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ARTICLE

Profiling Exosomal Metabolomics as a Means for Diagnosis and Researching Early-Stage Hypertensive Nephropathy

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Abstract

Aims/Background Hypertension (HT) is a prevalent medical condition showing an increasing incidence rate in various populations over recent years. Long-term hypertension increases the risk of the occurrence of hypertensive nephropathy (HTN), which is also a health-threatening disorder. Given that very little is known about the pathogenesis of HTN, this study was designed to identify disease biomarkers, which enable early diagnosis of the disease, through the utilization of high-throughput untargeted metabolomics strategies.

Methods The participants of this study were patients admitted to The Second Affiliated Hospital of Chengdu Medical College, Nuclear Industry 416 Hospital, who were randomly divided into three groups: Normal group (n = 11), HT group (n = 10), and HTN group (n = 12). Urine exosomes were extracted, purified, and subjected to untargeted metabolomics analysis. Differential metabolites and their significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified. The least absolute shrinkage and selection operator (LASSO) regression analysis was then employed to establish a diagnostic model for early-stage HTN. Finally, logistic regression and receiver operating characteristic (ROC) curve analysis were performed to identify biomarkers related to early HTN.

Results Orthogonal partial least squares-discriminant analysis (OPLS-DA) revealed significant differences in the metabolic profiles of the three patient groups. Compared to subjects of the Normal group, the HT and HTN groups exhibited significantly upregulated and downregulated profiles of differential metabolites, respectively. LASSO regression analysis results indicated that 4-hydroxyphenylacetic acid, bilirubin, uracil, and iminodiacetic acid are potential biomarkers for HTN or HT.

Conclusion With untargeted metabolomics analysis, we successfully identified differential metabolites in HTN. A further LASSO regression analysis revealed that four key metabolites, namely 4-hydroxyphenylacetic acid, bilirubin, uracil, and iminodiacetic acid, hold promise for the diagnosis of early-stage HTN.

Key words: hypertension; hypertensive nephropathy; exosomes; metabolomics analysis; biomarkers

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Introduction

Hypertension (HT) is a condition characterized by persistently high blood pressure in the arteries, which can cause damage to vital organs such as the brain, heart, and kidneys (Kućmierz et al, 2021). Hypertensive nephropathy (HTN) is defined as a change in the structure and function of the kidneys caused by poorly controlled

long-term hypertension (Duan et al, 2023). In its early stages, HTN may be asymptomatic, but its progression is accompanied by manifestations such as microalbuminuria and elevated serum creatinine, as well as increased risk for end-stage renal disease, cardiovascular events, and sudden death, posing a significant health threat to patients (Schmieder et al, 1995). Thus, prompt detection and intervention are vital for halting the progression of HTN and preventing the development of its complications. Currently, the diagnosis of HTN primarily relies on the assessment of blood pressure, serum creatinine levels, and proteinuria (O'Neal et al, 2016). However, these indicators often lack sensitivity and specificity for detecting the early stages of the disease (Hayashida et al, 2022). At present, the absence of sensitive and specific biomarkers for early-stage HTN hampers the early diagnosis efforts, thus delaying treatment and impacting patient outcomes.

Exosomes are cell-secreted nano-sized vesicles containing proteins, lipids, microRNAs, and DNA (Zou et al, 2023). Protected by phospholipid bilayer membrane, exosomes are released into the extracellular environment and play regulatory roles in cell signalling and immune responses (Simons and Raposo, 2009). Cambier et al (2018) demonstrated in an Angiotensin (Ang) II-induced hypertensive kidney injury mouse model that hypoxia stimulated the secretion of exosomes enriched with Y RNA fragments from cardiomyocyte-derived cells, which reduced the levels of urinary protein, macrophage marker protein cluster of differentiation68 (CD68), interleukin-6, and other pro-inflammatory factors, thus alleviating kidney inflammation and tissue fibrosis. Therefore, exosomes may have played a protective role in the progression of HTN.

Metabolomics, the comprehensive study of small-molecule metabolites, provides a powerful tool to characterize metabolite profiles associated with disease processes and identify potential biomarkers (Piirsalu et al, 2022; Qiu and Zhang, 2019). Existing research suggests that metabolomics could be a new approach for early HTN diagnosis (Piirsalu et al, 2022), but research on using exosomal metabolites from urine for diagnosing early-stage HTN and creating corresponding models remains scanty. Thus, this study aimed to fill this knowledge gap by investigating the diagnostic potential of urinary exosomal metabolites for early-stage HTN, identifying specific metabolic patterns, establishing diagnostic models, and providing new tools for early-stage HTN diagnosis.

This study employed techniques such as exosome isolation, untargeted metabolomics analysis, and machine learning techniques to identify specific metabolic patterns associated with HTN. We hypothesize that unique metabolic signatures in urinary exosomes to distinguish early-stage HTN patients from those with HT and healthy controls. This research contributes to the evolving field of exosome-based diagnostics, exploring its potential in early HTN detection. Identifying specific metabolite biomarkers and developing reliable diagnostic models could revolutionize early-stage HTN diagnosis, enabling timely intervention and potentially improving patient outcomes.

Methods

Sample Collection

Patients who were admitted to The Second Affiliated Hospital of Chengdu Medical College, Nuclear Industry 416 Hospital from February 2023 to December 2023 were recruited in this study and divided into three groups: hypertensive group (n = 10), hypertensive nephropathy group (n = 12), and Normal group (n = 11). The control group consisted of healthy individuals who underwent physical examination in The Second Affiliated Hospital of Chengdu Medical College, Nuclear Industry 416 Hospital during the same period. Informed consent was obtained from all subjects enrolled in this study. This study was conducted strictly according to the Declaration of Helsinki (revised in 2013) and was approved by the Medical Ethics Committee of The Second Affiliated Hospital of Chengdu Medical College, Nuclear Industry 416 Hospital (Approval No.: YJ-2024-014).

Urine samples of HT, HTN, and Normal groups, collected from February 2024 to June 2024, were used as experimental samples in our study. Urinary exosomes in each group were extracted and purified using Invitrogen™ Total Exosome Isolation Reagent (Item No.: 4484452, Thermo, Beijing, China) for untargeted metabolomics analysis. The specific steps are as follows: After thawing the samples slowly at 4 °C, an appropriate amount of sample was added to a pre-cooled methanol-acetonitrile-water solution (2:2:1, v/v/v) and vortexed. The mixture was then sonicated at a low temperature and incubated at –20 °C. The samples were then centrifuged at 14,000 × *g* for 20 min at 4 °C. The supernatant was collected and dried under vacuum. For mass spectrometry analysis, 100 μL of acetonitrile-water solution (1:1, v/v) was added to resuspend the samples, followed by vortexing and centrifugation at 14,000 × *g* for 15 min at 4 °C. The collected supernatant was used for subsequent analysis and stored at –80 °C.

Inclusion and Exclusion Criteria

The criteria for including patients for the HT group are as follows (Whelton et al, 2018): (1) patients who were diagnosed with HT, as per the latest international and domestic guidelines, after multiple repeated measurements, with the systolic blood pressure ≥140 mmHg or the diastolic blood pressure ≥90 mmHg (1 mmHg = 0.133 kPa); (2) patients who had been screened for secondary HT after hospitalization; (3) patients older than 18 and younger than 70 years old; and (4) patients with complete clinical data. Individuals with the following conditions were excluded from the HT group: (1) HT caused by other diseases (endocrine diseases such as hyperaldosteronism, chronic kidney disease, renal artery stenosis, etc.); (2) heart, brain, kidney and other complications caused by long-term HT; (3) body mass index (BMI) <18.5 or >30; (4) pregnancy or in lactation; (5) serious primary conditions impacting survival, such as malignant tumours, blood system disorders, immune system diseases, and immunodeficiencies; and (6) mental illness.

The inclusion criteria for the HTN group are as follows (Beck et al, 2023): (1) similar to the inclusion criteria (1) to (4) for the HT group; (2) patients with urinary albumin/creatinine >30 mg/g or estimated glomerular filtration rate (eGFR) <60

mL/(min·1.73 m^2) for 3 months, as per the guidelines for chronic kidney disease (CKD); (3) patients with HTN diagnosed via renal biopsy; and (4) patients with the history of HT was much longer than that of CKD. Patients with the following conditions were excluded from the HTN group: (1) kidney disease caused by other diseases such as diabetes, gout, rheumatic immune system diseases, and cardiac insufficiency; and (2) similar to the exclusion criteria (3) to (6) for the HT group.

Inclusion criteria for the Normal group are as follows: (1) individuals no history of HT, as defined by the systolic blood pressure in the range of 90 to 139 mmHg and the diastolic blood pressure in the range of 60 to 89 mmHg, according to the international standards for normal blood pressure; (2) individuals with no diseases that affect kidney function, such as diabetes and chronic kidney disease; and (3) individuals older than 18 and younger than 70 years old. Individuals with the conditions were excluded from the Normal group: (1) high blood pressure or other conditions related to hypertension; (2) diseases that affect kidney function, such as diabetes and chronic kidney disease; and (3) similar to the exclusion criteria (3) to (6) for the HT group.

UHPLC-Q-Exactive Orbitrap MS Analysis

Urine exosome samples from each group were separated using hydrophilic interaction liquid chromatography (HILIC) column based on Vanquish Ultra High Performance Liquid Chromatography (UHPLC) system (Thermo Fisher Scientific, Frankfurt, Hesse, Germany). The column temperature was set at 25 °C, with a flow rate of 0.3 mL/min and an injection volume of 2 µL. The mobile phase used consisted of two components: phase A was water with 25 mM ammonium acetate and 25 mM aqueous ammonia, and phase B was acetonitrile. The gradient elution procedure was as follows: from 0–1.5 min, phase B was maintained at 98%; from 1.5–12 min, phase B decreased linearly from 98% to 2%; from 12–14 min, phase B was maintained at 2%; from 14–14.1 min, phase B increased linearly from 2% to 98%; and from 14.1–17 min, phase B was maintained at 98%. The samples were kept in an autosampler set at 4 °C. To minimize the effects of instrument signal fluctuations, the samples were analyzed in a continuous and randomized order. To monitor system stability and ensure data reliability, quality-control samples were sporadically tested in the midst of testing the experimental samples.

Samples were analyzed using a Q Exactive series mass spectrometer (Thermo Fisher Scientific, Frankfurt, Hesse, Germany) for both the first and second stages of mass spectrometry, employing both positive and negative electrospray ionization modes for detection. The ion source was an electrospray ionization source with a curtain gas of 30 psi, employing a temperature of 600 °C and a spray voltage (Ion Spray Voltage Floating) of ±5500 V for both positive and negative ionization modes. The mass-to-charge ratio range for the first stage of mass spectrometry was 80–1200 Da, with a resolution of 60,000 and a scan accumulation time of 100 ms. Sequential acquisition was conducted during the second stage of mass spectrometry, employing a mass-to-charge ratio range of 70–1200 Da, a resolution of 30,000, a scan accumulation time of 50 ms, and a dynamic exclusion time of 4 s.

Raw data was transformed into. mzXML format via ProteoWizard Version 3.0 software (ProteoWizard Software Foundation, Los Angeles, CA, USA). Peak alignment, retention time correction, and peak area extraction were then performed via XCMS Version 3.7.1 software (Scripps Research Institute, La Jolla, CA, USA). The data downloaded from XCMS underwent initial processing for metabolite structure identification and pre-processing, after which a quality assessment of the experimental data was conducted.

Univariate Statistical Analysis

Univariate analysis was conducted on all detected metabolites, including those that were unidentified, under positive and negative ionization modes. The fold change of differential metabolites was visualized using a volcano plot.

Multivariate Statistical Analysis

Orthogonal partial least squares-discriminant analysis (OPLS-DA) was used for the multivariate statistical analysis. It helps in maximizing the variance that is related to class separation while minimizing the orthogonal (unrelated) variation. Model prediction parameters, including R^2Y (a metric evaluating model applicability) and Q^2 (model prediction capability), were used to assess the quality of OPLS-DA model. Values closer to 1 for both parameters indicate better model performance. To avoid overfitting during model construction, permutation testing was employed to verify the model validity.

Screening and Pathway Analysis of Differential Metabolites

The variable importance in projection (VIP) values, generated by OPLS-DA model, was used to assess the impact and explanatory power of each metabolite's expression pattern on the classification and discrimination of sample groups, helping to identify metabolites with biological significance. Typically, metabolites with VIP >1 were considered to have a significant contribution to the model interpretation. In this study, metabolites with a VIP value >1, a p-value < 0.05, and a fold change (FC) >1.5 or FC <0.67 were considered significantly different. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was conducted on the screened differential metabolites, with the results displayed in a bubble chart.

Constructing LASSO Model to Screen Biomarkers

The least absolute shrinkage and selection operator (LASSO) regression analysis, a statistical method used for variable selection and regularization in high-dimensional data analysis, was performed on differential metabolites using the "glmnet" (https://cran.r-project.org/web/packages/glmnet/index.html) and "survival" (https://cran.r-project.org/web/packages/survival/index.html) packages in R Version 4.1.1 software (The R Foundation, Auckland, Auckland Region, New Zealand). The minimum lambda value obtained from the calculation was used as the optimal reference value, representing the best variable included in the model. This process identified metabolites associated with early-stage hypertensive kidney damage. Then, a binary logistic regression model was used to generate a receiver operating characteristic (ROC) curve and calculate the area under the curve (AUC).

Table 1. Comparison of baseline information between HT, HTN, and Normal groups.

Variable	HT group (n = 10)	HTN group (n = 12)	Normal group (n = 11)	*p*-value
Age (years)				
<65	9 (90.00%)	8 (66.67%)	11 (100.00%)	0.078
≥65	1 (10.00%)	4 (33.33%)	0 (00.00%)	
Gender				
Male	4 (40.00%)	7 (58.33%)	5 (45.45%)	0.752
Female	6 (60.00%)	5 (41.67%)	6 (54.55%)	
Smoking status				
Yes	3 (30.00%)	5 (41.67%)	4 (36.36%)	0.903
No	7 (70.00%)	7 (58.33%)	7 (63.64%)	
Drinking status				
Yes	7 (70.00%)	8 (66.67%)	2 (18.18%)	0.029*
No	3 (30.00%)	4 (33.33%)	9 (81.82%)	
Frequent micturition				
Yes	2 (20.00%)	9 (75.00%)	1 (9.09%)	0.003*
No	8 (80.00%)	3 (25.00%)	10 (91.91%)	

Note: Data are expressed as count (percentage). Fisher's exact test was used for analyzing the data.
*$p < 0.05$.
HT, hypertension; HTN, hypertensive nephropathy.

The ROC curve was used to evaluate the performance of this LASSO disease diagnostic model. The AUC values are typically between 0.5 and 1.0, and a larger area indicates better predictive effect. An AUC value of greater than 0.85 is indicative of good accuracy and reliability showcased by the predictive model.

Statistical Analysis

Untargeted metabolomics and LASSO regression analysis were performed using R Version 4.1.1 software (The R Foundation, Auckland, Auckland Region, New Zealand), visualization was conducted using the R package "ggplot" (https://cran.r-project.org/web/packages/ggplot2/index.html), and image enhancements were carried out using Adobe Illustrator 29.0 software (Adobe Inc., San Jose, CA, USA). SPSS 26.0 software (IBM Corporation, Armonk, NY, USA) was used for statistical analysis of baseline information for all patients. Given the small sample size in this study, Fisher's exact test was used to analyze the classified data. This method is suitable for cases where the sample size is less than 40 ($n < 40$) or some theoretical frequencies are below 1 ($T < 1$) to avoid possible bias encountered in the traditional Chi-square tests. The p values < 0.05 were regarded as statistically significant.

Results

Baseline Information of Patients

Baseline information of the participants in this study, including age, sex, smoking status, drinking status, and micturition frequency, was statistically analyzed and compared across the three subject groups. The results are shown in Table 1.

Differential Metabolite Analysis for HT, HTN, and Normal Groups

Univariate analysis was employed to identify differential metabolites with FC >1.5 or <0.67, and $p < 0.05$, whose differential expressions were visualized using volcano plots. This analysis included all detected metabolites, including those that were unidentified, under positive and negative ionization modes. There were significant differences in the profiles of urinary exosomal metabolites between the HT and Normal groups (Fig. 1A,D). OPLS-DA is a multivariate statistical method commonly used to analyze and visualize group differences. It distinguishes between inter-group variation and intra-group variation by projecting the data onto orthogonal components, thereby enhancing the interpretability and separation of group-specific differences. The score plots of OPLS-DA models showed a clear distinction between the HT and Normal groups (Fig. 1B,E), indicating significant differences in their metabolite profiles. As shown in Table 2, the OPLS-DA model under the positive ionization mode had an R^2Y value of 0.994 and a Q^2 value of 0.614, while the OPLS-DA model under the negative ionization mode had an R^2Y value of 0.990 and a Q^2 value of 0.709, indicating the models' robustness and reliability. The permutation test plots of OPLS-DA models are displayed in Fig. 1C,F. As the permutation retention rate decreased, the R^2 and Q^2 values for the random models also dropped, indicating that the original models were not overfitted and showed high robustness.

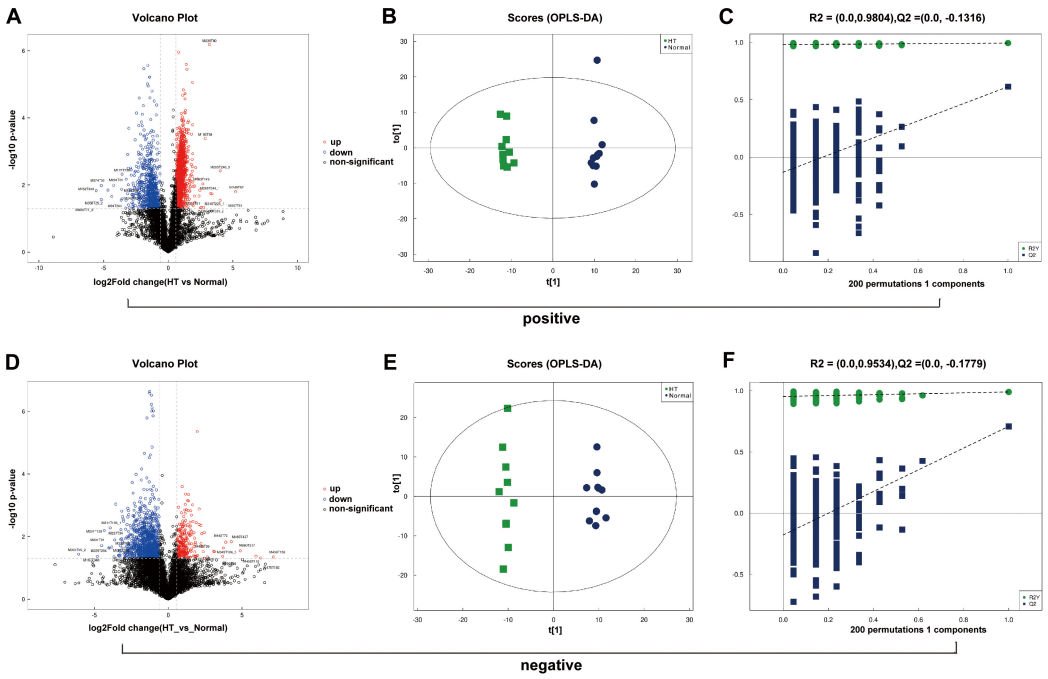

Fig. 1. Untargeted metabolomics analysis of HT and Normal groups. (A) Volcano plot of differential metabolites under positive ionization mode. (B) OPLS-DA plot under positive ionization mode. (C) Permutation test results for OPLS-DA under positive ionization mode. (D) Volcano plot of differential metabolites under negative ionization mode. (E) The results of OPLS-DA model under negative ionization mode. (F) The results of permutation test for OPLS-DA under negative ionization mode. HT, hypertension; OPLS-DA, Orthogonal partial least squares-discriminant analysis.

Table 2. Parameters of OPLS-DA model under positive and negative ionization modes.

Mode	Parameter	HT group vs Normal group	HTN group vs Normal group	HTN group vs HT group
Positive	R^2X	0.598	0.561	0.464
	R^2Y	0.994	0.993	0.995
	Q^2	0.614	0.691	0.645
Negative	R^2X	0.347	0.393	0.315
	R^2Y	0.990	0.994	0.929
	Q^2	0.709	0.876	0.730

Note: R^2X represents the model's explanatory power for the X variables; R^2Y represents the model's explanatory power for the Y variables; Q^2 indicates the model's predictive ability.
HT, hypertension; HTN, hypertensive nephropathy; OPLS-DA, Orthogonal partial least squares-discriminant analysis.

Significant differences in profiles of urinary exosomal metabolites were also observed between the HTN and Normal groups (Fig. 2A,D). The results of OPLS-DA exhibited a notable separation between the HTN and Normal groups (Fig. 2B,E). The R^2Y value was 0.993 and the Q^2 value was 0.691 for the positive ionization mode, while for the negative ionization mode, the R^2Y value was 0.994 and the Q^2 value was 0.876, demonstrating excellent model reliability (Table 2) and strong robustness (Fig. 2C,F).

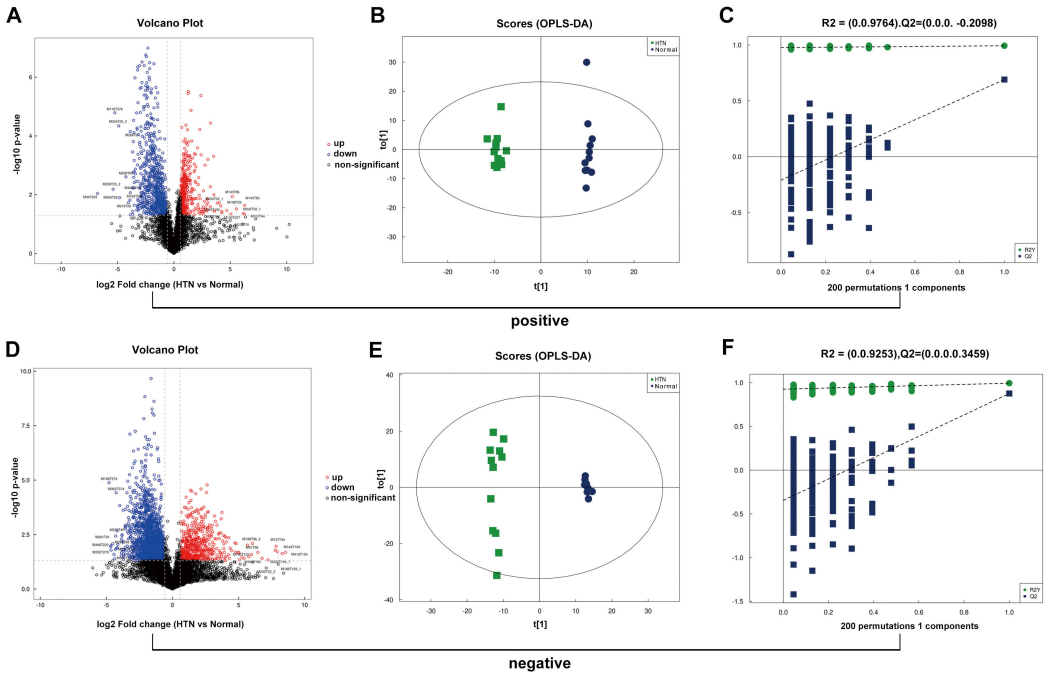

Fig. 2. Untargeted metabolomics analysis of HTN and Normal groups. (A) Volcano plot of differential metabolites under positive ionization mode. (B) OPLS-DA plot under positive ionization mode. (C) Permutation test results for OPLS-DA under positive ionization mode. (D) Volcano plot of differential metabolites under negative ionization mode. (E) OPLS-DA plot under negative ionization mode. (F) Permutation test plot for OPLS-DA model under negative ionization mode. HTN, hypertensive nephropathy; OPLS-DA, Orthogonal partial least squares-discriminant analysis.

The profiles of urinary exosomal metabolites also showed significant differences between the HTN and HT groups (Fig. 3A,D). The OPLS-DA results showed a remarkable separation between the HTN and HT groups (Fig. 3B,E). The R^2Y value was 0.995 and the Q^2 value was 0.645 for the positive ionization mode, while the R^2Y value was 0.929 and the Q^2 value was 0.730 for the negative ionization mode, demonstrating good model reliability (Table 2) and satisfactory robustness (Fig. 3C,F).

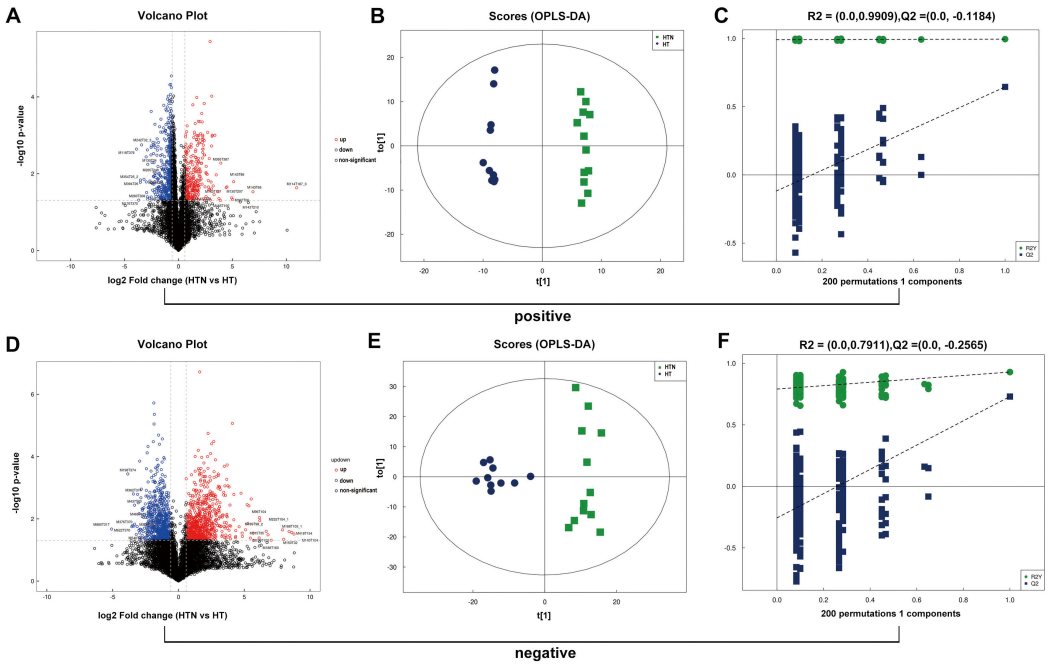

Fig. 3. Untargeted metabolomics analysis of HTN and HT groups. (A) Volcano plot of differential metabolites under positive ionization mode. (B) OPLS-DA plot under positive ionization mode. (C) Permutation test plot for OPLS-DA under positive ionization mode. (D) Volcano plot of differential metabolites in negative ionization mode. (E) OPLS-DA plot under negative ionization mode. (F) Permutation test plot for OPLS-DA under negative ionization mode. HT, hypertension; HTN, hypertensive nephropathy; OPLS-DA, Orthogonal partial least squares-discriminant analysis.

Screening and Identification of Differential Metabolites

The expression of differential metabolites was analyzed using OPLS-DA model, with $p < 0.05$ and VIP >1. The expressions of selected differential metabolites were compared against Human Metabolome Database (https://hmdb.ca/), and their fold changes were presented in the form of bar charts. In the HT and Normal groups, 94 differential metabolites were detected under positive ionization mode (**Supplementary Fig. 1A**), including 28 significantly elevated and 66 significantly decreased metabolites in the HT group. Under negative ionization mode, 66 differential metabolites were identified (**Supplementary Fig. 1B**), with 11 significantly elevated and 55 significantly decreased metabolites in the HT group. Compared to the Normal group, the HTN group features 124 differential metabolites that were detected under the positive ionization mode (**Supplementary Fig. 2A**),

including 24 significantly elevated and 100 significantly decreased metabolites. Under the negative ionization mode, 127 differential metabolites were identified (**Supplementary Fig. 2B**), with 26 significantly increased and 101 significantly reduced metabolites. Compared to the metabolite profile of the HT group, 66 differential metabolites were detected in the HTN group under the positive ionization mode (**Supplementary Fig. 3A**), including 29 significantly increased and 37 significantly decreased metabolites. Under the negative ionization mode, 53 differential metabolites were identified (**Supplementary Fig. 3B**), with 25 significantly elevated and 28 significantly decreased metabolites. These results indicated that there were significantly upregulated and downregulated metabolites in both hypertensive patients and patients with HTN when compared to healthy individuals.

Enrichment Analysis of Differential Metabolites in Metabolic Pathways

To further explore the overall metabolic changes during the development of HTN, KEGG enrichment analysis was performed on the identified differential metabolites. The differential metabolites in the HT and Normal groups were significantly enriched in pathways such as protein digestion and absorption, nicotinate and nicotinamide metabolism, phenylalanine metabolism, cyclic Adenosine Monophosphate (cAMP) signalling pathway, and caffeine metabolism (Fig. 4A). The differential metabolites in the HTN and Normal groups were significantly enriched in pathways such as nicotinate and nicotinamide metabolism, cAMP signalling pathway, alanine metabolism, pantothenate and Coenzyme A (CoA) biosynthesis, phenylalanine metabolism, and ATP-Binding Cassette (ABC) transporters (Fig. 4B). The differential metabolites in the HTN and HT groups were significantly enriched in pathways such as phenylalanine metabolism, and ABC transporters (Fig. 4C).

Biomarker Screening via LASSO Regression Analysis

Differential metabolites were further subjected to LASSO regression analysis and ten-fold cross-validation to identify potential biomarkers associated with HT and HTN. The logistic regression results of differential metabolites are shown in Table 3. It is worth noting that the metabolites regarded as promising disease biomarkers are probably endogenous metabolites synthesized in the context of HT and HTN, excluding those taken up from the external environment. These metabolites, including caffeine, 2-aminoanthraquinone, 1,2-benzenedicarboxylic acid, danazol and N-phenyl-1-naphthylamine, were considered to be ingested from the external environment and therefore were not included in the selection of biomarkers for disease. LASSO regression analysis of HT and Normal groups showed a reduction in the number of potential variables to 10 (Fig. 5A,B), including 4-hydroxyphenylacetic acid, isomaltose, xanthine, 5-hydroxyrosiglitazone, trihexyphenidyl, isopropalin, bilirubin, caffeine, 2-aminoanthraquinone, and quinoline-2,4-diol. Logistic regression revealed significant differences for 4-hydroxyphenylacetic acid ($p = 0.037$) and bilirubin ($p = 0.020$). The AUC values for these metabolites were 0.955 and 0.918, respectively (Fig. 5C). These results demonstrated a clear association of these two metabolites with hypertension, exhibiting high efficacy in distinguishing between normal and hypertensive samples, suggesting that they may be potential

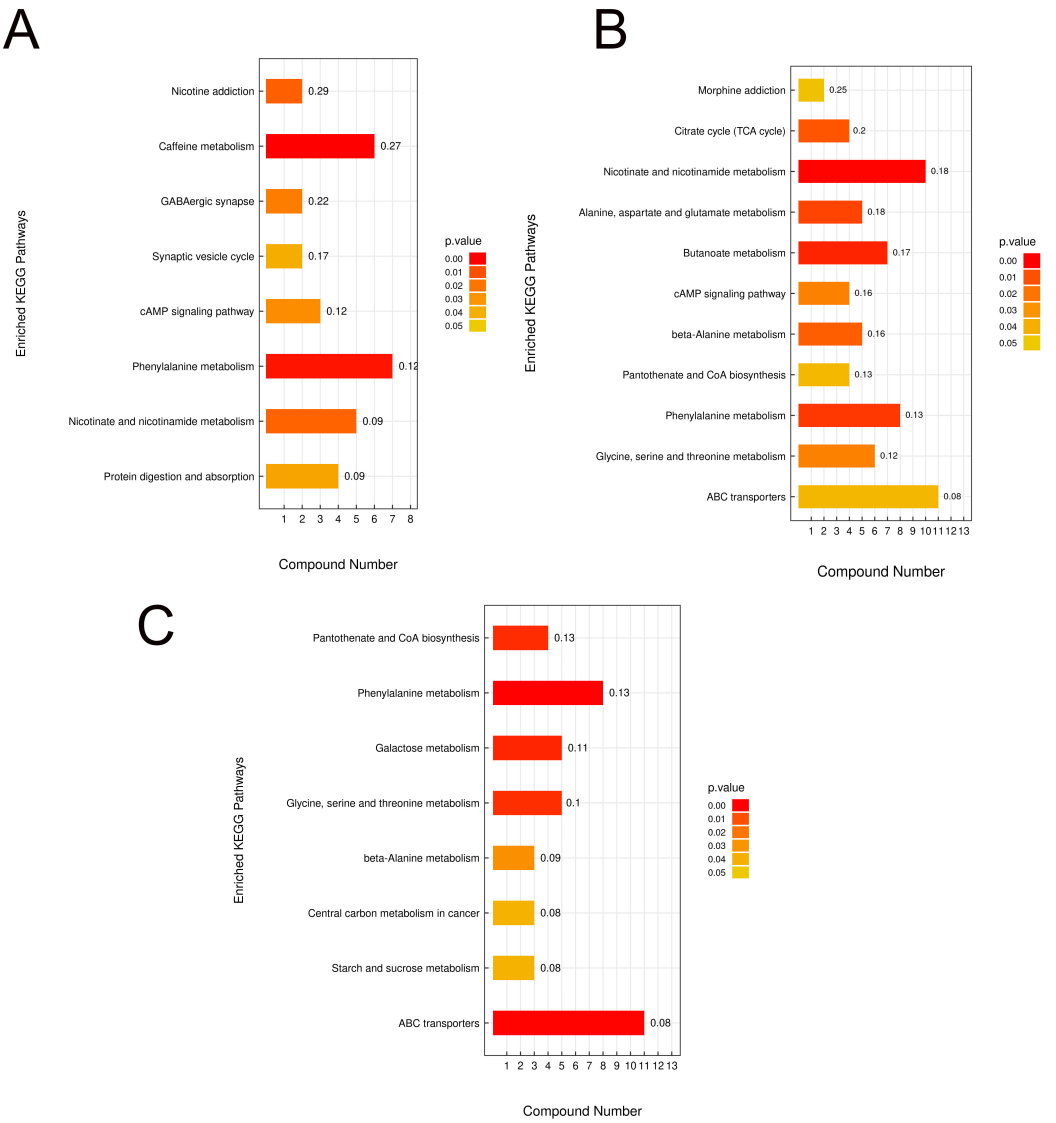

Fig. 4. KEGG pathway enrichment analysis of differential metabolites. (A) KEGG pathway enrichment analysis of differential metabolites in HT and Normal groups. (B) KEGG pathway enrichment analysis of differential metabolites in HTN and Normal groups. (C) KEGG pathway enrichment analysis of differential metabolites in HTN and HT groups. HT, hypertension; HTN, hypertensive nephropathy; KEGG, Kyoto Encyclopedia of Genes and Genomes; ABC, ATP-Binding Cassette; CoA, Coenzyme A; cAMP, cyclic Adenosine Monophosphate; TCA, Tricarboxylic Acid; GABA, Gamma-aminobutyric acid.

biomarkers for HT. LASSO regression analysis of the HTN and Normal groups yielded four variables (Fig. 5D,E), including uracil, 1,2-benzenedicarboxylic acid, trihexyphenidyl, and danazol. Logistic regression revealed a significant difference for uracil (p = 0.047). The AUC value for uracil was 0.917 (Fig. 5F). These results indicated that uracil has strong predictive accuracy for HTN, and could serve as a potential disease biomarker. LASSO regression analysis of the HTN and HT groups yielded four variables (Fig. 5G,H), including N-acetylglutamine, iminodiacetic acid, artemisinin, and N-phenyl-1-naphthylamine. Logistic regression revealed a significant difference for iminodiacetic acid (p = 0.041). With an AUC

value of 0.891 (Fig. 5I), iminodiacetic acid has a good accuracy in differentiating between the HTN and HT groups, with good potential as a biomarker for HTN.

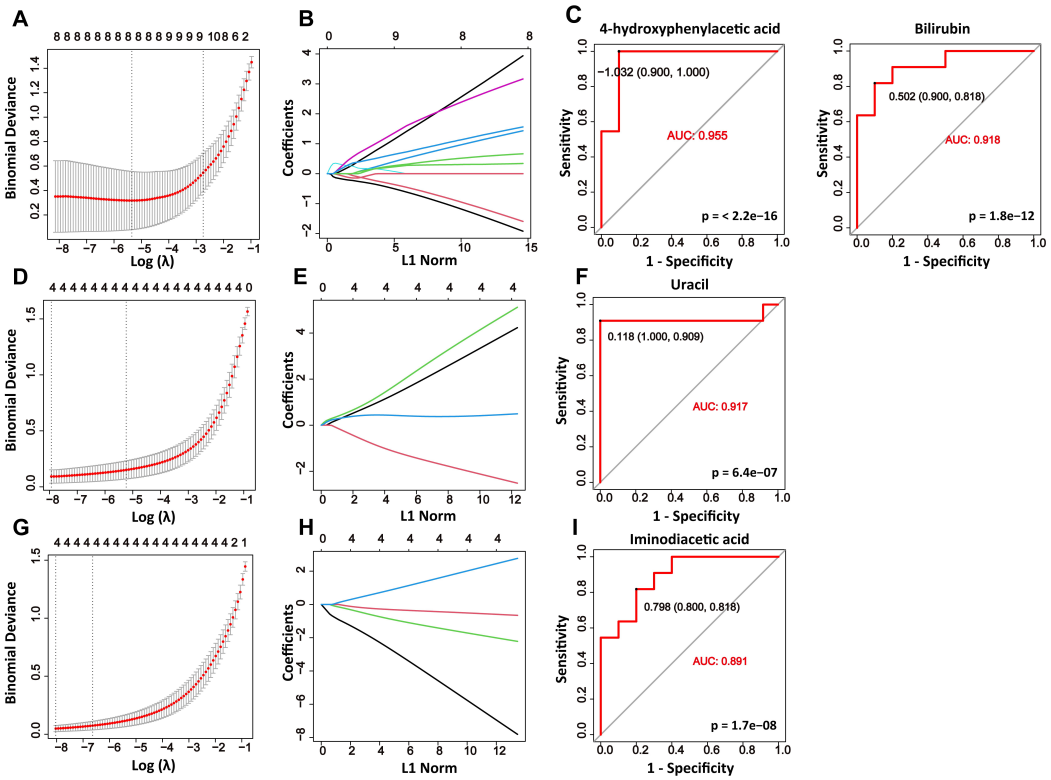

Fig. 5. Biomarker screening via LASSO regression analysis. (A) Distribution of feature coefficients for differential metabolites in HT and Normal groups. (B) Plot of binomial deviance as a function of parameter λ for HT and Normal groups. (C) ROC curve analysis of candidate biomarkers in HT and Normal groups. (D) Distribution of feature coefficients for differential metabolites in HTN and Normal groups. (E) Plot of binomial deviance as a function of parameter λ for HTN and Normal groups. (F) ROC curve analysis of candidate biomarkers in HTN and Normal groups. (G) Distribution of feature coefficients for differential metabolites in HTN and HT groups. (H) Plot of binomial deviance as a function of parameter λ for HTN and HT groups. (I) ROC curve analysis of candidate biomarkers in HTN and HT groups. AUC, area under the curve; HT, hypertension; HTN, hypertensive nephropathy; LASSO, least absolute shrinkage and selection operator; ROC, receiver operating characteristic.

Discussion

A shift in living environments and lifestyles, and aging, are the prime factors for the rising incidence of HT. The progression of HT is accompanied by the development of kidney damage, which is more narrowly defined as HTN (Cao et al, 2019). However, clear and distinctive clinical signs and symptoms are often less pronounced in the early stage of HTN, in which laboratory tests frequently yield negative results, making diagnosis challenging. In many cases, the time a definitive diagnosis becomes feasible often coincides with an expired treatment window, during which irreversible damage to the kidneys may have already occurred (Enevold-

Table 3. Results of logistic regression for differential metabolites.

Group	Characteristics	Estimate	SE	Z	*p*-value
HT group vs Normal group	4-hydroxyphenylacetic acid	2.977	1.427	2.087	0.037
	Isomaltose	–3.257	1.703	–1.913	0.056
	Xanthine	1.430	0.755	1.893	0.058
	5-hydroxyrosiglitazone	2.932	1.613	1.818	0.069
	Trihexyphenidyl	3.515	1.434	2.452	0.014
	Isopropalin	8.658	5.000	1.732	0.083
	Bilirubin	–2.963	1.271	–2.332	0.020
	Caffeine	–2.304	0.884	–2.605	0.009
	2-aminoanthraquinone	2.447	1.084	2.258	0.024
	Quinoline-2,4-diol	3.949	2.021	1.954	0.051
HTN group vs Normal group	Uracil	4.965	2.495	1.990	0.047
	1,2-benzenedicarboxylic acid	–2.732	0.993	–2.752	0.006
	Trihexyphenidyl	16.297	16.780	0.971	0.331
	Danazol	4.771	2.099	2.273	0.023
HTN group vs HT group	N-acetylglutamine	–107.928	111,589.995	–0.001	0.999
	Iminodiacetic acid	–2.632	1.289	–2.041	0.041
	Artemisinin	–8.251	4.897	–1.685	0.092
	N-phenyl-1-naphthylamine	4.296	1.940	2.214	0.027

HT, hypertension; HTN, hypertensive nephropathy; SE, Standard error.

sen et al, 2020; Lu et al, 2020). Therefore, early and accurate diagnosis is crucial in the management of HTN patients.

Exosomes are nano-sized vesicles actively secreted by cells into the extracellular environment, rich in lipids, proteins, and nucleic acids (Zhang et al, 2020). Their crucial role in disease onset and progression has garnered widespread research interest (Ludwig et al, 2019). Exosomes offer several advantages as biomarkers: Firstly, their phospholipid bilayer structure protects their contents from degradation; secondly, they can be detected in nearly all body fluids; and thirdly, their molecular characteristics reflect the phenotype of the originating cells. These attributes make exosomes promising candidates as simple, reliable, and stable molecular biomarkers. Untargeted metabolomics refers to the comprehensive and systematic analysis of the entire metabolome of a living organism via high-resolution analytical techniques, aiming to identify differential metabolites. Currently, this approach is widely applied in discovery of disease biomarkers, as well as the research on unravelling metabolic dynamics and mechanism research. The research by Yu et al (2024) revealed the potential of urinary exosomal mRNA (*RAB5B* and *WWP1*) as non-invasive biomarkers for early prostate cancer diagnosis. Trifonova et al (2022) utilized non-targeted metabolomics techniques to analyze blood plasma samples from patients to identify potential biomarkers for early diabetic nephropathy. These previous studies have laid a concrete foundation for identifying differential metabolites and their enriched pathways in this study through untargeted metabolomics analysis of purified urinary exosomes. LASSO regression analysis was then used

to establish a diagnostic model for early-stage HTN and to screen for potential biomarkers associated with early-stage HTN.

In our study, the OPLS-DA results of the HT, HTN, and Normal groups exhibited significant differences in the metabolite profiles of their exosomes. A comparison between the HT and Normal groups led to the identification of 94 and 66 differential metabolites under the positive and negative ionization modes, respectively. Comparing the HTN group with the Normal groups revealed 124 and 127 differential metabolites under the positive and negative ionization modes, respectively. Between the HTN and HT groups, 66 and 53 differential metabolites were identified under the positive and negative ionization modes, respectively. Further LASSO regression analysis identified four differential metabolites, including 4-hydroxyphenylacetic acid, bilirubin, uracil, and iminodiacetic acid, which may be associated with hypertension or early hypertensive nephropathy.

4-Hydroxyphenylacetic acid, a phenolic acid, has a phenyl ring with a hydroxyl group and an acetic acid group attached at the para position. It is an intermediate metabolite in tyrosine metabolism in humans. 4-Hydroxyphenylacetic acid is involved in oxidative stress and inflammation in the kidneys and may be a biomarker associated with kidney failure (Chen et al, 2012). An et al (2024) found that exogenous 4-hydroxyphenylacetic acid inhibits kidney pathological damage and cell apoptosis, alleviating sepsis-induced acute kidney injury. Godos et al (2017) observed a significant negative correlation between 4-hydroxyphenylacetic acid and HT, concurring with the finding of the present study that 4-hydroxyphenylacetic acid is significantly downregulated in the urinary exosomes of hypertensive patients. Additionally, in this study, 4-hydroxyphenylacetic acid was enriched in pathways like tyrosine metabolism and phenylalanine metabolism. Previous research has shown that phenylalanine can inhibit vascular smooth muscle cell proliferation in spontaneously hypertensive rats, leading to a reduction in blood pressure (Zhao et al, 2001). Hypertensive patients exhibit significantly lower phenylalanine levels, accompanied by reduced levels of tyrosine, norepinephrine, and other metabolites in the phenylalanine and tyrosine metabolic pathways (Hao et al, 2016). These metabolic abnormalities may contribute to the development of HT. Øvrehus et al (2018) discovered that patients with HTN experience disturbances in tyrosine metabolism and phenylalanine metabolism, characterized by impaired dopamine synthesis in the kidneys and reduced urinary excretion of tyrosine and phenylalanine. These metabolic abnormalities may lead to impaired kidney function, increased cardiovascular disease risk, and kidney fibrosis.

Made from heme through a complex catabolic process, bilirubin is a type of bile pigment, which is the main metabolite of iron porphyrin compounds in the human body. It presents in two types, namely direct bilirubin and indirect bilirubin, which are effective anti-inflammatory factors and antioxidants (Mancuso, 2017). Previous studies have shown that a significant decrease in serum bilirubin levels was found among patients with primary hypertension, suggesting that high bilirubin levels can reduce the incidence of hypertension (Wang and Bautista, 2015; Wang et al, 2020). Previous studies in the general Japanese population and type 2 diabetes mellitus groups revealed that higher serum bilirubin levels may indicate a lower risk of pro-

gression of type 2 diabetic nephropathy (Tanaka et al, 2014; Toya et al, 2014). It has been found that total bilirubin and indirect bilirubin in serum were positively correlated with eGFR in patients with type 1 diabetes mellitus (Nishimura et al, 2015). Zhao et al (2023) emphasized that elevated direct and indirect bilirubin levels had a protective effect on reducing CKD risk in a hypertensive patient population. The above studies suggest that higher serum levels of bilirubin may have a potential protective effect on HT and kidney disease. However, in our study, bilirubin levels in urine were elevated in hypertensive patients compared to the healthy individuals, challenging the narrative regarding the protective effect of higher serum bilirubin. This may be due to the complexity of bilirubin metabolism in the body. Bilirubin excretion in urine is not regarded as a normal physiological process and is typically linked to pathological issues related to bilirubin metabolism (Thomas et al, 2021). These results suggest that bilirubin plays a regulatory role in HT and various kidney diseases, and has the potential to be a good biomarker for HT and HTN. Furthermore, existing research indicates that serum bilirubin levels can act as a biomarker to aid in the early detection of pancreatic cancer and distinguish early-stage pancreatic cancer from benign periampullary conditions (Boyd et al, 2023).

Uracil is a member of the nucleotide base family and an essential component of RNA. In living organisms, uracil pairs with adenine to form base pairs of RNA (Hagen et al, 2006). Uracil in urine or plasma indicates the possibility of DNA damage and abnormal cell growth. Numerous studies have shown that uracil is a biomarker for various diseases, including metabolic disorders, and human cancers. Uracil has been used for the clinical diagnosis and monitoring of prostate cancer (Lima et al, 2021), multiple trauma combined with sepsis (Feng et al, 2022), and hereditary orotic aciduria (Ramesh et al, 2020). However, the studies on uracil in HT and HTN are relatively limited. Iminodiacetic acid is a type of chelating agent that features an ethylenediamine backbone linking an amino group and two acetic acid groups (Markowicz-Piasecka et al, 2017). It strongly binds with transition metals, forming stable complexes with metal ions (Repo et al, 2013) and participating in bile acid metabolism within the body (Jacobson et al, 2022). Iminodiacetic acid can serve as a biomarker for predicting the severity of acute respiratory distress syndrome (Lin et al, 2019), and it is potential for diagnostic and prognostic applications in disease management warrants further investigation. However, no studies have provided specific evidence or research results regarding the influence of iminodiacetic acid on kidney diseases.

Although this study has identified potential biomarkers for HT and HTN, several limitations that should be acknowledged. Firstly, the small sample size of this study may introduce some shortcomings. For example, a limited sample may result in statistical noise, making it more difficult to interpret complex metabolic profiles; it may reduce the generalizability of the study's results; and it may increase the risk of false positives and false negatives. Secondly, only urine samples were analyzed in this study, potentially yielding inconsistent results because the levels of metabolites detected in urine can be affected by renal processing to varying degrees. Thus, it is advisable to include serum samples for analysis to generate more accurate and meaningful. In addition, untargeted metabolomics, which was employed

in this study for screening potential disease biomarkers, entails the global, unbiased analysis of all unknown metabolites in the sample, but the data of which are poorly reproducible and fall within limited linear range. Therefore, it is necessary to surmount these shortcomings in future studies by, for example, using larger and more diverse cohorts, using serum samples, and conducting targeted metabolomics screening, coupled with targeted quantitative analysis, on possible biomarkers, in order to improve the accuracy and repeatability of the study' results. Additionally, further biological studies are needed to explore the function of metabolites and the pathogenesis of diseases, as well as to perform functional validation of disease-related targets.

Conclusion

The present study identified differential metabolites in the HT and HTN groups through untargeted metabolomics analysis of urinary exosomes. The results of LASSO regression analysis suggest that 4-hydroxyphenylacetic acid, bilirubin, uracil, and iminodiacetic acid are potential biomarkers for HTN or HT. These newly identified metabolites offer novel insights for the diagnostic and therapeutic strategies of early-stage HTN.

Key Points

- According to the OPLS-DA analysis of untargeted metabolomics findings, there were significant differences in metabolite profiles between the hypertensive patients, hypertensive nephropathy (HTN) patients, and healthy individuals.
- Compared to healthy individuals, both hypertensive patients and those with HTN showed significant upregulation and downregulation of differential metabolites.
- Metabolites such as 4-hydroxyphenylacetic acid, bilirubin, uracil, and iminodiacetic acid are potential biomarkers for HTN or hypertension.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

WC and MJ designed and performed the experiments, analyzed the data, and wrote the original draft. RY and CWZ contributed to the data acquisition and analysis. JCH and HY were involved in the interpretation of data for the work. QW conceived the project, supervised the research, and reviewed and revised the manuscript. All authors contributed to the important editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Medical Ethics Committee of The Second Affiliated Hospital of Chengdu Medical College, Nuclear Industry 416 Hospital (Approval No. YJ-2024-014). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), and informed consent was obtained from all subjects enrolled in this study.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://www.magonlinelibrary.com/doi/suppl/10.12968/hmed.2024.0568.

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