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Serum metabolic profiling in diabetic kidney disease patients using ultra-high performance liquid chromatography-tandem mass spectrometry

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Abstract

Background Diabetic kidney disease (DKD) remains one of the leading causes of end-stage renal failure. The currently available diagnostic and classification markers, such as the urinary albumin-to-creatinine ratio and estimated glomerular filtration rate, demonstrate inadequate precision in forecasting the onset and progression of DKD. This study aims to investigate the serum metabolic profile of patients with DKD, with the objective of identifying reliable biomarkers that can enhance the prediction of the transition from diabetes mellitus (DM) to DKD and distinguishing DKD from nondiabetic kidney disease (NDKD).

Methods Untargeted metabolomic analysis was performed on serum samples obtained from 53 DKD patients, 54 NDKD patients, 59 individuals diagnosed with simple diabetes mellitus (SDM), and 56 healthy controls utilizing ultra-high performance liquid chromatography-tandem mass spectrometry. Differential metabolites among the groups were identified, metabolic pathways were investigated, and the diagnostic efficacy of selected metabolites was evaluated.

Results The metabolic enrichment pathways shared between DKD and NDKD encompassed glycerophospholipid metabolism, glycerolipid metabolism, and tryptophan metabolism. In contrast, pyrimidine metabolism and arginine biosynthesis were uniquely enriched in DKD. Compared to the NDKD group, significantly elevated levels of phosphatidylglycerol (PG, 14:0) and D-Maltose were observed in DKD patients. Additionally, in comparison to the SDM group, the DKD group exhibited significant increases in lysophosphatidic acid (LPA, 16:3), LPA (18:5), LPA (22:5), phosphatidic acid (PA, 18:3), PG (26:4), L-Glutamine, Uridine, Cytidine, Formyl-N-acetyl-5-methoxykynurenamine, 2-Oxoadipate, Thymidine, L-Citrulline, and 5-Hydroxy-L-tryptophan, while PG (28:4) levels were markedly reduced. Among these, Uridine, Cytidine, Thymidine, and L-Citrulline were associated with pyrimidine metabolism, whereas L-Glutamine and L-Citrulline participated in the arginine biosynthesis pathway. Furthermore, the differential metabolites exhibited varying degrees of correlation with renal function indicators in DKD patients.

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Conclusions PG (14:0) and D-Maltose may help distinguish DKD from NDKD, while L-Glutamine, Uridine, Cytidine, Thymidine, and L-Citrulline are linked to the progression from DM to DKD. Larger studies are needed to validate these findings and assess their diagnostic and causal significance.

Keywords Candidate biomarkers, Diabetic kidney disease, Metabolic pathway analysis, Ultra-high performance liquid chromatography-tandem mass spectrometry, Untargeted metabolomics

Introduction

Epidemiological studies have consistently shown a substantial increase in the global prevalence of diabetes mellitus (DM), with the number of cases escalating from 151 million in 2000 to 537 million in 2021. Projections suggest that the number of DM cases worldwide is expected to reach 784 million by 2045 [1]. The etiology of DM is not yet fully elucidated, as its progression involves a complex interaction of diverse pathophysiological mechanisms. The primary complications associated with DM encompass microvascular impairments affecting the kidneys, retina, and nerves, as well as macrovascular complications that involve the coronary arteries, peripheral arteries, and cerebral vessels [2]. It has been estimated that approximately 30–40% of individuals diagnosed with DM may develop chronic kidney disease (CKD) as a consequence of microvascular complications, a condition classified as diabetic kidney disease (DKD) [3]. The etiology of CKD is complex and multifactorial, with commonly identified contributing factors such as hypertension, hyperuricemia, obesity, renal vascular disease, glomerular arteriosclerosis, nephron loss, and renal ischemia, in addition to DM [4]. Within the scope of this study, CKD arising from factors unrelated to DM is classified as nondiabetic kidney disease (NDKD).

Regarding kidney function indicators, urea (Ur), uric acid (UA), and neutrophil gelatinase-associated lipocalin (NGAL) are commonly employed in clinical settings to evaluate tubular function [5]. Complement 1q (C1q) serves as a marker for the detection of immune complex accumulation within the kidney tissue [6]. In contrast, creatinine (Cr), cystatin C (CysC), estimated glomerular filtration rate (eGFR), and the urinary albumin-to-creatinine ratio (UACR) are utilized to assess glomerular function [7]. The Kidney Disease: Improving Global Outcomes (KDIGO) guidelines have consistently recommended UACR and eGFR as critical biomarkers for both diagnostic evaluation and therapeutic monitoring in DKD [8]. Nevertheless, it has been observed that approximately 30% of patients with DKD do not present with albuminuria, and the presence of microalbuminuria does not necessarily predict progression to macroalbuminuria, thereby limiting the efficacy of UACR in accurately forecasting the onset and progression of DKD [9]. While UACR and eGFR remain valuable in the diagnosis of kidney disease (KD), encompassing both DKD and NDKD, their diagnostic specificity for DKD is insufficient, particularly given that eGFR is influenced by multiple factors [10]. Consequently, the identification of more sensitive and specific biomarkers for DKD prediction is imperative to facilitate early diagnosis and improve risk assessment for DKD progression.

DM is a multifaceted metabolic disorder characterized by dysregulation of glucose and lipid metabolism, often accompanied by complications such as DKD [11]. Clinical monitoring of lipid profiles typically involves the assessment of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), apolipoprotein A1 (ApoA1), and apolipoprotein B (ApoB). Previous studies have established an association between alterations in blood lipid profiles and the onset and progression of renal microvascular damage in patients with DM [12].

CKD patients with DM may present with DKD induced by DM, NDKD resulting from non-DM factors, or a combination of both DKD and NDKD. The distinct etiologies of CKD necessitate the implementation of tailored therapeutic strategies due to their differing pathogenic mechanisms [13, 14]. The American Diabetes Association (ADA) and KDIGO have established medication management guidelines for patients with DKD [15]. The identification of biomarkers capable of differentiating between DKD and NDKD would aid clinicians in selecting the most appropriate treatment strategies. In cases where the diagnosis of DKD remains uncertain, a kidney biopsy is required to rule out the presence of NDKD or the simultaneous occurrence of DKD and NDKD. However, kidney biopsy is an invasive procedure that many patients are reluctant to undergo, and it carries inherent risks, including bleeding, the formation of arteriovenous fistulas, and, in severe cases, mortality [16]. Previous attempts to establish predictive models for NDKD through the analysis of diabetic retinopathy, duration of diabetes, proteinuria, and hematuria have not yielded reliable methods for differentiating between DKD and NDKD [17]. Metabolomics technology has increasingly been employed to identify disease-specific biomarkers for a range of conditions, including Alzheimer's disease [18], chronic liver disease [19], sepsis [20], and renal cell carcinoma [21]. Utilizing untargeted metabolomics, Feng et al. analyzed the urinary metabolic profiles of DKD patients with and without albuminuria, leading to the identification of 65 differential metabolites [22]. Similarly, Trifonova et al. [23] demonstrated that plasma metabolomics

could identify a combination of 15 potential biomarkers with promising diagnostic performance for the early detection of DKD and differentiation between its early and late stages. Despite these advancements, none of the identified markers have yet been established as standalone diagnostic biomarkers for DKD, underscoring the ongoing need for the discovery of early diagnostic biomarkers for DKD.

In this study, ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was employed to analyze the serum metabolic profiles of patients with DM, DKD, NDKD, and healthy controls (HC). The study focused on identifying differential metabolites in serum that are associated with the onset of DKD and the differentiation between DKD and NDKD. Correlation analyses were performed between these metabolites and conventional clinical indicators of renal function and lipid profiles to investigate the potential metabolic pathways implicated in the progression of DKD. Additionally, the study aimed to identify reliable biomarkers for its early and differential diagnosis.

Materials and methods

Study subjects

Statistical analysis was conducted using PASS 11.0v Statistical Software (NCSS LLC, Kaysville, Utah, USA), which indicated that each group of subjects necessitates a minimum of 42 cases. To account for potential uncertainties, such as patient attrition and difficulties in follow-up, the sample size was increased by 20%, resulting in a requirement of no fewer than 51 subjects per group. Based on these sample size estimations, between January 2022 and December 2022, a total of 53 patients diagnosed with DKD, 54 patients with CKD, 59 individuals with simple diabetes mellitus (SDM), and 56 HC were selected from those who visited or underwent physical examinations at Mianyang Central Hospital. These participants were subsequently categorized into the DKD group, NDKD group, SDM group, and HC group, respectively.

Inclusion criteria: ① Participants aged ≥ 18 years; ② DKD group: diagnosed with DM according to the ADA diagnostic criteria [24] and confirmed DKD through kidney biopsy; NDKD group: diagnosed with CKD based on the 2024 KDIGO criteria for CKD [25] and confirmed NDKD through kidney biopsy; SDM group: diagnosed with DM per the ADA diagnostic criteria [24], without evidence of microvascular complications, and demonstrating at least two UACR measurements < 30 mg/g and eGFR ≥ 60 ml/min/1.73 m^2 during routine monthly screenings; HC group: individuals without renal impairment, exhibiting normal blood lipid levels, standard blood test results, and unremarkable electrocardiogram, ultrasound, and X-ray computed tomography findings. ③ No usage of medications or supplements with high concentrations of hormones or vitamins, such as contraceptives or nutritional supplements, in the preceding month; ④ Normal liver function parameters.

Exclusion criteria: ① Participants aged < 18 years; ② Women who are menstruating, pregnant, or lactating; ③ Presence of conditions capable of altering urinary albumin (UAlb) levels or contributing to eGFR decline, including both benign and malignant tumors, systemic lupus erythematosus, rheumatoid arthritis, connective tissue diseases, infections, thyroid disorders, genitourinary diseases, and other systemic or active conditions. ④ History of kidney transplantation; ⑤ Inability to collect urine or blood samples as required; ⑥ NDKD group: exclusion of CKD cases induced by DM.

This study protocol was approved by the Medical Ethics Committee of Mianyang Central Hospital (P2020030), and informed consent was obtained from all participants.

Sample collection

Blood samples were collected from subjects in the morning (8:00–10:00) following an overnight fasting period of 8–10 h using vacuum blood collection tubes containing SSTII inert separation gel, filled to the designated volume of approximately 5 ml. The collected blood was allowed to rest at room temperature for approximately 30 min to facilitate clot formation before being centrifuged at 2,000 g for 10 min to obtain the serum. An aliquot of approximately 1.0 ml of serum was then transferred into low-adsorption centrifuge tubes (Eppendorf, EP tubes) and subjected to further centrifugation at 12,000 g for 10 min at 4 °C. A minimum of 0.5 ml of the resulting supernatant was carefully aliquoted into 1.5 ml EP tubes and stored at -80 °C for subsequent metabolomic analysis. An additional 1.0 ml of serum was reserved for renal function, glucose, and lipid profile assessments, which were conducted within 2 h. Furthermore, within 1 h of blood collection, approximately 5 ml of random midstream urine was obtained from the subjects. The urine samples were centrifuged at 1,500 g for 10 min within 30 min of collection, and an aliquot of 1.0 ml of the supernatant was utilized for the analysis of UAlb and urinary creatinine (UCr) levels within 1 h.

Detection of common renal function indicators, glucose, and lipid parameters

Serum parameters associated with renal function, glucose, and lipid levels were analyzed using a Roche Cobas C701 automatic biochemical analyzer. The concentrations of Ur, Cr, UA, Glu, TC, TG, HDL-C, and LDL-C were measured through their respective enzymatic methods, while the levels of C1q, CysC, NGAL, ApoA1, and ApoB were quantified via immunoturbidimetric assays. The C1q reagent kit was obtained from Shanghai Beijia Biochemical Reagent Co., Ltd., the NGAL reagent kit was

provided by Maccura Biotechnology Co., Ltd., and the remaining kits were supplied by Roche Diagnostics Products (Shanghai) Co., Ltd. The eGFR was calculated using the CKD-EPI 2021 equation, incorporating Cr and CysC levels [26], as represented by the following formula:

$$eGFR = 135 \times min\ (Cr/\kappa,1)^{a} \times max\ (Cr/\kappa,1)^{-0.544} \times min\ (CysC/0.8,1)^{-0.323} \times max\ (CysC/0.8,1)^{-0.778} \times 0.9961^{Age} \times b$$

In the formula, Cr is expressed in mg/dL, while CysC is quantified in mg/L. The term "min" represents the minimum value between the two numbers within parentheses, whereas "max" denotes the maximum value between them. The κ-value is assigned as 0.9 for males and 0.7 for females, while the α-value is set at -0.144 for males and − 0.219 for females. Age is calculated in years, and the b-value is designated as 1 for males and 0.963 for females.

UAlb concentrations were measured using a modified immunoturbidimetric method, whereas UCr levels were quantified via the sarcosine oxidase method on a BioSystems BA400 specific protein analyzer (Spain), with reagents supplied by Chongqing Biostec Co., Ltd. The UACR was subsequently calculated based on the obtained values for UAlb and UCr, as described by the following formula:CysC levels [26], as represented by the following formula:

$$UACR\ (mg/g) = UAlb\ (mg/L)/UCr\ (g/L)$$

UPLC-MS/MS analysis

Sample preparation and analysis

Clenbuterol and chloramphenicol were utilized as internal standards for the positive and negative ion modes, respectively, and were thoroughly combined with methanol (> 99.9%) to prepare internal standard solutions at a concentration of 10 mg/L. Serum samples, previously stored at -80 °C, were gradually thawed on ice. Once completely dissolved, the samples were mixed thoroughly three times. A pipette was employed to transfer 190 µl of serum and 10 µl of the internal standard solution into a 1.5 ml low-adsorption centrifuge tube, followed by vortex mixing for 15 s and brief centrifugation for 10 s. Subsequently, 800 µl of a pre-chilled (4 °C) methanol-acetonitrile mixture was introduced, after which the sample was vortexed for 2 min and subjected to ultrasonication in a 4 °C water bath for 10 min. The centrifuge tubes were then placed in a -20 °C freezer for 1 h. Following centrifugation at 12,000 g for 15 min at 4 °C, at least 100 µl of the supernatant was withdrawn using a syringe and filtered through a 0.22 µm microporous membrane into sample vials. Serum metabolomic analysis was performed using the UPLC-MS/MS method previously established by the research group [27, 28]. Quality control (QC) samples were analyzed after every 10 test samples in the analytical sequence to evaluate the reliability of the large-scale metabolomic analysis and ensure the stability of the instrument [29, 30].

Metabolite identification and analysis

The raw data for the untargeted metabolomics analysis were acquired using Analyst TF 1.7 software (AB Sciex, USA) and processed via the One-MAP platform (Dalian, China; http://www.5omics.com). Peak matchi ng was conducted using the XCMS algorithm. The data acquired included the retention time (tR), primary mass spectrometry (MS), secondary mass spectrometry (MS/MS), and mass spectrometry intensity of metabolite chromatographic peaks. Utilize the standard sample database as confidence level 1, and employ databases such as KEGG (http://www.kegg.jp/kegg/pathway.html), PubChem (https://pubchem.ncbi.nlm.nih.gov/), HMDB (https://hmdb.ca), and LipidMaps (https://www.lipidmaps.org/) as confidence level 2 to match and annotate the molecular weights of metabolites [27, 28]. Conduct an analysis of differential metabolites and their associated metabolic pathways using MetaboAnalyst 6.0 (http://www.metaboanalyst.ca/) [28, 31]. Principal component analysis (PCA) was performed on the QC samples, followed by an evaluation of the relative standard deviation (RSD) of the mass spectrometry characteristics. Data quality was assessed based on the criterion that over 70% of the measurements should exhibit RSD values below 30%. Metabolic features with more than 20% missing values were excluded in accordance with the "80% rule", with missing values subsequently imputed using the minimum observed value for each variable. Normalization of the data was conducted using the QC-based MetNormalizer method. PCA and orthogonal partial least squares discriminant analysis (OPLS-DA) were applied to process the metabolic feature variables.

Statistical analysis

Statistical analyses were conducted utilizing SPSS 26.0 software, with statistical significance established at $P < 0.05$. For parametric data conforming to a normal distribution, values were expressed as means ± standard deviation. Between-group differences were assessed via one-way analysis of variance (ANOVA), with subsequent pairwise comparisons performed using post-hoc LSD-t tests. Non-parametric data were presented as medians (interquartile range), and between-group variations were examined using the Kruskal-Wallis H test, followed by appropriate post-hoc multiple comparison procedures. Categorical variables across multiple groups were compared utilizing the Chi-square test. Multivariate statistical analyses, including PCA and OPLS-DA, were executed with SIMCA 14.1 software (Umetrics AB,

Table 1 General clinical features of subjects

Group	DKD (*n* = 53)	NDKD (*n* = 54)	SDM (*n* = 59)	HC (*n* = 56)	χ^2/F, *P*
Male/Female (n)	33/20	25/29	38/21	29/27	5.003[a],0.172
Age (year)	61.34 ± 11.90	52.44 ± 12.86[c]	55.54 ± 12.64[c]	47.39 ± 13.25[c, d,e]	11.546[b], <0.001
CKD stage(n)					0.827[a], 0.843
I	2	2	-		-
II	17	20	-		-
III	28	24	-		-
IV	6	8	-		-

Note: n, case; DKD, group of diabetic kidney disease; NDKD, group of nondiabetic kidney disease; SDM, group of diabetes mellitus; HC, group of healthy control; a, the χ2 value; b, the value of F; c, compared with DKD group, *P*<0.05; d, compared with NDKD group, *P*<0.05; e, compared with SDM group, *P*<0.05

Table 2 Laboratory indicators of subjects

Group	DKD (*n* = 53)	NDKD (*n* = 54)	SDM (*n* = 59)	HC (*n* = 56)	χ^2/F/H, *P*
Ur(mmol/L)	7.62(6.27,10.92)	8.15(5.62,11.17)	5.46(4.84,6.49)[d, e]	4.93(4.25,5.96)[d, e]	60.961[c], <0.001
Cr(μmol/L)	104.30(72.30,142.85)	110.95(83.23,185.65)	64.80(54.60,71.60) [d, e]	66.95(58.13,78.73) [d, e]	78.711[c], <0.001
Glu(mmol/L)	7.81(6.02,10.54)	5.35(5.04,5.74)[d]	8.78(7.16,10.63)[e]	5.05(4.83,5.31) [d, f]	133.405[c], <0.001
UA(μmol/L)	370.70(322.85,441.75)	406.50(351.05,481.15)	306.30(268.40,339.90) [d, e]	330.90(272.53,404.90)[e]	37.367[c], <0.001
CysC(mg/L)	1.53(1.15,2.12)	1.50(1.13,2.36)	0.90(0.77,0.98)[d, e]	0.90(0.80,0.97)[d, e]	109.214[c], <0.001
eGFR(ml/min/1.73m^2)	51.08(32.84,73.86)	52.22(25.74,71.58)	100.38(89.36,117.10)[d, e]	101.65(90.47,110.44)[d, e]	109.413[c], <0.001
C1q(mg/L)	188.00(164.00,213.00)	192.00(176.25,220.50)	193.00(163.00,209.00)	184.50(160.25,202.75)	4.937[c], 0.176
NGAL(μg/L)	161.00(116.50,259.50)	166.50(123.00,265.50)	98.00(76.00,136.00)[d, e]	123.00(97.25,147.50)[d, e]	49.849[c], <0.001
TC(mmol/L)	4.67 ± 1.18	4.86 ± 1.16	4.87 ± 1.23	5.11 ± 0.86	1.445 [b],0.230
TG(mmol/L)	1.28(0.96,2.08)	1.35(1.00,1.83)	1.34(1.02,1.71)	1.27(0.95,1.58)	2.006 [c],0.571
HDL-C(mmol/L)	1.26(0.99,1.59)	1.29(1.06,1.57)	1.25(1.11,1.44)	1.46(1.21,1.88)[d, f]	11.430 [c],0.010
LDL-C(mmol/L)	2.67 ± 0.94	2.82 ± 0.91	2.99 ± 0.99	3.11 ± 0.78	2.433 [b],0.066
ApoA1(g/L)	1.44(1.29,1.68)	1.47(1.33,1.68)	1.42(1.31,1.66)	1.54(1.36,1.80)	4.437 [c],0.218
ApoB(g/L)	0.90(0.68,1.06)	0.85(0.72,0.97)	0.90(0.76,1.13)	0.90(0.75,1.04)	3.011 [c],0.390
UACR(mg/g)	52.41(15.12,824.20)	153.85(15.78,870.72)	10.61(4.87,28.86) [d, e]	3.42(2.42,4.56)[d, e,f]	29.907[c], <0.001

Note: n, case; DKD, group of diabetic kidney disease; NDKD, group of nondiabetic kidney disease; SDM, group of diabetes mellitus; HC, group of healthy control; Ur, serum urea; Cr, serum creatinine; Glu, serum glucose; UA, serum uric acid; CysC, serum cystatin C; eGFR, estimated glomerular filtration rate; C1q, serum complement 1q; NGAL, serum neutrophil gelatinase-associated lipocalin; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; UACR, urinary albumin-to-creatinine ratio; a, the χ2 value; b, the value of F; c, the value of H; d, compared with DKD group, *P*<0.05; e, compared with NDKD group, *P*<0.05; f, compared with SDM group, *P*<0.05

Umea, Sweden). The validity of the OPLS-DA model was verified through permutation testing with 1000 random iterations. In the analysis of inter-group differential metabolites, the P-values derived from the T-test have been adjusted for false discovery rate (FDR) and are referred to as P_{FDR}. All P values are corrected as FDR-adjusted P values using Benjamini–Hochberg procedure and are denoted as P_{FDR}. Receiver operating characteristic (ROC) curve analysis was employed to evaluate the diagnostic efficacy of differential metabolites. Area under the curve (AUC) > 0.7 is considered acceptable per general screening principles [32, 33]. Correlations between variables were determined using Spearman's rank correlation coefficient.

Results

Demographic characteristics and laboratory indicators of study subjects

The general clinical data of the subjects are presented in Table 1, while the analysis of laboratory indicators is summarized in Table 2. Moreover, no statistically significant differences were detected in the levels of C1q, TC, TG, LDL-C, ApoA1, ApoB, or in gender distribution across the four groups ($P > 0.05$). However, significant variations were observed in the levels of Ur, Cr, Glu, UA, CysC, eGFR, NGAL, HDL-C, UACR, and age among the groups ($P < 0.05$). Pairwise comparisons indicated that, compared to the NDKD group, the DKD group exhibited a higher age (t = 2.451, $P < 0.001$) and elevated Glu levels (z = 5.772, $P < 0.001$). The SDM group demonstrated lower levels of Ur (z = -4.387, $P < 0.001$), Cr (z = -7.306, $P < 0.001$), UA (z = -5.597, $P < 0.001$), CysC (z = -7.396, $P < 0.001$), NGAL (z = -6.235, $P < 0.001$), and UACR (z = -3.940, $P < 0.001$), but higher Glu (z = 7.658, $P < 0.001$) and eGFR levels (z = 7.545, $P < 0.001$). When compared to the SDM group, the DKD group displayed a greater age (t = 5.797, $P = 0.015$) and increased levels of Ur (z = 4.788, $P < 0.001$), Cr (z = 6.365, $P < 0.001$), UA (z = 3.910, $P < 0.001$), CysC (z = 7.358, $P < 0.001$), NGAL (z = 5.424, $P < 0.001$), and UACR (z = 3.989, $P < 0.001$), whereas eGFR was lower (z = -7.264, $P < 0.001$). Additionally, HDL-C levels in the DKD group were significantly lower than

those in the HC group (z = -3.094, $P = 0.002$), the NDKD group (z = -2.262, $P = 0.024$), and the SDM group (z = -2.661, $P = 0.008$). Furthermore, no significant differences were identified in common renal function indicators between the DKD and NDKD groups.

Multivariate statistical analysis of metabolites

UPLC-MS/MS metabolomic analysis was conducted on 222 serum samples collected from all subjects, along with 24 QC samples, under both positive electrospray ionization (ESI^+) and negative electrospray ionization (ESI^-) modes. Multivariate statistical analysis was applied to evaluate the metabolic mass spectrometry characteristics, including retention time, accurate mass-to-charge ratio, and peak intensity, across all samples. In ESI mode, the ionization efficiency of acidic compounds is significantly influenced by the pH and solvent composition, contingent upon the deprotonation capacity of metabolites. This influence can result in signal fluctuations, as anions present in the sample may compete with target metabolites for ionization, thereby exacerbating signal instability. Additionally, anionic metabolites are susceptible to oxidation or degradation. Collectively, these factors contribute to increased variability in ESI- mode samples. In this study, the total ion current (TIC) and base peak intensity (BPI) plots of QC samples demonstrated appropriate chromatographic gradients and robust instrument stability (Fig. 1). In QC samples, 93.81% of the metabolite characteristics exhibited a relative standard deviation (RSD) of less than 30% in ESI + mode (Fig. 2A), while 91.45% demonstrated an RSD of less than 30% in ESI- mode (Fig. 2B). Notably, the proportion of mass spectrometry features with a RSD of less than 30% exceeded 90% across different QC samples. Furthermore, the QC samples were predominantly located in the central region of the sample attachment in the QC specific principal component analysis (PCA) graph (Fig. 2E and F). These findings suggest that the data quality is high, and the analytical results are both accurate and reliable. The metabolites with an RSD < 30% were subsequently included in the downstream analysis. In contrast, subsequent OPLS-DA analysis identified a clear segregation of serum components between any two groups in both ESI^+ and ESI^- modes, as illustrated by the OPLS-DA score plots (Fig. 3). The Y-explanatory (R2Y) and predictive ability (Q2) values between groups, summarized in Table 3, highlight the robust predictive capability of the model. The permutation test results (Table 3), derived from 1000 random permutations conducted to evaluate model performance, confirmed that the OPLS-DA model was not subject to overfitting.

Screening of differential metabolites and pathway analysis

In ESI^+ mode, 1,151 metabolites were identified from 7,772 fragment peaks, whereas 975 metabolites were detected from 8,096 fragment peaks under ESI^- mode. Fold Change (FC) serves as a metric for quantifying the differential concentration or abundance of a metabolite between two distinct sample groups, such as an experimental group and a control group. It is calculated as the ratio of the mean metabolite concentration in the experimental group to that in the control group. Based on findings from prior literature [34, 35], this study adopts a threshold of FC > 1.5 or FC < 0.67 to mitigate the risk of false negatives associated with excessive screening and by applying the selection criteria of a T-test ($P_{FDR} < 0.05$), and variable importance in projection score > 1 derived from OPLS-DA [34, 35], a total of 62 differential metabolites were identified between the DKD and NDKD groups, 170 between the DKD and SDM groups, 242 between the NDKD and SDM groups, and 316 between the SDM and HC groups, as illustrated in Fig. 4. The outcomes of the metabolic pathway analysis are presented in Table 4; Fig. 5, with the differential metabolites enriched in metabolic pathways detailed in Table 5. The Venn diagram illustrates the distinctions in metabolic pathways

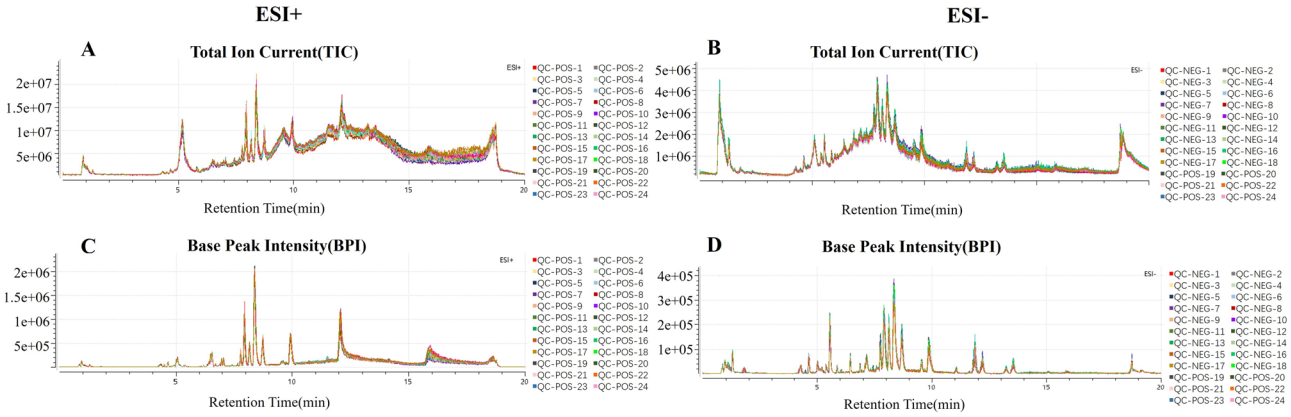

Fig. 1 Quality analysis of QC samples. Total ion current (**A**) and base peak intensity (**C**) in ESI + patterns of QC samples. Total ion current (**B**) and base peak intensity (**D**) in ESI- patterns of QC samples. QC, group of quality control. TIC, total ion current; BPI, base peak intensity

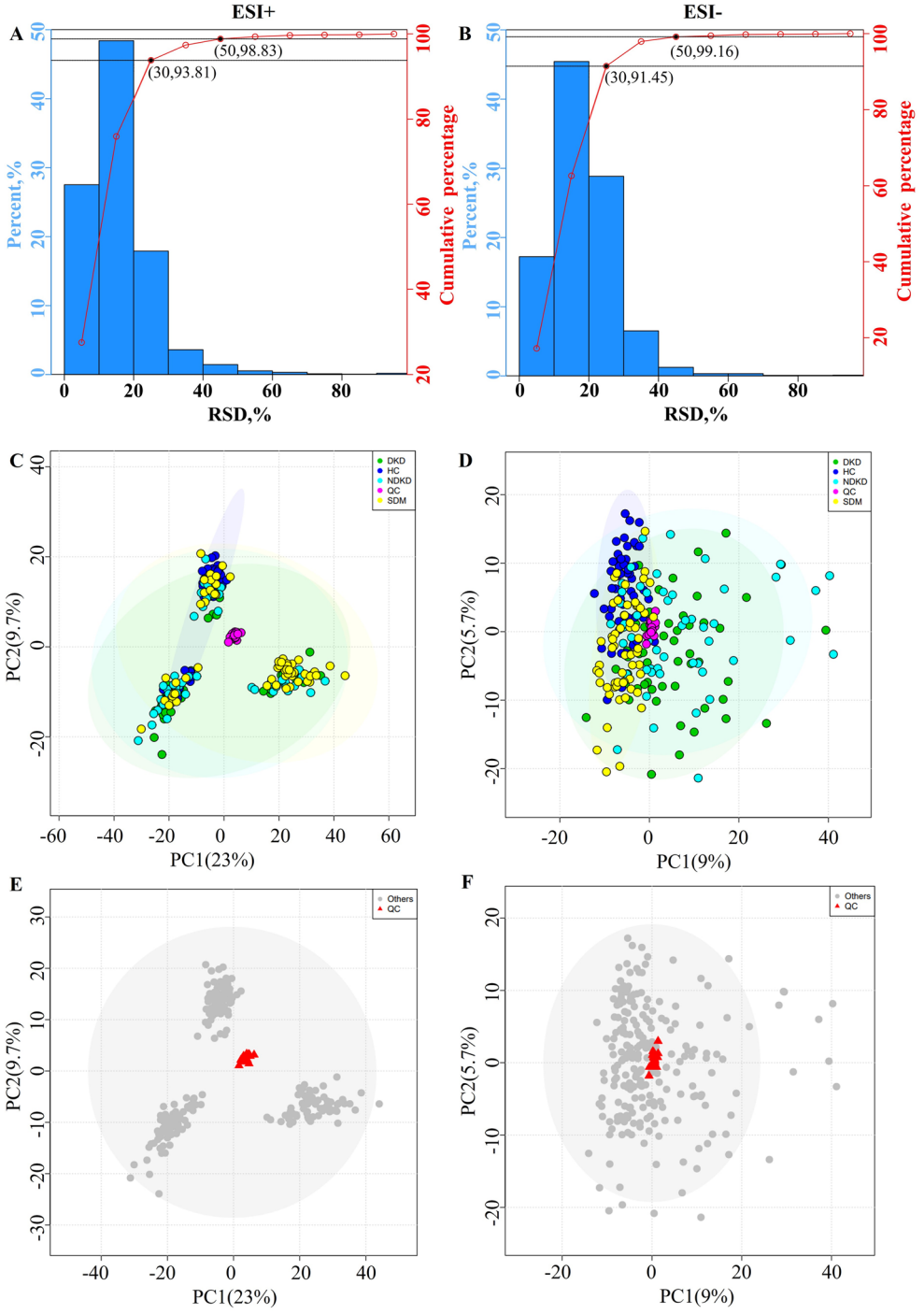

Fig. 2 RSD and PCA analysis of serum samples and QC samples. RSD of QC samples in ESI+ (**A**) and ESI- (**B**) patterns. PCA score plots in ESI+ (**C**) and ESI- (**D**) patterns among DKD, NDKD, SDM, HC, and QC groups. The QC specific PCA score plots in ESI+(**E**) and ESI-(**F**) patterns for visualizing the controls and QC replicates. DKD, group of diabetic kidney disease; NDKD, group of nondiabetic kidney disease; SDM, group of diabetes mellitus; HC, group of healthy control; QC, group of quality control; RSD, relative standard deviation

among the groups, as well as the metabolites associated with these pathways (Fig. 6). These results indicate that the metabolic pathways shared between DKD and NDKD encompass glycerophospholipid metabolism, glycerolipid metabolism, and tryptophan metabolism. The tryptophan metabolism pathway is likely to represent a common metabolic route in KD progression, whereas pyrimidine metabolism and arginine biosynthesis pathways appear to be distinct metabolic processes associated with the transition from DM to DKD.

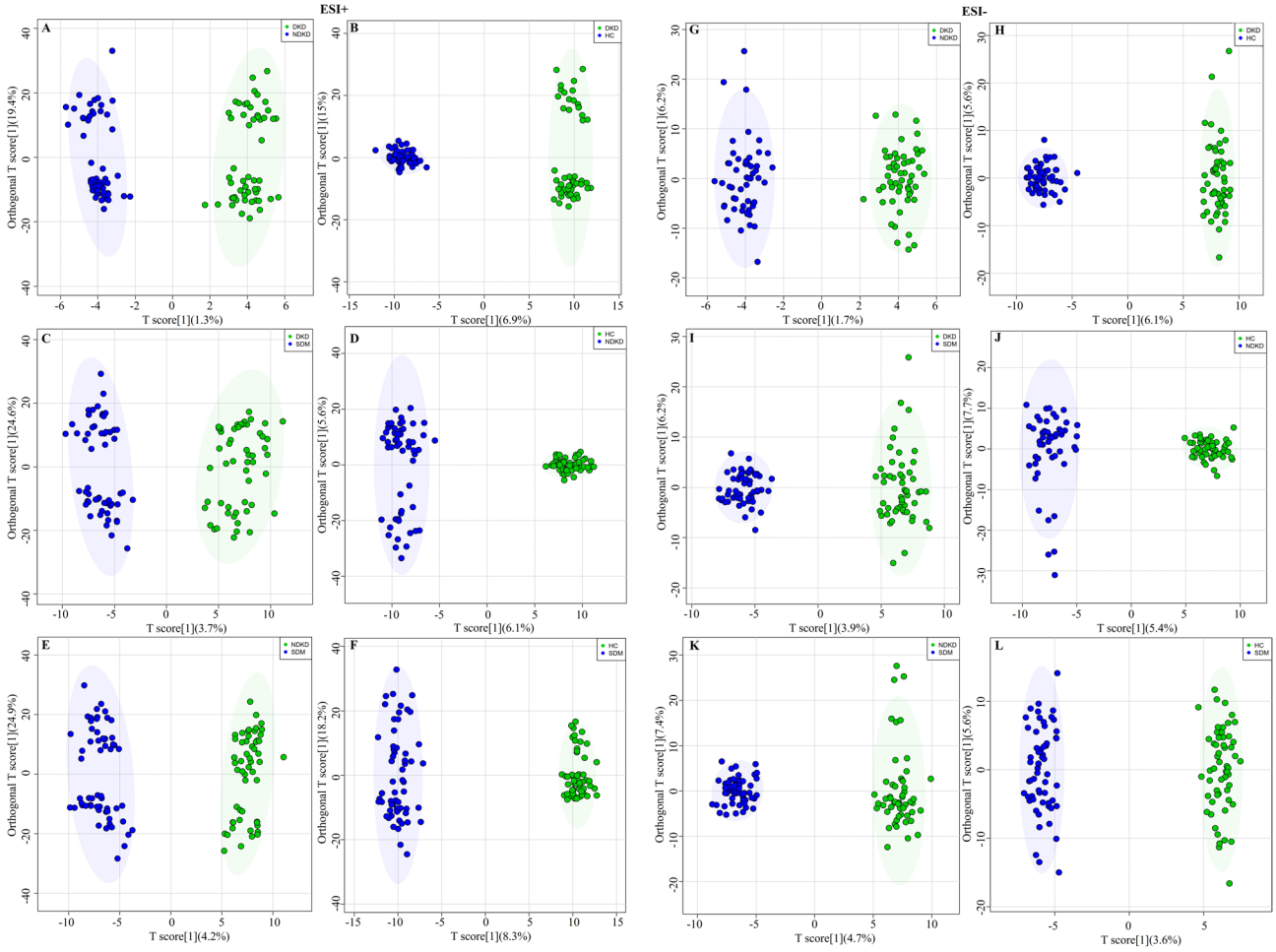

Fig. 3 OPLS-DA analysis of serum samples. OPLS-DA analysis was performed between DKD and NDKD (**A**), between DKD and HC (**B**), between DKD and SDM (**C**), between NDKD and HC (**D**), between NDKD and SDM (**E**), between SDM and HC (**F**) in ESI + patterns. OPLS-DA analysis was performed between DKD and NDKD (**G**), between DKD and HC (**H**), between DKD and SDM (**I**), between NDKD and HC (**J**), between NDKD and SDM (**K**), between SDM and HC (**L**) in ESI- patterns. DKD, group of diabetic kidney disease; NDKD, group of nondiabetic kidney disease; SDM, group of diabetes mellitus; HC, group of healthy control

Table 3 Permutation testing among groups in ESI + and ESI- patterns based on OPLS-DA analysis

Pattern	Group	R2Y, *p*	Q2, *p*
ESI+	DKD vs. NDKD	0.796,<0.001	0.475,<0.001
	DKD vs. SDM	0.922, 0.012	0.460,<0.001
	DKD vs. HC	0.967,<0.001	0.822,<0.001
	NDKD vs. SDM	0.982,<0.001	0.710,<0.001
	NDKD vs. HC	0.984,<0.001	0.752,<0.001
	SDM vs. HC	0.938,<0.001	0.784,<0.001
ESI-	DKD vs. NDKD	0.969, 0.011	0.422,<0.001
	DKD vs. SDM	0.756, 0.031	0.382,<0.001
	DKD vs. HC	0.974,<0.001	0.782,<0.001
	NDKD vs. SDM	0.953,<0.001	0.609,<0.001
	NDKD vs. HC	0.960,<0.001	0.653,<0.001
	SDM vs. HC	0.940,<0.001	0.675,<0.001

Note: DKD, group of diabetic kidney disease; NDKD, group of nondiabetic kidney disease; SDM, group of diabetes mellitus; HC, group of healthy control; R2Y, explainability of the model Y variable; Q2, the predictability of the model

Diagnostic performance of screened differential metabolites

ROC curve analysis was performed on the differential metabolites within the enriched metabolic pathways (Table 6), using an AUC > 0.7 to assess the diagnostic efficacy of the differential metabolites [32, 33]. The results of this analysis are provided in Table 6. The Venn diagram illustrating these differential metabolites distinctly highlights their specificity across various observational groups (Fig. 7). In comparison to the NDKD group (Table 6), significant elevations were identified in phosphatidylglycerol (PG, 14:0) and D-Maltose within the DKD group. When contrasted with the SDM group (Table 6), notable increases were observed in a lysophosphatidic acid (LPA, 16:3), LPA (18:5), LPA (22:5), phosphatidic acid (PA, 18:3), PG (26:4), L-Glutamine, Uridine, Cytidine, Formyl-N-acetyl-5-methoxykynurenamine, 2-Oxoadipate, 5-Hydroxy-L-tryptophan, Thymidine,

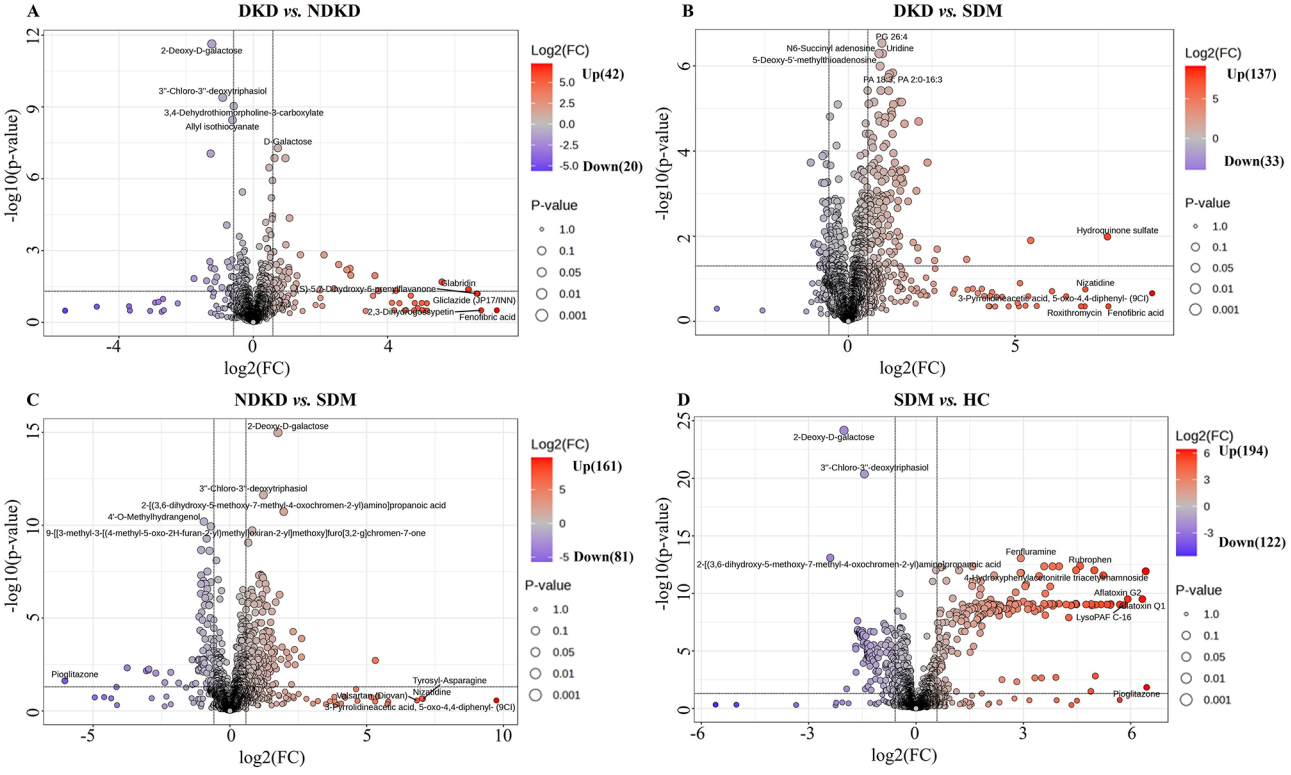

Fig. 4 Volcano plot of metabolites. Volcano plot of metabolites between DKD and NDKD (**A**), between DKD and SDM (**B**), between NDKD and SDM (**C**), between SDM and HC (**D**). DKD, group of diabetic kidney disease; NDKD, group of nondiabetic kidney disease; SDM, group of diabetes mellitus; HC, group of healthy control; FC, fold change; P-value: the significance value of T-test and corrected as false discovery rate (FDR)-adjusted P values using Benjamini–Hochberg procedure; n, the number of upregulated or down-regulated metabolites. The abscissa is equal to log2(FC), and the abscissa is equal to -log10(p). The two lines parallel to the Y-axis are x = 0.58 and x=-0.58, the point to the left of x=-0.58 is more than 1.5 times down-regulated metabolites, and the point to the right of x = 0.58 is more than 1.5 times up-regulated metabolites. A line parallel to the X-axis is Y = 1.30, and points above the line represent metabolites with a significant $p < 0.05$

Table 4 Analysis of different metabolite pathways between groups

Group	Pathway name	-Log(*P*)	Impact	Hits	Classes	KEGG.id
DKD vs. NDKD	Glycerophospholipid metabolism	5.85	0.29	6	Lipid metabolism	hsa00564
	Glycerolipid metabolism	2.28	0.69	2	Lipid metabolism	hsa00561
	Starch and sucrose metabolism	1.77	0.10	2	Carbohydrate metabolism	hsa00500
	Pentose phosphate pathway	1.57	0.20	2	Carbohydrate metabolism	hsa00030
DKD vs. SDM	Glycerophospholipid metabolism	4.64	0.33	7	Lipid metabolism	hsa00564
	Glycerolipid metabolism	2.14	0.13	3	Lipid metabolism	hsa00561
	Ether lipid metabolism	1.86	0.33	3	Lipid metabolism	hsa00565
	Pyrimidine metabolism	1.77	0.11	4	Nucleotide metabolism	hsa00240
	Tryptophan metabolism	1.70	0.13	4	Amino acid metabolism	hsa00380
	Arginine biosynthesis	1.31	0.23	2	Amino acid metabolism	hsa00220
NDKD vs. SDM	Glycerophospholipid metabolism	4.57	0.44	7	Lipid metabolism	hsa00564
	Tryptophan metabolism	2.41	0.14	5	Amino acid metabolism	hsa00380
	Glycerolipid metabolism	2.11	0.21	3	Lipid metabolism	hsa00561
	Galactose metabolism	1.48	0.50	3	Carbohydrate metabolism	hsa00052
SDM vs. HC	Glycerophospholipid metabolism	9.49	0.45	10	Lipid metabolism	hsa00564
	Glycerolipid metabolism	5.10	0.24	5	Lipid metabolism	hsa00561
	Ether lipid metabolism	2.20	0.33	3	Lipid metabolism	hsa00500
	Sphingolipid metabolism	1.63	0.22	3	Lipid metabolism	hsa00600

Note: DKD, group of diabetic kidney disease; NDKD, group of nondiabetic kidney disease; SDM, group of diabetes mellitus; HC, group of healthy control; -Log(P), the negative log of the p-value; Impact, weight calculation based on topology analysis; Hits, the number of metabolites that match the metabolic pathway; Classes, types of metabolic pathways; KEGG, kyoto encyclopedia of genes and genomes

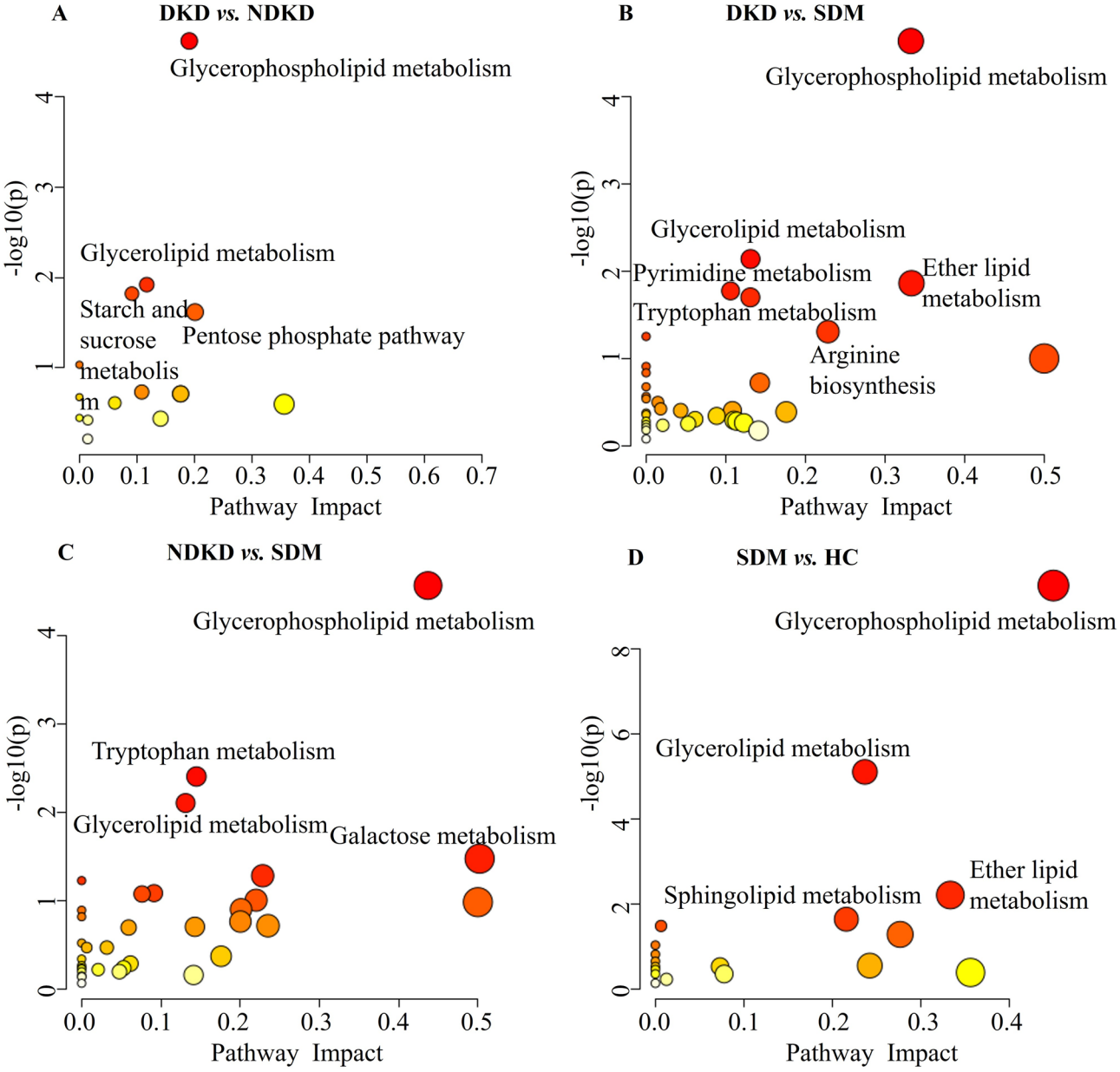

Fig. 5 Pathway analysis of differential metabolites. Pathway analysis of differential metabolites between DKD and NDKD (**A**), between DKD and SDM (**B**), between NDKD and SDM (**C**), between SDM and HC (**D**). DKD, group of diabetic kidney disease; NDKD, group of nondiabetic kidney disease; SDM, group of diabetes mellitus; HC, group of healthy control

and L-Citrulline in the DKD group, whereas a significant reduction was detected in PG (28:4). In the NDKD group, substantial elevations were identified in LPA (16:3), LPA (18:5), LPA (22:5), PA (18:3), PA (24:4), PG (26:4), 2-Oxoadipate, 5-Hydroxyindoleacetate, 5-Hydroxy-L-tryptophan, and Indole-3-acetate, while a marked decline in D-Galactose levels was observed. These results indicate that PG (14:0) and D-Maltose may serve as potential biomarkers for differentiating DKD from NDKD. In contrast, PG (28:4), L-Glutamine, Uridine, Cytidine, Formyl-N-acetyl-5-methoxykynurenamine, Thymidine, and L-Citrulline exhibit strong specificity for diagnosing DKD and may act as potential risk indicators for the progression of DM to DKD.

Correlation analysis between screened differential metabolites and conventional biochemical indicators

Spearman correlation analysis was performed to examine the associations between the identified differential metabolites and conventional biochemical indicators, with the results summarized in Table 7. PG (14:0) exhibited a positive correlation with Cr ($r = 0.139$, $P = 0.039$)

Table 5 Differential metabolites enriched in metabolic pathways between groups

Group	Pathway name	Differential metabolites
DKD vs. NDKD	Glycerophospholipid metabolism	LPA(17:2),LPA(20:0),LPG(14:0),LPG(18:3),LPI(17:1),PA(27:4),PA(29:4),PC(17:0),PC(36:4),PG(14:0).
	Glycerolipid metabolism	LPA(17:2),LPA(20:0),PA(27:4),PA(29:4).
	Starch and sucrose metabolism	Maltose, D-Fructose 6-phosphate.
	Pentose phosphate pathway	D-Fructose 6-phosphate,6-Phospho-D-gluconate.
DKD vs. SDM	Glycerophospholipid metabolism	LPG(20:4),LPG(16:3),LPG(21:0),LPA(16:3),LPA(22:5),LPA(18:5),LPA(18:4),PC(12:0),PC(17:0),PG(20:3),PG(21:5),PG(26:4),PG(28:4),LPI(20:1),PS(20:0),PS(21:0/22:1(11Z)), PA(20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), PA(24:4),PA(18:3),PA(23:5),PA(19:2),PA(23:3),PA(24:5),PA(27:6),PA(29:4).
	Glycerolipid metabolism	LPA(16:3),LPA(22:5),LPA(18:5),LPA(18:4),PA(20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), PA(24:4),PA(18:3),PA(23:5),PA(19:2),PA(23:3),PA(24:5),PA(27:6),PA(29:4),TAG(52:10),TAG(54:10).
	Ether lipid metabolism	PC(18:1(9Z)e/2:0),PC(34:8e), PE(25:6e).
	Pyrimidine metabolism	L-Glutamine, Uridine, Cytidine, Thymidine.
	Tryptophan metabolism	5-Hydroxy-L-tryptophan, 2-Oxoadipate, Formyl-N-acetyl-5-methoxykynurenamine, Indole-3-acetate.
	Arginine biosynthesis	L-Citrulline, L-Glutamine.
NDKD vs. SDM	Glycerophospholipid metabolism	PE(17:0e), PC(12:0),PC(13:0),PC(36:4),PC(47:6),PS(43:1),LPA(16:3),LPA(18:4),LPA(18:5),LPA(22:5),LPG(16:3),LPG(20:4),LPG(21:0),LPG(9:0),PG(20:3),PG(21:5),PG(26:4),PG(28:4),PG(20:2(11Z,14Z)/22:4(7Z,10Z,13Z,16Z)), PA(24:4),PA(18:3),PA(23:5),PA(19:2),PA(23:3),PA(24:5),PA(24:6).
	Tryptophan metabolism	5-Hydroxyindoleacetate, 5-Hydroxy-L-tryptophan, 2-Oxoadipate, Formyl-N-acetyl-5-methoxykynurenamine, Indole-3-acetate.
	Glycerolipid metabolism	LPA(16:3),LPA(18:4),LPA(18:5),LPA(22:5),PA(24:4),PA(18:3),PA(23:5),PA(19:2),PA(23:3),PA(24:5),PA(24:6),TAG(52:10).
	Galactose metabolism	Melibiitol, D-Galactose, Lactose.
SDM vs. HC	Glycerophospholipid metabolism	PE(18:1),PE(44:11),PE(35:7),PE(16:0),PE(46:7),PE(12:0/15:1(9Z)), PE(34:2),PE(20:4(5Z,8Z,11Z,14Z)/24:1(15Z)), PE(27:3),PE(22:6),PE(22:4(7Z,10Z,13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), PE(16:1(11Z)/15:0),LysoPC(26:2),LysoPC(20:4(5Z,8Z,11Z,14Z)), PA(17:1(9Z)/22:4(7Z,10Z,13Z,16Z)), PA(12:0/20:5(5Z,8Z,11Z,14Z,17Z)), PA(12:0/20:3(8Z,11Z,14Z)), PA(12:0/14:1(9Z)), PA(17:0/14:1(9Z)), PA(15:1(9Z)/22:4(7Z,10Z,13Z,16Z)), PA(20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), PA(18:4(6Z,9Z,12Z,15Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), PA(12:0/17:2(9Z,12Z)), PA(12:0/17:1(9Z)), PG(12:0/16:1(9Z)), PG(20:0(11Z,14Z)/22:4(7Z,10Z,13Z,16Z)), PG(20:3),PG(22:1(11Z)/0:0),PG(14:0),PG(16:1(9Z)/16:0),PG(13:0/17:1(9Z)), PG(28:4),PG(36:8),PG(38:10),PG(41:9),PG(P-16:0/14:1(9Z)), PG(12:0/17:1(9Z)), PS(22:2(13Z,16Z)/0:0),PS(44:10),PS(O-16:0/14:1(9Z)), PS(19:0/21:0),PS(O-16:0/15:1(9Z)), PS(O-16:0/16:1(9Z)), PS(21:0/21:0),PS(17:0/21:0),PS(O-18:0/19:1(9Z)), PS(O-16:0/22:1(11Z)), PS(43:1),PS(O-16:0/17:1(9Z)), PS(21:0/22:1(11Z)), PS(18:1(9Z)/18:2(9Z,12Z)), PS(P-16:0/12:0),PC(25:3),PC(18:0/11:1(10E)), PC(34:2),PC(42:7),PC(18:4),PC(47:6),PC(20:5(5Z,8Z,11Z,14Z,17Z)/19:0),PC(MonoMe(9,5)/DiMe(9,3)),PC(20:4),PC(41:7),PI(38:5),PI(18:1/8,9-EpETE), PI(12:0/15:1(9Z)), PI(21:0/22:4(7Z,10Z,13Z,16Z)), LPG(12:0),LPG(18:3),LPG(21:2).
	Glycerolipid metabolism	TAG(36:5),TAG(36:6),TAG(39:8),TAG(40:6),TAG(46:10),TAG(52:10),TAG(53:13),TAG(54:17),TAG(54:10),TAG(56:10),TAG(63:19),PA(17:1(9Z)/22:4(7Z,10Z,13Z,16Z)), PA(12:0/20:5(5Z,8Z,11Z,14Z,17Z)), PA(12:0/20:3(8Z,11Z,14Z)), PA(12:0/14:1(9Z)), PA(17:0/14:1(9Z)), PA(15:1(9Z)/22:4(7Z,10Z,13Z,16Z)), PA(20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), PA(18:4(6Z,9Z,12Z,15Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), PA(12:0/17:2(9Z,12Z)), PA(12:0/17:1(9Z)), PG(12:0/16:1(9Z)),1-Acylglycerol, Digalactosyldiacylglycerol, MGDG(24:6),MGDG(30:7),MGDG(30:6),MGDG(31:6),MGDG(32:6),MGDG(35:6),MGDG(37:1),MGDG(18:1),MGDG(22:1),MGDG(34:8),MGDG(39:1),MGDG(40:6),MGDG(42:6),MGDG(48:10).
	Ether lipid metabolism	PE(17:0e), PC(24:5e), PC(34:8e), PC(18:1(9Z)e/2:0).
	Sphingolipid metabolism	SM(d26:0),SM(d18:0/16:1(9Z)(OH)), Sulfatide, Cer-BDS(d35:8), Cer-NP t35:2, Cer-BDS(d39:8).

Note: DKD, group of diabetic kidney disease; NDKD, group of nondiabetic kidney disease; SDM, group of diabetes mellitus; HC, group of healthy control

and Glu ($r = 0.155$, $P = 0.021$). A positive correlation was also detected between D-Maltose and Glu ($r = 0.458$, $P < 0.001$), as well as between D-Maltose and UACR ($r = 0.170$, $P = 0.011$). D-Galactose demonstrated a positive correlation with Glu ($r = 0.319$, $P < 0.001$) but showed a negative correlation with HDL-C (r = -0.151, $P = 0.025$) and ApoA1 (r = -0.149, $P = 0.027$). Moreover, LPA (16:3), LPA (18:5), LPA (22:5), PA (18:3), PG (26:4), L-Glutamine, Uridine, Cytidine, 2-Oxoadipate, 5-Hydroxy-L-tryptophan, Thymidine, L-Citrulline, PA (24:4),

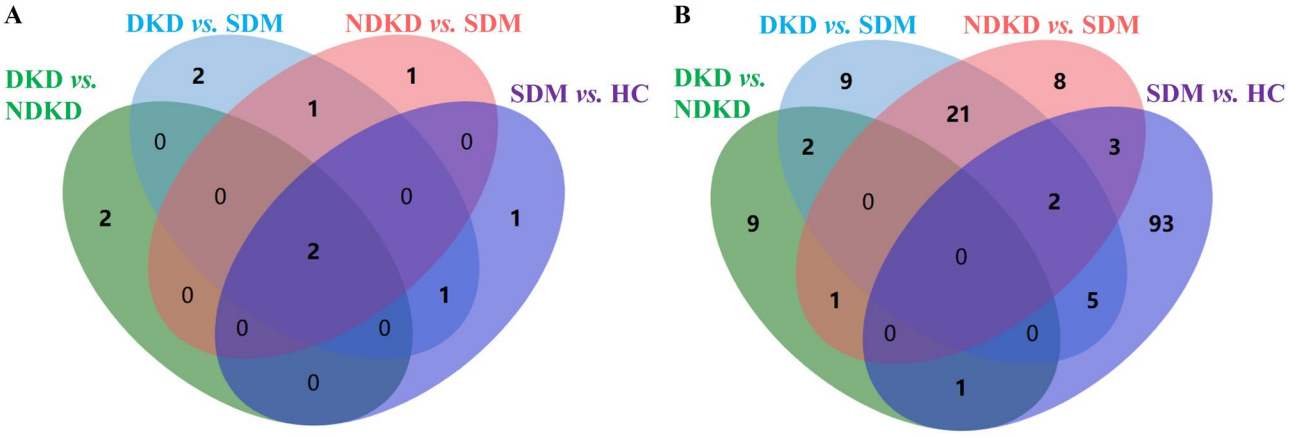

Fig. 6 Venn diagrams of different metabolic pathways and metabolites in the subjects. Venn diagram for differential pathways (**A**) and venn diagram for metabolites on differential metabolic pathways (**B**). Figure 6A shows the number of metabolic pathways in the region, while Fig. 6B displays the number of metabolites in that area. DKD, group of diabetic kidney disease; NDKD, group of nondiabetic kidney disease; SDM, group of diabetes mellitus; HC, group of healthy control

5-Hydroxyindoleacetate, and Indole-3-acetate all displayed positive correlations with Urea, Cr, CysC, NGAL, and UACR, while exhibiting a negative correlation with eGFR. These findings indicate that fluctuations in the concentrations of the differential metabolites identified in DKD patients are associated with alterations in renal function and blood lipid indicators.

Discussion

DM is a metabolic disorder with multifactorial origins, and its associated renal complications have emerged as the leading cause of CKD. In recent years, research has predominantly focused on the early diagnosis and treatment of DKD, whereas fewer investigations have examined the metabolic alterations occurring during the transition from DM to DKD or those distinguishing DKD from NDKD. By employing UPLC-MS/MS-based untargeted metabolomics, significant variations in serum metabolite profiles have been identified among the DKD, NDKD, SDM, and HC groups. Both DKD and NDKD are characterized by renal dysfunction, leading to the involvement of overlapping metabolic enrichment pathways, including glycerophospholipid metabolism, glycerolipid metabolism, and tryptophan metabolism. Notably, the tryptophan metabolism pathway may function as a shared metabolic route in the progression of KD, whereas pyrimidine metabolism and arginine biosynthesis pathways may be specifically linked to the transition from DM to DKD.

In this study, the glycerophospholipid metabolism pathway was identified as one of the shared metabolic enrichment pathways between DKD and NDKD. As fundamental constituents of cell membranes, glycerophospholipids interact with membrane proteins and participate in signal transduction, small molecule transport, and enzymatic processes essential for maintaining cellular homeostasis [36]. In individuals with DM, elevated glucose levels promote the hepatic synthesis of saturated fatty acid chains and glycerol-3-phosphate from excess glucose, leading to an increase in triglyceride production [37]. Consequently, the accumulation of metabolic intermediates is induced, thereby disrupting lipid metabolism through the glycerophospholipid metabolism pathway. Prior research has established a strong association between phospholipid metabolism and the molecular mechanisms underlying insulin resistance [38, 39]. Through experimental studies in murine models, Rietjens RGJ et al. [40] demonstrated that disruptions in phosphatidylinositol lipid metabolism within proximal tubular segments preceded pathological alterations in renal tissue. Likewise, Shen et al. [41] confirmed in rat models that the molecular mechanism by which Salvia miltiorrhiza ameliorates renal function is primarily linked to modifications in glycerophospholipid and sphingolipid metabolism. These findings provide further evidence supporting the current observation that renal dysfunction in DM patients is likely to induce alterations in glycerophospholipid metabolic pathways. Furthermore, additional studies have documented changes in glycerophospholipid metabolism pathways in various conditions, including organophosphate poisoning-induced kidney injury [42], IgA nephropathy [43], lupus nephritis [30], and renal cell carcinoma [21]. However, the specific lipid species implicated in these metabolic pathway alterations differ from those observed in DM-related kidney injury, potentially offering novel insights into the mechanisms underlying kidney damage across different etiologies.

In this study, significant alterations in the tryptophan metabolism pathway were identified between the DKD and SDM groups, as well as between the NDKD

Table 6 Evaluation of differential metabolite diagnostic performance

Pathway name	Metabolite	VIP	FC	Trend	AUC(95%CI)	Se(%)	Sp(%)	YI	Z	*P*
DKD vs. NDKD										
Glycerophospholipid metabolism	PG(14:0)	2.52	1.96	↑	0.740(0.647,0.820)	83.02	62.96	0.46	4.983	<0.001
Starch and sucrose metabolism	D-Maltose	4.15	1.94	↑	0.838(0.754,0.902)	79.25	81.48	0.61	8.301	<0.001
DKD vs. SDM										
Glycerophospholipid metabolism	LPA(16:3)	2.00	2.17	↑	0.793(0.706,0.864)	77.36	79.66	0.57	6.586	<0.001
	LPA(18:5)	2.45	2.48	↑	0.822(0.738,0.888)	67.92	89.83	0.58	7.862	<0.001
	LPA(22:5)	1.87	3.47	↑	0.825(0.742,0.890)	75.47	77.97	0.53	8.441	<0.001
	PA(18:3)	2.69	2.51	↑	0.847(0.767,0.908)	81.13	72.88	0.54	9.582	<0.001
	PG(26:4)	2.84	2.01	↑	0.854(0.775,0.913)	73.58	88.14	0.62	9.586	<0.001
	PG(28:4)	1.45	0.55	↓	0.733(0.641,0.812)	60.38	77.97	0.38	4.940	<0.001
Pyrimidine metabolism	L-Glutamine	1.78	2.78	↑	0.765(0.676,0.840)	73.58	67.80	0.41	5.969	<0.001
	Uridine	2.77	2.03	↑	0.881(0.806,0.934)	84.91	79.66	0.65	11.460	<0.001
	Cytidine	2.44	1.53	↑	0.807(0.722,0.876)	75.47	74.58	0.50	7.476	<0.001
	Thymidine	2.19	1.83	↑	0.725(0.633,0.805)	58.49	86.44	0.45	4.464	<0.001
Tryptophan metabolism	5-Hydroxy-L-tryptophan	2.40	2.15	↑	0.781(0.693,0.853)	66.04	84.75	0.51	6.281	<0.001
	2-Oxoadipate	2.51	2.08	↑	0.787(0.700,0.859)	66.04	83.05	0.49	6.659	<0.001
	Formyl-N-acetyl-5-methoxykynurenamine	2.10	2.91	↑	0.744(0.652,0.821)	49.06	91.53	0.41	5.218	<0.001
Arginine biosynthesis	L-Citrulline	2.49	1.55	↑	0.767(0.677,0.841)	62.26	81.36	0.44	5.885	<0.001
NDKD vs. SDM										
Glycerophospholipid metabolism	LPA(16:3)	1.72	2.87	↑	0.808(0.723,0.876)	79.63	81.36	0.61	7.268	<0.001
	LPA(18:5)	2.03	2.57	↑	0.857(0.778,0.916)	77.78	79.66	0.57	10.155	<0.001
	LPA(22:5)	1.47	4.32	↑	0.783(0.695,0.855)	66.67	83.05	0.50	6.508	<0.001
	PA(18:3)	2.19	2.91	↑	0.840(0.759,0.902)	81.48	76.27	0.58	9.017	<0.001
	PA(24:4)	2.30	2.27	↑	0.805(0.717,0.874)	64.81	84.75	0.50	7.419	<0.001
	PG(26:4)	2.36	1.85	↑	0.809(0.724,0.876)	59.26	93.22	0.52	7.676	<0.001
Tryptophan metabolism	5-Hydroxyindoleacetate	2.81	1.59	↑	0.773(0.685,0.847)	64.81	84.75	0.50	6.077	<0.001
	5-Hydroxy-L-tryptophan	1.99	2.52	↑	0.791(0.704,0.861)	70.37	86.44	0.57	6.533	<0.001
	2-Oxoadipate	1.91	1.78	↑	0.753(0.663,0.830)	68.52	79.66	0.48	5.394	<0.001
	Indole-3-acetate	2.01	1.89	↑	0.792(0.705,0.863)	70.37	77.97	0.48	7.010	<0.001
Galactose metabolism	D-Galactose	2.80	0.62	↓	0.831(0.749,0.895)	79.63	76.27	0.56	8.508	<0.001

Note: DKD, group of diabetic kidney disease; NDKD, group of nondiabetic kidney disease; SDM, group of diabetes mellitus; HC, group of healthy control; VIP, variable influence on projection based on orthogonal partial least squares discriminant analysis; FC, fold change; AUC, area under the curve; Se, sensitivity; Sp, specificity; YI, youden index

and SDM groups. Conversely, no such alterations were detected between the DKD and NDKD groups or between the SDM and HC groups, suggesting that modifications in the tryptophan metabolism pathway may constitute a shared pathogenic mechanism underlying kidney injury. Tryptophan metabolism gives rise to various bioactive compounds that regulate multiple physiological processes, including neurological activity, immune responses, metabolism, and inflammation, with key pathways encompassing indole metabolism, serotonin metabolism, and kynurenine metabolism [44]. The accumulation of these tryptophan metabolites, classified as uremic toxins, is regarded as a pivotal mechanism contributing to CKD progression [45, 46].

Through an analysis of serum tryptophan metabolites in DKD patients, Debnath et al. [47] demonstrated that impaired renal function in DKD patients is associated with the accumulation of tryptophan metabolites such as kynurenine, kynurenic acid, and quinolinic acid. Similarly, Zeng et al. [48] investigated the serum metabolic profiles of CKD patients with diverse etiologies and identified substantial variations in the amino acid metabolic profiles among individuals with DKD, hypertensive nephropathy, and chronic nephritis. This study employed patients with NDKD and those with SDM as disease controls, in addition to healthy controls and patients with DKD, for the purpose of metabolomics analysis. The results indicated that 2-Oxoadipate was commonly accumulated in the tryptophan metabolism pathway in both the DKD and SDM groups, as well as in the NDKD and SDM groups. These results suggest that even when the research subjects belong to the same category (i.e.,

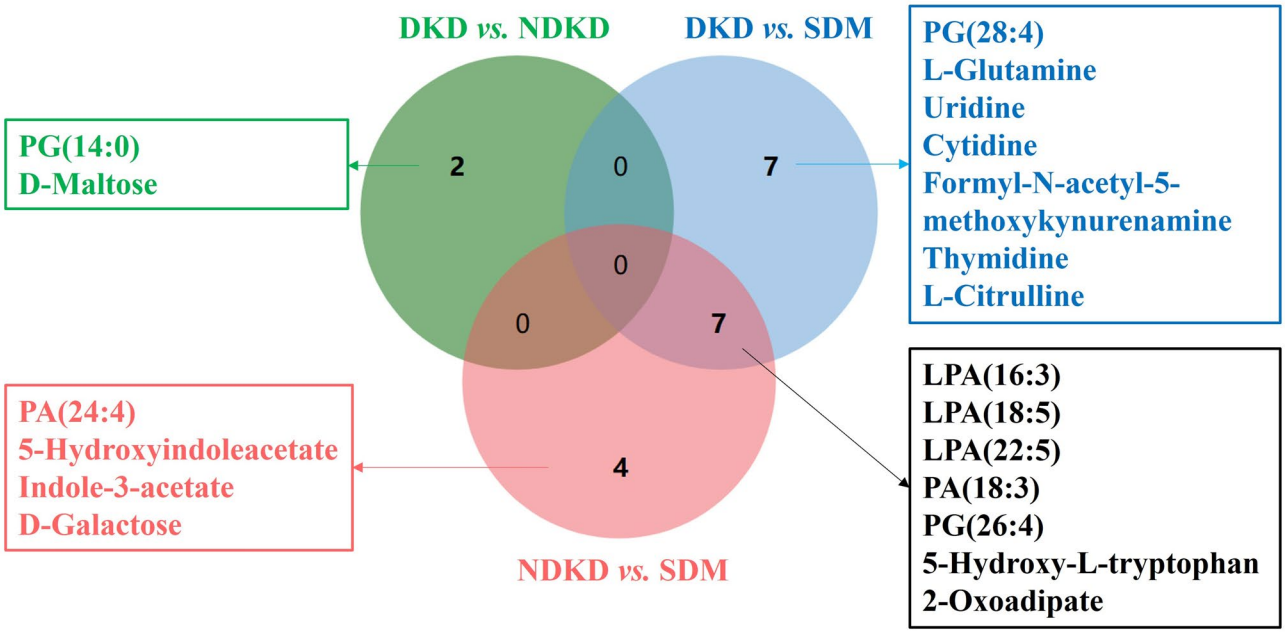

Fig. 7 Venn diagram illustrating differential metabolites with an AUC greater than 0.7 among the subjects. PG(14:0) and D-Maltose exist only in DKD vs. NDKD; PG(28:4), L-Glutamine, Uridine, Cytidine, Formyl-N-acetyl-5-methoxykynurenamine, Thymidine and L-Citrulline exist only in DKD vs. SDM; LPA(16:3), LPA(18:5), LPA(22:5), PA(18:3), PG(26:4), 5-Hydroxy-L-tryptophan and 2-Oxoadipate coexist in DKD vs. SDM and NDKD vs. SDM. The numeral "0" indicates no common differential metabolites in this region. DKD, group of diabetic kidney disease; NDKD, group of nondiabetic kidney disease; SDM, group of diabetes mellitus; HC, group of healthy control

DKD patients), the control groups—particularly the disease control group—differ significantly. Consequently, the samples analyzed vary, leading to distinct differential metabolites identified through metabolomics, with minimal overlap among them [22, 48]. Within mitochondria, 2-Oxoadipate undergoes oxidative decarboxylation to generate Glutaryl-CoA via 2-oxoadipate dehydrogenase, simultaneously producing superoxide and hydrogen peroxide [49]. When abnormalities in the tryptophan metabolism pathway result in excessive 2-Oxoadipate accumulation, the subsequent elevation of superoxide and hydrogen peroxide exacerbates oxidative stress by disrupting the oxidation-antioxidation balance. This process may serve as one of the pathogenic mechanisms contributing to oxidative stress-induced renal dysfunction in DM patients [50].

In this study, 5-Hydroxyindoleacetate was found to be selectively accumulated in the tryptophan metabolism pathway between the NDKD and SDM groups. Dai et al. [51] developed rat models of acute kidney injury and chronic renal failure, analyzing serum tryptophan metabolites via UPLC-MS/MS, and identified 5-Hydroxyindoleacetate as a potential biomarker for both acute kidney injury and chronic renal failure. Likewise, Li et al. [52] established a mouse model of early kidney injury induced by hyperuricemia and observed markedly elevated serum levels of 5-Hydroxyindoleacetate in these mice. The present study revealed that UA levels in NDKD patients were significantly higher than those in SDM patients, indicating that the aberrant serum levels of 5-Hydroxyindoleacetate observed between the NDKD and SDM groups might be associated with hyperuricemia. Nevertheless, further investigations are required to confirm this hypothesis.

Non-targeted metabolomics, through the analysis of metabolic pathways of small molecule metabolites, holds the potential to elucidate underlying pathogenic mechanisms of various human diseases and assess the viability of small molecule metabolites as biomarkers [53, 54]. The metabolic distinctions among DKD, NDKD, and SDM can be understood as a hierarchical representation of the "etiological-metabolic pathway-kidney injury". SDM is predominantly characterized by systemic disorders in glucose and lipid metabolism, with the kidneys remaining unaffected at this stage. In contrast, DKD represents a kidney-specific metabolic catastrophe driven by hyperglycemia, which is exacerbated by various factors including polyol pathway activation, the accumulation of advanced glycation end products, lipid-mediated cytotoxicity, among others. The metabolic abnormalities observed in NDKD are primarily associated with the underlying disease, wherein hyperglycemia is not the principal mechanism. This study identifies that the metabolic pathway differences between DKD and NDKD are chiefly related to lipid and carbohydrate metabolism. Furthermore, the distinctions in metabolic pathways

Table 7 Correlation analysis of differential metabolites and biochemical markers(r, P)

Metabolite	Ur	Cr	Glu	UA	CysC	eGFR	C1q	NGAL	TC	TG	HDL-C	LDL-C	ApoA1	ApoB	UACR
PG(14:0)	0.065, 0.333	0.139, 0.039[a]	0.155, 0.021[a]	-0.013, 0.851	0.123, 0.067	-0.109, 0.104	-0.091, 0.177	-0.025, 0.706	-0.057, 0.394	-0.071, 0.294	-0.052, 0.443	-0.046, 0.495	-0.050, 0.463	-0.030, 0.652	0.003, 0.964
D-Maltose	0.053, 0.432	-0.069, 0.309	0.458, <0.001	-0.080, 0.237	-0.013, 0.843	0.011, 0.867	-0.038, 0.573	-0.181, 0.007[a]	-0.043, 0.529	-0.013, 0.844	-0.132, 0.049[a]	-0.033, 0.625	-0.128, 0.056	0.047, 0.490	0.170, 0.011[a]
LPA(16:3)	0.162, 0.016[a]	0.238, <0.001	-0.111, 0.099	0.272, <0.001	0.299, <0.001	-0.286, <0.001	0.068, 0.315	0.182, 0.007[a]	-0.028, 0.673	0.004, 0.952	-0.085, 0.206	-0.083, 0.215	0.001, 0.989	-0.060, 0.371	0.178, 0.008[a]
LPA(18:5)	0.328, <0.001	0.303, <0.001	-0.046, 0.497	0.183, 0.006[a]	0.377, <0.001	-0.372, <0.001	0.159, 0.018[a]	0.288, <0.001	-0.071, 0.289	0.049, 0.468	-0.090, 0.183	-0.162, 0.015[a]	-0.082, 0.223	-0.134, 0.047[a]	0.283, <0.001
LPA(22:5)	0.302, <0.001	0.311, <0.001	-0.006, 0.934	0.264, <0.001	0.350, <0.001	-0.340, <0.001	0.071, 0.292	0.248, <0.001	-0.052, 0.442	0.072, 0.287	-0.006, 0.930	-0.130, 0.052	0.017, 0.800	-0.108, 0.110	0.196, 0.003[a]
PA(18:3)	0.271, <0.001	0.374, <0.001	-0.054, 0.425	0.265, <0.001	0.400, <0.001	-0.393, <0.001	0.036, 0.594	0.275, <0.001	-0.069, 0.306	0.032, 0.632	-0.078, 0.247	-0.122, 0.069	-0.057, 0.396	-0.101, 0.132	0.272, <0.001
PG(26:4)	0.328, <0.001	0.349, <0.001	0.043, 0.521	0.249, <0.001	0.429, <0.001	-0.410, <0.001	0.022, 0.747	0.267, <0.001	-0.091, 0.177	0.000, 0.998	-0.111, 0.100	-0.134, 0.046[a]	-0.118, 0.079	-0.075, 0.265	0.341, <0.001
PG(28:4)	-0.107, 0.111	-0.246, <0.001	0.148, 0.028[a]	-0.154, 0.022[a]	-0.281, <0.001	0.267, <0.001	-0.073, 0.280	-0.153, 0.023[a]	-0.072, 0.289	-0.064, 0.342	0.081, 0.232	-0.044, 0.518	0.050, 0.460	-0.054, 0.423	-0.104, 0.123
L-Glutamine	0.225, 0.001[a]	0.214, 0.001[a]	0.044, 0.511	0.203, 0.002[a]	0.346, <0.001	-0.356, <0.001	0.075, 0.267	0.154, 0.022[a]	-0.018, 0.789	0.008, 0.911	-0.039, 0.560	-0.090, 0.182	-0.008, 0.902	-0.043, 0.525	0.334, <0.001
Uridine	0.360, <0.001	0.367, <0.001	-0.042, 0.533	0.287, <0.001	0.467, <0.001	-0.470, <0.001	0.124, 0.065	0.248, <0.001	-0.111, 0.100	0.019, 0.783	-0.091, 0.175	-0.168, 0.012[a]	-0.076, 0.262	-0.106, 0.116	0.342, <0.001
Cytidine	0.291, <0.001	0.300, <0.001	0.156, 0.020[a]	0.193, <0.001	0.397, <0.001	-0.397, <0.001	0.054, 0.425	0.180, 0.007[a]	-0.045, 0.508	0.088, 0.193	-0.151, 0.025[a]	-0.084, 0.214	-0.127, 0.058	0.001, 0.991	0.352, <0.001
Thymidine	0.170, 0.011[a]	0.222, 0.001[a]	-0.148, 0.028[a]	0.185, 0.006[a]	0.267, <0.001	-0.270, <0.001	0.016, 0.814	0.193, 0.004[a]	-0.071, 0.290	-0.034, 0.613	-0.023, 0.735	-0.135, 0.045[a]	-0.036, 0.597	-0.165, 0.014[a]	0.211, 0.002[a]
5-Hydroxy-L-tryptophan	0.330, <0.001	0.278, <0.001	0.027, 0.689	0.208, 0.002[a]	0.357, <0.001	-0.374, <0.001	0.072, 0.287	0.177, 0.008[a]	0.027, 0.690	0.141, 0.036[a]	-0.125, 0.064	-0.049, 0.464	-0.089, 0.185	0.054, 0.424	0.305, <0.001
2-Oxoadipate	0.215, 0.001[a]	0.281, <0.001	-0.025, 0.716	0.221, 0.001[a]	0.360, <0.001	-0.365, <0.001	-0.006, 0.927	0.174, 0.009[a]	-0.055, 0.412	0.108, 0.108	-0.194, 0.004[a]	-0.062, 0.358	-0.125, 0.062	0.012, 0.857	0.191, 0.004[a]
Formyl-N-acetyl-5-methoxykynurenamine	0.216, 0.001[a]	0.273, <0.001	0.076, 0.257	0.118, 0.080	0.292, <0.001	-0.301, <0.001	-0.095, 0.158	0.131, 0.051	-0.078, 0.248	0.004, 0.957	-0.066, 0.328	-0.120, 0.075	-0.005, 0.937	-0.066, 0.326	0.131, 0.052
L-Citrulline	0.289, <0.001	0.235, <0.001	-0.124, 0.066	0.208, <0.001	0.288, <0.001	-0.321, <0.001	0.119, 0.077	0.178, 0.008[a]	-0.114, 0.090	-0.070, 0.302	0.003, 0.968	-0.182, 0.007[a]	0.022, 0.750	-0.196, 0.003[a]	0.162, 0.016[a]
PA(24:4)	0.246, <0.001	0.332, <0.001	-0.170, 0.011[a]	0.140, 0.037[a]	0.347, <0.001	-0.367, <0.001	-0.020, 0.765	0.176, 0.008[a]	-0.058, 0.388	0.029, 0.668	-0.038, 0.569	-0.154, 0.022[a]	0.026, 0.697	-0.126, 0.060	0.241, <0.001
5-Hydroxyindoleacetate	0.195, 0.004[a]	0.229, 0.001[a]	-0.062, 0.356	0.222, 0.001[a]	0.257, <0.001	-0.271, <0.001	0.006, 0.930	0.215, 0.001[a]	-0.006, 0.925	0.092, 0.172	-0.019, 0.784	-0.061, 0.368	0.029, 0.668	-0.022, 0.740	0.296, <0.001
Indole-3-acetate	0.190, 0.005[a]	0.223, 0.001[a]	-0.086, 0.200	0.193, 0.004[a]	0.225, 0.001[a]	-0.239, <0.001	0.027, 0.694	0.211, 0.002[a]	-0.060, 0.372	-0.041, 0.547	-0.012, 0.860	-0.110, 0.101	-0.039, 0.565	-0.105, 0.118	0.273, <0.001
D-Galactose	0.003, 0.960	-0.061, 0.366	0.319, <0.001	-0.058, 0.391	-0.003, 0.959	-0.003, 0.965	-0.075, 0.267	-0.128, 0.056	-0.040, 0.553	0.037, 0.585	-0.151, 0.025[a]	-0.021, 0.758	-0.149, 0.027[a]	0.055, 0.415	0.099, 0.141

Note: DKD, group of diabetic kidney disease; NDKD, group of nondiabetic kidney disease; SDM, group of diabetes mellitus; LPA, lysophosphatidic acid; PA, phosphatidic acid; PG, phosphatidylglycerol; Ur, serum urea; Cr, serum creatinine; Glu, serum glucose; UA, serum uric acid; CysC, serum cystatin C; eGFR, estimated glomerular filtration rate; C1q, serum complement 1q; NGAL, serum neutrophil gelatinase-associated lipocalin; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; UACR, urinary albumin-to-creatinine ratio; a, $P<0.05$

among DKD, NDKD, and SDM also encompass nucleoside and amino acid metabolism. The findings of this study indicated that the concentration levels of 13 differential metabolites distinguishing the DKD and SDM groups were elevated, except for PG (28:4), which exhibited reduced levels. These differential metabolites demonstrated strong diagnostic capabilities in differentiating DKD from SDM, with all AUC values exceeding 0.7. LPA (16:3), LPA (18:5), LPA (22:5), PA (18:3), PG (26:4), and PG (28:4) were identified as intermediates within the glycerophospholipid metabolism pathway, while 5-Hydroxy-L-tryptophan, 2-Oxoadipate, and Formyl-N-acetyl-5-methoxykynurenamine were intermediates in the tryptophan metabolism pathway. Additionally, L-Glutamine, Uridine, Cytidine, and Thymidine were identified as intermediates within the pyrimidine metabolism pathway, whereas L-Citrulline was classified as an intermediate in arginine biosynthesis. The accumulation of these metabolites suggests an early onset of renal function impairment in DM patients [39–42, 48, 49]. Among these, LPA (16:3), LPA (18:5), LPA (22:5), PA (18:3), PG (26:4), 5-Hydroxy-L-tryptophan, and 2-Oxoadipate also exhibited high diagnostic performance in differentiating NDKD from SDM, with AUC values exceeding 0.7. PG (28:4), L-Glutamine, Uridine, Cytidine, Formyl-N-acetyl-5-methoxykynurenamine, Thymidine, and L-Citrulline may serve as potential specific kidney injury markers for DKD. Additionally, LPA (16:3), LPA (18:5), LPA (22:5), PA (18:3), PG (26:4), 5-Hydroxy-L-tryptophan, and 2-Oxoadipate may function as potential universal biomarkers for kidney injury. Furthermore, metabolites such as LPA (16:3), LPA (18:5), LPA (22:5), PA (18:3), PG (26:4), L-Glutamine, Uridine, Cytidine, 2-Oxoadipate, 5-Hydroxy-L-tryptophan, Thymidine, and L-Citrulline demonstrated positive correlations with renal function markers, including Ur, Cr, CysC, NGAL, and UACR, in DKD patients, while negative correlations were observed with eGFR. These findings suggest that differences in the concentrations of these metabolites between DKD and SDM patients are associated with alterations in renal function indicators, highlighting their potential role as predictive biomarkers for the progression of DM to DKD. However, further validation through studies with larger sample sizes and targeted metabolomics approaches is necessary to confirm whether these differential metabolites are directly implicated in the transition from DM to DKD.

In this study, two differential metabolites were identified between the DKD and NDKD groups. The levels of PG (14:0) (AUC = 0.740) and D-Maltose (AUC = 0.838) were significantly elevated in the DKD group compared to the NDKD group. PG (14:0) functions as an intermediate in the glycerophospholipid metabolism pathway, whereas D-Maltose serves as an intermediate in the starch and sucrose metabolism pathways. The increased concentrations of these metabolites may result from disruptions in glucose metabolism among DKD patients or stem from distinct mechanisms of kidney injury between DKD and NDKD. However, the precise underlying cause remains undetermined, as no conclusive evidence has been documented in the literature to date. Despite this, the differential expression of serum PG (14:0) and D-Maltose between the DKD and NDKD groups suggests that these metabolites may hold potential as biomarkers for differential diagnosis.

This study offers novel insights into the metabolic mechanisms associated with DKD. This study employed UPLC-MS/MS technology to conduct a comparative analysis with a control group comprising patients with NDKD and SDM as disease controls, alongside a HC population. The research not only conducted a preliminary screening of potential biomarkers for the diagnosis of DKD but also identified possible similarities and differences in metabolic substances between DKD and NDKD patients. Furthermore, these findings may offer objective evidence to aid in the differentiation of DKD from NDKD in preliminary clinical diagnoses. Nonetheless, its conclusions should be considered preliminary due to several limitations. Firstly, the use of a single-center sample introduces geographical constraints, and the small sample size may not adequately represent the broader population characteristics. Secondly, although untargeted UPLC-MS/MS technology allows for comprehensive screening of differential metabolites, the structural identification of certain metabolites relies on database matching, which may result in false positives or ambiguous annotations. Thirdly, the clinical applicability is limited by potential confounding factors: sex and age disparities between the DKD group and other cohorts may influence the results, the study does not distinguish between type 1 and type 2 diabetes, the cross-sectional design precludes the establishment of causal relationships between metabolite changes and disease progression, and the absence of dynamic follow-up data hinders the validation of their longitudinal predictive value. Furthermore, an additional critical limitation that warrants discussion is the fact that the metabolites screened are downstream molecules, thereby raising uncertainty regarding whether they are causative factors in the development of diabetic nephropathy or merely consequences of the condition. Consequently, it is crucial for future research to utilize large-scale, multicenter cohorts in conjunction with targeted metabolomics techniques to validate the direct role of these metabolites in disease pathogenesis and to evaluate their efficacy as independent diagnostic markers. Despite existing limitations, the identified associations between differential metabolites and clinical indicators highlight their potential utility, thereby laying

a foundation for further studies into the early detection and differential diagnosis of diabetic kidney disease (DKD) through metabolic profiling.

Conclusions

The findings of this study indicate that multiple metabolic pathways may undergo alterations during the transition from DM to DKD, with distinct metabolic abnormalities potentially corresponding to different etiologies of kidney injury. Given the results obtained, it is pertinent to consider whether the treatment approaches for these patients should be modified. This consideration warrants careful reflection by clinicians and researchers alike, with the aim of preventing individuals with DM from developing DKD or delaying the progression of the disease in those already affected. By employing UPLC-MS/MS technology, this study identified shared metabolic enrichment pathways between DKD and NDKD, including glycerophospholipid metabolism, glycerolipid metabolism, and tryptophan metabolism. The tryptophan metabolism pathway may function as a common metabolic route in the progression of various forms of CKD, whereas the pyrimidine metabolism and arginine biosynthesis pathways may be specifically implicated in the transition from DM to DKD. Metabolites such as L-Glutamine, Uridine, Cytidine, Thymidine, and L-Citrulline, which are associated with these pathways, showed significant differences between DKD and SDM patients and correlated with kidney function indicators, suggesting their potential as candidate biomarkers for predicting the progression from DM to DKD. Additionally, PG (14:0) and D-Maltose exhibit potential as biomarkers for distinguishing DKD from NDKD, underscoring their prospective utility as differential indicators for these conditions. However, are these findings representative or deterministic, given the limited sample size from a single center? Further research is necessary to validate these results through targeted metabolomics, which should encompass multiple centers and larger sample sizes.

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Author contributions
BZ and JFF designed and conceived the study. BZ and CMD were the major contributors in writing the manuscript. JYJ and JW received, investigated and analyzed patient data. BZ and YWY sorted out the data and performed the statistical analysis. BZ and CMD wrote the original draft. JFF reviewed and edited the final draft. All authors read and approved final version of manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All research procedures were conducted with approval of the Ethics Committee of Mianyang Central Hospital (P2020030). All patients and/or legal guardians signed the informed consent documentation prior to experiments.

Consent for publication
All authors contributed to the article and approved the submitted version.

Non-use of artificial intelligence
The authors affirm that no artificial intelligence (AI) tools or technologies were employed at any stage of this research or during the preparation of the manuscript.

Competing interests
The authors declare no competing interests.

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