Original Paper

Metabolite Profiling of Feces and Serum in Hemodialysis Patients and the Effect of Medicinal Charcoal Tablets

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Key Words

UPLC/MS • Fecal metabolomics • Serum metabolomics • Medicinal charcoal tablets • Hemodialysis

Abstract

Background/Aims: Recently, the colon has been recognized as an important source of various uremic toxins in patients with end stage renal disease. Medicinal charcoal tablets are an oral adsorbent that are widely used in patients with chronic kidney disease in China to remove creatinine and urea from the colon. A parallel fecal and serum metabolomics study was performed to determine comprehensive metabolic profiles of patients receiving hemodialysis (HD). The effects of medicinal charcoal tablets on the fecal and serum metabolomes of HD patients were also investigated. **Methods:** Ultra-performance liquid chromatography/mass spectrometry was used to investigate the fecal and serum metabolic profiles of 20 healthy controls and 31 HD patients before and after taking medicinal charcoal tablets for 3 months. **Results:** There were distinct metabolic variations between the HD patients and healthy controls both in the feces and serum according to multivariate data analysis. Metabolic disturbances of alanine, aspartate and glutamate metabolism, arginine and proline metabolism figured prominently in the serum. However, in the feces, alterations of tryptophan metabolism, lysine degradation and beta-alanine metabolism were pronounced, and the levels of several amino acids (leucine, phenylalanine, lysine, histidine, methionine, tyrosine, and tryptophan) were increased dramatically. Nineteen fecal metabolites and 21 serum metabolites were also identified as biomarkers that contributed to the metabolic differences. Additionally, medicinal charcoal treatment generally enabled the serum and fecal metabolomes of the HD patients to draw close to those of the control subjects, especially the serum metabolic profile. **Conclusion:** Parallel fecal and serum metabolomics uncovered the systematic metabolic variations of HD

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patients, especially disturbances in amino acid metabolism in the colon. Medicinal charcoal tablets had an impact on the serum and fecal metabolomes of HD patients, but their exact effects still need to be studied further.

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Introduction

Chronic kidney disease (CKD) has become a global public health problem, and most patients with CKD progress to end stage renal disease (ESRD). Due to the loss of renal function, numerous waste products accumulate in the body, and these compounds are typically called “uremic retention molecules.” Recent evidence [1, 2] has suggested that the colon is an important source of numerous uremic retention molecules such as indoxyl sulfate and p-cresol. Many factors, such as a reduced intake of dietary fibres and increased blood ammonia concentrations, contribute to disturbances of the colonic microbiota and metabolism in patients with uremia [2, 3]. Thus, it is essential to study colonic co-metabolism in uremic patients. In addition, due to the limitation of current hemodialysis strategies to remove protein-bound toxins, other therapies are needed for more efficacious toxin elimination. Drug therapies targeting the colon, mainly including modulating the composition of the microflora and adsorbing uremic solutes in the gut, have raised a number of concerns. Due to its strong adsorption ability, activated carbon has been used in CKD patients to reduce serum uremic solutes. Medicinal charcoal tablets (Changtian Pharmaceutical Co., Ltd., Hebei, China) are an oral activated carbon adsorbent that have been used widely in CKD patients in China as an adjuvant therapy to decrease creatinine and urea levels. These tablets are insoluble in water and cannot be degraded by digestive juices or intestinal bacteria. Every gram of medicinal charcoal has a specific surface area of 1000–1400 m² and can strongly adsorb small/medium-sized molecular toxins in the colon to help the body remove these toxins.

Metabolomics, an important evolving tool of systems biology, aims to acquire an overview of metabolic status and to identify novel potential biomarkers [4]. Metabolomics approaches have already been applied to study the metabolic profile of serum, urine, and hemodialysate in CKD patients at different stages [5–7]. However, metabolomics analyses of fecal samples in CKD patients are still rare. Since fecal samples can be collected easily and noninvasively, they are a good choice for the study of metabolism in the colon. In addition, fecal samples can well reflect the status of the colorectum [8]. It is believed that fecal water interacts directly with colonic epithelial cells [9], and in colonic tissue, the compounds in fecal water seem to have a more important influence than those in the blood [10]. Thus, in this study, we enrolled patients undergoing hemodialysis (HD) who were to be administered medicinal charcoal therapy and healthy controls to investigate the fecal and serum metabolic profiles by ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) in order to characterize colonic co-metabolism in uremic patients and to investigate the effects of medicinal charcoal in HD patients.

Materials and Methods

Study population

We enrolled 31 maintenance HD outpatients who were to be administered medicinal charcoal tablets (Changtian Pharmaceutical Co., Ltd., Hebei, China) in the Nephrology Department of Hanzhong Central Hospital from November to December 2015. Only stable chronic HD patients without gastrointestinal diseases, diabetes, recent infections, or intake of antibiotics, probiotics, or immunosuppressive agents in the last 4 weeks before the study were included. All of them were prescribed medicinal charcoal tablets (1.5 g each, 3 times daily) according to the instructions. The medicinal charcoal was administered after a lag of 60 min or between meals and at least 1 h after the administration of other drugs. All patients were followed

up for 3 months. Blood and fecal samples were collected at baseline and after treatment for 3 months. Samples collected at baseline and after treatment were marked as the disease (D) group and treatment (T) group, respectively. Additionally, 20 healthy individuals were recruited as the control (CT) group from the medical examination center, and blood and fecal samples were obtained from these subjects. The study protocol complied with the Declaration of Helsinki. The ethical Committees of First Affiliated Hospital of Xi'an Jiaotong University on research involving human subjects approved this study, and informed consent was obtained from each patient and each healthy volunteer.

Sample Preparation

All of the subjects had been fasting at the time of blood sampling. Serum was obtained by centrifuging the blood specimen for 10 min at 3000×g, and was then stored at –80°C. Before analysis, 100 µl serum was centrifuged at 14000×g for 20 min at 4°C. Next, methanol (pre-chilled to –80°C) was added to the supernatant to make a final 80% (v/v) methanol solution, and the mixture was thoroughly mixed, incubated at –80 °C for 1 h and centrifuged at 14000×g for 10 min at 4°C. The supernatant was transferred to a new Eppendorf tube, and another 200 µl methanol (pre-chilled at –80°C) was taken to vortex for 1 min at 4°C, incubate at –80°C for 1 h and centrifuge at 14000×g for 20 min at 4°C. Finally, the supernatant from both extractions were transferred and combined together.

All fecal samples were aseptically taken and stored immediately at –80°C. Before analysis, fecal water was extracted via taking a weighed sample of thawed stool and mixing with methanol (pre-chilled at –80°C) in the ratio 1:10(g:vol = feces: methanol). The mixtures were ground for 2 min, vortexed for 3 min, incubate at –80°C for 1 h and centrifuged at 14000×g for 20 min at 4°C. The supernatants were transferred and filtered through a membrane (0.22 µm pore size), and the filtered fecal water was directly injected onto the UPLC.

UPLC/MS measurement for metabolome analysis

The metabolites were separated with a Dionex Ultimate 3000 Ultra Performance LC system (Thermo Fisher Scientific, Waltham, MA) on an ACQUITY UPLC BEH Amide column (1.7 µm, 2.1×100 mm, Waters) at 0.25 ml/min with the column maintained at 35°C. Next, 1µl supernatant was loaded onto the normal phase chromatography column, and then the sample was eluted into an orbitrap mass spectrometer with an aqueous solution containing 10 mmol/l ammonium acetate as an eluent from 1% to 99% within 15 min. The stationary phase of the positive mode was 95% acetonitrile with 10 mmol/l ammonium acetate (PH=9). The stationary phase of the negative mode was 95% acetonitrile with 10 mmol/l ammonium formate (PH=3).

The UPLC system was coupled to a Q-Exactive plus orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a heated electrospray ionization (HESI) probe. The data with mass ranges of m/z 70-1050 and m/z 80-1200 were acquired at positive ion mode and negative ion mode with data-dependent MS/MS acquisition. The full scan and fragment spectra were collected with resolution of 70000 and 17500, respectively. The source parameters are as follows: spray voltage: 3000 V; capillary temperature: 320°C; heater temperature: 300°C; sheath gas flow rate: 35; and auxiliary gas flow rate: 10. Ammonium acetate, acetonitrile, ammonium formate and HPLC-MS grade solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Data processing and statistical analysis

TraceFinder software (Thermo Fisher Scientific, Waltham, MA) was used to acquire and process the data. Three-dimensional (3D) data arrays of paired retention time-m/z, sample names, and intensity data were obtained. Metabolite identification was based on a TraceFinder search with a home-built database, with an MS1 mass accuracy error within 8 ppm and MS2 mass accuracy error within 15 ppm for all metabolites. Differential peak identities were confirmed further by retention time and compared with the available mass spectra of standard compounds from a home-built standards database. The data arrays were exported to Simca-P 14.0 software (Umetrics, Umeå, Sweden), where orthogonal partial least squares discriminate analysis (OPLS-DA) was performed. Unit variance scaling was used to normalize the spectral intensity data. Differential biomarkers were selected based on a threshold of variable influence on projection (VIP) values greater than 1.0 according to OPLS-DA, where maximum variations playing important roles in the separation were revealed. To globally analyze metabolomics differences between the CT and D groups and D and T groups, nonparametric Mann-Whitney U test analysis and fold change (D/CT group and T/D group) were

performed by SPSS 18.0. The false discovery rate (FDR) was used to identify the differential metabolites further ($p < 0.05$). The area under the curve of the selected biomarkers was calculated by MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca/>).

Results

Fecal and serum samples from 31 HD patients at baseline and after taking medicinal charcoal tablets for 3 months and from 20 healthy individuals were collected and analyzed by UPLC/MS. The clinical characteristics of the study population are listed in Table 1.

Distinct fecal and serum metabolic patterns in HD patients

Non-targeted metabolomics analyses based on UPLC/MS were applied to investigate serum and fecal metabolites in healthy controls and HD patients before and after taking medicinal charcoal tablets for 3 months. After the 80% rule was used to exclude missing values and data filtering, a total of 298 and 295 metabolites were identified in feces and serum, respectively, and used for subsequent statistical analysis. To highlight the holistic distribution, clustering, and trends, and to identify maximum variations in the data set, OPLS-DA was performed on the preprocessed UPLC/MS data arrays. The score plots between the CT and D groups in the feces and serum (Supplementary Fig. 1) showed clear profile separation between both groups. For all supplemental material see www.karger.com/doi/10.1159/000489912. The two-dimensional (2D) and 3D OPLS-DA score plots of fecal and serum metabolite profiling among the CT, D, and T groups (Fig. 1) showed that the three groups could be distinguished clearly by serum metabolite profiling with good model fitness and predictability (R^2 and Q^2); however, the fecal profiles of the D and T groups overlapped partially with each other. Additionally, according to the gaps of the congeries between the three groups in the 3D score plots, the T group was broadly located in the middle of the CT and D groups, indicating that medicinal charcoal treatment enabled the serum and fecal metabolomes of the HD patients to draw close to those of the control subjects.

Fecal and serum metabolic differences between HD patients and healthy individuals

A nonparametric test was conducted to identify the significantly altered metabolites in the HD patients. Compared with the healthy individuals, 192 and 65 compounds in the serum and feces, respectively, showed a remarkable change in the HD group. The results are exhibited in heat maps shown in Supplementary Fig. 2A and 2B. The relative intensity of compounds in region I was decreased in the HD patients with FC (D group vs. CT group) < 0.5 and $FDR < 0.05$, and the other compounds in region II were increased in the HD patients with FC (D group vs. CT group) > 2 and $FDR < 0.05$. The significantly altered metabolites ($FDR < 0.05$) in the feces and serum were then used to analyze further the differential metabolic pathways in the HD patients by MetaboAnalyst 3.0. The results revealed that the metabolic pathways of amino acids were significantly altered in both the serum and feces

Table 1. Clinical characteristics of the study population. Values shown are the mean $\pm$ standard deviation; NA, not applicable; BMI, body mass index; ^a $p > 0.05$ vs. D group and CT group; ^b $p < 0.05$ vs. D group and CT group; *bowel habits were obtained via the gastrointestinal symptom rating scale; in the D group, there were 3 cases with self-reported constipation, and all subjects in the CT group had normal bowel habits

Variable	D group	CT group
Number	31	20
Age (year)	47.0 $\pm$ 10.3 ^a	48.7 $\pm$ 3.2
Sex (M/F)	15/16 ^a	10/10
BMI (kg/m ²)	20.4 $\pm$ 1.7 ^a	20.57 $\pm$ 1.11
Creatinine (μ mol/L)	1000.67 $\pm$ 299.281 ^b	83.05 $\pm$ 10.92
Hemodialysis age (months)	23.73 $\pm$ 26.82	NA
Etiology of ESRD	26 glomerular disease, 1 hypertension, 1 polycystic kidney, 1 vasculitis, 1 obstructive nephropathy, 1 unknown	NA
Bowel habits*	3 with constipation	Normal

Fig. 1. OPLS-DA 2D and 3D score plots of the metabolic profiles from the CT, D, and T groups. (A) and (B) 2D and 3D score plots of serum samples from the CT, D, and T groups. $R^2X = 0.522$, $R^2Y = 0.964$, $Q^2 = 0.923$. (C) and (D) 2D and 3D score plots of fecal samples from the CT, D, and T groups. $R^2X = 0.364$, $R^2Y = 0.842$, $Q^2 = 0.536$.

(Fig. 2A and 2B). Metabolic disturbances of alanine, aspartate and glutamate, arginine and proline metabolism figured prominently in the serum; however, alterations of tryptophan metabolism, lysine degradation and beta-alanine metabolism were more pronounced in the feces. In addition, perturbations of the pathways of tryptophan metabolism, lysine degradation and phenylalanine metabolism were prominent in both the serum and feces (Fig. 2C).

Biomarkers were selected between the CT and D groups using VIP values (>1.0) from OPLS-DA and FDR (<0.05). When a VIP value is greater than 1.0, the variable is considered to make a contribution to the clustering of different groups on OPLS-DA. As shown in Table 2, 21 and 19 differential metabolites in the serum and feces, respectively, were picked as biomarkers of HD patients. In the serum metabolic profile, indoxyl sulfate, creatinine, citric acid cycle intermediates (citric acid and cis-aconitic acid), acetylcarnitine, choline, hippuric acid, asymmetric dimethylarginine (ADMA), N^1 -acetyl- N^2 -formyl-5-methoxykynuramine (AFMK), 3-methylhistidine, 2-keto-6-aminocaproate, L-proline, and citrulline were up-regulated in the HD patients; glycerophosphocholine, 3-methyl-2-oxovaleric acid, betaine, hypoxanthine, L-arginine, L-glutamine, L-lysine, and L-tryptophan were down-regulated in the HD patients. In the fecal metabolic profile, uracil, 3-methyl-2-oxovaleric acid, creatine, succinic acid, 5-aminovalerate, urocanic acid, imidazoleacetic acid, tryptamine, and several amino acids (L-leucine, L-phenylalanine, L-lysine, beta-alanine, L-histidine, L-methionine, L-tyrosine, and L-tryptophan) were increased in the HD patients; conversely, 5-acetamidovalerate, 3-phenylpropionic acid, and 5-methoxytryptamine were decreased. The area under the receiver operating characteristic curve of the biomarkers was also calculated (Table 2 and Supplementary Fig. 3).

Fig. 2. Metabolic pathway comparison between the controls and HD patients. (A) Changed pathways in HD patients in the serum. (B) Changed pathways in HD patients in the feces. (C) Detailed changes in three important metabolic pathways (tryptophan metabolism, lysine degradation, and phenylalanine metabolism), which figured prominently in both the serum and feces. Red represents metabolites detected in the serum; blue represents metabolites detected in the feces; green represents metabolites detected both in the serum and feces. The red arrows in the boxes represent the metabolites significantly changed in the serum (ratio D/CT > 2 or < 0.5, FDR < 0.05). The blue arrows in the boxes represent the metabolites significantly changed in the feces (ratio D/CT > 2 or < 0.5, FDR < 0.05). The dotted arrows indicate multiple processes, while the solid arrows indicate a single process.

Additionally, it is worth mentioning that three oxidative stress-associated metabolites were significantly increased (FDR < 0.0001) in the serum of the HD patients, namely, N¹-acetyl-5-methoxykynuramine (AMK), L-methionine S-oxide (MetSO), and the aforementioned biomarker AFMK (Fig. 3). AFMK produced by melatonin oxidation is formed actively in inflammation [11]. In turn, AMK is formed by the deformylation of AFMK [12]. Both of them have been reported to be strong free radical scavengers and exert antioxidant activity [11, 13, 14]. MetSO is a marker of oxidative damage to proteins [15]. Methionine side chains are highly susceptible to oxidation and can react directly with reactive oxygen species to generate oxidative modifications, resulting mainly in MetSO [16]. Free MetSO in serum primarily originates from the proteolysis of oxidized proteins [16].

Impact of medicinal charcoal tablets on the differential fecal and serum metabolites of HD patients

As shown in the heat map (Supplementary Fig. 2B), in region IV, the metabolites that were increased in the feces of the HD patients were slightly decreased after treatment, but in regions I and III, the relative intensity of the changed metabolites in the feces was basically stable in the D and T groups. By contrast, the alterations of serum metabolites in the HD patients were more visible after treatment. In region III of Supplementary Fig. 2A, the metabolites that were decreased in the HD patients demonstrated a certain recovery after

Table 2. Biomarkers identified in the serum and fecal metabolic profiles of HD patients. ^aArea under the receiver operating characteristic (ROC) curve of the biomarkers; ^bsensitivity and ^cspecificity were calculated from the ROC curve; AMDA: asymmetric dimethylarginine; AFMK: N¹-acetyl-N²-formyl-5-methoxykynuramine; AUC: area under the curve

Sample	Metabolite	VIP	D vs. CT		T vs. D		ROC analysis		
			FC	FDR	FC	FDR	AUC ^a	Sensitivity ^b	Specificity ^c
Serum	Indoxyl sulfate	9.04	17.05	<0.001	1.27	0.111	1.0	1.0	1.0
	Creatinine	8.57	11.54	<0.001	1.12	0.206	1.0	1.0	1.0
	Citric acid	6.77	13.62	<0.001	0.16	<0.001	0.98	0.97	1.0
	Glycerophosphocholine	6.61	0.14	<0.001	4.36	<0.001	1.0	1.0	1.0
	Acetylcarnitine	4.45	193.88	<0.001	0.53	0.005	1.0	1.0	1.0
	Choline	3.69	2.45	<0.001	1.05	0.658	0.99	1.0	0.95
	3-Methyl-2-oxovaleric acid	2.93	0.66	<0.001	0.95	0.896	0.84	0.65	1.0
	L-Proline	2.71	2.24	<0.001	0.64	<0.001	0.95	0.90	0.85
	cis-Aconitic acid	2.63	21.33	<0.001	0.43	<0.001	0.99	0.97	1.0
	Hippuric acid	2.43	33.25	<0.001	1.50	0.073	0.98	0.90	1.0
	AMDA	2.09	4.87	<0.001	1.53	0.078	0.99	0.97	0.95
	Betaine	2.04	0.78	<0.001	0.97	0.962	0.83	0.71	0.9
	L-Arginine	1.76	0.32	<0.001	2.73	<0.001	0.99	0.90	1.0
	AFMK	1.75	49.10	<0.001	1.13	0.550	0.99	0.97	1.0
	2-Keto-6-aminocaproate	1.74	40.27	<0.001	0.44	0.011	1.0	1.0	1.0
	3-Methylhistidine	1.55	15.01	<0.001	0.98	0.745	1.0	1.0	1.0
	L-Glutamine	1.53	0.74	<0.001	0.96	0.756	0.87	0.82	0.85
	Citrulline	1.11	4.76	<0.001	1.12	0.485	1.0	1.0	1.0
	L-Lysine	1.10	0.43	<0.001	1.37	<0.001	0.97	0.97	1.0
	L-Tryptophan	1.10	0.27	<0.001	1.42	0.002	1.0	1.0	1.0
	Hypoxanthine	1.08	0.40	<0.001	1.44	<0.001	0.99	0.97	1.0
Feces	Succinic acid	7.03	4.60	0.047	0.68	0.891	0.74	0.71	0.67
	5-Acetamidovalerate	6.10	0.05	<0.001	2.95	0.818	0.89	0.86	0.87
	L-Leucine	3.25	4.33	0.002	0.52	0.237	0.86	0.86	0.73
	L-Phenylalanine	3.24	5.10	<0.001	0.62	0.502	0.89	0.86	0.8
	3-Phenylpropanoic acid	2.90	0.36	0.006	1.07	0.921	0.82	0.82	0.73
	Uracil	2.89	3.67	0.004	0.77	0.319	0.82	0.71	0.87
	L-Lysine	2.83	3.24	0.006	0.55	0.187	0.81	0.75	0.87
	3-Methyl-2-oxovaleric acid	2.69	10.43	0.025	0.77	0.735	0.76	0.71	0.87
	Creatine	2.63	4.32	0.038	1.15	0.921	0.75	0.82	0.67
	5-Aminovalerate	2.54	3.49	0.001	0.69	0.410	0.86	0.89	0.73
	beta-Alanine	1.91	3.69	<0.001	0.59	0.187	0.92	0.89	0.87
	L-Histidine	1.83	8.39	0.004	0.42	0.203	0.83	0.79	0.73
	Urocanic acid	1.75	3.96	0.017	0.84	0.900	0.78	0.71	0.73
	L-Methionine	1.58	3.03	0.013	0.59	0.239	0.79	0.71	0.73
	Imidazoleacetic acid	1.53	4.04	0.017	0.79	0.387	0.78	0.64	0.93
	Tryptamine	1.39	2.62	0.016	0.18	0.187	0.78	0.79	0.73
	5-Methoxytryptamine	1.17	0.03	<0.001	0.88	0.866	0.93	0.86	0.87
	L-Tyrosine	1.15	3.66	0.007	0.64	0.672	0.81	0.82	0.73
	L-Tryptophan	1.09	3.25	0.011	0.45	0.061	0.79	0.86	0.8

treatment. In region IV of Supplementary Fig. 2A, the up-regulated metabolites in the serum were decreased after treatment in the HD patients.

As illustrated in Table 2, we found that the selected biomarkers of the fecal metabolic profile were scarcely influenced by medicinal charcoal tablets. However, among the biomarkers of the serum metabolic profile, the abundances of citric acid, cis-aconitic acid, glycerophosphocholine, acetylcarnitine, L-proline, L-arginine, L-lysine, L-tryptophan, 2-keto-6-aminocaproate, and hypoxanthine were changed after treatment for 3 months to levels that were significantly opposite to those found at baseline. Furthermore, among the three metabolites that might be biomarkers of oxidative stress, AMK showed a decrease in the serum of HD patients after treatment (FDR < 0.1, Fig. 3). In addition, AFMK and MetSO had a tendency to be down-regulated (Fig. 3).

Fig. 3. Oxidative stress-associated metabolites identified in the serum. Three oxidative stress-associated metabolites (AFMK, AMK, and MetSO) were significantly higher in the D group (FDR adjusted $p < 0.0001$). After treatment with medicinal charcoal for 3 months, AMK was decreased in HD patients (FDR adjusted $p = 0.09$). AFMK and MetSO also had a tendency to be down-regulated (FDR adjusted $p > 0.1$). AMK: N¹-acetyl-5-methoxykynuramine; MetSO: L-methionine S-oxide; AFMK: N¹-acetyl-N²-formyl-5-methoxykynuramine.

Discussion

Serum metabolism has been studied in different stages of CKD; however, the fecal metabolic profile of uremic patients has been explored rarely. In the current study, parallel metabolomics analysis of the feces and serum was conducted to comprehensively profile metabolic variations and identify differential metabolites between HD patients and healthy controls, and attempt to understand gut microbial and host metabolism in HD patients. Additionally, the effects of medicinal charcoal tablets, an adjunctive therapy to eliminate toxins by adsorption, on the fecal and serum metabolomes of HD patients were also investigated.

HD patients display distinct fecal metabolic features and prominent perturbations of the pathways of amino acid metabolism

According to the results of OPLS-DA, the fecal metabolic profile of HD patients was obviously different from the control group. Compared with healthy controls, the altered pathways in the colon of HD patients mainly involved amino acids metabolism. Among the selected biomarkers of the fecal metabolic profile in HD patients, the levels of several amino acids (leucine, phenylalanine, lysine, histidine, methionine, tyrosine, and tryptophan) were increased dramatically. Most of these amino acids are essential amino acids. This may indicate the impaired digestion and absorption of dietary proteins and amino acids in the upper digestive tract of uremic patients, due to disturbances related to protein breakdown, synthesis, and oxidation [17, 18]. Previous studies have shown that proteolytic bacteria, such as *Peptostreptococcaceae*, *Clostridium*, and *Enterobacteriaceae*, were more abundant in ESRD patients compared with controls [19, 20]. The large amount of undigested amino acids reaching the colon increases the availability of nitrogen, which may favor these proteolytic bacteria and leads to the formation of toxic metabolites such as phenolic compounds and amines [19, 20]. 5-Aminovalerate and 3-methyl-2-oxovalerate are degradation products of lysine and isoleucine, respectively, which could be generated by gut microflora metabolism [21, 22]. Especially, 5-aminovalerate is thought to be mainly a microbial metabolite produced by the gut microflora [21, 23]. The elevation in the fecal content of 5-aminovalerate and 3-methyl-2-oxovalerate suggests increased bacterial processing of dietary proteins in uremic patients. 5-Acetamidovalerate is also a metabolite in the lysine degradation pathway that can react reversibly with H₂O to generate acetate, which is a short chain fatty acid (SCFA). Vaziri and colleagues also demonstrated that *Lactobacillaceae* and *Prevotellaceae*, which are SCFA-producing bacteria, were at a low abundance in ESRD samples [19, 20]. The down-regulation of 5-acetamidovalerate in HD patients may be a result of the compensatory generation of acetate. In addition, the significant increase of succinic acid in the feces of

HD patients may be consistently related to the high levels of *Bacteroides* in the colon of HD patients [24], which produce succinic acid as a specific fermentation product [25]. These metabolic features describe that the alteration of gut microbial populations may have a direct influence on metabolic products.

In addition, compared with the CT group, 3-phenylpropionic acid was significantly down-regulated in the feces of the HD group. 3-Phenylpropionic acid is an endogenous and microbial product in the phenylalanine metabolic pathway [23, 26]. The reason for its decrease is unclear, but is probably due to the alteration of intestinal flora populations in uremic patients. Karlsson et al. found that 3-phenylpropionic acid contributed to the cyclooxygenase-2 (COX-2) protein inhibitory effect of human fecal water on colonic cells [27]. Normally, COX-2 is not expressed in the majority of cells. It can be induced in response to stimuli, such as growth factors, tumor necrosis factor- α , and lipopolysaccharides, and is involved mainly in the regulation of inflammatory responses [28-30]. It is worth investigating further whether the decrease of 3-phenylpropionic acid in the feces plays a role in the formation of regional intestinal inflammation in uremic patients [3] by regulating the expression of COX-2.

Serum metabolic profile of HD patients differs significantly from healthy subjects

As shown above, indoxyl sulfate, creatinine, ADMA, and hippuric acid, as well-known uremic toxins, were significantly increased in HD patients and played an important role in distinguishing the serum metabolic patterns between HD patients and healthy controls. Indoxyl sulfate and hippuric acid originate, respectively, from tryptophan and phenylalanine metabolism by gut microflora [1, 2]. Both of them belong to protein-bound uremic solutes and are difficult to remove by hemodialysis. The concentrations of indoxyl sulfate and hippuric acid may well reflect the retention of uremic toxins, especially protein-bound uremic solutes. Among the biomarkers identified in the serum, citric acid and cis-aconitic acid are formed in the tricarboxylic acid (TCA) cycle. Impaired kidney function may result in the alteration of TCA cycle intermediates [31]. AFMK is a newly identified biomarker in the serum of HD patients. It has been reported to be a strong free radical scavenger, to exert antioxidant activity, and can eliminate reactive oxygen and nitrogen species [11, 13]. The elevation of serum AFMK may indicate enhanced oxidative stress in HD patients. For the serum biomarkers, the increased levels of proline and citrulline and decreased levels of arginine, glutamine, lysine, and tryptophan reflected the disturbance of the serum amino acid profile in HD patients, which has been reported previously [32].

The carnitines and choline also showed significant changes in HD patients. Serum free carnitine, as a small molecular, high polarity solute, is lost to a significant degree in the dialysate after hemodialysis [33]. In the present study, the concentration of serum free carnitine in HD patients (D/CT ratio = 1.524, FDR = 0.526) was almost equal to that of the healthy subjects, which may be due to intravenous supplementation after hemodialysis. In contrast, acetylcarnitine was increased markedly in HD patients. Renal failure and low hemodialysis clearance contribute to its elevation in serum [33]. Similar to carnitine, choline can also be removed from the circulation to dialysate during hemodialysis [34]; however, the concentration of serum free choline was elevated in HD patients before dialysis. Conversely, glycerophosphocholine, one of the two major forms of choline storage in the cytosol, was decreased in HD patients. Glycerophosphocholine can react with H₂O to generate choline by glycerophosphohydrolase. The kidney plays a major role in choline homeostasis [35]. Rennick et al. proposed that when renal failure occurs, the choline homeostatic mechanism might be reset, leading to a higher “normal” plasma concentration for reasons that are unclear [35]. It was suggested that there might exist a transient, dialysis-induced choline deficiency in uremic patients [34]. Thus, the decreased level of glycerophosphocholine in HD patients may be attributed to the higher “normal” plasma concentration of choline.

Betaine is an essential tissue osmolyte, especially in the kidney, and a source of methyl groups, and almost entirely comes from either the betaine in our food (mainly cereals and

vegetables of the beet family) or dietary choline by oxidation [36]. The decreased plasma concentration of betaine and elevated urinary betaine excretion have been reported in CKD patients, which were considered to be stress-related and compensated for by homeostatic control [37]. Betaine is also highly water soluble and not protein-bound [36]. The decrease of betaine in HD patients is also likely due to its loss in dialysate. Betaine insufficiency is associated with metabolic syndrome, lipid disorders, and increased vascular risk [37]. Betaine supplementation has already been explored in some pathologic conditions, including liver disease, atherosclerosis, and hyperhomocysteinemia in CKD patients [38-41].

Medicinal charcoal tablets have an impact on the serum and fecal metabolomes of HD patients

In this study, we also investigated the effect of medicinal charcoal tablets on the fecal and serum metabolomes of HD patients. According to the results of OPLS-DA, medicinal charcoal treatment generally enabled the serum and fecal metabolomes of HD patients to draw close to those of the control subjects, especially the serum metabolite profile. As illustrated in Table 2, the abnormalities of TCA cycle intermediates (citric acid and cis-aconitic acid), glycerophosphocholine, acetylcarnitine, and several amino acids (proline, arginine, lysine, and tryptophan) were partially reversed after charcoal tablets treatment. However, the uremic toxins indoxyl sulfate, creatinine, hippuric acid, and ADMA, also as serum biomarkers of HD patients, were not significantly decreased after treatment. In addition, charcoal tablets treatment did not significantly reverse the abnormalities of the fecal biomarkers.

The administration of activated carbon was previously observed to attenuate markers of oxidative stress and inflammation in uremic rats [42]. In the present study, after treatment, three oxidative stress-associated metabolites (AFMK, AMK, and MetSO) tended to be down-regulated in the serum of HD patients, which also demonstrated that activated charcoal may have a role in reducing the oxidative stress reaction in patients with uremia.

The present study has several limitations that merit discussion. Firstly, this study was performed with a small sample size in a single center. Further studies should be conducted with a greater number of patients and in multiple centers. Secondly, this study was an observational study and not a strict randomized clinical trial. There were limitations including observational design to confounding and insufficient statistical power. To determine the exact effects of medicinal charcoal tablets still needs well-designed randomized clinical trials. Thirdly, SCFAs are an important group of compounds produced by bacterial metabolism in the colon and play an important pathophysiological role in uremic patients [2, 20]. However, SCFAs were not detected in our study, which was due to a limitation of the UPLC/MS platform. To investigate SCFAs, a gas chromatography/mass spectrometry (GC/MS) platform, which works best with nonpolar, volatile molecules (for example free fatty acids and steroids), is the best approach [31]. UPLC/MS combined with GC/MS should be performed in future research to attain more comprehensive metabolomes in ESRD patients.

Conclusion

The parallel metabolomics study of feces and serum is a promising tool to reveal systematic metabolic variations related to renal failure. In this study, there were obvious metabolic distinctions between HD patients and healthy controls in the feces and serum according to OPLS-DA. Our findings indicate that protein malabsorption occurs along with disturbances of amino acid metabolism in the gut and various metabolic disorders exist in uremic patients. Generally, medicinal charcoal tablets had an impact on the serum and fecal metabolomes of HD patients; however, their specific effects still need to be studied further.

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Disclosure Statement

The authors of this manuscript state that they do not have any conflicts of interest and nothing to disclose.

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