



## ARTICLE

# Metabolomics analysis of the serum from children with urolithiasis using UPLC-MS

Junxiang Wen<sup>1</sup> | Yinyin Cao<sup>2</sup> | Yang Li<sup>1</sup> | Fenhua Zhu<sup>1</sup> | Meifen Yuan<sup>1</sup> | Jin Xu<sup>1</sup> | Jian Li<sup>1,3</sup>

<sup>1</sup>Clinical Laboratory Center, Children's Hospital of Fudan University, Shanghai, China

<sup>2</sup>Cardiovascular Center, Children's Hospital of Fudan University, Shanghai, China

<sup>3</sup>Shanghai Key Laboratory of Birth Defect, Shanghai, China

## Correspondence

Jin Xu and Jian Li, Clinical Laboratory Center, Children's Hospital of Fudan University, 399 Wanyuan Road, Shanghai 201102, China.

Email: xujinekjy@163.com and lijianjulia@fudan.edu.cn

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## Abstract

Pediatric urolithiasis is a common urologic disease with high morbidity and recurrence rates. Recent studies have shown that metabolic dysfunction plays a vital role in the pathogenesis of urolithiasis, especially in children, but the specific mechanism is still unclear. Metabolomics is an ideal technology for exploring the mechanism of metabolic disorders in urolithiasis. In the present study, a serum metabolomics based on ultra-performance liquid chromatography mass spectrometry was performed. A total of 50 children subjects were recruited for the study, including 30 patients with kidney stones and 20 normal controls (NCs). Principal component analysis and orthogonal partial least-squares determinant analysis were carried, and 40 metabolites were found to be significantly altered in patients with kidney stones, mainly involving retinol metabolism, steroid hormone biosynthesis, and porphyrin and chlorophyll metabolism. The kidney stone group appeared to have a lower serum level of bilirubin, but a relative higher level of retinal, all-transretinoic acid, progesterone, and prostaglandin E2 compared with those of the NC group. All the findings suggest that patients with urolithiasis have several metabolic characteristics, which are related to stone formation or compensation. These metabolites and pathways are very likely associated with development of kidney stones and should be considered as potential novel targets for treatment and prevention.

## Study Highlights

### WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Metabolic disorders can be found in most children with kidney stones, suggesting that it plays a vital role in the pathogenesis of pediatric urolithiasis. Metabolomics is an ideal strategy to explore the mechanism of metabolic disorders in kidney stones.

### WHAT QUESTION DID THIS STUDY ADDRESS?

We aimed to identify the changes of serum metabolites in children with urolithiasis compared with normal controls by using ultra-performance liquid chromatography mass spectrometry.

### WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

We found the special metabolic characteristics in patients with pediatric urolithiasis, which are related to stone formation or compensation.

## HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Our findings indicate that the metabolic phenotype of serum in pediatric patients with urolithiasis is significantly different from that in normal controls. These metabolites and metabolic pathways associated with stone formation will help to develop novel therapeutic strategies and preventive interventions.

## INTRODUCTION

Urolithiasis is a common urologic disease with a high incidence and recurrence rate in children, and the incidence of urolithiasis in children has increased rapidly in the past decades.<sup>1</sup> Traditionally, urolithiasis is considered a urologic disease; however, recent studies have suggested that urolithiasis might be a chronic metabolic condition punctuated by symptomatic and preventable stone events, and be linked to hypertension, cardiovascular diseases, diabetes, and metabolic syndrome.<sup>2</sup> Notably, among the patients with urolithiasis, the metabolic disorder rate is higher in children than adults, suggesting that it plays a more critical role in the pathogenesis of pediatric urolithiasis.<sup>3</sup>

Owing to innovative developments in informatics and analytical technologies, metabolomic profiling has been established to elucidate the mechanisms of diseases for exploring novel biomarkers and therapeutic targets.<sup>4</sup> Several studies have applied metabolomics to urolithiasis for evaluating the metabolic disorders associated with stone formation. The preliminary metabolic profiling of the urine from patients with kidney stones found four metabolic pathways closely related with urolithiasis, including glyoxylate and dicarboxylate metabolism, glycine, serine and threonine metabolism, phenylalanine metabolism, and citrate cycle.<sup>5</sup> A later metabolomics study based on the urine of calcium oxalate (CaOx) stone formers found 18 differential metabolites with potential for biomarkers, which mainly involved caffeine, phenylalanine, galactose, and tyrosine metabolism.<sup>6</sup> These results provided metabiologic data for adult patients with urolithiasis, but they are limited for only focusing on urinary metabolomics. As a primary biofluid carrying small molecules in the body, serum is readily accessible and informative, and serum metabolomics is an ideal method for revealing homeostatic imbalances in the biological system.<sup>7</sup> Thus, we believed that using serum for untargeted metabolomics could comprehensively record metabolic changes in urolithiasis, providing valuable insights into the mechanisms of kidney stones.

In the present study, we investigated and compared the metabolite profiles of serum from children with urolithiasis and normal controls via ultra-performance liquid chromatography mass spectrometry (UPLC-MS). We identified 40 small molecules and 3 metabolic pathways associated with kidney stones. We believe that the data obtained will improve our understanding of the metabolic changes in the serum of pediatric urolithiasis.

## METHODS AND MATERIALS

### Sample collection and clinical information

A total of 50 children were enrolled in this study, among which there were 20 normal controls and 30 patients with urolithiasis. Patients with kidney stones and normal controls were recruited from January 2018 to July 2019 in Children's Hospital of Fudan University. All patients were diagnosed with upper urinary tract stones in the department of nephrology, and normal controls were from the physical examination center. According to stone composition, patients were divided into groups, the CaOx group ( $n = 10$ ), the cystine stone (cystine group,  $n = 10$ ), and the calcium carbonate stone (CA group,  $n = 10$ ). The detailed information is shown in Table 1. Venous blood (10 ml) from children with urolithiasis and normal controls was collected from Children's Hospital of Fudan University (Shanghai, China). The blood was clotted at room temperature, and serum was obtained by centrifugation and stored at  $-80^{\circ}\text{C}$ . The same standard operation procedures were used for all samples. The study was designed and performed as per the Declaration of Helsinki of the World Medical Association and was approved by the Ethics Committee of Children's Hospital of Fudan University (approval number 2019-258). Informed consent forms were signed by the enrolled subjects or their parents/guardians.

### Serum sample pretreatment

Serum sample pretreatment was performed according to the prior study.<sup>8</sup> The serum samples were thawed at room

**TABLE 1** Characteristics of kidney stone patients and normal controls

Characteristic	Patients with kidney stones	Normal controls	<i>t</i> -test
Number of samples	30	20	-
Age range, years	5.02 ± 4.13	4.23 ± 3.35	$p > 0.05$
Sex (F/M)	0.47	0.35	$p > 0.05$
BMI <sup>a</sup>	18.09 ± 2.74	16.54 ± 0.71	$p = 0.03$
Calcium oxalate	10	-	-
Cystine	10	-	-

Abbreviation: BMI, body mass index.

<sup>a</sup>The  $p < 0.05$ , patients with urolithiasis versus normal controls.

temperature, and 300  $\mu$ l methanol was used to extract 100  $\mu$ l of the sample, followed by the addition of 5  $\mu$ l internal standard (2.8 mg/ml, DL-o-chlorophenylalanine). After vortex for 1 min, the samples were incubated for 1 h at  $-20^{\circ}\text{C}$ . Then, the samples were centrifuged at 13,780 g at  $4^{\circ}\text{C}$  for 15 min, and 200  $\mu$ l of the supernatant was collected for liquid chromatography mass spectrometry (LC-MS) analysis.

## Data acquisition

Data acquisition was performed according to the prior study.<sup>9</sup> The 10  $\mu$ l of the supernatant were injected into the LC-MS (Ultimate 3000 LC, Q Exactive; Thermo Scientific) in random order. A reverse-phase 2.1\*100 mm 1.9  $\mu$ m C18 column (Hyper gold) was used for separation. The mobile phases were (A) acetonitrile in water (5:95, v/v) containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. The mobile-phase gradient elution procedure was shown in Table S1. The mass parameters were as follows: electrospray ionization (ESI) positive: heater temp  $300^{\circ}\text{C}$ ; sheath gas flow rate, 45 arb; aux gas flow rate, 15 arb; sweep gas flow rate, 1 arb; spray voltage, 3.0 kV; capillary temp,  $350^{\circ}\text{C}$ ; and S-lens RF level, 30%. ESI negative: heater temp  $300^{\circ}\text{C}$ , sheath gas flow rate, 45 arb; aux gas flow rate, 15 arb; sweep gas flow rate, 1 arb; spray voltage, 3.2 kV; capillary temp,  $350^{\circ}\text{C}$ ; and S-lens RF level, 60%.

## Data analysis

The data were subjected to feature extraction, preprocessed with Compound Discoverer software (Thermo Scientific), and then normalized and edited into a two-dimensional data matrix by Microsoft Excel 2010 software (Microsoft Corporation), including the retention time, compound molecular weight, observations (samples), and peak intensity. A total of 3451 features in the (ESI positive) ion mode and 2698 features in the (ESI negative) ion mode were identified in this experiment and the data after editing were subjected to multivariate analysis using SIMCA-P software (version 14.0; Umetrics). Variable Importance in Projection generated from orthogonal partial least-squares determinant analysis (OPLS-DA) and false discovery rate (FDR)-adjusted  $p$  values were used to select metabolites distinguishing patients with kidney stones from normal controls. Permutation tests were used to check whether the models were overfitting. The raw data have been uploaded to the MetaboLights Database (URL [www.ebi.ac.uk/metabolights/MTBLS2231](http://www.ebi.ac.uk/metabolights/MTBLS2231)), and the ID number was MTBLS2231.<sup>10</sup>

The candidate metabolites were identified by searching the METLIN Database (<https://metlin.scripps.edu/>). Metabolic

pathway analysis was performed by MetaboAnalyst version 4.0 using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.kegg.jp/>).<sup>11</sup>

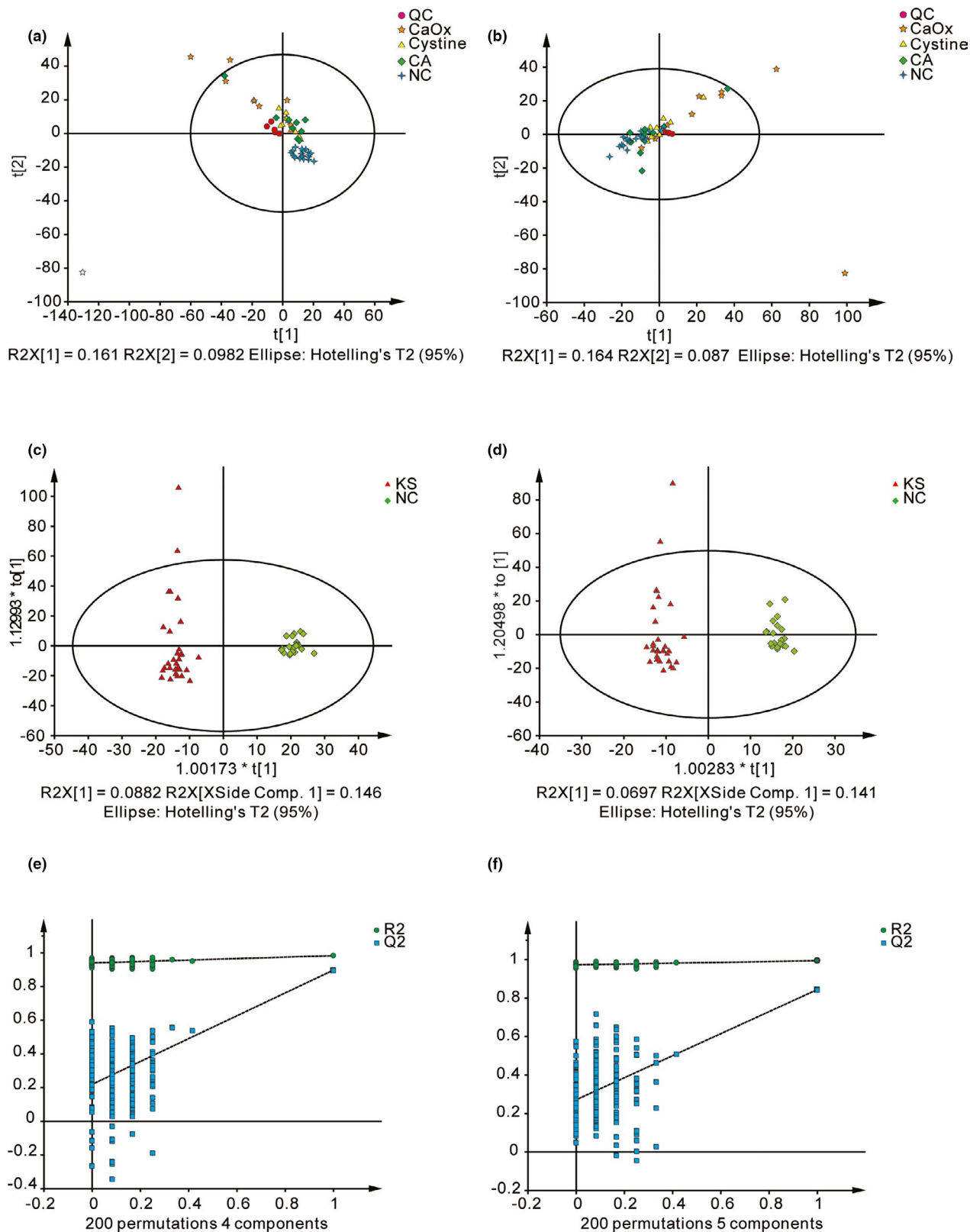
## RESULTS

### Clinical characteristics of the participants

In this study, 30 patients with urolithiasis and 20 normal controls aged from 6 months to 15 years in Children's Hospital of Fudan University were recruited. Urolithiasis was diagnosed by imaging examination, and the composition of stones was identified by infrared spectroscopy. One-way analysis of variance (ANOVA) showed no significant difference in age or sex between the two groups. Compared with normal controls, the body mass index of the kidney stone group was higher, suggesting that being overweight might affect stone formation. The clinical characteristics of participants are presented in Table 1.

### Metabolomics analysis of serum samples

Serum samples were collected and subjected to metabolomics analysis, as described in the Materials and Methods. Quality control samples clustered together tightly, indicating great repeatability and analysis system stability of experiments. Visualization of the features performed by principal component analysis (PCA) showed clear separation between the kidney stone and normal control groups, suggesting that there was a significant difference between the two metabolic profiles. However, partial overlaps among the CaOx, CA, and cystine groups implied that partial differences exist within the kidney stone group (Figure 1a,b). The cumulative interpretation rate of the PCA model was  $R^2X = 0.57$  and  $Q^2 = 0.0765$  for the positive mode and  $R^2X = 0.531$  and  $Q^2 = 0.0566$  for the negative mode. OPLS-DA were then applied to compare metabolite profiles between the kidney stone and normal control groups (Figure 1c,d). The results showed a clear separation between them. In addition,  $R^2Y$  was used to assess fitness and  $Q^2$  to indicate the prediction ability of the model ( $R^2Y = 0.982$ ,  $Q^2 = 0.863$  for the positive mode and  $R^2Y = 0.981$ ,  $Q^2 = 0.812$  for the negative mode). The results indicated that no overfitting was found (Figure 1e,f). Differential metabolites were obtained in the standard of FDR-adjusted  $p$  value less than 0.05 and VIP greater than 1, with a total of 139 metabolites detected. Based on this detection platform, the main types of metabolites we obtained were fatty acyls, carboxylic acids and derivatives, steroids and steroid derivatives, and glycerophospholipids, as shown in Table S2. The molecular information and confidence of identification of these metabolites are included in Table S3.



**FIGURE 1** Significant disturbed metabolites analysis. (a, b) Principal component analysis score plots based on the data from liquid chromatography mass spectrometry under the electrospray ionization (ESI)-positive mode (a) and ESI-negative mode (b). (c, d) The orthogonal partial least-squares determinant analysis (OPLS-DA) between the normal controls (NCs) and the patients with kidney stones (KS) under the ESI-positive mode (c) and ESI-negative mode (d). Samples from the KS group are marked with red triangle and samples from the NC group are marked with green rhombus. (e, f) Permutation test of the OPLS-DA model under the ESI-positive mode (e) and ESI-negative mode (f). The values of  $R^2Y$  and  $Q^2$  represent the goodness of fit and predictability of the model, respectively. CA, calcium carbonate; QC, quality control

**TABLE 2** List of significant metabolites in serum from KS compared with NCs

Name	VIP	FDR-adjusted <i>p</i> value	Fold change	Metabolic pathway
14,15-DiHETE	1.83786	0.002321692	8.206696603	
Prostaglandin E2	1.93064	0.002183181	7.155911687	Arachidonic acid metabolic pathway
12-HEPE	1.59364	0.002086709	6.891761202	
Progesterone	2.30579	3.48E-06	6.875274069	Steroid hormone biosynthesis
Resolvin D5	1.96848	0.002183181	6.714337917	
7alpha-Hydroxypregnenolone	2.23074	6.40E-06	6.36947986	Steroid hormone biosynthesis
all-trans-Retinoic acid	1.60242	0.002059783	6.237153534	Retinol metabolism
19,20-DiHDDPA	1.71094	0.004443925	6.138256788	
14-HDoHE	1.67492	0.001594106	6.101611693	
Stearidonyl carnitine	2.25607	5.57E-06	6.040409414	
Dihydroandrosterone	1.55958	0.002430166	5.688303828	
Epipregnanolone	1.58585	0.002102015	5.438231908	
Retinal	1.65778	0.001623226	5.399985673	Retinol metabolism
Pregnenolone	1.59871	0.002059783	5.331412687	Steroid hormone biosynthesis
3-Hydroxyhexadecadienoylcarnitine	2.08407	4.07783E-05	5.263996188	
Prostaglandin J2	1.3448	0.009278387	4.956518406	
7-Ketocholesterol	1.4987	0.003862658	4.738107879	
Tetradecanoylcarnitine	1.65977	0.001623226	4.660562725	
Heptanoylcarnitine	2.13465	2.33506E-05	4.453388797	
Tetrahydrodeoxycortisol	1.60022	0.002059783	4.227149803	
3-Hydroxy-9-hexadecenoylcarnitine	1.97393	0.000120279	4.179010327	
Vitamin D3	1.72711	0.001062085	4.160717912	Steroid biosynthesis
MG(P-18:0/0:0/0:0)	2.07303	0.001094262	4.056197582	
17,18-EpETE	1.60072	0.002059783	3.939868312	
Tetrahydrocortisol	1.62949	0.001941933	3.89128564	Steroid hormone biosynthesis
Prostaglandin E1	1.61982	0.006939291	3.832785008	
Tocopheronic acid	1.76242	0.003644001	3.800301475	
LysoPA(i-14:0/0:0)	1.34796	0.009278387	3.76117369	
3-Hydroxyhexadecanoylcarnitine	2.03894	6.55712E-05	3.640922816	
9-OxoODE	1.38374	0.008055574	3.584966457	
Stearidonic acid	1.47385	0.004612203	3.520644742	
Prostaglandin A1	1.7922	0.003104045	3.419913784	
Tetrahydrodeoxycorticosterone	1.38872	0.007948791	3.341225478	Tetrahydrodeoxycorticosterone
Leucylleucine	1.88416	0.000295522	3.200143002	
Glycylleucine	1.37698	0.008111066	2.785439389	
9,12-Hexadecadienoylcarnitine	2.0249	6.9379E-05	2.500742344	
Cholestenone	1.91604	0.000222369	2.478446854	
L-beta-aspartyl-L-leucine	1.7162	0.001119926	2.402849132	
Prostaglandin B1	1.62935	0.006817424	-3.351922979	
Bilirubin	2.94423	5.78E-14	-5.823064548	Porphyrin and chlorophyll metabolism

Note: The cutoff value for FDR-adjusted *p* value was set at 0.01.

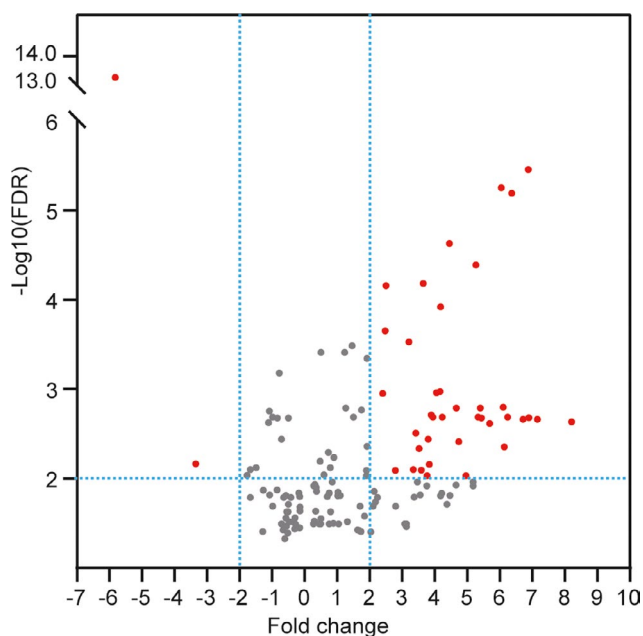
Abbreviations: FDR, false discovery rate; KS, kidney stone; NC, normal control; VIP, Variable Importance in Projection.

## Significant metabolites in serum from children with kidney stones

To further identify small molecules showing the greatest changes in the serum of patients with kidney stones, 139 metabolites screened in the previous stage were further selected by the standard of FDR-adjusted  $p$  value less than 0.01 and fold change greater than 2. A total of 40 small molecules with the most obvious differences were identified (Table 2). Among these, 37 molecules were significantly upregulated, and 2 molecules downregulated in the kidney stone group (Figure 2). The heatmap in Figure 3 further showed the relative level difference and variation trend of the screened metabolites. The CaOx was the main group distinguishing the normal controls, and bilirubin exhibited excellent consistency in distinguishing the patients with kidney stones from the normal controls.

## Disturbed metabolic pathways in pediatric urolithiasis

MetaboAnalyst version 4.0 was used to analyze metabolic pathways among the 40 metabolites, which revealed 6 metabolic pathways possibly related to urolithiasis (Table S4). According to the  $p$  value and pathway impact, three metabolic pathways, porphyrin and chlorophyll metabolism, steroid hormone biosynthesis, and retinol metabolism, were



**FIGURE 2** Volcano plot representing the relationship between the fold change and significance of the metabolites. The red dots represent the 40 metabolites selected according to fold change greater than 2 and false discovery rate (FDR)-adjusted  $p$  value  $< 0.01$ . The gray dots represent filtered metabolites

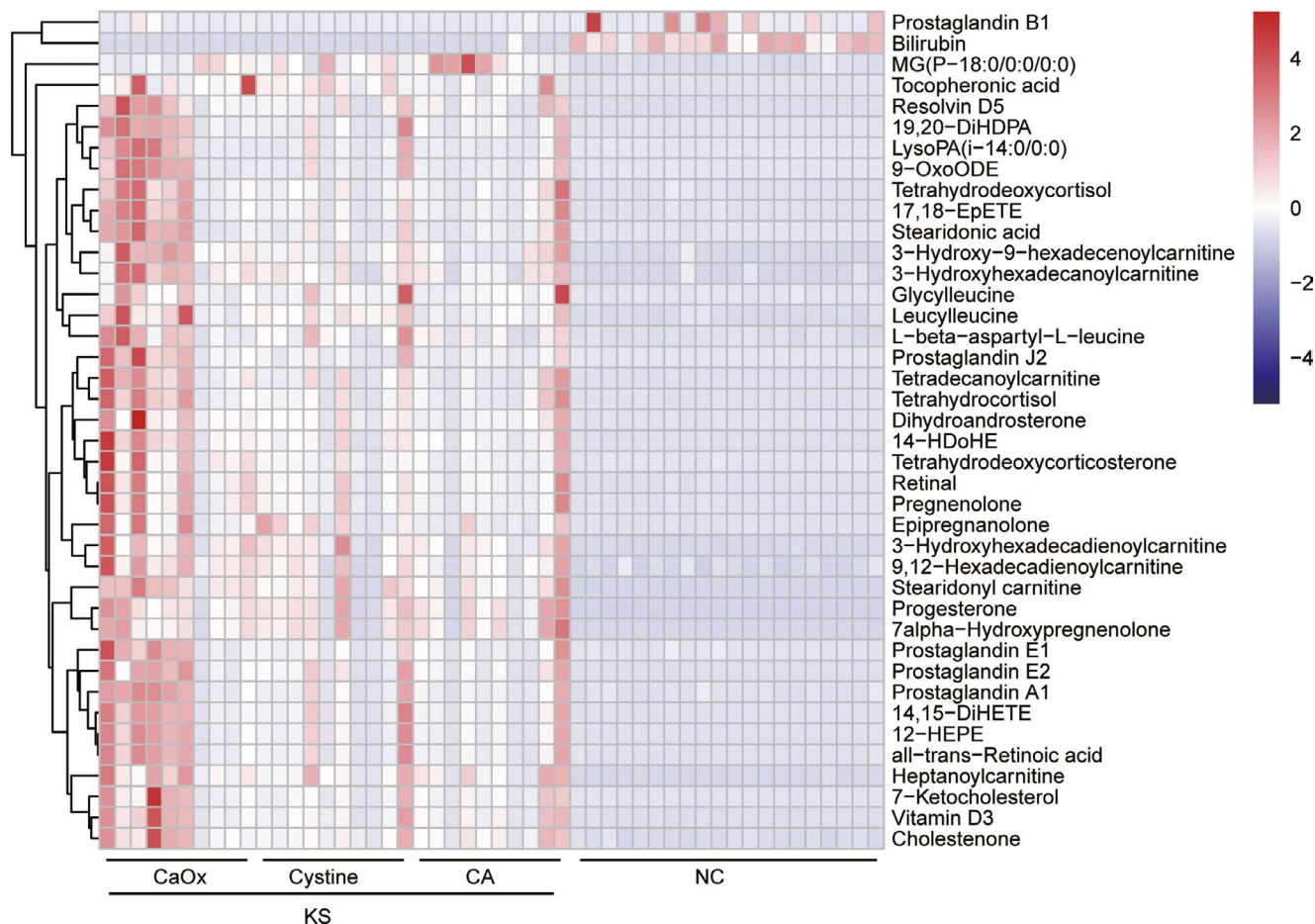
considered to have significant abnormalities in urolithiasis (Figure 4). The schematic diagrams of the main disturbed metabolic pathways and corresponding metabolites were constructed (Figure 5).

## DISCUSSION

In the past few decades, the incidence of pediatric urolithiasis has significantly increased, with a high recurrence rate in which  $\sim 50\%$  of patients with kidney stones experience a relapse within 3 years.<sup>12</sup> The cause of urolithiasis in children has switched from urinary tract infection to metabolic disorder over the past few years, and metabolic disorders can now be found in most children with urolithiasis.<sup>13</sup> However, the specific mechanism of metabolic disorders in kidney stones is unclear. Therefore, monitoring methods based on metabolic analysis may be an ideal strategy to identify the causes of kidney stones and predict recurrence risk. Nevertheless, existing detection methods, such as 24-hour urine analysis, have many drawbacks and cannot meet the requirements.<sup>14</sup>

Metabolomics is a method to measure changes within the entire metabolome of samples.<sup>4</sup> It has previously been performed to analyze the serum and urine of animal models with CaOx stones, by which potential biomarkers and disturbed metabolic pathways in rats with stones were identified.<sup>15</sup> Recently, metabolomics studies using the urine of patients with kidney stones have also been published,<sup>5,6</sup> which are of great significance for the prevention and treatment of kidney stones. However, the previous researches only focused on adults, ignoring children and adolescents. To our knowledge, our study is the first metabolomics research focusing on the serum of children with urolithiasis. We believe that the findings of this study provide a deeper understanding of urolithiasis in children.

The main metabolites we obtained were lipids, and the involved metabolic pathways in our findings did not completely match to previous studies. These differences may be related to filtration, reabsorption, secretion, synthesis, and degradation of the kidneys.<sup>16</sup> Because the glomerular filtration barrier prevents lipoprotein particles from being filtered into the original urine, serum characterizes a more stable and abundant lipid profile than urine.<sup>17</sup> Therefore, serum metabolomics can reveal metabolic disorders, especially lipids metabolism disorders, without being affected by the kidneys. Although the finding that porphyrin and chlorophyll metabolism altered in urolithiasis is not completely new, the previous study ignored the potential protective effect of bilirubin for only detecting glycine in urine.<sup>5</sup> We hypothesized that the excretion of bilirubin in serum and bile through the liver might mask the change in urine of patients with kidney stones.<sup>18</sup> Therefore, we believed



**FIGURE 3** Heatmap showing the expression of the 40 selected metabolites in 30 patients with urolithiasis and 20 normal controls. Significant differences were detected between the patients and controls. Red indicates an increase and blue a decrease. CA, calcium carbonate; CaOx, calcium oxalate; KS, kidney stone; NC, normal control

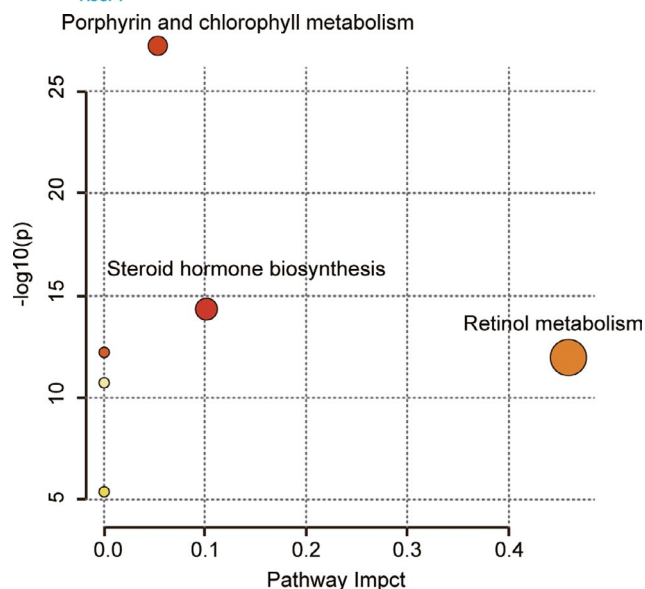
that serum and urine metabolomics were complementary for urolithiasis. The identification of small molecules that are closely related to urolithiasis and independent of renal excretion, may reveal metabolites and pathways that are related to stone formation.<sup>16</sup>

It is known that CaOx is the major component of the stones in children with urolithiasis. In addition, the rate of cystine stones and infectious stones is also high in children.<sup>13</sup> Therefore, serum was collected from patients with three major forms of stones (CaOx, cystine, and CA). Untargeted metabolomics was performed to comprehensively measure metabolic profiles associated with urolithiasis, by which three major metabolic disorder pathways, retinol metabolism, porphyrin and chlorophyll metabolism, and steroid hormone biosynthesis were identified.

The most obvious metabolic disorder in kidney stones based on our results was with regard to the retinol metabolic pathway. In this pathway, retinol is metabolized as retinal, which is further metabolized as all-transretinoic acid (tRA), as shown in Figure 5a. Notably, although we found no significant difference in retinol levels between the patients

with kidney stones and the normal controls, its downstream products, retinal, and tRA, were significantly increased in the patients (Figure 5h,i). tRA, as the main active ingredient of retinol, is known as a kidney-protective factor.<sup>19,20</sup> It is thought to protect the kidneys by diminishing expression of inflammatory cytokines, protecting proximal renal tubular epithelial cells against hypoxia damage, and promoting the differentiation of renal tubular epithelial cells in kidney function reconstruction.<sup>21</sup> Studies have shown that nephrotic fibrosis occurring in the chronic kidney diseases, including kidney stones, has the effect of kidney protection and repair reconstruction in response to renal tubular injury, which is tRA-dependent.<sup>22</sup> Taken together, we believe that enhancement of retinol metabolic pathway activity is a protective mechanism in response to crystal damage in the renal tubule.

There is a significant sex difference in the incidence of urolithiasis, which is three times higher in men than in women.<sup>23</sup> Studies have suggested that androgens had a significant role in stone formation, whereas estradiol might strongly inhibit it, indicating that sex hormones might be key in kidney stones.<sup>24</sup> Coincidentally, in our study, steroid



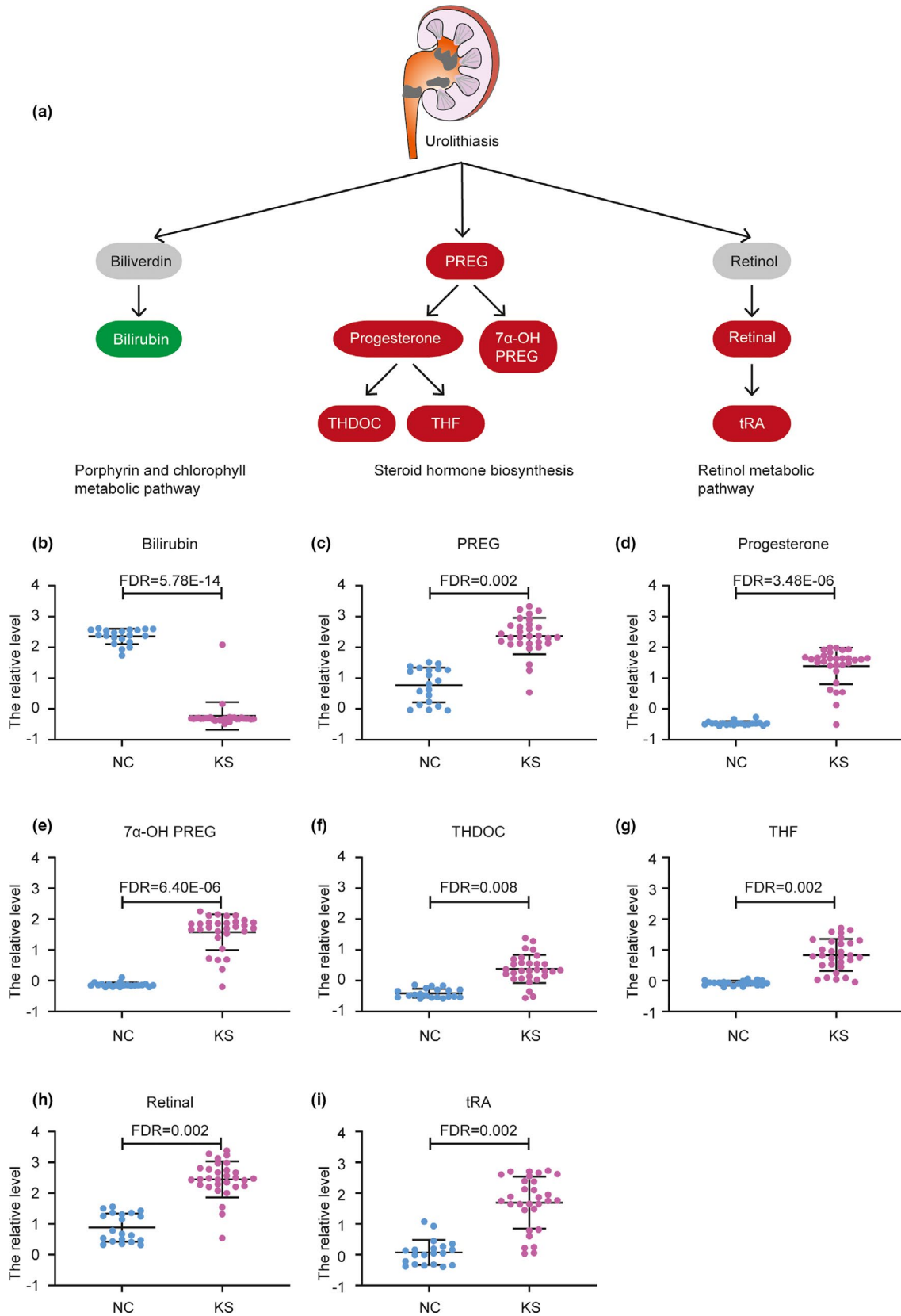
**FIGURE 4** Metabolic pathway analysis was performed with MetaboAnalyst version 4.0. The calculated  $p$  value was established based on pathway enrichment analysis, and the pathway impact value was based on pathway topology analysis. The most relevant metabolic pathways were the retinol metabolic pathway, porphyrin and chlorophyll metabolic pathway, and steroid hormone biosynthesis pathway

hormone biosynthesis, the main synthetic metabolism of sex hormones, was obviously disturbed in children with urolithiasis. The results showed that both androgens (dihydrotestosterone [DHT]) and estradiol (pregnenolone [PREG]), progesterone, 7 $\alpha$ -hydroxypregnenolone [7 $\alpha$ -OH PREG], tetrahydrocorticosterone, and tetrahydrocortisol) significantly elevated in children with stone (Figure 5 and Table S3). DHT is the main androgen in the body. Many studies have demonstrated that androgens promoted stone formation by inducing apoptosis of renal tubular epithelial cells and providing apoptotic fragments as stone matrix.<sup>25</sup> In addition, its role in leading to hyperoxaluria, a risk factor for kidney stones, has been reported, indicating that the elevated DHT may be one of the reasons for kidney stone formation.<sup>26</sup> As for estradiol, its role in protecting the kidneys against crystal damage has been proved, based on the mechanism of decreasing surface expression of CaOx crystal receptors, reducing intracellular metabolism, and enhancing cell proliferation and tissue healing.<sup>27</sup> In addition, progesterone has been reported to affect parathyroid hormone transcription, which regulated the balance of water and salt.<sup>28</sup> Besides, by promoting bone formation and bone turnover, progesterone lowered calcium levels in blood and urine.<sup>29</sup> However, in our study, estrogen levels were significantly elevated in children with kidney stones. Therefore, we speculate that estrogen may be not the initiating factor of urolithiasis but rather part of the self-protection mechanism of the body after stone formation.

Most of the differential metabolites in the patients with kidney stones were elevated. On the contrary, bilirubin, a key molecule in the porphyrin and chlorophyll metabolic pathway, was significantly decreased (Figure 5a,b). As an endogenous antioxidant, bilirubin may play a significant antioxidant effect at the physiological concentration,<sup>30</sup> and oxidative stress induced by crystals in renal tubular epithelial cells is essential for the pathogenesis of urolithiasis.<sup>31</sup> Recent studies have confirmed that bilirubin not only attenuated the renal injury by inhibition oxidative stress and apoptosis,<sup>32</sup> but also dose-dependently promoted the proliferation of renal tubular epithelial cells.<sup>33</sup> All the results highlight the protective effect of bilirubin on renal tubular epithelial cells. Gilbert syndrome (GS) is a benign hyperbilirubinemia with mild unconjugated hyperbilirubinemia due to decreased uridine 5'-diphospho-glucuronosyl transferase (UGT) activity.<sup>34</sup> Several clinical studies have shown that the incidence of type 2 diabetes, coronary heart disease, and metabolic syndrome is significantly reduced in the population with GS.<sup>35</sup> However, there is no clinical study on the incidence of kidney stones in GS. Here, we propose an interesting conjecture that bilirubin could attenuate oxidative stress damage caused by crystals of renal tubular epithelial cells as an antioxidant. Bilirubin deficiency in children with kidney stones may be the initiating factor, worsening crystal damage in renal tubular epithelial cells, and eventually promoting urolithiasis. Inhibition of UGT activity may be a theoretical prophylaxis by modulating serum bilirubin level. Many natural compounds commonly used as nutraceuticals could inhibit UGT activity, such as anthocyanidin and pterostilbene.<sup>36,37</sup>

Arachidonic acid (AA) is a major component of cell membrane lipids and could be converted into various metabolites that trigger several inflammatory responses, which are thought to be closely related to kidney stone formation. In our study, the major metabolites generated from AA, prostaglandin E2 (PGE2), and prostaglandin F2a (PGF2a), were found to be significantly elevated in the patients with kidney stones (Figure 3 and Table S3). PGF2a is a lipid peroxidation product that partially reflects the level of oxidative stress in the body,<sup>38</sup> and its effect of kidney stone formation has been reported in CaOx rats. Animal models confirmed that serum PGF2a was significantly increased in rats with kidney stones.<sup>39</sup> Therefore, we speculate that the elevated PGF2a might represent the increase of oxidative stress in renal tubular epithelial cells, which is considered one of the key factors in kidney stone formation.<sup>40</sup> Besides, the role of PGE2 that is elevated in patients with kidney stones has been studied. Specifically, more PGE2 was secreted by the renal epithelial cells in the presence of CaOx monohydrate crystals.<sup>41</sup> What is worse, the elevated PGE2 further increased calcium excretion by affecting renal tubular function and intestinal calcium absorption, and mediated apoptosis of renal tubular epithelial cells by inducing oxidative stress, all of which





**FIGURE 5** Metabolic pathways and metabolites associated with pediatric urolithiasis. (a) Schematic of abnormal metabolic pathways in patients with urolithiasis. Red or green represent metabolites that are elevated or reduced in the patients with kidney stones (KSs) compared with the normal controls (NCs), respectively. (b–i) The relative levels of disordered metabolites. The relative level was the log of the relative concentration. The cutoff value for false discovery rate (FDR)-adjusted  $p$  value was set at 0.01. PREG, pregnenolone; THDOC, tetrahydrodeoxycorticosterone; THF, tetrahydrocortisol; tRA, all-transretinoic acid

promoted kidney stone formation.<sup>42</sup> Based on our results and other studies on PGE<sub>2</sub>, we assume that there is a positive feedback mechanism between PGE<sub>2</sub> and kidney stone formation, which may promote the development and recurrence of kidney stones.

## CONCLUSIONS

In the present study, we investigated and identified the metabolic characteristics of serum samples from children with urolithiasis and normal controls via UPLC-MS-based metabolomics. Forty differential metabolites were identified, mainly involved in retinol metabolism, steroid hormone biosynthesis, and porphyrin and chlorophyll metabolism. These results indicated that the metabolic phenotype of serum in patients with kidney stones was significantly different from that in normal controls. Our study provides a new insight into the potential pathogenesis of urolithiasis, which may help to develop novel therapeutic strategies and preventive interventions.

## CONFLICT OF INTEREST

The authors declared no competing interests for this work.

## AUTHOR CONTRIBUTIONS

J.W. wrote the manuscript. J.X. and J.L. designed the research. J.W., Y.C., Y.L., F.Z., M.Y., J.X., and J.L. performed the research. J.W., Y.C., and J.L. analyzed the data. Y.L., F.Z., M.Y., and J.X. contributed new reagents/analytical tools.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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