Associations of Plasma Amino Acid and Acylcarnitine Profiles with Incident Reduced Glomerular Filtration Rate

Feijie Wang, Liang Sun, Qi Sun, Liming Liang, Xianfu Gao, Rongxia Li, An Pan, Huaxing Li, Yueyi Deng, Frank B. Hu, Jiarui Wu, Rong Zeng, and Xu Lin

Abstract

Background and objectives Metabolomics is instrumental in identifying novel biomarkers of kidney function to aid in the prevention and management of CKD. However, data linking the metabolome to incident eGFR are sparse, particularly in Asian populations with different genetic backgrounds and environmental exposures. Therefore, we aimed to investigate the associations of amino acid and acylcarnitine profiles with change in eGFR in a Chinese cohort.

Design, setting, participants, & measurements This study included 1765 community-living Chinese adults aged 50–70 years with baseline eGFR ≥60 ml/min per 1.73 m². At baseline, 22 amino acids and 34 acylcarnitines in plasma were quantified by gas or liquid chromatography coupled with mass spectrometry. Annual rate of change in eGFR was calculated, and incident eGFR decline was defined as eGFR < 60 ml/min per 1.73 m² by the end of 6 years of follow-up.

Results The mean (SD) unadjusted annual change in eGFR was 2.2 ± 1.25 per SD increment of metabolites; nitines (C3DC and C10), were significantly associated with annual change in eGFR. After multivariable adjustment of baseline covariates, including baseline eGFR, seven of the 13 metabolites, including cysteine, long-chain acylcarnitines (C14:1OH, C18, C18:2, and C20:4), and other acylcarnitines (C3DC and C10), were significantly associated with incident reduced eGFR (relative risks ranged from 1.16 to 1.25 per SD increment of metabolites; P = 3.8E-03 after Bonferroni correction of multiple testing of the 13 metabolites). Moreover, principal component analysis identified two factors, consisting of cysteine and long-chain acylcarnitines, respectively, that were associated with incident reduced eGFR.

Conclusions Elevated plasma levels of cysteine and a panel of acylcarnitines were associated with a higher incidence of reduced eGFR in Chinese adults, independent of baseline eGFR and other conventional risk factors.


Introduction

As a major public health challenge, CKD affects about 8%–16% of the world population and contributes to tremendous disease and socioeconomic burdens (1,2). The prevalence of CKD is expected to accelerate over the next few decades, particularly in countries undergoing rapid nutrition and lifestyle transitions, such as China, which has the largest population living with diabetes and hypertension in the world (3,4). Although CKD is preventable through early detection and intervention (5), the commonly used creatinine-based eGFR is not sensitive to detect incipient kidney dysfunction, and circulating concentrations of creatinine may be influenced by factors that are unrelated to kidney function (6,7). Therefore, it is necessary to identify novel biomarkers for early detection of the onset and progression of CKD.

With recent advances in metabolomics technology, it is now feasible to identify novel markers to predict eGFR change and therefore gain a deeper understanding of CKD pathophysiology (8). However, to date, data linking the metabolome to incident CKD are sparse (9–12). Moreover, most studies have investigated individuals of European or African descent, rather than Asians, who have different genetic backgrounds and environmental exposures and therefore different baseline risks of developing CKD.

To fill these knowledge gaps, we used a targeted and quantitative metabolomics approach to profile plasma levels of amino acids and multiple acylcarnitines in a well-characterized Chinese cohort, and examined prospective associations between these metabolites and change in eGFR.

Materials and Methods

Study Population

The Nutrition and Health of Aging Population in China study is a prospective cohort study designed to investigate environmental and genetic factors of metabolic diseases (13). Briefly, the study was initiated in 2005 among 3289 residents living in Beijing or Shanghai, aged 50–70 years. After 6 years, 2529 participants completed the follow-up visit, and 760 persons were considered lost to follow-up due to loss of contact (n=554) or refusal to participate (n=206). Information on baseline and follow-up surveys is described elsewhere.
amino acids were detected by gas chromatography-tandem mass spectrometry (Agilent Technologies Inc., CA), respectively. Plasma acylcarnitines were measured using an Agilent 1260 HPLC system and 5975C inert MSD system (Agilent Technologies, Germany) on an automatic analyzer (Hitachi 7080, Tokyo, Japan) with good linearity within a wide range, lower limit of quantitation, and better precision (Supplemental Methods, 13,14). In this analysis, we excluded participants without creatinine data at follow-up (n=261) or baseline metabolite data (n=459). When examining associations between metabolites and incident reduced eGFR, we further excluded 44 participants who had eGFR<60 ml/min per 1.73 m² at baseline. The study protocol was approved by the Institutional Review Board of the Institute for Nutritional Sciences, Chinese Academy of Sciences. All participants provided written, informed consent.

Data Collection
Information on demographic, health status, dietary, and lifestyle factors was collected at baseline by trained staff using a standard questionnaire (13). Educational attainment (0–6, 7–9, or >10 years), current smoking (yes or no), current drinking (yes or no), antihypertensive medication use (yes or no), lipid-lowering medication use (yes or no), physical activity (low, moderate, or high), cardiovascular disease (yes or no), hypertension (yes or no), and type 2 diabetes (yes or no) were previously defined (13). All participants underwent a physical examination by trained staff. Body weight, height, waist circumference, and BP were measured following a standardized protocol (13). Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters.

At the time of baseline and follow-up surveys, fasting venous blood samples were collected after overnight fasting. Baseline plasma glucose, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, γ-glutamyltransferase, uric acid, and C-reactive protein were measured as previously described (13). Plasma creatinine levels at baseline and follow-up survey were measured by an alkaline picrate method (Roche Diagnostics, Mannheim, Germany) on an automatic analyzer (Hitachi 7080, Tokyo, Japan), with intra- and interassay coefficients of variation<3%, and then calibrated traceable to isotope dilution mass spectrometry standards (15).

Targeted Metabolomics
Plasma acylcarnitines were profiled using liquid chromatography-tandem mass spectrometry, as previously described (16). Briefly, chromatographic separation and mass spectrometric analysis of acylcarnitines were performed on an Agilent 1260 HPLC system and 6410B QQQ mass spectrometer (Agilent Technologies Inc., CA), respectively. Plasma amino acids were detected by gas chromatography-mass spectrometry on an Agilent 7890A gas chromatography system and 5975C inert MSD system (Agilent Technologies Inc., CA), with good linearity within a wide range, lower limit of quantitation, and better precision (Supplemental Methods, Supplemental Table 1). The validity of both metabolic platforms has been previously described (16,17).

Outcome Ascertainment
eGFR was calculated using the Modification of Diet in Renal Disease (MDRD) study equation for Chinese individuals: eGFR (ml/min per 1.73 m²)=175×creatinine (mg/dl)−1.234 (Jaffe’s kinetic method) × age−0.179 × 0.79 (if female), which was derived from native Chinese individuals and showed stronger correlation with measured GFR compared with other equations (18,19). The primary outcome was annual eGFR change, defined as the difference in eGFR between baseline and follow-up visits divided by the time between visits in years. Moreover, incident reduced eGFR was defined as the onset of eGFR<60 ml/min per 1.73 m² during follow-up (20). In sensitivity analyses, a more stringent outcome, incident reduced eGFR plus annual eGFR decline>3%, was examined to reduce misclassification near the eGFR threshold (21). Additionally, eGFR was calculated using another definition, the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (19,22).

Statistical Analyses
t tests or Wilcoxon’s Signed Rank tests were used for continuous variables, and chi-squared tests were used for categoric variables, for the comparison of baseline characteristics between participants with and without incident reduced eGFR. Spearman partial correlation coefficients (r) were calculated to examine relationships among metabolites, as well as between the metabolites and covariates, after adjustment for age, sex, region, and residence. R package heatmap.2 was used to construct colored blocks representing correlation levels.

The cross-sectional associations of individual metabolites (scaled to SD of 1) with baseline eGFR were calculated using multivariable linear regression. Model 1 was adjusted for age, sex, region, and residence; model 2 was further adjusted for educational attainment, current smoking, current drinking, physical activity, BMI, lipid-lowering medication use, HDL, LDL, cardiovascular disease, hypertension, or type 2 diabetes. Longitudinal associations of metabolites with annual eGFR change were also assessed using multivariable linear regression, with additional adjustment for baseline eGFR. After Bonferroni correction, metabolites were considered significant at P<0.05/56=8.9E-04 corrected for multiple testing of the 56 metabolites in the fully adjusted model. Metabolites that were significantly associated with annual eGFR change were then analyzed with incident reduced eGFR using the log-Poisson model (23,24), with α=3.8E−03 after Bonferroni correction for multiple testing of 13 metabolites associated with annual eGFR change.

In secondary analyses, we performed a principal component analysis. The number of retained factors was determined by the scree plot. Varimax rotation was used to produce interpretable factors. Metabolites with a factor load ≥0.5 were reported as composing factors. Factor scores for each participant were calculated by summing the standardized values of metabolites weighted by their factor loadings. We then applied the abovementioned methods to examine associations of the factors with change in eGFR.

For metabolites significantly associated with incident reduced eGFR, analyses were stratified by age, sex, region, BMI, hypertension, type 2 diabetes, and eGFR at baseline. Potential interactions were examined by likelihood ratio test. All analyses were conducted using R (version 3.1.1; www.r-project.org). A two-sided P value <0.05 was considered statistically significant, unless specified otherwise.

Results
Baseline Characteristics
During 6 years of follow-up, the incidence of reduced eGFR was 16% (274 of 1765). The mean (SD) annual eGFR change was −2.2±2.0 ml/min per 1.73 m². Compared with noncases,
participants with incident reduced eGFR were older, had lower educational attainment and higher uric acid levels, and were more likely to have hypertension and cardiovascular disease ($P<0.05$). The cases also had lower eGFR levels and became significant (Figures 1B and 2B). After adjustment in model 2. When additionally controlling for lifestyle, anthropometrics, and medical history (model 2). The eight amino acids were glutamic acid, valine, tyrosine, tryptophan, glutamine, serine, lysine, and alanine, with effect sizes per SD ranging from 1.25 to 2.04 ml/min per 1.73 m$^2$ (all $P<8.9E-04$). The seven acylcarnitines were C3DC, C4, C5OH, C5:1, C6DC, C7DC, and C12OH, with effect sizes per SD ranging from −1.10 to −2.56 ml/min per 1.73 m$^2$ (all $P<8.9E-04$; Supplemental Table 3).

## Associations between Metabolites and Change in eGFR

Longitudinal associations with annual eGFR change were detected among 12 metabolites after multivariable adjustment in model 2. When additionally controlling for baseline eGFR, the positive associations of amino acids were attenuated toward the null, whereas the inverse associations of several amino acids and short- and medium-chain acylcarnitines were strengthened and became significant (Figures 1B and 2B). After Bonferroni correction, 13 metabolites were significantly associated with annual eGFR change, including three amino acids (cysteine, glutamate, and phenylalanine)
and ten acylcarnitines (C3DC, C5OH, C8, C10, C14:1OH, C16:2, C18, C18:2, C20, and C20:4). Higher levels of all of these metabolites were associated with larger annual eGFR reduction (effect sizes per SD ranged from −0.15 to −0.25 ml/min per 1.73 m², all P<8.9E-04; Table 2). Effect sizes for all metabolites are shown in Supplemental Table 4.

Figure 1. Associations of plasma amino acids and their ratios with baseline eGFR (eight amino acids significantly associated) and annual change in eGFR (three significantly associated) in the Nutrition and Health of Aging Population in China study. (A) Difference in eGFR per SD increment in metabolite (ml/min per 1.73 m²). (B) Difference in change in eGFR per SD increment in metabolite (ml/min per 1.73 m² per year). Effect sizes (β) (left y axis), with corresponding P values (right y axis), for the amino acids arranged by ascending β (model 3) for annual eGFR change along the x axis are shown. The dotted horizontal line shows the cutoff for P=8.9E-04 after Bonferroni correction for multiple testing of the 56 metabolites. Model 2 was adjusted for age, sex, region, residence, educational attainment, physical activity, current smoking, current drinking, body mass index, lipid-lowering medication use, HDL, LDL, cardiovascular disease, hypertension, and type 2 diabetes. Model 3 was further adjusted for baseline eGFR only for outcome of annual eGFR change.
Regarding incident reduced eGFR, seven of these 13 metabolites, including cysteine, short- and medium-chain acylcarnitines (C3DC and C10), and long-chain acylcarnitines (C14:1OH, C18, C18:2 and C20:4), showed positive associations, with relative risks (RRs) ranging from 1.17 to 1.25 (all \( P < 3.8E-03 \)) per SD increment of metabolites (Table 2).
Table 2. Associations of plasma metabolites with change in eGFR in the Nutrition and Health of Aging Population in China study

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Change in eGFR (mL/min per 1.73 m² per year)</th>
<th>Incident Reduced eGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β (95% Confidence Interval)</td>
<td>P Value</td>
</tr>
<tr>
<td>Cysteine</td>
<td>−0.25 (−0.33 to −0.17)</td>
<td>2.7E−09</td>
</tr>
<tr>
<td>C3DC</td>
<td>−0.19 (−0.27 to −0.11)</td>
<td>5.8E−06</td>
</tr>
<tr>
<td>C10</td>
<td>−0.17 (−0.25 to −0.10)</td>
<td>1.6E−05</td>
</tr>
<tr>
<td>C20</td>
<td>−0.18 (−0.26 to −0.09)</td>
<td>3.8E−05</td>
</tr>
<tr>
<td>C14:1OHH</td>
<td>−0.17 (−0.25 to −0.09)</td>
<td>4.5E−05</td>
</tr>
<tr>
<td>C18</td>
<td>−0.17 (−0.26 to −0.09)</td>
<td>5.6E−05</td>
</tr>
<tr>
<td>C18:2</td>
<td>−0.17 (−0.25 to −0.09)</td>
<td>1.0E−04</td>
</tr>
<tr>
<td>C16:2</td>
<td>−0.16 (−0.24 to −0.07)</td>
<td>2.0E−04</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>−0.16 (−0.24 to −0.07)</td>
<td>2.0E−04</td>
</tr>
<tr>
<td>Glutamine</td>
<td>−0.15 (−0.23 to −0.03)</td>
<td>3.0E−04</td>
</tr>
<tr>
<td>C8</td>
<td>−0.14 (−0.22 to −0.06)</td>
<td>4.0E−04</td>
</tr>
<tr>
<td>C20:4</td>
<td>−0.15 (−0.23 to −0.07)</td>
<td>4.0E−04</td>
</tr>
<tr>
<td>C5OH</td>
<td>−0.15 (−0.23 to −0.06)</td>
<td>4.0E−04</td>
</tr>
</tbody>
</table>

Data are annual change in eGFR or relative risk per SD increase in metabolite concentration, after adjustment for age, sex, region (Beijing/Shanghai), residence (urban/rural), education, current smoking, current drinking, physical activity, body mass index, lipid-lowering medication use, HDL, LDL, cardiovascular disease, hypertension, type 2 diabetes, and eGFR at baseline. Only metabolites associated with annual eGFR change are shown.

Discussion

Using targeted metabolomic approaches, we found that plasma levels of cysteine and a panel of acylcarnitines, especially long-chain species, were significantly associated with change in eGFR in a Chinese cohort, independent of established risk factors including baseline eGFR.

Previously, most circulating short- and medium-chain acylcarnitines and amino acids, including tryptophan, ornithine, citrulline, and homocysteine, as well as ratios such as tyrosine-to-phenylalanine and serine-to-glycine, were reported to change with the progression of CKD or to correlate with eGFR in cross-sectional studies (25–28). Consistently, significant associations of the majority of these metabolites with eGFR were also indicated in our study. To date, only four longitudinal studies have investigated the link between metabolomic markers and change in eGFR in populations of European or African ancestry, with mixed findings (9–12). The null associations of cysteine and acylcarnitines with change in eGFR in these studies might be ascribed to ethnic diversities in genetic predisposition, diet, and lifestyle. In addition, only one study examined cysteine using a semiquantitative non-targeted method (9); however, three of these studies had a relatively narrow coverage of acylcarnitines (from <10 to 20) (9–11). In contrast, our study measured 34 acylcarnitines using a method involving chromatographic separation. This wider coverage of acylcarnitines apparently allowed us to discover more novel acylcarnitines and their associations. Besides the independent association with incident reduced eGFR, our earlier study previously demonstrated for the first time that a panel of acylcarnitines was associated with risk of incident type 2 diabetes in the same population (16).

Of all of the metabolites, plasma cysteine levels showed the strongest association with incident reduced eGFR, after multivariable adjustment including baseline eGFR. Cysteine and its oxidized form cystine are the most abundant small-molecular-weight thiol/disulfide couple in plasma, and their imbalance may amplify oxidative stress which is closely related to CKD risk (29,30). Previously, higher plasma levels of total cysteine, including free cystine, cysteine, and their protein-bound forms, were shown in patients with declined kidney function before receiving hemodialysis (31,32). Our study further suggested different associations of cysteine and cystine with CKD risk. In vitro studies have revealed detrimental effects of cysteine on various cell types, probably through generating reactive...
acylcarnitines, the initial metabolites of membrane for cytosolic long-chain fatty acids across the inner mitochondrial in kidney dysfunction. Long-chain acylcarnitines transport another important group of metabolites that might play a role in kidney dysfunction, especially during the early-stages. Cysteine might be a potential marker of incident kidney dysfunction (6). Cysteine might be a potential marker of incident kidney dysfunction (6). Col-
tively, cysteine might be a potential marker of incident kid-
ey dysfunction (6). Col-

Interestingly, unlike long-chain acylcarnitines, the associa-
tions of medium- and short-chain species C10 (decanoylcarni-
nite) and C3DC (malonylcarnitine) with incident reduced eGFR became significant only after baseline eGFR was adjusted, implying that baseline eGFR might confound their associations. Indeed, medium- but not long-chain acylcarnitines were shown to decrease from kidney arterial to venous levels in individuals undergoing catheterization, suggesting that reduced eGFR could influence kidney uptake or elimination of these metabolites (42). The positive associations of C3DC and C10 with incident kidney dysfunction might be attributed to their effects on dysregulating FAO and mitochondrial stress, which subsequently impairs kidney function (43,44). It was also interesting that the associations of C3DC and C10 with future CKD risk were pronounced only in overweight/obese indi-

Table 3. Principal components analysis of plasma metabolites and change in eGFR in the Nutrition and Health of Aging Population in China study

| Factor | Description | Components | Eigenva
<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Long-chain acylcarnitines</td>
<td>C18:2, C18, C14OH, C20:4, C20, C18:1, C16:2, C14:1OH, C16:1, C18:0</td>
<td>11.39</td>
</tr>
<tr>
<td>2</td>
<td>Medium-chain acylcarnitines</td>
<td>C12, C12:1, C14, C12OH, C8, C10, C16, C6, C3DC, C2, C6OH, C10DC</td>
<td>8.11</td>
</tr>
<tr>
<td>3</td>
<td>Branched-chain and aromatic amino acids</td>
<td>Leucine, valine, isoleucine, alanine, tyrosine, phenylalanine, tryptophan, glutamic acid, proline, methionine, lysine</td>
<td>5.95</td>
</tr>
<tr>
<td>4</td>
<td>Short-chain acylcarnitines</td>
<td>C0, C3, C4, C5, C5OH</td>
<td>3.02</td>
</tr>
<tr>
<td>5</td>
<td>Miscellaneous</td>
<td>Serine, glutamine, glycine, ornithine, histidine</td>
<td>2.02</td>
</tr>
<tr>
<td>6</td>
<td>Miscellaneous</td>
<td>Cysteine, threonine</td>
<td>1.94</td>
</tr>
</tbody>
</table>

Six factors were retained according to the scree plot and varimax rotation performed to produce interpretable factors. Metabolites with a factor load $>$0.5 were reported as composing a factor. Data are annual change in eGFR or relative risk per SD increase in factor scores, after adjustment for age, sex, region (Beijing/Shanghai), residence (urban/rural), education, current smoking, current drinking, physical activity, body mass index, lipid-lowering medication use, HDL, LDL, cardiovascular disease, hypertension, type 2 diabetes, and eGFR at baseline.

<table>
<thead>
<tr>
<th>Value</th>
<th>P Value</th>
<th>Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>1.32 (1.18 to 1.47)</td>
<td>&lt;0.001</td>
<td>1.32 (1.18 to 1.47)</td>
</tr>
<tr>
<td>0.002</td>
<td>1.14 (1.03 to 1.27)</td>
<td>&lt;0.01</td>
<td>1.13 (0.99 to 1.28)</td>
</tr>
<tr>
<td>0.02</td>
<td>1.09 (0.98 to 1.21)</td>
<td>0.06</td>
<td>1.09 (0.98 to 1.21)</td>
</tr>
<tr>
<td>0.01</td>
<td>1.03 (0.91 to 1.16)</td>
<td>0.67</td>
<td>1.03 (0.91 to 1.16)</td>
</tr>
<tr>
<td>0.01</td>
<td>1.03 (0.91 to 1.16)</td>
<td>0.67</td>
<td>1.03 (0.91 to 1.16)</td>
</tr>
<tr>
<td>0.02</td>
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<td>0.67</td>
<td>1.03 (0.91 to 1.16)</td>
</tr>
<tr>
<td>0.01</td>
<td>1.03 (0.91 to 1.16)</td>
<td>0.67</td>
<td>1.03 (0.91 to 1.16)</td>
</tr>
<tr>
<td>0.001</td>
<td>1.32 (1.18 to 1.47)</td>
<td>&lt;0.001</td>
<td>1.32 (1.18 to 1.47)</td>
</tr>
</tbody>
</table>

oxygen species or forming an adduct with nitric oxide (33–35). Thus, the cytotoxic properties of cysteine may impair endothelial function and induce kidney dysfunction (36). Another interesting finding of our study was that the association of cysteine with incident reduced eGFR was more pronounced in participants with normal kidney function (eGFR $>$90 ml/min per 1.73 m$^2$) than those with mildly impaired kidney function (eGFR 60–90 ml/min per 1.73 m$^2$) (20). The kidney has a compensatory ability to maintain GFR, and the creatinine-based eGFR may not be sensitive enough to indicate incipient kidney dysfunction (6). Collectively, cysteine might be a potential marker of incident kidney dysfunction, especially during the early-stages.

Our study also highlighted long-chain acylcarnitines as another important group of metabolites that might play a role in kidney dysfunction. Long-chain acylcarnitines transport cytosolic long-chain fatty acids across the inner mitochondrial membrane for $\beta$-oxidation (37). Accumulation of long-chain acylcarnitines, the initial metabolites of $\beta$-oxidation, might reflect mitochondrial dysfunction induced by lipotoxicity, which could drive the progression of kidney impairment (38,39). In fact, dramatic mitochondrial damage in multiple kidney cell types was found in mice fed a high-fat diet, possibly due to suppressing AMP kinase activity and subsequently hindering fatty acid oxidation (FAO) in the kidney (40,41). Nevertheless, future studies are needed to elucidate the role of long-chain acylcarnitines in the pathogenesis of kidney disease.

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Disclosures

None.
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