 (116,303, 313,946.25)	261,616.5 (167,321.75, 786,099.25)	HMDB00020	C00642	Tyrosine metabolism
L-Threonine	Amino Acid	1.50E-02	1.7	1.1	61,327 (28,033.75, 150,853)	66,284 (28,502, 105,775)	HMDB00167	C00188	Glycine and serine metabolism, threonine and 2-oxobutanoate degradation, transcription/translation
L-Phenylalanine/Phenylpyruvic acid	Amino acid metabolism	6.13E-04	1.7	0.3	30.1 (11.584, 61.208)	8.09 (4.657, 15.047)	HMDB00159/HMDB00205	C00079/C00166	Phenylalanine, tyrosine and tryptophan biosynthesis
L-Serine	Amino Acid	1.00E-02	1.7	0.9	92,666 (71,705.5, 163,200)	79,442.5 (36,488, 107,665.25)	HMDB00187	C00065	Ammonia recycling, glycine and serine metabolism, homocysteine degradation, methionine metabolism, sphingolipid metabolism
Galactonic acid	Carbohydrates	1.00E-03	1.6	0.7	54,048 (37,692.25, 93,398.25)	35,796.5 (19,514.75, 57,736)	HMDB00565	C00880	Galactose metabolism; metabolic pathways; microbial metabolism in diverse environments
Phenylpyruvic acid/L-Phenylalanine	Amino acid metabolism	1.20E-02	1.6	3.7	0.03 (0.016, 0.088)	0.12 (0.066, 0.215)	HMDB00205/HMDB00159	C00166/C00079	Phenylalanine metabolism
D-Glucose/Trehalose	Carbohydrate metabolism	9.97E-04	1.6	0.3	4.64 (2.208, 8.466)	1.5 (0.677, 2.537)	HMDB00122/HMDB00975	C00031/C01083	Starch and sucrose metabolism
Xanthine	Nucleotide	7.00E-03	1.6	1.7	8,609.5 (5,751.25, 12,169.5)	15,018 (7,303.5, 26,031.75)	HMDB00292	C00385	Purine metabolism
D-Galactose	Carbohydrates	2.00E-03	1.6	0.6	247,989.5 (167,010.5, 353,676.75)	151,497 (88,382, 237,218.75)	HMDB00143	C00984	Galactose metabolism, lactose degradation, nucleotide sugars metabolism
Ornithine/L-Arginine	Amino acid metabolism	2.20E-02	1.5	0.7	33.3 (9.814, 65.809)	23.33 (14.106, 29.792)	HMDB00214/HMDB00517	C00077/C00062	Arginine biosynthesis; arginine and proline metabolism
L-Lactic acid	Organic Acids	4.50E-02	1.5	0.5	430,096.5 (201,968.75, 737,092.5)	200,346.5 (91,188.75, 407,080)	HMDB00190	C00186	Gluconeogenesis, pyruvate metabolism
Phenylpyruvic acid	Organic Acids	8.00E-03	1.5	3.1	6,991 (3,393.5, 19,416.25)	21,715 (12,861, 39,259.75)	HMDB00205	C00166	Phenylalanine and tyrosine metabolism
Fumaric acid/Succinic acid	Carbohydrate metabolism	7.00E-03	1.5	0.3	1.87 (0.783, 3.783)	0.58 (0.233, 1.478)	HMDB00134/HMDB00254	C00122/C00042	Citrate cycle (TCA cycle)
Oxalic acid	Organic Acids	4.63E-04	1.4	0.5	15,214.5 (7,031.5, 29,221.25)	6,881.5 (4,141.25, 9,998)	HMDB02329	C00209	Purine metabolism; chloroalkane and chloroalkene degradation; glyoxylate and dicarboxylate metabolism; metabolic pathways; microbial metabolism in diverse environments
D-Ribose	Carbohydrates	5.00E-03	1.4	0.7	304,110.5 (148,998.75, 452,514.75)	199,112 (160,690.25, 289,500.5)	HMDB00283	C00121	Pentose phosphate pathway
L-Glutamic acid	Amino Acid	3.60E-02	1.4	1.0	29,932.5 (18,307, 60,635)	29,173.5 (15,021.5, 48,765.75)	HMDB00148	C00025	Alanine metabolism, amino sugar metabolism, ammonia recycling, arginine and proline metabolism, cysteine metabolism, folate metabolism, glucose-alanine cycle, glutamate metabolism, glutathione metabolism, glycine and serine metabolism, histidine metabolism, malate-aspartate shuttle, transcription/translation, urea cycle
Hippuric acid	Organic Acids	2.50E-02	1.4	0.7	1,331,521 (545,393.5, 2,606,043)	928,768 (330,140.25, 1,618,823.25)	HMDB00714	C01586	Phenylalanine metabolism
Cytidine	Nucleotide	2.00E-02	1.4	0.4	434.5 (148.5, 720)	174.5 (84.75, 439.25)	HMDB00089	C00475	Pyrimidine metabolism
L-Xylonate	Organic Acids	2.50E-02	1.4	0.6	319,783.5 (226,142.25, 471,741.5)	192,075.5 (124,227.5, 342,688)	HMDB60256	C05411	Pentose and glucuronate interconversions; ascorbate and aldarate metabolism
Mannitol	Carbohydrates	3.00E-03	1.4	1.6	257,485.5 (177,979, 411,349.25)	399,367 (266,932.25, 641,879.75)	HMDB00765	C00392	Fructose and mannose metabolism; ABC transporters; phosphotransferase system (PTS)
Normetanephrine	Hormone	1.50E-02	1.4	1.6	5,526 (2,798.5, 10,694.25)	8,911.5 (3,673.25, 19,318.5)	HMDB00819	C05589	Tyrosine Metabolism
Uridine	Nucleotide	1.50E-02	1.4	0.6	3,777 (2,104.75, 6,605.5)	2,230.5 (1,601.25, 4,315.25)	HMDB00296	C00299	Pyrimidine Metabolism
D-Fructose/Sucrose	Carbohydrate metabolism	1.20E-02	1.3	2.1	0.11 (0.059, 0.259)	0.23 (0.071, 0.763)	HMDB00660/HMDB00258	C02336/C00089	Starch and sucrose metabolism
D-Threitol	Carbohydrates	8.00E-03	1.3	0.8	45,409.5 (31,440, 66,992.5)	34,121 (19,136, 47,399.5)	HMDB04136	C16884	NA
Ketoleucine	Amino Acid	8.00E-03	1.3	2.8	8,137 (4,966, 16,225.25)	22,470.5 (4,557.75, 49,945.5)	HMDB00695	C00233	Valine, leucine and isoleucine degradation
1,2,3-Trihydroxybenzene	Phenols	3.50E-02	1.3	1.4	23,996 (6,720, 48,837)	33,284.5 (8,821.25, 85,409.75)	HMDB13674	C01108	Aminobenzoate degradation; microbial metabolism in diverse environments; catecholamine transferase inhibitors
Uracil/Uridine	Nucleotide metabolism	1.70E-02	1.2	2.2	2.01 (0.726, 3.555)	4.38 (1.92, 9.464)	HMDB00300/HMDB00296	C00106/C00299	Pyrimidine metabolism
Hydroxyphenyllactic acid	Organic Acids	2.40E-02	1.2	2.1	83,610 (39,609.25, 132,933.5)	178,418.5 (75,759, 275,474.25)	HMDB00755	C03672	NA
Citramalic acid	Organic Acids	3.00E-02	1.2	0.5	20,538.5 (9,175.25, 43,236.75)	11,130.5 (6,810.25, 25,437.25)	HMDB00426	C00815	NA
L-Arabinose	Carbohydrates	2.90E-02	1.2	0.8	442,924 (337,962, 666,296.25)	350,838.5 (184,538.25, 468,567.25)	HMDB00646	C00259	Pentose and glucuronate interconversions; ascorbate and aldarate metabolism; amino sugar and nucleotide sugar metabolism; metabolic pathways; ABC transporters
D-Fructose	Carbohydrates	1.70E-02	1.2	0.6	104,449.5 (49,230.25, 185,290)	64,712.5 (30,731.75, 106,221)	HMDB00660	C02336	Amino sugar metabolism, fructose and mannose degradation, galactose metabolism, starch and sucrose metabolism
Inosine/Adenosine	Nucleotide metabolism	1.30E-02	1.1	1.5	0.35 (0.19, 0.599)	0.51 (0.301, 1.445)	HMDB00195/HMDB00050	C00294/C00212	Purine metabolism
Inosine	Nucleotide	2.50E-02	1.1	1.6	2,835.5 (1,850, 5,864.75)	4,552.5 (2,281, 14,824.75)	HMDB00195	C00294	Purine metabolism

Differential metabolites were selected according to VIP >1 and P<0.05. Values are expressed as medians (IQRs). P value were calculated from t test for continuous variables and adjusted by FDR method; FC, fold change; VIP, variable influence on projection.

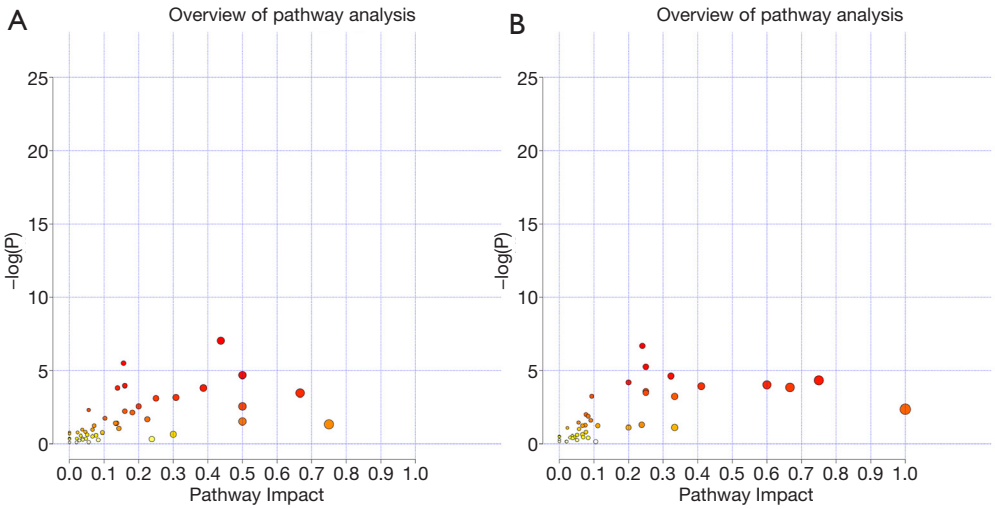

Figure S2 Metabolic pathway analysis. (A) Serum metabolic pathway analysis. (B) Urine metabolic pathway analysis. All the matched pathways are displayed as circles. The colour and size of each circle are based on the P value and pathway impact value, respectively. The graph was obtained by plotting on the y-axis the log of P values from the pathway enrichment analysis and on the x-axis the pathway impact values derived from the pathway topology analysis.