Journal of Inflammation Research

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ORIGINAL RESEARCH

Metabolic Assessment in Non-Dialysis Patients with Chronic Kidney Disease

Hao Hong[1,*], Suya Zhou[2,*], Junyao Zheng[3], Haimin Shi[3], Yue Chen[4], Ming Li[3]

[1]Department of Intensive Care Unit, The First Affiliated Hospital of Soochow University, Soochow, Suzhou, People's Republic of China; [2]Laboratory Nephrology, Jinshan hospital of Fudan University, Shanghai, People's Republic of China; [3]Laboratory Nephrology, The First Affiliated Hospital of Soochow University, Soochow, Suzhou, People's Republic of China; [4]Laboratory Nephrology, The First People's Hospital of Kunshan, Soochow, Suzhou, People's Republic of China

*These authors contributed equally to this work

Correspondence: Ming Li, Email 1925924373@qq.com

Purpose: The aim of this study was to investigate the changes of different metabolites in the body fluids of non-dialysis patients with chronic kidney disease (CKD) using a metabolomics approach. The goal was to identify early biomarkers of CKD progression through metabolic pathway analysis.

Patients and Methods: Plasma samples from 47 patients with stages 1–4 CKD not requiring dialysis and 30 healthy controls were analyzed by liquid chromatography-mass spectrometry (LC-MS). Using multivariate data analysis, specifically a partially orthogonal least squares discriminant analysis model (OPLS-DA), we investigated metabolic differences between different stages of CKD. The sensitivity and specificity of the analysis were evaluated using the Area Under Curve (AUC) method. Furthermore, the metabolic pathways were analyzed using the Met PA database.

Results: Plasma samples from CKD patients and controls were successfully differentiated using an OPLS-DA model. Initially, twenty-five compounds were identified as potential plasma metabolic markers for distinguishing CKD patients from healthy controls. Among these, six compounds (ADMA, D-Ornithine, Kynurenine, Kynurenic acid, 5-Hydroxyindoleacetic acid, and Gluconic acid) were found to be associated with CKD progression It has been found to be associated with the progression of CKD. Changes in metabolic pathways associated with CKD progression include arginine and ornithine metabolism, tryptophan metabolism, and the pentose phosphate pathway.

Conclusion: By analyzing the metabolic pathways of different metabolites, we have identified the significant impact of CKD progression. The main metabolic pathways involved are Arginine and Ornithine metabolism, Tryptophan metabolism, and Pentose phosphate pathway. ADMA, D-Ornithine, L-Kynurenine, Kynurenic acid, 5-Hydroxyindoleacetic acid, and Gluconic acid could serve as potential early biomarkers for CKD progression. These findings have important implications for the early intervention and treatment of CKD, as well as for further research into the underlying mechanisms of its pathogenesis.

Keywords: arginine and ornithine metabolism, tryptophan metabolism, pentose phosphate pathway, biomarkers

Introduction

Chronic kidney disease (CKD) is a prevalent condition in the general population and a significant global public health concern. According to Hill et al, the estimated global prevalence of CKD ranges from 10.6% to 13.4%.[1] In the early stages of CKD, symptoms may not be obvious and can be easily overlooked. However, the progression of CKD towards end-stage renal disease (ESRD) is associated with high costs, mortality, and a significant decline in a patient's quality of life, particularly when dialysis treatment is introduced. The widely used plasma biochemical marker of glomerular filtration rate (GFR), plasma creatinine concentration (sCr), is often considered insensitive for detecting early stages of CKD. Hence, early recognition of CKD is crucial in order to slow down disease progression, reduce morbidity, and improve survival.

Metabolomics is a systematic analysis of metabolites in a biological specimen, focusing on the dynamic changes, interactions, and responses of metabolites in various metabolic pathways. It has become a commonly used approach in systems biology research, particularly for identifying new diagnostic and prognostic biomarkers for human diseases.[2,3]

Received: 26 January 2024
Accepted: 30 July 2024
Published: 17 August 2024

Liquid chromatography-tandem mass spectrometry (LC-MS) is a powerful tool that enables accurate identification and quantification of compounds due to its greater sensitivity and specificity. Currently, LC-MS is primarily utilized to uncover the complexity of plasma metabolome and provide various biomedical applications based on the obtained results. The primary objective of this study was to assess plasma metabolic status in non-dialysis patients with early CKD compared with controls using LC-MS.

Material and Methods

Study Design

This study included 47 patients with non-Dialysis CKD stage 1–4 (13, 11, 11, 12, respectively) and 30 healthy volunteers. CKD was diagnosed from patient medical records and CKD severity was assessed by estimated glomerular filtration rate (eGFR) calculated using the CKD-EPI formula.[4] Inclusion criteria: 1, Patients diagnosed with non-dialysis CKD for the first time and who have not undergone any drug treatment; 2, All patients were adult. The studied groups were matched based on certain exclusion criteria, such as secondary glomerulonephritis, severe heart failure, cardiac arrhythmias, liver cirrhosis, hematopathy, infectious diseases, vegetarianism, solitary kidney, professional sports involvement, pregnancy, lactation, menstruation, and mental illness. This study was approved by the Ethics Research Society of the First Affiliated Hospital of Soochow University (no. 079). All participants signed an informed consent form. Our study was complied with the Declaration of Helsinki.

Sampling and Sample Preparation

Plasma samples were collected according to the ethical protocol of the Ethics Research Society of the First Affiliated Hospital of Soochow University. Informed consent was obtained from all participants. The study was carried out according to the principles set forth by the World Medical Association in Helsinki. Blood samples were collected under fasting conditions and then centrifuged at 3000 rpm for 10 minutes. The supernatant was separated and stored at −80°C until assayed. Prior to analysis, all samples were thawed at 4°C and vortexed for 60 seconds after adding 800 μL of methanol (OKA, China). The supernatant was then centrifuged for 10 minutes at 12,000 rpm at 4°C. The prepared sample was dissolved in 300L methanol aqueous solution (4:1, 4°C), filtered through a 0.22m filter membrane, and then used for LC-MS analysis. Quality control (QC) samples were prepared to evaluate the bias in the results of the analysis of the cell mixture caused by the analyzer itself.

LC-MS Conditions

Chromatographic separations were performed on a Thermo Ultimate 3000 system with an ACQUITY UPLC®HSS T3 column (150 x 2.1 mm, 1.8 μm, Waters) maintained at 40 °C. The autosampler temperature was 8 °C. Gradient elution was performed at a flow rate of 0.25 mL/min using 0.1% formic acid in water (Merck, Germany) (A) and 0.1% formic acid in acetonitrile (Thermo, USA) (B) or 5 mM ammonium formate in water (Sigma, Germany) (C) and acetonitrile (D). After equilibration, inject 2 μL of each sample. Solvent B (v/v) linear gradient 0–1min, 2% B/D; 1–9 minutes, 2–50% B/T; 9–14 minutes, 50–98% B/D; 14–15 minutes, 98% B/D; 15–15.5 minutes, 98–2% B/D; 15.5–17 minutes, 2% B/D.

ESI-MSn experiments were performed using a fine-focus Thermo Q mass spectrometer. The spray voltages for positive and negative modes were set to 3.8 kV and −2.5 kV, respectively. The sheath gas and auxiliary gas were set to 45 and 15 arbitrary units, respectively. The capillary temperature was maintained at 325 °C. The Orbitrap analyzer scans the mass range from m/z 81 to 1000 with a resolution of 70,000. Correlative Acquisition (DDA) mass spectrometry experiments were performed by HCD scanning. The normalized collision energy is 30 eV. Redundant spectral information is removed by dynamic subtraction.

Data Preprocessing and Statistical Comparison

Data sets for LC-MS analysis were converted using Proteowizard (v3.0.8789). R (v3.3.2) xcms was used for peak identification, filtering and alignment. Batch normalization was employed to compare orders of magnitude. Subsequently, multivariate statistical analysis was conducted using R (ropls). Principal component analysis (PCA) was used to assess data quality, while orthogonal projections to latent structures discriminant analysis (OPLS-DA) was used to observe differences between groups studied. Both one-way ANOVA p-value ($P < 0.01$) were applied.

Identification of Metabolites and Signaling Pathways

Identification of statistically significant metabolites in positive and negative ionization modes was performed through LC-MS analyses using databases such as HMDB, Metlin, Massbank, LipidMaps, and Mzcloud. MetPA was then applied to identify possible disturbed metabolic pathways.

Statistical Analysis of Clinical Data

For nonnormal variables, the median and interquartile range were given, while for normal variables, the mean and standard deviation were given. Subnormal variables were log-transformed and tested for normality. Normally distributed continuous variables were analyzed using one-way ANOVA with Fisher's least significant test, and skewed continuous variables were analyzed using the Kruskal–Wallis test. Categorical variables were analyzed using the chi-squared test. Data analysis was performed using SPSS software, version 22.0. $P < 0.05$ was considered significant.

Results

Clinical and Biochemical Parameters

Clinical and biochemical parameters of recruited subjects were summarized in Table 1. BMI, Creatinine, blood urea nitrogen (BUN) and total cholesterol (TC) were transformed as logarithmic data to normalize the data allowing the use of parametric tests.

Plasma Metabolomics and Analytical Reproducibility Testing

The chromatogram of the plasma peak measured by LC-MS is shown in Figure 1. The OPLS-DA model was created using filtered and normalized data sets obtained from LC-MS analytical measurements (Figure 2). Check the stability of the analytical system, the reproducibility of the procedure and the test method using a QC sample of the PCA plot (Figure 2).

Statistical Comparison and Metabolic Changes Associated with CKD Patients and Controls

Among the variables measured by LC-MS positive and negative ionization modes, 147 metabolites were significantly distributed in CKD patients with stage 1 to stage 4 and controls according to one-way ANOVA ($P < 0.05$),

Table 1 Clinical Characteristics of Control Group and 47 Non-Dialysis Patients with Stage 1 to Stage 4

	Control Group (n=30)	Stage 1 (n=13)	Stage 2 (n=11)	Stage 3 (n=11)	Stage 4 (n=12)	*P*
Age (years)	48.1±12.1	47.4±16.4	51.0±21.3	55.8±16.7	58.4±13.9	0.232
Sex	10M,20F	5M,8F	7M,4F	7M,4F	7M,5F	0.223
BMI (kg/m^2) *	1.37±0.07	1.38±0.06	1.36±0.58	1.40±0.04	1.38±0.05	0.755
Creatinine (umol/L)*	1.80±0.08	1.74±0.10	1.97±0.10	2.14±0.09	2.37±0.07	0.000
Albumin (g/L)	48(46,49)	36(29,41)	40(37,43)	43(39,44)	41(38,43)	0.000
BUN (mmol/L) *	0.67±0.10	0.66±0.14	0.79±0.12	0.95±0.12	1.09±0.16	0.000
TC (mmol/L)	4.5(3.9,5.2)	5.2(4.1,5.6)	4.7(4.5,5.5)	4.5(4.2,5.1)	4.5(3.8,5.2)	0.419
TG (mmol/) *	0.12±0.23	0.20±0.29	0.16±0.17	0.20±0.11	0.24±0.19	0.493
Glucose (mmol/L)	4.9(4.6,5.2)	4.6(4.5,4.8)	4.6(4.1,4.9)	4.7(4.1,4.8)	4.6(4.3,5.0)	0.086
HB (g/L)	140.0±11.4	128.5±17.7	126.6±11.4	121.9±12.8	111.3±17.9	0.000
UA (umol/L)	344.5±91.0	379.8±122.7	430.3±59.8	431.2±129.7	420.4±138.4	0.060
Blood phosphorus (mmol/L)	1.15±0.23	1.58±0.25	1.83±0.18	2.03±0.15	2.32±0.43	0.000
NLR	1.75±0.61	2.13±0.63	2.04±0.55	2.59±1.07	2.74±1.64	0.023

Notes: *Logarithmic data transformation, Values were given as mean standard deviation, median (interquartile range).
Abbreviations: BUN, blood urea nitrogen; TC, total cholesterol; TG, triglyceride; HB, hemoglobin; UA, Uric acid; NLR, Neutrophil-to-Lymphocyte Ratio; M, male; F, female.

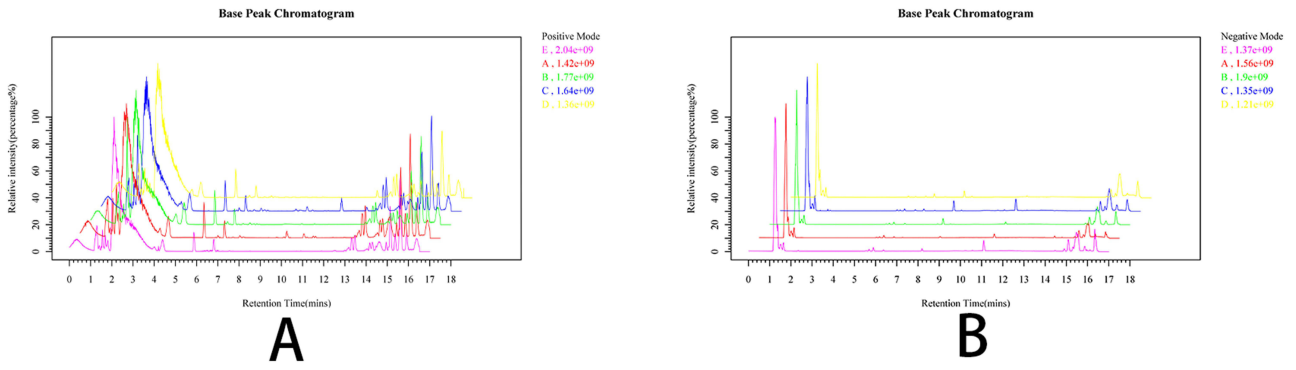

Figure 1 Base peak chromatogram of CKD patients and control group obtained with LC-MS positive (**A**), negative (**B**)ionization mode.
Notes: E control group; A-D: CKD stage 1 to stage 4.

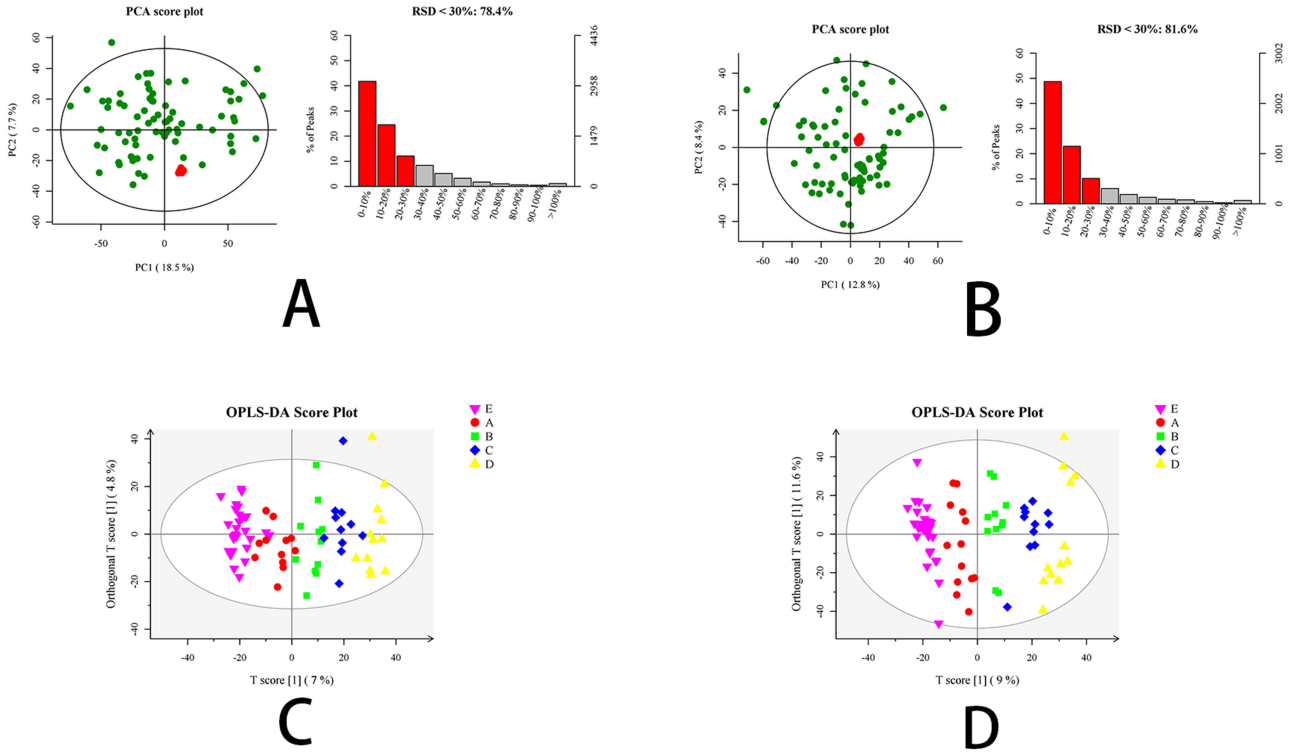

Figure 2 PCA models built on QC data obtained with LC-MS analysis in (**A**) positive ionization mode, (**B**) negative ionization mode; OPLS-DA models built on plasma data obtained with LC-MS analysis in (**C**) positive ionization mode, (**D**) negative ionization mode, respectively.

78 metabolites according to VIP value > 1 and 67 metabolites were selected according to AUC value > 0.7 (Figure 3).

25 metabolites were selected after sifting chemicals and unmatched metabolites in KEGG pathways.6 metabolites showed a continuous upward trend period (Figure 4). The identified statistically significant metabolites and the differences between groups were shown in Table 2. The AUC values of 6 metabolites between control group and CKD patients were > 0.7 (P <0.01) (Figure 5).

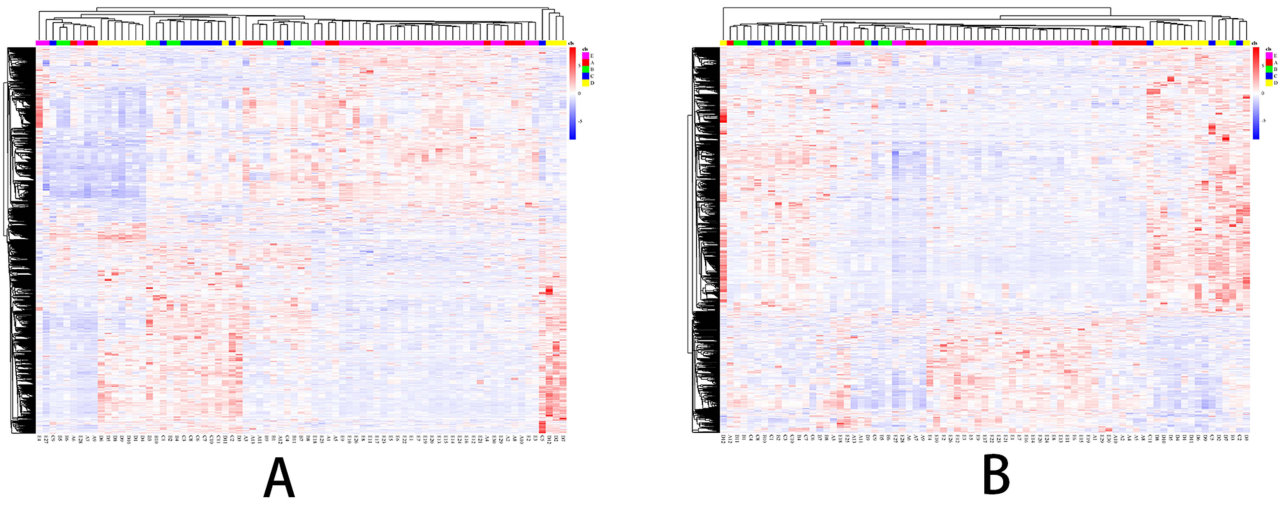

Figure 3 Heatmap of discriminating metabolites between CKD patients with stage 1 to stage 4 and controls with positive (**A**), negative (**B**) ionization mode.
Notes: E control group; A-D: CKD stage.

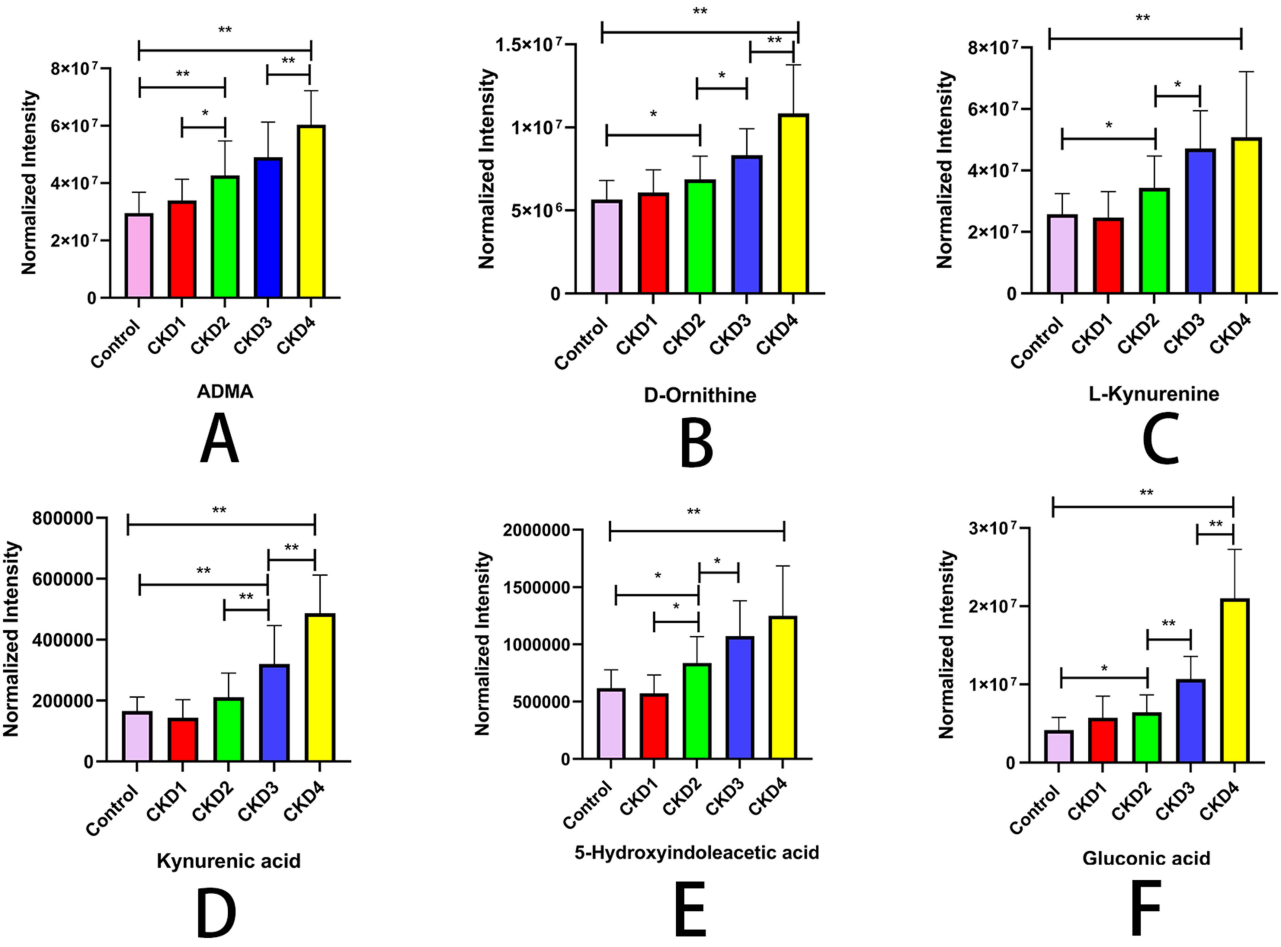

Figure 4 Differences in normalized intensity of selected metabolites between 6 compared groups.
Notes: Based on One-way ANOVA and the Fisher least significant difference test. (**A**) ADMA, (**B**) D-Ornithine, (**C**) L-Kynurenine, (**D**) Kynurenic acid, (**E**) 5-Hydroxyindoleacetic acid, (**F**) Gluconic acid. 1–4, CKD stage 1 to stage 4; *: P <0.05, **: P <0.01.

Table 2 Statistical Analysis of 6 Main Discriminating Metabolites

Metabolites	Exact Mass	Mass Error [ppm]	RT [min]	Formula	Biochemical Pathway
ADMA	202.25428	2	100.86100	C8H18N4O2	Arginine metabolism
D-Ornithine	132.16106	8	94.37390	C5H12N2O2	Arginine and Ornithine metabolism
L-Kynurenine	208.21390	7	348.71000	C10H12N2O3	Tryptophan metabolism
Kynurenic acid	189.16750	2	368.44100	C10H7NO3	Tryptophan metabolism
5-Hydroxyindoleacetic acid	190.1834	4	331.46300	C10H9NO3	Tryptophan metabolism
Gluconic acid	196.15528	3	86.67005	C6H12O7	Pentose phosphate pathway

Metabolic Pathway

The main metabolic pathways were illustrated in Figure 6. Notably, the arginine and proline metabolism, tryptophan metabolism, and pentose phosphate pathway were identified as the most significant pathways, which were enhanced in the early stages and the progression of CKD.

Correlation Between 6 Discriminating Metabolites and Various Clinical Parameters

The correlation analysis between the six metabolites and CKD-related clinical parameters revealed significant associations (P <0.05) (Figure 7). These metabolites were mainly related to inflammation and oxidative stress in CKD.

Discussion

In this study, we employed an untargeted metabolomic approach to identify biomarkers for patients with non-dialysis CKD. We observed distinct differences in plasma metabolic profiles between patients with non-dialysis CKD and healthy controls. Notably, ADMA, D-Ornithine, L-Kynurenine, Kynurenic acid, 5-Hydroxyindoleacetic acid, and Gluconic acid exhibited significant changes.

One advantage of our study was the prospective inclusion of patients with non-dialysis CKD. This approach reduced the metabolic deviation caused by dialysis and allowed for the exploration of the original metabolic changes in CKD patients at an early stage. This provides valuable insights for the early diagnosis, prevention, and progression of CKD.

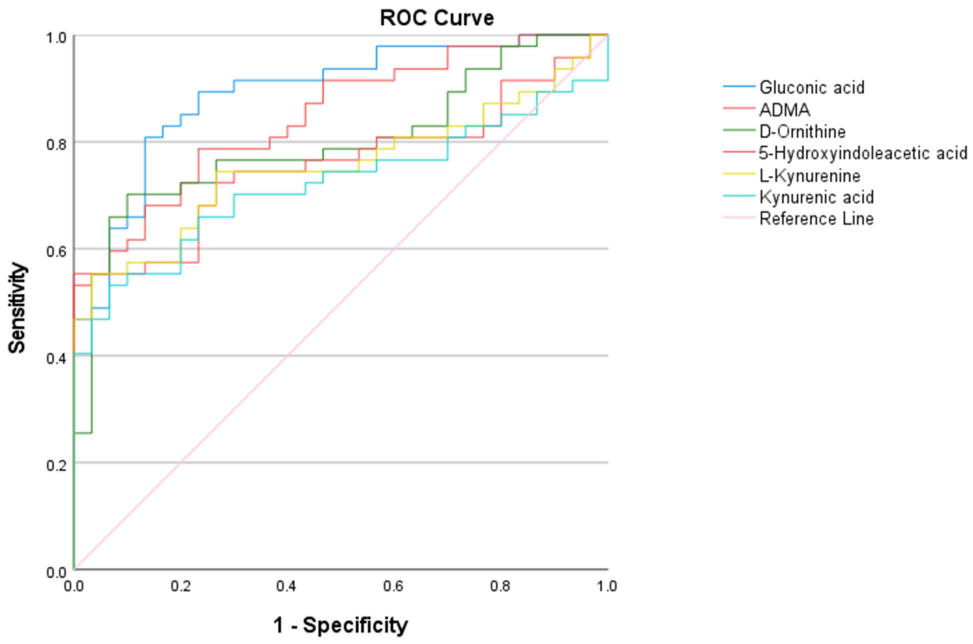

Figure 5 ROC curve of 6 metabolites between 6 between control group and CKD patients.

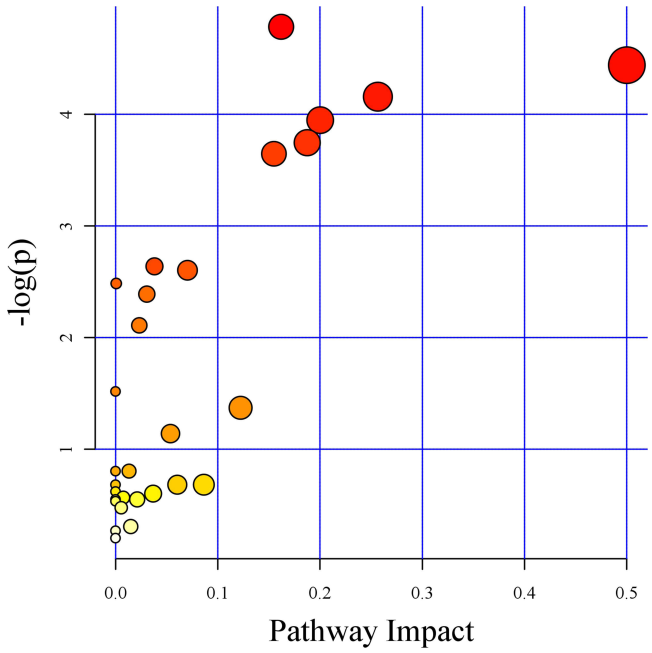

Figure 6 Pathway identification between CKD patients and controls.

Our OPLS-DA analysis revealed a significant difference between patients with non-dialysis CKD and healthy controls, highlighting the presence of a distinct metabolic pattern in non-dialysis CKD patients.

ADMA is an endogenous endothelial nitric oxide synthase inhibitor derived from arginine residues and catalyzed by protein arginine methyltransferase. Approximately 80% of ADMA is degraded via dimethylarginine dimethylaminohydrolase (DDAH), while the remaining ADMA is excreted by the kidney.[5] Fliser et al reported an association between plasma ADMA and early-stage CKD patients, while Ravani et al identified plasma ADMA as an independent risk factor for patients with end-stage renal disease.[6,7] Our results support these findings and suggest that this could be attributed to decreased renal excretion or reduced DDAH activity in CKD. ADMA reduces the level of nitric oxide (NO) by decreasing the activity of endothelial nitric oxide synthase (NOS), leading to impaired vascular tone and endothelial dysfunction. This could result in decreased renal perfusion and increased cardiovascular disease (CVD) events, which may further complicate CKD.[8] Some studies have indicated that drugs such as EPO, vitamin E, statins, and ACE inhibitors can potentially interfere with the level of ADMA. However, these studies have not been able to explain whether the benefit observed in CKD patients is due to the reduction in ADMA or other aspects of these drugs.[9–11] Further research is needed to elucidate the underlying mechanism. Age plays an important role in vascular aging. An imbalance between oxidative and antioxidant systems leads to increased reactive oxygen species production, NO inactivation, increased nitrosative stress, and ultimately age-related endothelial dysfunction.[12] Our findings demonstrate a positive correlation between ADMA and age ($r=0.471$, $P<0.01$). Accumulation of ADMA with age leads to endothelial damage in CKD patients. The Neutrophil-to-Lymphocyte Ratio (NLR) is currently recognized as a biomarker for inflammation and atherosclerosis progression.[13,14] The simultaneous increase of ADMA and NLR may be associated with inflammatory activity.[15] In this study, ADMA showed a positive correlation with NLR ($r=0.274$, $P<0.05$). Similar findings were reported by Ibrahim et al, who found that NLR was significantly different between ESRD hemodialysis patients and controls, and NLR was positively correlated with ADMA.[16] These results suggest that ADMA could potentially serve as a predictive marker for the early occurrence, progression, and end-stage of CKD. Further investigation into the role of ADMA in inflammation and endothelial injury is crucial for the diagnosis and treatment of CKD. In addition, it may be an interesting clinical intervention to explore the potential impact of relatively safe drugs, such as vitamin C consumption at different ages, on ADMA levels to potentially improve outcomes for patients with CKD.

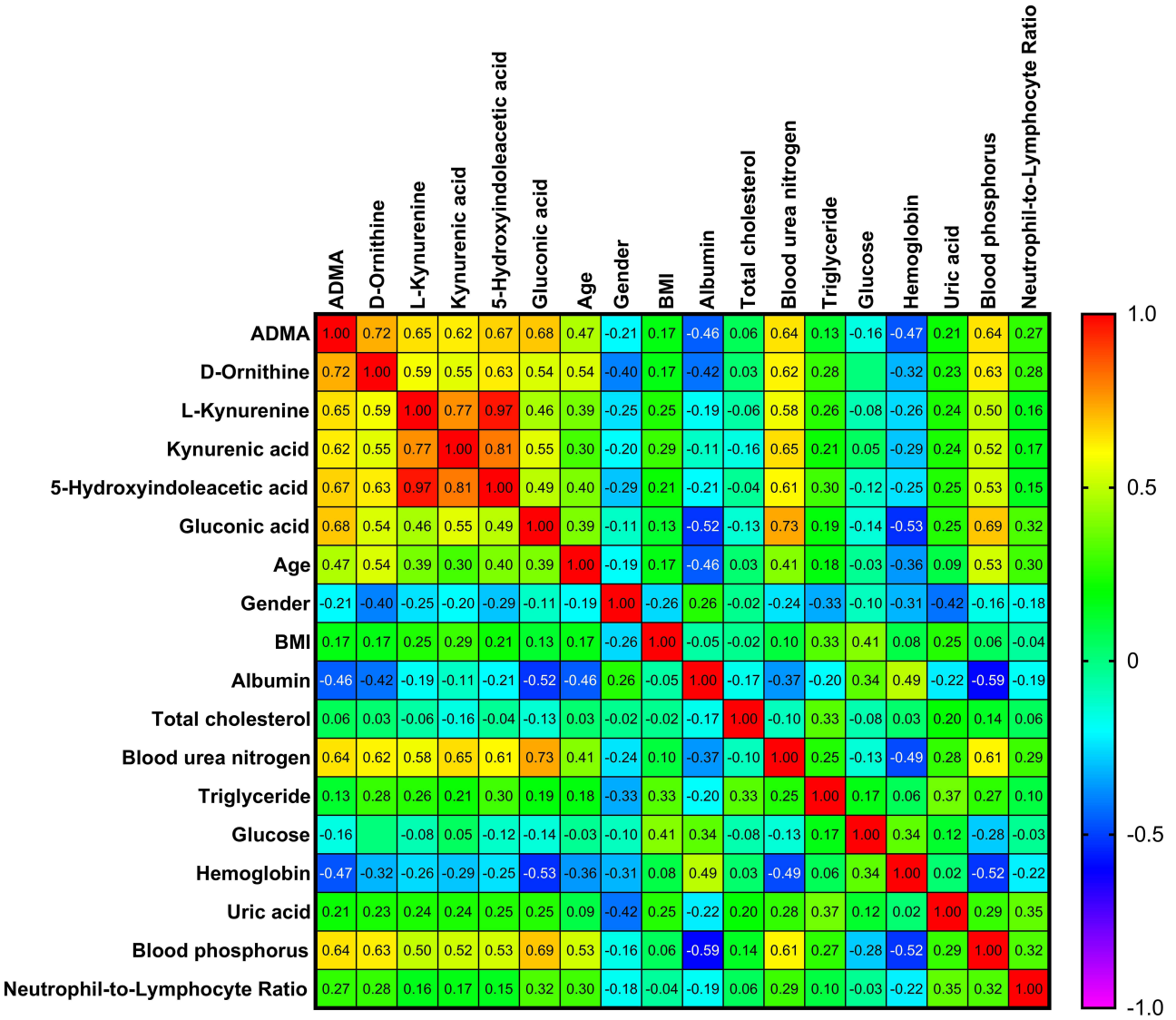

Figure 7 Heatmap of correlation analysis between 6 discriminating metabolites and various clinical parameters.

D-Ornithine, an intermediate in the urea cycle, has been identified as a significant factor in hyperphosphatemia. This condition leads to increased expression of arginase 2, resulting in elevated levels of D-Ornithine.[17] Several studies have reported the negative effects of elevated D-Ornithine levels in hyperphosphatemia on endothelial cells, including reduced production of nitric oxide and disruption of mitochondrial function.[18,19] The concentration of blood phosphorus gradually increases with the progression of chronic kidney disease (CKD) (P <0.01) and shows a positive correlation with D-Ornithine (r=0.62, P <0.01). We speculate that the increase in blood phosphorus concentration in CKD patients may enhance the activity of arginase 2, leading to an elevation in ornithine concentration. However, we have not yet measured the activity of arginase 2 or other potential factors that may interfere with the enzyme. The relationship between phosphorus-arginase 2 and D-ornithine still requires further elaboration. This is of great significance in understanding the mechanism of ornithine as a potential therapeutic target for treating hyperphosphatemic-related cardiovascular events andCKD. The mechanism of cardiorenal syndrome in CKD patients is not yet fully understood. Given the role of D-Ornithine in both the kidneys and heart, investigating the impact of interfering with D-Ornithine on the development of cardiorenal syndrome in CKD patients could be an interesting research topic.

Statistically significant differences were observed in tryptophan metabolism between CKD patients and the control group. Tryptophan, an essential amino acid in humans, is primarily metabolized through two pathways: the kynurenine

pathway, which produces L-Kynurenine and Kynurenic acid, and the serotonin pathway, which produces 5-Hydroxyindoleacetic acid. The kidney plays a crucial role in the metabolism and excretion of tryptophan. L-Kynurenine and Kynurenic acid act as endogenous agonists of the aromatic compound receptor (AHR), which is considered to be a significant factor in inducing immune response tolerance and promoting the reprogramming of Th17 cells into regulatory T cells.[20,21] L-Kynurenine has the ability to interact with AHR in dendritic cells, leading to an upregulation in the expression of Indoleamine 2.3-dioxygenase. This, in turn, results in the further decomposition of tryptophan and the production of L-Kynurenine.[22] In an inflammatory environment, the L-Kynurenine and AHR pathway exhibit protective effects.[23] Increased levels of L-Kynurenine and Kynurenic acid have been observed in various complications of CKD, such as thrombosis, oxidative stress, mesangial cell proliferation, CKD-MBD, and dyslipidemia.[24–29] These findings suggest a potential association between inflammation, immune disorders, and CKD. In this study, we found that the levels of L-Kynurenine, Kynurenic acid, and 5-Hydroxyindoleacetic acid increased with the progression of CKD (P <0.05). Additionally, L-Kynurenine and 5-Hydroxyindoleacetic acid showed a positive correlation with triglycerides (r=0.259, P <0.05, r=0.301, P <0.05). Furthermore, the key rate-limiting enzyme of the kynurenine pathway, Indoleamine 2.3-dioxygenase, was found to be overexpressed in CKD.[30] Previous studies by Sekula P et al have demonstrated that Indoleamine 2.3-dioxygenase serves as a reliable indicator of impaired renal function.[31] Our previous study has identified the alterations in Indoleamine 2.3-dioxygenase levels among CKD patients.[32,33] However, further investigation is required to explore the impact of these changes on the tryptophan metabolic pathway and its role in the inflammatory and immune regulation mechanisms of CKD. Moreover, Clinicians have recognized the significance of depressive symptoms in certain CKD patients. Exploring the potential impact of modulating the serotonin metabolism pathway of tryptophan on the depressive state of CKD patients could be an intriguing topic.

Gluconic acid is enzymatically formed from glucose by the activity of glucose oxidase, which releases hydrogen peroxide. Previous research has shown an association between gluconate and decreased kidney function in patients with end-stage chronic kidney disease.[34] In a rat nephrotoxicity model, gluconic acid levels changed in a dose- and time-dependent manner, suggesting that they may be related to the severity of renal injury.[35] Additionally, gluconic acid has been found to be correlated with early Diabetic retinopathy and type 2 diabetes.[36,37] Uric acid, on the other hand, is the end product of purine metabolism in the body. It has extracellular antioxidant effects and has been implicated in oxidative stress.[38,39] Our study revealed a significant increase in gluconic acid levels in stage 2 of CKD (P <0.05), indicating its association with disease progression. Furthermore, we observed a positive correlation between gluconic acid and uric acid (r=0.251, P <0.05). Interestingly, our findings also showed an enhancement of the pentose phosphate pathway, which is primarily involved in gluconic acid metabolism. This pathway is considered to have potential protective effects, as its metabolite NADPH can effectively reduce reactive oxygen species levels, thereby counteracting oxidative stress in the kidneys.[40] Weng et al demonstrated that increased NADPH production through the pentose phosphate pathway can alleviate oxidative stress in renal ischemia-reperfusion injury.[41] These findings suggest that oxidative stress occurs in early CKD stages, triggering a protective mechanism. Metabolic pathways were analyzed using LC-MS, revealing changes in arginine and ornithine metabolism, tryptophan metabolism, and the pentose phosphate pathway. Metabolites identified in these pathways included ADMA, D-ornithine, L-kynurenine, kynurenic acid, 5-hydroxyindoleacetic acid, and Gluconic acid. These findings have potential implications for the early detection of CKD in patients. Furthermore, exploring interventions involving common clinical drugs targeting these metabolic pathways and metabolites may hold promise for the treatment of CKD.

Acknowledgments

We would like to thank Boxi Youth Natural Science Foundation.

Disclosure

All authors report no conflicts of interest in this work.

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