MEDICAL SCIENCE MONITOR

CLINICAL RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2019; 25: 7235-7242
DOI: 10.12659/MSM.915735

Received: 2019.02.17
Accepted: 2019.06.13
Published: 2019.09.26

Differences in Serum Amino Acid Phenotypes Among Patients with Diabetic Nephropathy, Hypertensive Nephropathy, and Chronic Nephritis

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Funds Collection G

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Source of support: This work was supported by the China Postdoctoral Science Foundation (2018M633630XB), the Science and Technology Research Program of Chongqing Municipal Education Commission (KJQN201800403)

Background: We assessed levels of circulating amino acids in different etiologies of chronic kidney disease (CKD) and the association of amino acids with risk factors of CKD progression.

Material/Methods: High-performance liquid chromatography-based analysis was used to determine amino acid profiles in patients with diabetic nephropathy (DN, n=20), hypertensive nephropathy (HN, n=26), and chronic nephritis (CN, n=33), and in healthy controls (HC, n=25).

Results: All 3 types of CKD patients displayed decreased serum levels of serine, glycine, GABA, and tryptophan compared with healthy controls. Moreover, serine and tryptophan were positively correlated with glucose in DN cohorts. Total cholesterol was positively correlated with tryptophan levels in the DN cohort and negatively correlated with serine levels in the CN cohort. In the HN cohort, glycine was negatively correlated with triglyceride levels, and systolic blood pressure (SBP) was negatively correlated with GABA levels.

Conclusions: Patients with different etiologies of CKD have significantly different amino acids profiles, and this indicates specific supplementary nutritional needs in CKD patients.

MeSH Keywords: Amino Acids • Diabetic Nephropathies • Glomerulonephritis • Hypertension, Renal • Renal Insufficiency, Chronic

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/915735

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Background

Chronic kidney disease (CKD) is a widespread disease arising from multiple pathogeneses, which irreversibly alters the function and structure of the kidney. It is estimated that the prevalence of CKD is 8–16% worldwide, which corresponds to nearly 500 million individuals [1]. Since decreased renal function from CKD is predictive of increased hospitalization rates, decreased cognitive function, and poorer quality of life [2], CKD has become increasingly recognized as a global public health concern [3]. In developed countries, the most important risk factors for CKD are diabetes, obesity, and hypertension, and the pathogenesis is mostly related to unhealthy lifestyles and poor nutrition management [2]. Thus, in addition to pharmacological therapy, nutrition management is recognized as an important independent factor to slow the progression of CKD.

In the last decade, many studies have reported that diet therapy is a main factor in conservative treatment for CKD, which aims to ameliorate the metabolic effects of CKD, reduce the uremic toxemia, suppress the progression of renal damage, and delay dialysis [4]. In this respect, various types of diet are used to preserve renal function, such as Mediterranean diet, moderately restricted low-protein diets (LPD), and very-low-protein diet supplemented with amino acids and ketoacids (s-VLPD) [5]. However, clinical trials have reported varying results on the benefit of diet on CKD.

Differences among individuals in metabolism in the pathogenesis of the CKD necessitate individualized nutritional plans. A GC-MS study of plasma samples from patients revealed that the metabolites (3 hydroxy isovalerate, aconitate, citrate, 2 ethyl, 3 hydroxy propionate, glycolate, 3 hydroxy isobutyrate, 2 methyl acetoacetate, 3 methyl adipic acid, 3 hydroxy propionate, and uracil) are related to mitochondrial disfunction in diabetic kidneys [6]. Another NMR study reported that patients with focal segmental glomerulosclerosis (FSGS) had a different metabolite signature compared to patients with other CKD etiologies [7]. FSGS disturbs several metabolic pathways, including chondroitin sulfate degradation, eicosanoid metabolism, keratan sulfate biosynthesis, vitamin B6 metabolism, and amino acid metabolism [8]. Moreover, several amino acids and their intermediates, nucleotide metabolites, lipid metabolites, and sugar metabolites are associated with CKD progression [9].

Amino acid is the basic element synthesized for proteins. Nine of the 21 amino acids are essential because they cannot be synthesized in the body and require supplementation: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Moreover, many amino acids are metabolized to produce small molecules important in basic physiological functions and neurotransmission. In some disease states, excessive nutritional supplementation or increased proteolysis can disturb amino acids metabolism. Certain amino acids, such as isoleucine, phenylalanine, and tyrosine, are significantly associated with incident type 2 diabetes mellitus (T2DM) [10]. Moreover, higher phenylalanine levels are associated with increased macrovascular risk and mortality, and higher levels of tyrosine and alanine are negatively associated with microvascular risk in obesity and T2DM [11].

These data suggest differences in amino acid associations with disease outcomes, and some may play causal roles in disease etiology. Based on this, the objective of the present study was to use high-performance liquid chromatography (HPLC) to characterize serum amino acid profiles in CKD patients with different etiologies: diabetic nephropathy, hypertensive nephropathy, and chronic nephritis. Our findings may provide guidance in the development of precision nutrition strategies for CKD.

Material and Methods

Ethics statement

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University (No. (2019) 250). All persons recruited for this study provided written informed consent prior to participation.

Subjects

A total of 79 patients (48 males and 31 females) with advanced (stage 4–5) CKD diagnosed at the Department of Nephrology at the Second Affiliated Hospital of Chongqing Medical University were enrolled from October 2016 to December 2016. We recruited adult patients: 1) aged 18 to 80 years diagnosed with advanced CKD (stage 4–5) according to the 2012 Kidney Disease: Improving Global Outcomes (KDIGO) guidelines with an estimated glomerular filtration rate (eGFR) of less than 20 ml/min/1.73² calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula [12,13]; and 2) who received 4-h standard hemodialysis 3 times a week. Creatinine levels were assessed by the sarcosine oxidase enzymatic method [14]. Low-density lipoprotein cholesterol (LDL-C) levels were assessed by the direct peroxidase-based enzymatic method [15]. We excluded patients with a history of hepatobiliary or thyroid disease, kidney transplant recipients, active malignancies, those requiring immunosuppressive therapy, and those with body weight changes exceeding 10% in the past 3 months.

CKD enrollees were segregated into 3 cohorts by pathoetiology: diabetic nephropathy (DN cohort, n=20), hypertensive nephropathy (HN cohort, n=26), and chronic nephritis (CN cohort, n=33). In addition, sex-matched, otherwise healthy adults (with no

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Table 1. High-performance liquid chromatography gradient.

	Time (min)								
	0	3.0	10.5	52.5	67.5	99	110	111	120
Mobile phase A (%)	20	20	80	65	50	0	0	20	20
Mobile phase B (%)	80	80	20	35	50	100	100	80	80

Mobile phase A: 9 mM NaH_2PO_4, 4% DMF, and 0.16% TEA (adjusted to pH 6.55 with phosphoric acid). Mobile phase B: 80% (v/v) CAN.

history of obesity, hypertension, or dyslipidemia) were selected as healthy controls (HC cohort, n=25). DN cohort enrolled individuals had: 1) a diagnosis of type 2 diabetes; 2) diabetic retinopathy; and 3) hyperglycemia controlled with insulin. Individuals with a history or presence of glomerulonephritis were excluded. HN cohort enroll individuals had: 1) non-malignant hypertension; 2) macroalbuminuria (urine albumin-to-creatinine ratio >200 mg/g Cr), and 3) hypertension retinopathy. Exclusion criteria were: 1) known primary kidney disease; 2) ≥3 red blood cells per high-powered field of urine or urine cellular casts; 3) history of diabetes or fasting blood glucose ≥110 mg/dl; 4) a known history or presence of glomerulonephritis. CN cohort enrolled individuals had biopsy-proven primary glomerulopathy and exclusions were as follows: 1) reflux nephropathy; 2) HIV-associated nephropathy; 3) autoimmune diseases; 4) infection; and 5) hereditary kidney diseases. All patients received antihypertensive therapy, including angiotensin receptor blockers, angiotensin-converting enzyme inhibitors, or calcium channel blockers.

Plasma samples were obtained 30 min before dialysis, between 8 a.m. and 10 a.m. after an overnight fast. All blood samples were then centrifugated at 3000 g for 15 min and immediately stored at –80°C.

Chemicals and reagents

Water was purified using the MilliQ™ System (Millipore, Bedford, MA, USA). Amino acid standards (cat. no AAS18), DABS-Cl (cat. no. 502219), hydrochloric acid (cat. no. 30721), sodium bicarbonate (cat. no. 31437), sodium phosphate (cat. no. 71504), triethylamine (cat. no. 90340), and N, N-dimethylformamide (cat. no. 270547) were purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile (cat. no. 114291) and ethanol (cat. no. 107017) were purchased from Millipore.

Derivatization

Sample pretreatment was performed based on previously described procedures [16]. Briefly, serum (100 µl aliquots) or standard were added to 400 ul of 90% methanol solution containing 250 nmol/ml norleucine as an internal standard. The mixture was homogenized and ice-cooled for 10 min to fully precipitate serum proteins. The supernatant was centrifuged at 15 000 g for 15 min and dried in a heated vacuum concentrator (Thermo Electron Corporation, Waltham, MA, USA). The residue was reconstituted in 100 µl of 0.15 M $NaHCO_3$ (pH 8.6), vortexed, and centrifuged for 10 min at 10 000 g at 4°C. We diluted 20 µl of supernatants in 180 µl dilution buffer (acetonitrile/ethanol/mobile phase A, 50/25/25, v/v/v). After thorough vortex mixing, DABS-Cl (12.4 mM in acetone, 200 µl) was added to the solutions and heated at 70°C for 15 min with constant vortexing. The vials were then cooled in an ice bath for 5 min. After centrifugation at 15 000 g for 15 min, 100 µl supernatants were mixed with 400 µl dilution buffer.

HPLC analysis

HPLC analysis was performed on an Agilent 1260 High-Performance Liquid Chromatograph system (Agilent Technologies, Waldbronn, Germany). Separation was achieved using a ZORBAX Extent C18 column (250×4.6 mm; 5-µm particle size). The derivatized samples or amino acid standards (20 µl aliquots) were injected into the unit. Two eluents were used: (i) mobile phase A (9 mM sodium dihydrogen phosphate, 4% dimethylformamide and 0.16% triethylamine (TEA), titrated to pH 6.55 with phosphoric acid) and (ii) mobile phase B (80% (v/v) aqueous acetonitrile). The column was thermostated at 50°C. Employing the gradient system detailed in Table 1, elution was performed at a 1 ml/min flow rate. Detection was performed with a UV-vis detector (436 nm) and a data acquisition rate of 5 Hz.

Statistical analysis

Data analysis was performed using SPSS 24.0. Differences between groups were assessed using one-way ANOVA with Fisher's least significant difference test. Significant differences identified using the above tests are indicated in the figures as follows: * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Correlations were assessed by stepwise regression analyses and Spearman rank correlation analyses. We first used a stepwise regression method on all CKD patients to screen for explanatory variables (with an inclusion criterion of 0.10 and a rejection criterion of 0.15), taking age, sex, calcium, phosphorus, and PTH as controlling variables. We then performed Spearman rank correlation analyses by the CKD cohort.

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Table 2. Demographic and clinical characteristics by cohort.

Parameter	DN cohort (n=20)	HN cohort (n=26)	CN cohort (n=33)	HC cohort (n=25)
Age	63.5±20.5*,**	69±12*,**	46±38	54±10
Sex (M/F)	15/5	15/11	18/15	15/10
eGFR (ml/min/1.73²)	5.90±3.38*,**	5.32±2.77*,**	4.02±2.61*	110.52±21.28
Creatinine (μmol/l)	788.9±478.9*	703.7±324.9*,**	954.6±484.4*	51.7±10.9
BUN (mmol/l)	21.5±7.4*	17.7±7.6*	22.1±7.0*	3.9±0.9
Albumin (g/l)	36.85±5.95*	37.00±4.10*	39.00±7.70*	46.30±2.80
Glucose (mmol/l)	8.6±4.3*,**,#	5.7±1.5	5.6±1.3	4.6±0.5
Triglycerides (mmol/l)	1.62±1.23*	1.31±1.26*	1.21±0.87	1.12±0.32
Total cholesterol (mmol/l)	2.75±1.29*,**,#	3.85±1.01*,**	3.73±1.21	4.12±0.57
HDL (mmol/l)	0.94±0.42*	1.08±0.38*	1.11±0.40	1.28±0.16
LDL (mmol/l)	1.29±0.69*,**,#	1.71±1.00	2.02±1.26	2.09±0.15
PTH (pg/ml)	97.10±110.98**,#	278.50±310.20	209.20±381.70	–
Calcium (mmol/l)	2.13±0.32*	2.17±0.49*	2.13±0.26*	2.34±0.06
Phosphate (mmol/l)	1.41±0.70*	1.60±0.80*	1.68±0.76*	1.10±0.26
Insulin, n(%)	20 (100%)	–	–	–
Acarbose, n(%)	7 (35%)	–	–	–
Repaglinide, n(%)	2 (10%)	–	–	–
ACEI/ARB, n(%)	8 (40%)	16 (62%)	–	–
β-blocker, n(%)	7 (35%)	7 (27%)	–	–
CCB, n(%)	15 (75%)	21 (81%)	–	–

Data reported as means with associated standard deviations (SDs). Comparisons performed via one-way ANOVA or chi-square testing. * $p<0.05$ *vs.* HC cohort; ** $p<0.05$ *vs.* CN group; # $p<0.05$ *vs.* HN cohort. DN – diabetic nephropathy; HN – hypertensive nephropathy; CN – chronic nephritis; HC – healthy control; eGFR – estimated glomerular filtration rate; BUN – blood urea nitrogen; HDL – high-density lipoprotein; LDL – low-density lipoprotein; FFA – free fatty acid; PTH – parathyroid hormone. ACEI – ACE inhibitor; ARB – angiotensin-renin blocker; CCB – calcium channel blocker.

Results

Participant characteristics

Seventy-nine participants were enrolled in the study: 20 with DN, 26 with HN, and 33 with CN, and the participant characteristics are detailed in Table 2. There were no significant differences in sex distribution between the 3 CKD cohorts (p>0.05). As expected, the 3 CKD cohorts exhibited significantly lower eGFR, calcium, and albumin levels, and higher serum creatinine, BUN, and phosphate levels relative to the control group (p<0.05). However, there were no significant differences in these parameters between the 3 CKD cohorts (p>0.05).

Due to the earlier age of onset for CN, the mean age of the CN cohort (years) was significantly lower than in the DN cohort (years) and the HN cohort (years) (p<0.05). The DN cohort had significantly higher glucose levels accompanied by significantly lower total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) levels relative to the control group (p<0.05). The DN cohort had significantly higher triglyceride levels relative to the HC cohort (p<0.05). Moreover, the DN cohort displayed significantly lower PTH levels relative to the other 2 CKD cohorts (p<0.05).

Serum amino acid profile is associated with cause of CKD

We next performed a one-way ANOVA with Fisher's least significant difference test to explore serum amino acid differences

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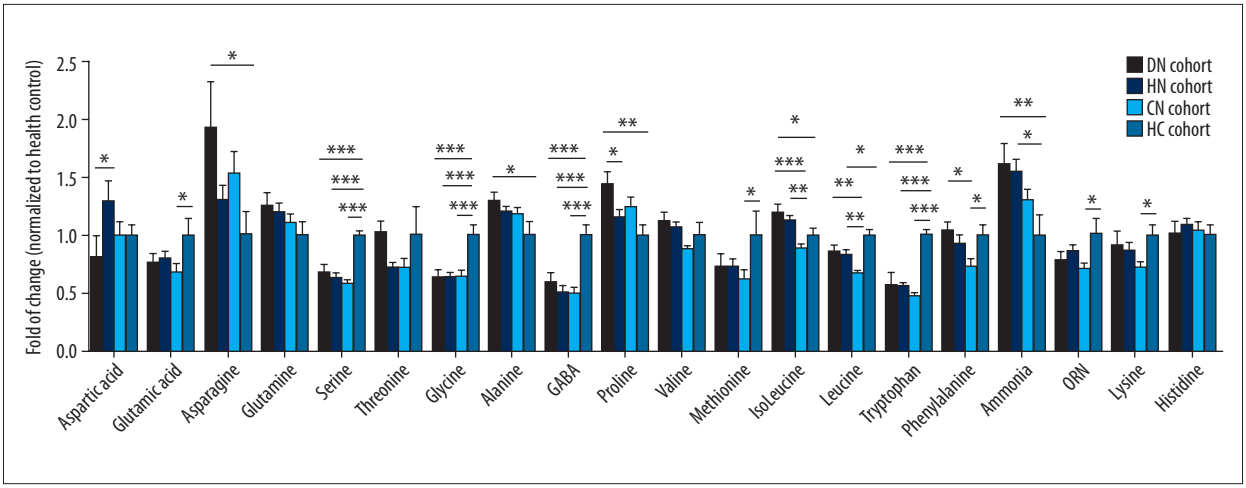

Figure 1. Serum amino acid profiling in CKD patients with different etiologies. Fold changes in serum amino acid levels for the 4 cohorts (normalized to the HC cohort). Data represent the means ±SEM. * p<0.05, ** p<0.01, *** p<0.001.

among the 4 groups, and representative fold changes of the amino acids of with different CKD subjects and healthy controls are shown in Figure 1. As compared to HC subjects, DN subjects exhibited higher levels of asparagine, alanine, proline, isoleucine, and ammonia and lower levels of serine, glycine, GABA, and tryptophan; HN subjects exhibited higher levels of ammonia and lower levels of serine, glycine, GABA, leucine, and tryptophan; CN subjects exhibited higher levels of glutamic acid, and lower levels of serine, glycine, GABA, methionine, tryptophan, phenylalanine, ornithine (ORN), and lysine.

When compared across the CKD cohorts, some amino acids displayed differential trends. DN subjects exhibited higher levels of proline and lower levels of aspartic acid compared to HN subjects. The levels of isoleucine, leucine, and phenylalanine were significantly higher in DN subjects compared to CN subjects. Levels of isoleucine and leucine were significantly higher in HN subjects compared to CN subjects (Figure 1).

Serum amino acid levels relative to poor prognostic indicator for CKD

We used Spearman correlation analyses to identify potential relationships between serum amino acid levels and other serum markers on a cohort-by-cohort basis. The statistically significant correlations are detailed in Figure 2. In the DN cohort, blood glucose levels were positively correlated with serine (Figure 2A, r=0.554, p=0.013) and tryptophan (Figure 2B, r=0.473, p=0.041). In addition, total cholesterol levels were positively correlated with tryptophan levels (Figure 2C, r=0.522, p=0.021). In the HN cohort, triglyceride levels were negatively correlated with glycine levels (Figure 2D, r=–0.703, p<0.001). Moreover, systolic blood pressure (SBP) was negatively correlated with GABA (Figure 2E, r=–0.406, p<0.017). In the CN cohort, total cholesterol levels were negatively correlated with serine levels (Figure 2F, r=–0.51, p=0.003).

Discussion

Amino acids play complex and often contrary roles in clinical research and practice. In this investigation, we used HPLC-UV to identify and characterize the serum amino acid profiles of CKD patients with diabetic nephropathy, hypertensive nephropathy, and chronic nephritis. We found significantly different metabolic phenotypes with different etiologies of CKD.

We found that all 3 CKD cohorts showed significantly lower levels of serine, glycine, GABA, and tryptophan relative to healthy controls. Serine and glycine are major metabolic sources for generating one-carbon units that connect major catabolic and anabolic pathways [16]. Serine can convert to glycine by donating the carbon atom from its side-chain to folate. They can be synthesized from a glycolysis metabolite intermediate and broken down to synthesize several important biomolecules, including nucleotides, lipids, and glutathione. Moreover, serine appears to be an allosteric activator of pyruvate kinase M2 (PKM2) [17]. Activation of PKM2 can induce glucose flux via glycolysis and decrease glucose metabolism via sorbitol, methylglyoxal, and DAG synthesis pathways [18]. In diabetic nephropathy, PKM2 activation decreases toxic glucose-derived end-product accumulation in podocytes and improves mitochondrial function [17,19]. In our study, serine was positively correlated with serum glucose in DN cohort, indicating that it is a protective factor in the activation of PKM2 and attenuates hyperglycemia-induced elevations in toxic glucose metabolites [20].

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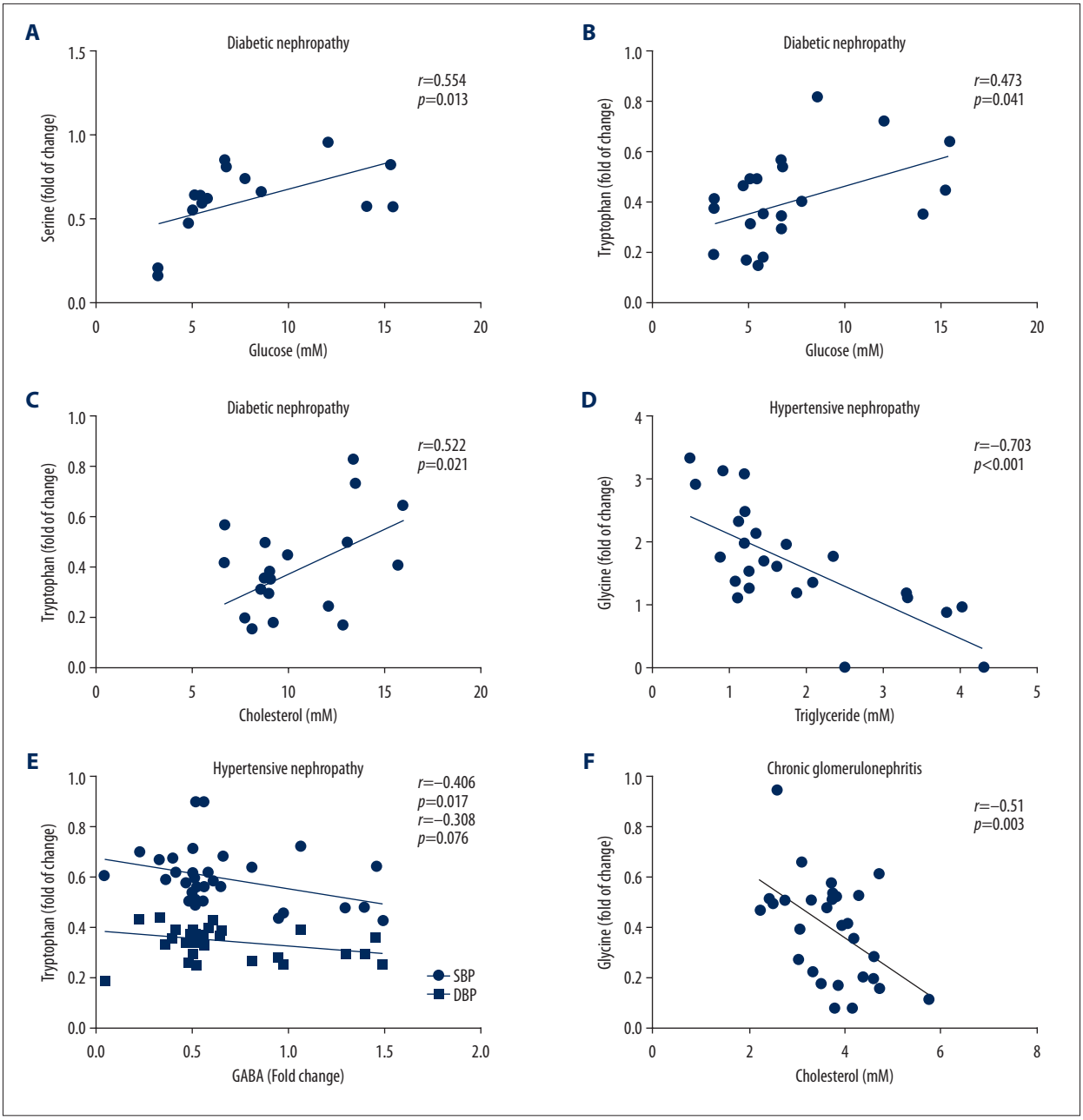

Figure 2. Correlation analysis between risk factor and amino acid. (**A, B**) Serine and tryptophan were positively correlated with glucose in the DN cohort. (**C**) Tryptophan was positive correlated with cholesterol in the DN cohort. (**D**) Glycine was negatively correlated with triglyceride in the HN cohort. (**E**) GABA was negatively correlated with neither SBP nor DBP in the HN cohort. (**F**) Serine was negatively correlated with cholesterol in the CN cohort.

In addition, serine was negatively correlated with cholesterol in the CN cohort, which has been noted in previous obesity and diabetes studies [21]. Previous research has observed serine deficiency concomitant with lipid metabolism disorder [22]. Supplementation of serine in drinking water increases the synthesis of GSH and S-glutathionylation of AMPK, which further activates AMPK [23]. AMPK activation by serine supplementation alleviates lipid accumulation with a high-fat diet [24]. Moreover, AMPK has been demonstrated to be involved in glucose metabolism, the activation of which improves insulin sensitivity and glucose tolerance [25]. These results indicate that serine deficiency decreases insulin sensitivity and increases lipid accumulation in ESRD patients.

Glycine is the only amino acid that effectively attenuates uptake of the triglyceride-rich VLDL and triglyceride biosynthesis

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in macrophages, which is associated with decreased CVD risk and anti-atherogenic effects [26]. Glycine was negatively correlated with triglyceride levels in our HN cohort, indicating that HN subjects may benefit from glycine supplementation. Previous research has demonstrated that orally supplemented glycine can decrease triglyceride and homocysteine levels, increase total NO concentration, and has a cardio-protective effect [27].

GABA has been reported to reduce cerebrospinal fluid in hypertensive CKD rats, and oral administration of a GABA analog increased periphery GABA levels and decreased blood pressure [28]. Consistent with this animal research, we found that GABA levels were negative correlated with blood pressure in CKD patients, suggesting that GABA supplementation may be beneficial in CKD patients with hypertension.

Tryptophan is upregulated in several catabolic pathways in CKD, particularly the kynurenine and uremic toxin pathways, resulting in chronic kidney inflammation and uremic symptoms [29]. In our study, tryptophan was significantly decreased in all 3 CKD cohorts and was positively correlated with glucose and cholesterol in the DN cohort. Genetic inhibition of the tryptophan metabolic pathway improves insulin sensitivity, decreases chronic inflammation, and regulates lipid metabolism [30]. Rothhammer et al. observed that a low-tryptophan diet decreased toxic tryptophan metabolites, which may suggest a new nutritional method to prevent the progression of CKD [31].

Leucine and isoleucine are other essential amino acids which are branched-chain amino acids (BCAAs) along with valine. Metabolite profiles reveal that BCAAs were significantly higher in those who subsequently developed future diabetes, and the restriction of BCAAs in the diet can alleviate insulin resistance and improve glucose tolerance [10]. Previous research has also reported decreased BCAAs in both early and end-stage CKD and this is likely associated with uremic malnutrition. However, in the current study, leucine and isoleucine were significantly increased in the DN and HN groups compared to the CN group, with no significant change compared to the HC group. These results indicate that, compared to CN patients, DN and HN patients are at increased risk for insulin resistance.

There are several limitations to this study. First, the findings were limited to patients recruited from a single clinical population in China. Second, serum amino acid levels were determined at a single time-point in advanced-stage CKD patients. Therefore, our findings do not reflect dynamic changes in circulating amino acid levels that can result from CKD progression. Thirdly, the HC cohort was significantly younger than the DN and HN cohorts, which may have affected our conclusions. Finally, medical intervention may affect circulating amino acids.

Conclusions

In conclusion, CKD patients display significant changes in their serum amino acid profiles, and these profiles were differentially regulated based on their pathoetiology.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University (No. (2019) 250). All persons recruited for this study provided written informed consent prior to participation.

Acknowledgments

We would like to thank all Fellows and Staff of the Institute of Ultrasound Imaging of Chongqing Medical University.

Conflict of interests

None.

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