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# Blood metabolism study on protection of residual renal function of hemodialysis patients by traditional Chinese medicine Kidney Flaccidity Compound

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**Abstract:** In recent years, metabolomics using high-performance liquid chromatography (UPLC) has been used to study the metabolic profiles in plasma, urine, stool and tissue in animal model of chronic kidney disease (CKD). In the previous work, we found that traditional Chinese medicine (TCM) "Kidney Flaccidity Compound" (KFC) based on "kidney flaccidity theory" can improve renal function and quality of life of patients with kidney disease. This study aimed to investigate the metabolic profiles in peripheral blood of hemodialysis patients administrated by KFC for 1.5 and 3 months and explore the potential metabolic mechanism using UPLC. Results showed that 121 metabolites were different between KFC 3-months group and untreated control, of which 75 were significantly upregulated and 46 were significantly downregulated. In the 1.5-months treatment group, there were 365 metabolites, of which 164 were significantly upregulated and 192 downregulated. There were 6 metabolites and 15 metabolites upregulated 3-fold in 3-months and 1.5-months KFC treatment group, respectively. In addition, more than 60 new metabolites were identified in the peripheral blood in KFC treated patients, including two potential diagnostic markers MGDG 30:8 and 2-(hydroxymethyl)-6-[[(1R,4S) -2,2,4-trimethyl-3-oxabicyclo[2.2.2]octan-5-yl]oxy]oxane-3,4,5-triol. The pathway enrichment analysis showed thce differential metabolites mainly enriched in Arginine and proline metabolism, Urea cycle, Tyrosine metabolism, Methionine metabolism, Tricarboxylic acid cycle, and Androgen and estrogen metabolism. The findings are helpful to reveal the mechanism of KFC protects CKD, and to provide a new strategy for recovery renal function in hemodialysis patients.

***Key words:*** Traditional chinese medicine; Kidney flaccidity compound; Hemodialysis; Metabolomics.

## Introduction

The incidence of chronic kidney disease (CKD) has been rapidly increasing worldwide and has become a major public health problem (1). Most patients with CKD was maintained through dialysis or kidney transplantation (2, 3). However, it was demonstrated that maintenance hemodialysis (MHD) is an incomplete renal replacement therapy that cannot completely correct the metabolic disorder of uremia and clear the accumulation of toxic substances in the body, which will cause several chronic complications with treatment prolongs. Therefore, improving the diagnosis, prevention and treatment of complications in patients with MHD is of great importance to reduce the incidence of complications and patients' mortality, improve patients' quality of life, and prolong life of patients.

In recent years, metabolomics using high-performance liquid chromatography (UPLC) has been used to study the metabolic profiles in plasma, urine, stool and tissue in animal model of chronic kidney disease (CKD) (4-12). Zhao et al. found that adenine-induced chronic renal failure (CRF) rats showed different metabolite concentrations compared with normal control rats by serum UPLC (6, 7). The concentration of phosphatidylcholine (16: 0/18: 2), hemolytic phosphatidylcholine (18: 1), creatinine, hemolytic phosphatidylcholine (17: 0), hemolytic phosphatidylcholine (16: 0) in CRF rats were higher than normal rats. In addition, in serum metabolite profiles of patients with CRF, levels of tryptophan, phenylalanine, lysophosphatidylcholine, creatinine, and kynurenine can be used as early biomarkers of CRF. The serum concentrations of lysophosphatidylcholine (16: 0), creatinine, phenylalanine and kynurenic acid in CRF patients were higher than healthy subjects (11). These results indicate that CRF has specific amino acid and phospholipid metabolic abnormalities.

At present, a large number of studies show that tra-

ditional Chinese medicine can effectively improve the quality of life of dialysis patients and reduce complications. In our previous work, we found that traditional Chinese medicine (TCM) "Kidney Flaccidity Compound" (KFC) based on "kidney flaccidity theory" can improve renal function and quality of life of patients with kidney disease (19). KFC can reduce proteinuria, serum creatinine and urea nitrogen in rats with CKD, inhibit renal fibrosis, and delay the progression of CKD, thereby protecting renal function (20). It was demonstrated that KFC might inhibit renal interstitial fibrosis through the TGF-β1/Smad signaling pathway (21).

In this study, we aimed to observe the outcome of KFC in residual renal function of patients with renal hemodialysis, investigate the metabolic profiles in peripheral blood of hemodialysis patients administrated by KFC and explored the potential metabolic mechanism using UPLC.

## Materials and Methods

### General data

A single-center randomized controlled trial was designed. The trial was approved by the ethics committee of Sichuan University (No. K2014025) and was registered in Clinical Trial Center of China (No. ChiCTR-IOR-14005644). All subjects signed the informed consent. Patients with end-stage renal disease who underwent maintenance hemodialysis (GFR<10.5ml/(min*1.73m$^2$); blood creatinine Scr > 707 μmol /L) from June 2015 to June 2017 in the Blood Purification Center of Chinese Medicine Hospital Affiliated to Southwest Medical University were selected. Inclusion criteria: (1) 18-75 years old; (2) meet the diagnostic criteria of chronic renal failure; (3) maintenance hemodialysis. Exclusion criteria: (1) patients with severe infection, heart failure, hypertension, diabetes, poor control of hypotension, or mental illness; (2) patients with severe drug allergies or hemodialysis anaphylaxis. Finally, a total of 100 patients were randomly divided into three groups: KFC 1.5-months treatment (n=30), KFC 3-months treatment (n=30), and control groups (n=40). (age 57.22±2.18, 55.34±2.07, 56.11±2.36; Male: Female 19:21, 17:13, 14:16; control, KFC 1.5-months, and KFC 3-months, P>0.05;). Both groups were given basic treatment. The KFC treatment groups were treated with traditional Chinese medicine KFC for 1.5 or 3 months, respectively. The control group was treated with traditional Chinese medicine placebo. After 3 months, the changes in residual renal function, metabolism, nutrition and oxidative stress in the two groups were detected by blood tests (Hirson Meikang, XN series), semiconductor laser, flow cytometry, nucleic acid fluorescence staining, hypersensitivity c-reactive protein, immunoturbidimetry and chemiluminescence to analysis the outcome of KFC. The patients were treated with Campbell F14 dialyzer (polysulfone membrane, membrane area 1.2) with bicarbonate buffer and ultrapure dialysate, 3 times per week.

### KFC treatment

All KFC water pan pills were prepared by the Chinese Medicine Hospital Affiliated Southwest Medical University as water pan granulation, three times a day, each 10 g. The compositions of KFC were monarch astragalus 50 g, Chen Yan Panax powder(Blunt) 10 g, Angelica 10 g, kelp 12 g, oyster 20 g, Rhubarb 6 g, and Achyranthes 30 g.

### Metabolomics detection and data analysis

For sample preparation, 200 μL of serum was added to 400 μL of methanol and vortex for 30 s. After placed 2h at -20 ℃, the samples were centrifuged at 13,000 rpm for 15 min at 4 ° C, vacuum pumping dry. Then, 200 μl of 50% aqueous methanol solution was added and dissolved by vortexed for 30s, centrifuged at 13000 rmp for 15 min, and filtered through 0.22 μm filter. 20 μl of each sample was mixed to make a QC sample. Ultra Efficient Liquid Phase was performed by Ekspert UltraLC 110 (AB Sciex) with water: acetonitrile: formic acid 900: 100: 1, and acetonitrile: water: formic acid 900:100:1. Column was ACQUITY UPLC HSS T3 1.8 μm 2.1 × 100 mm (Waters). Primary and secondary mass spectrometry data acquisition were performed by the AB 5600+ Triple TOF mass spectrometer (AB Sciex) based on the IDA function, under the control of software (Analyst TF 1.7, AB Sciex) with a primary sweep span of m/z (50-1000), a secondary sweep span of m/z (25-1000), secondary bombardment energy: 30eV. The ESI source parameters were set as follows: atomization pressure (GS1): 55 Pa; assist pressure: 55 Pa; air curtain pressure: 40 Pa; temperature: 550 ° C; spray voltage: 5500V (positive ion mode).

For the data identified by MSDIAL alignment, CV value was controlled less than 30% using QC sample, and then the ion peak with >50% missing value was deleted. The Pareto-scaling method was used for normalization and the MetaboAnalyst software was used for difference analysis and enrichment analysis. P<0.05 was considered as statistical significant.

## Results

### Clinical outcome of KFC

A total of 100 patients with end-stage renal disease who underwent maintenance hemodialysis were selected and randomly divided into three groups. The residual Renal function, metabolism, nutrition and oxidative stress in control and KFC 3-month treatment groups were detected and data showed significant differences in urea reduction ratio (URR), Creatinine, and urea between the two groups (Table 1), suggesting KFC protects renal function.

### PCA analysis

As shown in Figure 1, we examined the serum metabolites of hemodialysis patients with KFC treatment. To identify the differential metabolites from different groups (control vs. KFC 3-months, control vs. KFC 1.5-months, KFC 3-months vs. KFC 1.5-months), we analysis the sample by PCA method and found the metabolites were different among the treatments.

### Identification of differential metabolites

In order to identify the differential metabolites, we conducted a volcano plot analysis of the metabolites (Figure 2). There were 121 differential metabolites in KFC 3-months group, compared with untreated

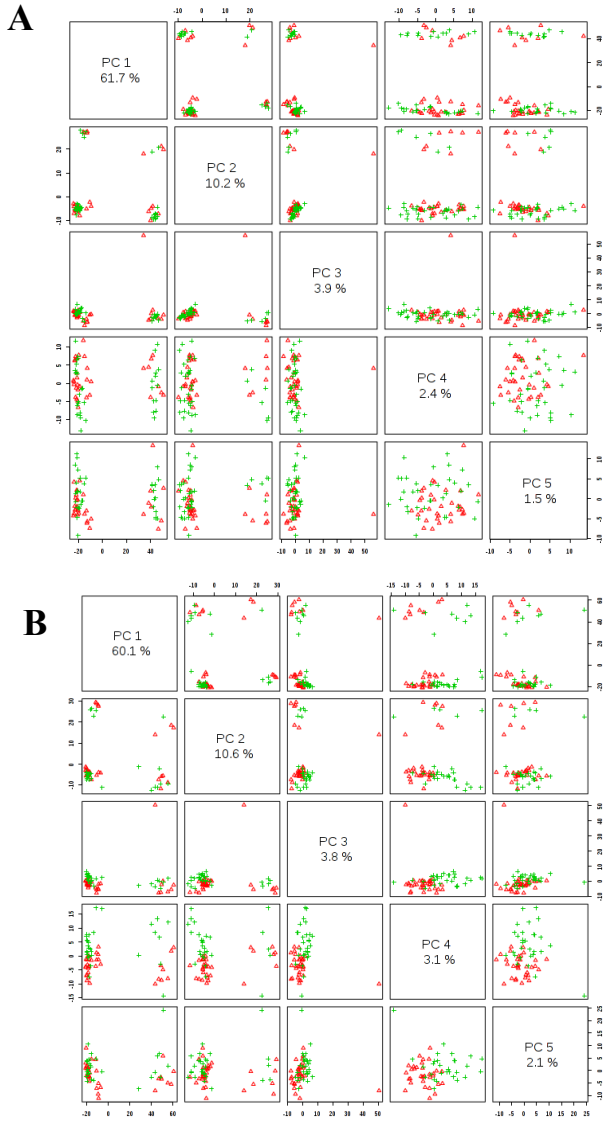


**Figure 1.** PCA analysis. The corresponding diagonal cells show the value of the variance of the major components. A: control vs. KFC 3-months; B: control vs. KFC 1.5-months.

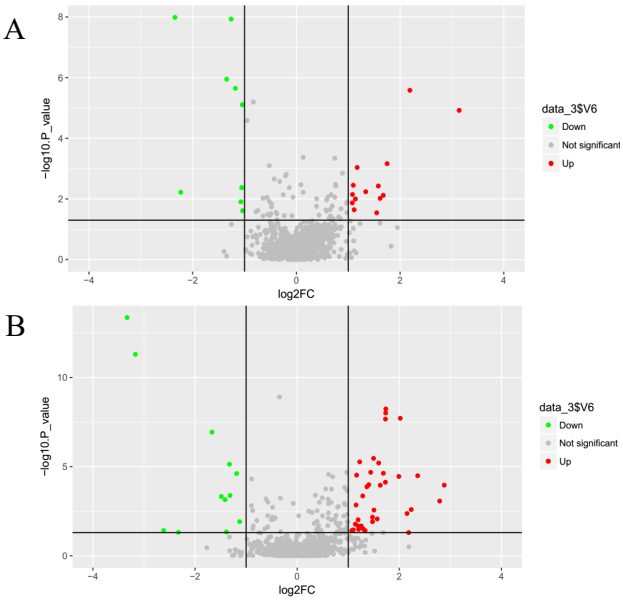


**Figure 2.** Volcano Plot. Red dots represent significant differential metabolites (FC> 2, P <0.05). A: control vs. KFC 3-months; B: control vs. KFC 1.5-months.

**Table 1.** The clinical results between control group and KFC treatment group (3-months) (x±s).

| Indicators | Control | KFC |
|---|---|---|
| age | 58.18±12.65 | 55.77±12.86 |
| height | 1.62±0.07 | 1.63±0.08 |
| body weight | 57.16±9.54 | 59.27±12.84 |
| BMI | 21.76±3.31 | 22.28±3.69 |
| Body surface area | 1.69±0.14 | 1.72±0.19 |
| Dialysis time | 3.99±0.09 | 3.97±0.12 |
| Ultrafiltration volume | 2.78±1.11 | 2.65±1.28 |
| URR | 65.71±7.25 | 34.05±6.94* |
| spKt/V | 1.28±0.25 | 1.22±0.2 |
| leukocyte | 5.85±1.55 | 6.5±1.95 |
| Hemoglobin | 105.52±18.01 | 106.11±16.07 |
| Hematocrit | 33.44±5.48 | 33.73±4.7 |
| Platelets | 178.67±66.47 | 164.46±57.82 |
| Blood total calcium | 2.11±0.26 | 2.12±0.14 |
| Phosphorus if = s | 1.89±0.57 | 1.94±0.56 |
| iPTH | 435.27±461.21 | 403.13±361.65 |
| Serum iron | 9.83±3.31 | 12.79±6.4 |
| Total iron bond | 39.89±14.17 | 39.83±5.14 |
| Transfer saturation | 27.15±13.9 | 31.96±14.69 |
| Ferritin | 252.56±237.94 | 221.2±163.78 |
| Urea (mmol / L) | 15.27±3.76 | 7.89±2.87* |
| Creatinine (μmol / L) | 994.03±278.26 | 187.09±95.52* |
| Blood total protein | 63.15±6.78 | 65.23±4.72 |
| Serum albumin | 38.68±3.58 | 39.31±2.94 |
| AST aspartate aminotransferase | 14.71±6.32 | 16.31±12.86 |
| ALT alanine aminotransferase | 16.24±12.61 | 18.91±13.51 |
| Total bilirubin | 7.65±2.19 | 7.97±2.54 |
| Triglycerides | 1.78±1.07 | 1.81±1.18 |
| Total cholesterol | 3.96±1.53 | 3.76±0.98 |
| Low-density lipoprotein | 2.49±1.41 | 2.37±0.79 |
| High-density lipoprotein | 1.05±0.25 | 1.06±0.31 |
| blood sugar | 7.47±2.76 | 8.03±3.31 |
| Potassium | 4.64±0.91 | 4.92±0.71 |
| Blood sodium | 137.5±3.04 | 136.46±2.08 |
| Blood chlorine | 100.92±4.93 | 101.43±3.8 |
| carbon dioxide | 20.33±2.91 | 18.66±3.4 |
| C-reactive protein | 4.08±6.27 | 4.92±5.97 |
| Urine volume (ml) | 238.53±382.84 | 325.14±293.22 |

control, of which 75 were significantly upregulated and 46 were significantly downregulated. In the KFC 1.5-months group, there were 365 metabolites, of which 164 were significantly upregulated and 192 downregulated. If threshold set as P<0.05, fold change greater than 2, and VIP>1, there were 6 metabolites upregulated in KFC 3-months group including cis-Aconitate, Nigrosporapyrone D, PS 27:6, Petunidin, MGDG 30:8, and 2-(hydroxymethyl)-6-[[(1R,4S)-2,2,4-trimethyl-3-oxabicyclo[2.2.2]octan-5-yl]oxy]oxane-3,4,5-triol (Table 2). Among them, MGDG 30:8 and 2-(hydroxymethyl)-6-[[(1R,4S)-2,2,4-trimethyl-3-oxa-

**Table 2.** Maximum differential metabolites between control and KFC 3-months group.

| Metabolite name | FC | raw.pval | VIP |
|---|---|---|---|
| cis-Aconitate | 2.1397 | 0.0035332 | 2.3984 |
| Nigrosporapyrone D | 2.1639 | 0.022728 | 1.8964 |
| PS 27:6 | 2.2026 | 0.010026 | 2.132 |
| Petunidin | 3.063 | 0.0095707 | 2.1446 |
| MGDG 30:8 | 4.5594 | 0.000002617 | 3.6755 |
| 2-(hydroxymethyl)-6-[[(1R,4S)-2,2,4-trimethyl-3-oxabicyclo[2.2.2]octan-5-yl]oxy]oxane-3,4,5-triol | 8.8146 | 0.000011996 | 3.4606 |

**Table 3.** Maximum differential metabolites between control and KFC 1.5-months group.

| Metabolite name | FC | raw.pval | VIP |
|---|---|---|---|
| lysoPC 18:1 | 2.24 | 0.000029789 | 3.6016 |
| lysoPE 18:0 | 2.3363 | 5.3882E-06 | 3.8736 |
| Nadolol | 2.4343 | 0.00043716 | 3.0961 |
| Nigrosporapyrone D | 2.4469 | 0.030483 | 1.9661 |
| lysoPC 18:0 | 2.5754 | 0.00013442 | 3.3317 |
| lysoPC 18:0 | 2.6432 | 0.00010247 | 3.3827 |
| Dorzolamide hydrochloride130693-82-2 | 2.7797 | 0.0068174 | 2.4318 |
| lysoPC 18:0 | 2.8198 | 3.3996E-06 | 3.9415 |
| lysoPC 18:0 | 3.0243 | 6.1945E-06 | 3.8526 |
| cis-Aconitate | 3.09 | 0.00011035 | 3.3689 |
| MGDG 30:8 | 3.2219 | 0.000023446 | 3.6417 |
| Putative_Alantolactone | 4.4424 | 0.00424 | 2.5613 |
| (2S)-2-[(1S)-1-hydroxypentyl]-4-methoxy-2,3-dihydropyran-6-one | 4.7079 | 0.002533 | 2.6939 |
| lysoPC 18:1 | 5.1349 | 0.000032213 | 3.5884 |
| 2-(hydroxymethyl)-6-[[(1R,4S)-2,2,4-trimethyl-3-oxabicyclo[2.2.2]octan-5-yl]oxy]oxane-3,4,5-triol | 7.3634 | 0.00010889 | 3.3714 |

bicyclo[2.2.2]octan-5-yl]oxy]oxane-3,4,5-triol were more than three-fold (Table 2). In the KFC 1.5-months group, there were 15 metabolites upregulated 2-fold, including lysoPC 18:1, lysoPE 18:0, Nadolol, Nigrosporapyrone D, lysoPC 18:0, lysoPC 18:0, Dorzolamide hydrochloride130693-82-2, lysoPC 18:0, lysoPC 18:0, cis-Aconitate, MGDG 30:8, Putative_Alantolactone, (2S)-2-[(1S)-1-hydroxypentyl]-4-methoxy-2,3-dihydropyran-6-one, lysoPC 18:1, and 2-(hydroxymethyl)-6-[[(1R,4S)-2,2,4-trimethyl-3-oxabicyclo[2.2.2]octan-5-yl]oxy]oxane-3,4,5-triol (Table 3). Among them, 2-(hydroxymethyl)-6-[[(1R,4S)-2,2,4-trimethyl-3-oxabicyclo[2.2.2]octan-5-yl]oxy]oxane-3,4,5-triol upregulated 7-fold (Table 3). Moreover, more than 60 new metabolites were identified in the peripheral blood in KFC treated groups. Among them, MGDG 30:8 and 2-(hydroxymethyl)-6-[[(1R,4S)-2,2,4-trimethyl-3-oxabicyclo[2.2.2]octan-5-yl]oxy]oxane-3,4,5-triol were two potential diagnostic markers.

## Cluster analysis

Cluster analysis of all metabolites was performed (Figure 3). Results showed that the differential metabolites separated the control and KFC 3-months group (Figure 3A), and the control and KFC 1.5-months group (Figure 3B), suggesting that metabolites clustered in the same cluster may be involved in the same metabolic process.

## KEGG pathway enrichment

The KEGG database was used to analyze the enrichment pathways of metabolites with VIP> 1 by PLSDA. The results showed that the differential metabolites mainly involved in Arginine and proline metabolism, Urea cycle, Tyrosine metabolism, Methionine metabolism, Tricarboxylic acid cycle, and Androgen and estrogen metabolism. The main metabolic pathways were shown in Figure 4.

## Discussion

We here investigated the metabolic profiles in peripheral blood of hemodialysis patients administrated by KFC and explored the potential metabolic mechanism using UPLC. Improving the diagnosis and prevention and treatment of complications in patients with MHD is of great importance to reduce the incidence of complications and patient mortality. In recent years UPLC-MS and 1H NMR techniques have been used to study

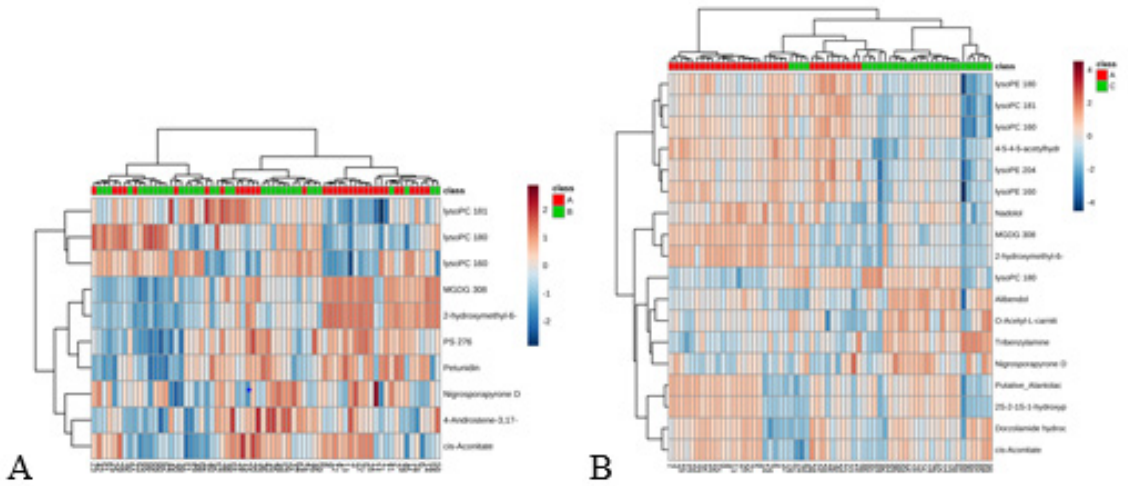


**Figure 3.** Hierarchical clustering results of differential metabolites. A: control vs. KFC 3-months; B: control vs. KFC 1.5-months.

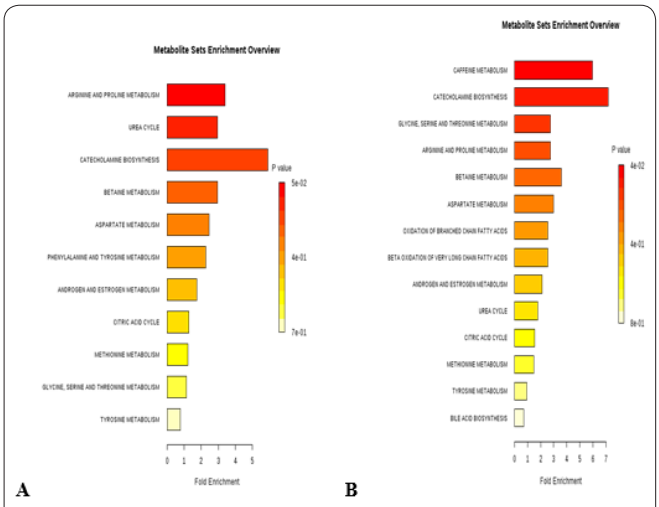


**Figure 4.** KEGG Enrichment**.** A: control vs. KFC 3-months; B: control vs. KFC 1.5-months.

serum or plasma metabolites in CRF patients. Researchers developed an UPLC-MS method to analyze plasma samples from 10 ESRD patients undergoing hemodialysis and 16 healthy subjects. 1-methylinosine was found to be a valid candidate biomarker for adequate doses of hemodialysis (13). Known uremic retention solutes, such as urea, creatinine, inositol, and trimethylamine-N-oxide, are all increased in dialysis patients (14). The 1H NMR-based metabolomics method was applied to the serum CKD profiles of 80 patients with stage 4 CKD and 28 healthy controls. Glucose, lactate, valine, alanine, glutamic acid, glycine, betaine, inositol, taurine and glycerophosphocholine are considered important endogenous metabolites that differentiate CKD at different stages (15, 16). The results show that glycolysis, amino acid and organic infiltration of metabolic abnormalities affect the process of CKD. Based on these metabolites, the diagnostic sensitivity and specificity for patients with CKD reach 100% (17). The study shows that the serum metabolic profile changes in renal insufficiency and CKD progression (18). In summary, the metabolic phenotype of CKD has been identified in several fields by metabonomics.

In these studies, the use of metabolic markers in the diagnosis of CKD demonstrates the immense potential of metabolites in clinical applications. Rapid peripheral blood markers can be used to diagnose or eliminate CKD rapidly and to assess various post-treatment evaluations. However, few researches focus on the diagnostic markers of CKD peripheral blood in the past, especially the evaluation of the residual renal function in hemodialysis patients after various medical treatments. With the development of HPLC-MS technology, people know more about small-molecule metabolic pathways and the small-molecule databases are gradually perfected. Therefore, more and more attention has been paid to peripheral blood metabolic markers. In the previous work, we found that the KFC can improve renal function and quality of life in patients with kidney disease (19). KFC can reduce proteinuria, serum creatinine, and urea nitrogen in rats with chronic kidney disease (20), thereby protecting renal function. KFC may inhibit renal interstitial fibrosis through the TGF-β1/Smad signaling pathway (21).

In this study, we found that 121 metabolites were different between KFC treated group (3 months) and untreated control, of which 75 were significantly upregulated and 46 were significantly downregulated. In the treatment for 1.5 months, there were 365 metabolites, of which 164 were significantly upregulated and 192 downregulated. There were 6 metabolites and 15 metabolites upregulated 3-fold in 3-months and 1.5-months KFC treatment group, respectively. In addition, more than 60 new metabolites were identified in the peripheral blood in KFC treated patients, including two potential diagnostic markers MGDG 30:8 and 2-(hydroxymethyl)-6-[[(1R,4S) -2,2,4-trimethyl-3-oxabicyclo[2.2.2]octan-5-yl]oxy]oxane-3,4,5-triol. The pathway enrichment analysis showed the differential metabolites mainly enriched in Arginine and proline metabolism, Urea cycle, Tyrosine metabolism, Methionine metabolism, Tricarboxylic acid cycle, and Androgen and estrogen metabolism.

This study has some limitations, trapped in research capacity and research funding, this article did not detect the CKD-related protein markers and blood-related protease after TCM treatment that reported in literatures. Whether the changes in CKD-related protein markers and blood-related protease induced by TCM is still unclear, this should be clarified in the future.

To sum up, by detecting the changes in metabolites and metabolism pathway, 60 new compounds with two potential diagnostic markers were identified. In addition, we found that the protective effect of traditional Chinese medicine KFC on residual renal function in hemodialysis patients may be related to Arginine and proline metabolism, Urea cycle and Tyrosine metabolism pathways. The findings are helpful to reveal the mechanism of KFC protects CKD, and to provide a new strategy for recovery renal function in hemodialysis patients.

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