# Plasma and Urinary Amino Acid Metabolomic Profiling in Patients with Different Levels of Kidney Function

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## Summary

**Background and objectives** Patients with CKD display altered plasma amino acid profiles. This study estimated the association between the estimated GFR and urinary and plasma amino acid profiles in CKD patients.

**Design, setting, participants, & measurements** Urine and plasma samples were taken from 52 patients with different stages of CKD, and plasma samples only were taken from 25 patients on maintenance hemodialysis. Metabolic profiling was performed by liquid chromatography coupled with tandem mass spectrometry after phenylisothiocyanate derivatization.

**Results** Most plasma amino acid concentrations were decreased in hemodialysis patients, whereas proline, citrulline, asparagine, asymmetric dimethylarginine, and hydroxykynurenine levels were increased ($P<0.05$). Both plasma levels and urinary excretion of citrulline were higher in the group of patients with advanced CKD (CKD stages 2 and 3 versus CKD stages 4 and 5; in plasma: $35.9\pm16.3$ versus $61.8\pm23.6$ $\mu$mol/L, $P<0.01$; in urine: $1.0\pm1.2$ versus $7.1\pm14.3$ $\mu$mol/mol creatinine, $P<0.001$). Plasma asymmetric dimethylarginine levels were higher in advanced CKD (CKD stages 2 and 3, $0.57\pm0.29$; CKD stages 4 and 5, $1.02\pm0.48$, $P<0.001$), whereas urinary excretion was lower ($2.37\pm0.93$ versus $1.51\pm1.43$, $P<0.001$). Multivariate analyses adjusting on estimated GFR, serum albumin, proteinuria, and other covariates revealed associations between diabetes and plasma citrulline ($P=0.02$) and between serum sodium and plasma asymmetric dimethylarginine ($P=0.03$). Plasma tyrosine to phenylalanine and valine to glycine ratios were lower in advanced CKD stages ($P<0.01$).

**Conclusion** CKD patients have altered plasma and urinary amino acid profiles that are not corrected by dialysis. Depending on solutes, elevated plasma levels were associated with increased or decreased urinary excretion, depicting situations of uremic retention (asymmetric dimethylarginine) or systemic overproduction (citrulline). These results give some insight in the CKD-associated modifications of amino acid metabolism, which may help improve their handling.

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## Introduction

The kidney plays an important role in the metabolism of proteins and amino acids (AA). Small molecules, including AAs and peptides, are constantly filtered and reabsorbed. Tubular AA reabsorption is almost complete (97%–98%) (1). The dynamics of renal peptide breakdown and protein synthesis as well as kidneys' capacity for AA interconversions modify the pool of AAs found in renal cells and also influence plasma concentrations (1). In patients with CKD, quantitative and qualitative alterations in the plasma AA profile have been observed (2,3). At an early stage of CKD, plasma concentrations in citrulline, cystine, and taurine have been shown to increase, whereas serine and ornithine were reduced (2). At more advanced stages of CKD and after dialysis initiation, alterations in the plasma profile were more pronounced (2,4–6). It has been shown that clinical features associated with CKD, such as inflammation or metabolic acidosis, influenced AA and protein metabolism (5,7). However, their specific influence on single AA concentrations has not been extensively studied. Furthermore, as CKD progresses, filtration and reabsorption may be altered and lead to AA losses or proteinuria. Dietary interventions have been successful in improving the nutritional state and AA profiles of CKD patients, but protein malnutrition may result from protein restriction aimed to prevent CKD progression (6,8). Given the role of the kidneys in AA and protein metabolism, we hypothesized that CKD severity would progressively affect plasma concentrations and urinary excretion of AAs. We performed a study in patients encompassing all the levels of CKD from CKD stage 2 patients to patients on maintenance hemodialysis (HD).

## Materials and Methods

### Study Participants

The study population consisted of 77 patients with CKD. Fifty-two CKD patients not treated with HD

(range of estimated GFR [eGFR]=9–90 ml/min per 1.73 $m^2$) were recruited from the Department of Nephrology, Transplantation and Dialysis of the University Hospital of Montpellier and the Public Hospital of Sète. Twenty-five patients on maintenance HD were recruited from the Néphrologie Dialyse Saint Guilhem Dialysis Unit in Sète. No patients received particular recommendations for protein or energy intake. Severe CKD patients were recommended to avoid potassium- and phosphate-rich foods. Mild CKD patients were not given specific dietary recommendations. Written informed consent was obtained from all participants before the study. The study was approved by the Comité de Protection des Personnes of Montpellier and declared to the French Ministry (reference number DC-2008–417). Recruitment was performed between February and June of 2008.

Plasma samples were obtained from all patients, and routine analyses were performed by the hospital laboratory. Urinary samples were obtained from nondialyzed patients, and protein and creatinine concentrations were determined by the hospital laboratory. eGFR was calculated based on serum creatinine using the simplified Modification of Diet in Renal Disease equation. Patient characteristics are summarized in Table 1. Laboratory differences between groups were consistent with increasing CKD severity. Low albumin levels were used as a marker of malnutrition indistinctive of causes. Clinical malnutrition defined as albumin levels below 3.5 g/dl was observed in 87% of HD patients, 33% of CKD stages 4 and 5 (CKD4–5) patients, and none of the CKD stages 2 and 3 (CKD2–3) patients. Diabetic patients were evenly distributed across groups.

### AA and Amine Determinations

Concentrations in 21 AAs and 3 biogenic amines (asymmetric dimethyl arginine [ADMA], symmetric dimethyl arginine [SDMA], and hydroxykynurenine [OH-Kyn]) were determined by liquid chromatography coupled with tandem mass spectrometry with electrospray ionization using the Absolute IDQ p180 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria).

Blood samples were collected during medical visits for non-HD patients or before a dialysis session for HD patients in EDTA-containing tubes. Sober blood samples could not be ensured. Blood was put on ice and immediately centrifuged (10 minutes at 2000×*g* or following the tube manufacturer's instructions) at 4°C. Plasma was obtained (500 μl) and stored at −80°C until analysis. Midstream urine samples were collected from non-HD patients during medical visits and centrifuged. The supernatant (2 ml) was stored at −80°C until analysis.

Samples were dried, and derivatization was performed by the addition of 20 μl 5% phenylisothiocyanate reagent. Excess liquids were removed by dehydration at room temperature, and 300 μl extraction solvent was added. Samples were shaken at 450 rpm at room temperature for 30 minutes to extract analytes and centrifuged for 2 minutes at 500×*g*. Using the CTC PAL autosampler (Agilent Technologies, Santa Clara, CA) and the Agilent 1100 Series Binary Pump (Agilent Technologies), 10 μl sample was loaded onto a C18 column (3.0×100 mm, particle size=3.5 μm; Agilent Eclipse XDB) for analysis. Mobile phases were milli-Q water (A) and HPLC-grade acetonitrile (B), and each phase contained 0.2% formic acid; the gradient used for metabolites separation was 0 minutes, 0% B; 0.5 minutes, 0% B; 5.5 minutes, 95% B; 6.5 minutes, 95% B; 7 minutes, 0% B; 9.5 minutes, 0% B. The column oven temperature was set to 50°C. The HPLC system was interfaced with a linear ion trap instrument (Sciex API 4000 QTrap LC-MS/MS; Applied Biosystems, Foster City, CA) equipped

**Table 1. Clinical characteristics of the patients**

| Patient Characteristics | CKD Group | | | *P* Values | |
|---|---|---|---|---|---|
| | CKD2–3 (*n*=24) | CKD4–5 (*n*=28) | HD (*n*=25) | CKD2–3 Versus CKD4–5 | Pre-HD Versus HD |
| Sex (men/women) | 14/10 | 15/13 | 20/5 | 0.73 | 0.04 |
| Diabetes (yes/no) | 11/13 | 15/13 | 12/13 | 0.87 | 0.58 |
| Age (yr) | 65.6±14.0 | 74.5±7.0 | 73.0±13.2 | 0.008 | 0.19 |
| eGFR (ml/min per 1.73 $m^2$) | 51.6±16.9 | 19.9±6.5 | — | <0.001 | — |
| Dialysis vintage (yr) | — | — | 3.1±3.5 | — | — |
| Plasma concentrations | | | | | |
| Protein (g/dl) | 7.4±0.5 | 7.2±0.5 | 6.4±0.7 | 0.10 | <0.001 |
| Albumin (g/dl) | 4.1±0.3 | 3.7±0.4 | 3.2±0.4 | <0.001 | <0.001 |
| Hemoglobin (g/dl) | 13.9±1.4 | 12.8±1.7 | 11.2±1.9 | 0.02 | <0.001 |
| BUN (mg/dl) | 26.2±10.2 | 59.7±17.3 | 59.9±21.3 | <0.001 | 0.004 |
| Uric acid (mg/dl) | 6.3±1.5 | 7.9±1.6 | 5.9±1.1 | <0.001 | 0.21 |
| Creatinine (mg/dl) | 1.7±0.5 | 3.7±1.2 | 7.9±2.4 | <0.001 | <0.001 |
| $Na^+$ (mEq/L) | 139.7±2.2 | 139.7±3.1 | 136.7±3.1 | 0.94 | <0.001 |
| $HCO_3^-$ (mEq/L) | 26.9±2.8 | 24.2±3.5 | 23.7±3.1 | 0.004 | 0.04 |
| PTH (pmol/L) | 5.3±3.1 | 27.7±25.4 | 33.7±24.6 | <0.001 | <0.001 |
| CRP (mg/L) | 2.8±2.5 | 9.3±21.1 | 31.4±81.2 | 0.01 | <0.001 |
| Urinary protein to creatinine ratio (g/g) | 0.55±1.5 | 1.79±2.4 | — | <0.001 | — |

All values are mean ± SD unless stated otherwise. CKD2–3, CKD stages 2 and 3; CKD4–5, CKD stages 4 and 5; HD, hemodialysis; eGFR, estimated GFR; ND, not determined; PTH, parathyroid hormone; CRP, C-reactive protein.

**Table 2. Plasma concentrations of amino acids and amines in nondialyzed patients with CKD stages 2–5 and hemodialyzed patients (micromoles per liter)**

| Variables | CKD2–5 ($n$=52) | Hemodialysis ($n$=25) | $P$ Value |
|---|---|---|---|
| Total amino acid | 2558.3±441.7 | 2162.9±615.4 | 0.01[a] |
| Total essential amino acid | 989.8±237.9 | 762.1±284.0 | <0.001[a] |
| Total nonessential amino acid | 1568.5±272.4 | 1400.8±379.8 | 0.06 |
| Total branched chain amino acid | 405.4±138.1 | 314.7±127.2 | 0.01[a] |
| Alanine | 320.9±102.8 | 232.7±90.4 | <0.001[a] |
| Arginine | 91.0±29.9 | 68.0±29.6 | <0.001[a] |
| Asparagine | 40.9±10.0 | 40.5±14.6 | 0.58 |
| Aspartate | 5.8±19.5 | 15.7±23.1 | 0.01[a] |
| Citrulline | 49.9±24.2 | 67.5±26.1 | 0.01[a] |
| Glutamine | 526.6±70.3 | 473.2±117.8 | 0.04[a] |
| Glutamate | 84.9±83.4 | 58.3±49.0 | 0.09 |
| Glycine | 187.6±62.2 | 166.8±54.0 | 0.27 |
| Histidine | 76.4±14.9 | 67.8±25.6 | 0.03[a] |
| Isoleucine | 75.9±28.6 | 61.5±25.4 | 0.03[a] |
| Leucine | 114.0±50.0 | 87.3±47.6 | 0.02[a] |
| Lysine | 189.6±45.1 | 150.3±62.9 | 0.001[a] |
| Methionine | 37.5±11.4 | 26.8±12.0 | <0.001[a] |
| Ornithine | 60.9±22.9 | 56.1±19.3 | 0.39 |
| Phenylalanine | 67.0±15.8 | 59.2±18.0 | 0.06 |
| Proline | 228.9±70.5 | 279.7±108.7 | 0.04[a] |
| Serine | 82.0±19.3 | 65.9±27.6 | 0.003[a] |
| Threonine | 100.6±29.9 | 75.8±36.5 | 0.01[a] |
| Tryptophan | 48.4±14.4 | 24.0±14.1 | <0.001[a] |
| Tyrosine | 65.0±22.7 | 43.5±17.8 | <0.001[a] |
| Valine | 215.4±69.0 | 165.9±62.1 | 0.003[a] |
| Asymmetric dimethylarginine | 0.81±0.4 | 1.79±0.6 | <0.001[a] |
| Symmetric dimethylarginine | 1.17±2.4 | 0.64±0.4 | 0.03[a] |
| Hydroxykynurenine | 2.07±1.2 | 5.50±1.8 | <0.001[a] |

All values are mean ± SD.
[a]$P$<0.05.

with an electrospray interface operated in MRM mode. Tandem mass spectrometry spectra were acquired in the positive mode. Molar concentrations were calculated with Analyst 1.4.2 Software (Applied Biosystems). Determinations were performed in a blind manner. Identity was revealed after depositing the results in the central server of the consortium.

### Statistical Analyses

Patients were grouped by CKD severity as patients receiving or not receiving HD, and nondialyzed patients were grouped into CKD2–3 (eGFR≥30 ml/min per 1.73 $m^2$) and CKD4–5 (eGFR<30 ml/min per 1.73 $m^2$). Differences between groups in continuous variables were tested by $t$ test or Wilcoxon signed-rank test after checking for normality and chi-squared test for class variables. Differences between CKD2–3, CKD4–5, and HD patients were analyzed by ANOVA and *post hoc* tests adjusted for multiple comparisons using Bonferroni correction.

The influence of clinical features was similarly tested after grouping on median serum levels of albumin (≤3.8 or >3.8 g/dl), C-reactive protein (CRP; ≤3.7 or >3.7 mg/L), bicarbonate (≤25 or >25 mEq/L), and urinary protein excretion (≤0.28 or >0.28 g/g urine protein-to-creatinine ratio) as surrogates of nutritional state, inflammation, acidosis, and proteinuria. Univariate and multivariate linear regressions with age, sex, diabetes, weight, eGFR, proteinuria, serum albumin, sodium, bicarbonate, and CRP concentrations as explanatory variables were performed to explain plasma and urine AA levels (urinary AA levels, proteinuria, and CRP levels were log-transformed).

The total concentration in AA was defined as the sum of 19 available proteogenic AAs. The total concentration in essential AA (EAAs) was defined as the sum of 10 EAAs (histidine, isoleucine, leucine, lysine, methionine, phenylalanine [Phe], threonine, tryptophan [Trp], tyrosine [Tyr], and valine [Val]), and the total concentration in nonessential AAs (NEAAs) was the sum of 9 NEAAs (alanine [Ala], arginine [Arg], asparagine, aspartate, glutamine, glutamate, glycine [Gly], proline [Pro], and serine [Ser]). The total concentration in branched chain AAs (BCAAs) was defined as the sum of leucine, isoleucine, and Val.

A type 1 error of 5% was considered for statistical analyses, which were performed using SAS version 9.2 (SAS Institute, Cary, NC). Results are given as the mean ± SD.

## Results

### Plasma AAs and Amines

**Dialyzed Versus Nondialyzed Patients.** The AA profiles of nondialyzed and dialyzed patients showed significant differences in 18 of 23 AAs and amines (Table 2). HD

**Table 3. Plasma concentrations (micromoles per liter) and urinary excretion (micromoles per mole creatinine) of amino acids and amines in nondialyzed CKD patients**

| Variables | Plasma Concentration | | | Urinary Excretion | | |
|---|---|---|---|---|---|---|
| | CKD2–3 | CKD4–5 | *P* Value | CKD2–3 | CKD4–5 | *P* Value |
| Total AA | 2512.4±411.0 | 2597.7±470.2 | 0.49 | 246.5±107.1 | 324.8±303.2 | 0.90 |
| Total essential AA | 1006.0±234.1 | 975.9±244.5 | 0.68 | 71.8±35.4 | 104.7±101.4 | 0.80 |
| Total nonessential AA | 1506.4±249.4 | 1621.8±284.4 | 0.13 | 200.1±126.8 | 220.1±204.5 | 0.56 |
| Total branched chain AA | 421.4±133.5 | 391.6±142.9 | 0.41 | 6.1±7.2 | 7.1±15.8 | 0.18 |
| Alanine | 305.3±100.0 | 334.3±105.0 | 0.32 | 23.8±21.5 | 30.7±29.2 | 0.54 |
| Arginine | 88.5±25.7 | 93.1±33.3 | 0.58 | 7.8±6.6 | 8.2±6.0 | 0.42 |
| Asparagine | 38.6±10.4 | 43.0±9.2 | 0.11 | 7.2±3.3 | 14.7±14.7 | 0.07 |
| Aspartate | 6.1±23.4 | 5.6±15.9 | 0.56 | 12.7±15.0 | 9.0±22.4 | 0.08 |
| Citrulline | 35.9±16.3 | 61.8±23.6 | <0.001[a] | 1.0±1.2 | 7.1±14.3 | <0.001[a] |
| Glutamine | 514.8±68.3 | 536.6±71.6 | 0.27 | 25.0±15.8 | 31.4±44.2 | 0.40 |
| Glutamate | 72.1±45.3 | 96.0±105.4 | 0.83 | 29.8±61.5 | 19.3±25.1 | 0.50 |
| Glycine | 185.3±48.1 | 189.5±73.1 | 0.71 | 69.9±44.8 | 67.6±69.6 | 0.32 |
| Histidine | 74.9±11.2 | 77.7±17.5 | 0.61 | 34.9±23.4 | 30.0±26.0 | 0.30 |
| Isoleucine | 76.7±26.9 | 75.2±30.4 | 0.80 | 0.9±1.9 | 0.6±2.9 | 0.11 |
| Leucine | 124.8±45.1 | 104.9±53.0 | 0.12 | 2.2±3.5 | 2.2±5.9 | 0.23 |
| Lysine | 186.3±45.7 | 192.4±45.2 | 0.53 | 10.4±10.3 | 26.5±30.7 | 0.11 |
| Methionine | 36.6±11.8 | 38.3±11.2 | 0.59 | 1.2±1.1 | 1.3±1.9 | 0.50 |
| Ornithine | 53.9±22.6 | 66.9±21.8 | 0.01[a] | 15.4±57.1 | 1.8±5.1 | 0.33 |
| Phenylalanine | 62.9±12.0 | 70.5±17.9 | 0.15 | 4.9±2.9 | 6.7±7.1 | 0.94 |
| Proline | 212.4±71.8 | 243.0±67.5 | 0.12 | 2.6±2.6 | 13.8±18.5 | <0.001[a] |
| Serine | 83.4±20.7 | 80.7±18.1 | 0.62 | 21.3±10.2 | 25.4±19.7 | 0.73 |
| Threonine | 101.6±31.9 | 99.8±28.6 | 0.92 | 11.1±10.5 | 20.9±29.1 | 0.43 |
| Tryptophan | 55.5±12.3 | 42.3±13.4 | <0.001[a] | 4.5±2.5 | 4.8±3.9 | 0.88 |
| Tyrosine | 66.9±22.8 | 63.3±22.9 | 0.59 | 6.5±3.9 | 7.5±6.9 | 0.94 |
| Valine | 219.9±64.6 | 211.6±73.6 | 0.69 | 3.0±2.6 | 4.3±7.9 | 0.54 |
| ADMA | 0.57±0.29 | 1.02±0.48 | <0.001[a] | 2.37±0.93 | 1.51±1.43 | <0.001[a] |
| SDMA | 1.54±3.48 | 0.85±0.58 | 0.69 | 2.85±0.93 | 2.89±2.91 | 0.06 |
| Hydroxykynurenine | 1.19±0.39 | 2.82±1.22 | <0.001[a] | 0.13±0.10 | 0.45±0.90 | 0.15 |

All values are mean ± SD. CKD2–3, CKD stages 2 and 3; CKD4–5, CKD stages 4 and 5; AA, amino acid; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine.
[a]$P<0.05$.

patients had lower total AA (−15.5%, $P=0.01$) and EAA concentrations (−23.0%, $P<0.001$). Apart from phenylalanine, the plasma concentrations of all EAAs were significantly reduced in dialyzed patients. Additionally, HD patients had lower concentrations in alanine ($P<0.001$), arginine ($P<0.001$), serine ($P=0.003$), and glutamine ($P=0.04$). In contrast, they had increased plasma concentrations in aspartate ($P=0.01$), citrulline ($P=0.01$), and proline ($P=0.04$). Finally, ADMA and hydroxykynurenine concentrations were increased in HD patients ($P<0.001$), whereas SDMA concentrations were reduced ($P=0.03$).

**CKD2–3 Versus CKD4–5.** The plasma AA concentrations of patients with CKD2–3 and CKD4–5 were similar in terms of total AA, EAA, and NEAA (Table 3). Plasma tryptophan concentrations were significantly lower in CKD4–5 compared with CKD2–3 ($P<0.001$). Furthermore, patients with CKD4–5 had significantly higher plasma concentrations of citrulline ($P<0.001$), ADMA ($P<0.001$), hydroxykynurenine ($P<0.001$), and ornithine ($P=0.01$). eGFR was significantly correlated with plasma citrulline (Spearman $\rho=-0.65$, $P<0.001$), ADMA ($\rho=-0.64$, $P<0.001$), tryptophan (Pearson $r=0.58$, $P<0.001$), and hydroxykynurenine ($\rho=-0.90$, $P<0.001$) concentrations (Figure 1).

**Clinical Features.** Patients with low albuminemia had increased plasma citrulline but lower tryptophan concentrations (both $P\leq0.001$) (Supplemental Figure 1A). Plasma ADMA and OH-Kyn levels were increased in patients with either malnutrition or greater proteinuria (all $P<0.001$). Patients with a higher extent of inflammation had lower plasma histidine concentrations ($P<0.001$).

In multivariate regression models, the plasma levels of citrulline, tryptophan, ADMA, and OH-Kyn were significantly associated with eGFR (Table 4). Independent of eGFR, plasma citrulline concentrations were significantly increased in diabetic patients ($P=0.02$), and both plasma ADMA and OH-Kyn levels were directly associated with serum sodium levels ($P=0.03$ and $P=0.01$, respectively). Finally, phenylalanine concentrations were independent of eGFR, but they were positively correlated with body weight and surrogates of inflammation and metabolic acidosis ($P=0.04$).

## Plasma AA and Amine Ratios

**CKD Stage.** Plasma AA ratios were calculated to evaluate the overall nutritional state (EAA/NEAA, Ala/BCAA, and Val/Gly), the kidney's ability for AA interconversions

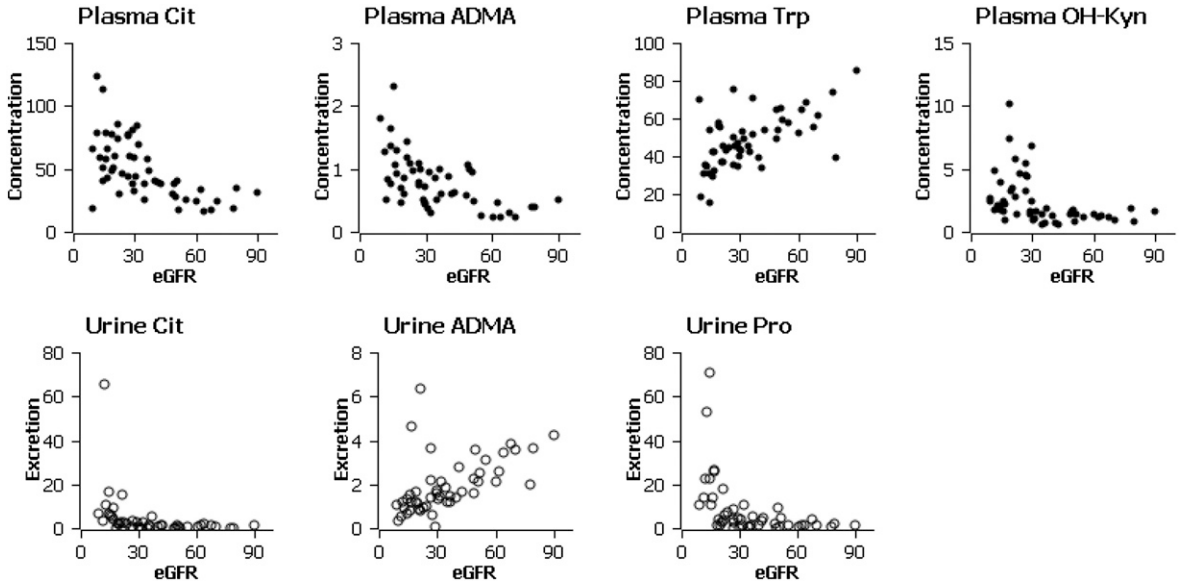


**Figure 1. | Plasma concentration (in micromoles per liter) and urinary excretion (in micromoles per mole urinary creatinine) of amino acids and amines significantly associated with estimated GFR (eGFR; milliliters per minute per 1.73 $m^2$).** ADMA, asymmetric dimethylarginine; Cit, citrulline; OH-Kyn, hydroxykynurenine; Pro, proline; Trp, tryptophan.

(Tyr/Phe, Ser/Gly, and Arg/citrulline [Cit]), the presence of urea cycle dysfunctions (ornithine [Orn]/Arg and Cit/Orn) (Supplemental Figure 2), the importance of the kynurenine pathway (OH-Kyn/Trp), and the relative presence of ADMA (Arg/ADMA and ADMA/SDMA). Patients from different CKD groups showed no differences in Ala/BCAA, Val/Gly, and Ser/Gly ($P>0.05$), whereas other plasma ratios were significantly associated with CKD stage ($P\leq0.01$) (Table 5) and showed monotonic variations along the course of the disease (Figure 2). The ratios of EAA/NEAA, Tyr/Phe, Arg/Cit, and Arg/ADMA were lower in more severe CKD stages, whereas Orn/Arg, Cit/Orn, ADMA/SDMA, and OH-Kyn/Trp were higher (Figure 2).

**Clinical Features.** A reduction in Arg/ADMA was found in patients with a greater degree of inflammation, malnutrition, or proteinuria (all $P\leq0.001$) (Supplemental Figure 1B). Those patients with worse nutritional states or proteinuria also had lower Arg/Cit and higher OH-Kyn/Trp (both $P<0.001$). Finally, ADMA/SDMA was increased in patients with malnutrition ($P<0.001$). In multivariate models, none of these ratios were associated with albuminemia, CRP, or proteinuria (Table 4). Instead, these ratios as well as EAA/NEAA and Cit/Orn were significantly associated with eGFR. Finally, Val/Gly was independently associated with eGFR, serum bicarbonate, and body weight.

### Urine AAs and Amines

**CKD2–3 Versus CKD4–5.** Total urinary AA excretion was not associated with CKD stage (Table 3). Urinary excretions of Pro and Cit were higher in CKD4–5 compared with CKD2–3 (both $P<0.001$), whereas urinary excretion of ADMA was lower ($P<0.001$). There were significant correlations between eGFR and urinary excretion of Cit, ADMA, and Pro ($\rho=-0.74$, 0.65, and $-0.68$, respectively, all $P<0.001$) (Figure 1).

**Clinical Features.** Total urinary AA excretion independently decreased with age ($P=0.03$) and increased with proteinuria ($P=0.01$) (Supplemental Table 1). Urinary excretion of EAA also decreased with age ($P=0.05$) but was not associated with proteinuria (Table 4). There were significant correlations between proteinuria and urinary excretion of 12 AAs ($\rho=0.74$–$0.29$ for proline, citrulline, asparagine, valine, alanine, threonine, lysine, phenylalanine, methionine, serine, tyrosine, and tryptophan; $P<0.05$). In multivariate analyses, urinary excretions of proline and citrulline were independently associated with eGFR and proteinuria (Table 4). Valine excretion was associated with both proteinuria and decreasing sodium levels. Lysine excretion was consistently increased in patients with lower serum albumin levels (Supplemental Figure 1B, Table 4). Multivariate regressions additionally showed that it was lower in patients with higher CRP levels (Table 4).

## Discussion

In this study, we have shown that CKD defined by eGFR and clinical features accompanying the disease was associated with alterations in plasma and urinary AA and amine profiles. Applying a metabolomic approach, we simultaneously measured targeted compounds with high selectivity and sensitivity (9) and obtained a clear idea of patients' metabolic states. We showed that plasma AA alterations appeared early in CKD and were more frequent and more pronounced in advanced stages. In HD patients, we observed significant alterations in plasma levels of many compounds, including serine, aspartic acid, valine, leucine, lysine, threonine, tyrosine, and SDMA, which had been shown to differ from healthy controls (2,3,6,10,11), but we did not find them to be correlated with eGFR in the nondialyzed patients. Despite having no dietary protein

**Table 4. Multivariate regressions on plasma concentrations (micromoles per liter), plasma ratios (no unit), and urinary excretion (micromoles per mole creatinine) of amino acids and amines in nondialyzed CKD patients**

| Dependent Variables | Explanatory Variable | Change for 1 Unit Increase | 95% Confidence Interval | *P* Value | Model *P* Value |
|---|---|---|---|---|---|
| Plasma concentration | | | | | |
| Citrulline | log(eGFR) | −27.2 | −42.4 to −12.1 | 0.001 | 0.002 |
| | Diabetes (yes) | 16.8 | 3.1 to 30.4 | 0.02 | |
| Phenylalanine | $HCO_3^-$ | −2.0 | −3.7 to −0.2 | 0.03 | 0.01 |
| | log(CRP) | 6.1 | 0.5 to 11.6 | 0.04 | |
| | Weight | 0.32 | 0.03 to 0.61 | 0.04 | |
| Tryptophan | eGFR | 0.31 | 0.04 to 0.58 | 0.03 | 0.02 |
| ADMA | log(eGFR) | −0.47 | −0.69 to −0.24 | <0.001 | <0.001 |
| | $Na^+$ | 0.05 | 0.01 to 0.09 | 0.03 | |
| Hydroxykynurenine | log(eGFR) | −1.7 | −2.3 to −1.2 | <0.001 | <0.001 |
| | $Na^+$ | 0.14 | 0.05 to 0.22 | 0.01 | |
| Plasma ratios | | | | | |
| EAA/NEAA | eGFR | 0.005 | 0.002 to 0.01 | 0.001 | 0.01 |
| | Weight | 0.003 | 0.0004 to 0.01 | 0.03 | |
| Arg/Cit | eGFR | 0.04 | 0.02 to 0.06 | <0.001 | 0.001 |
| Cit/Orn | eGFR | −0.01 | −0.01 to −0.001 | 0.02 | 0.01 |
| Val/Gly | eGFR | 0.02 | 0.01 to 0.03 | 0.002 | 0.004 |
| | Weight | 0.02 | 0.01 to 0.03 | 0.01 | |
| | $HCO_3^-$ | −0.07 | −0.13 to −0.01 | 0.03 | |
| Arg/ADMA | eGFR | 2.2 | 0.1 to 4.3 | 0.04 | 0.03 |
| ADMA/SDMA | log(eGFR) | −0.76 | −1.29 to −0.23 | 0.01 | 0.002 |
| | $Na^+$ | 0.10 | 0.01 to 0.19 | 0.04 | |
| OH-Kyn/Trp | log(eGFR) | −0.59 | −0.93 to −0.25 | 0.002 | 0.003 |
| Urinary excretion (logtransformed) | | | | | |
| Total EAA | Age | −0.02 | −0.05 to −0.001 | 0.05 | 0.01 |
| Alanine | log(proteinuria) | 0.22 | 0.08 to 0.36 | 0.004 | 0.04 |
| Asparagine | log(proteinuria) | 0.17 | 0.05 to 0.30 | 0.01 | <0.001 |
| Citrulline | log(eGFR) | −1.0 | −1.7 to −0.4 | 0.01 | <0.001 |
| | log(proteinuria) | 0.25 | 0.02 to 0.48 | 0.04 | |
| Lysine | log(CRP) | −0.6 | −0.99 to −0.21 | 0.01 | 0.003 |
| | Albumin | −0.18 | −0.30 to −0.06 | 0.01 | |
| Phenylalanine | log(proteinuria) | 0.2 | 0.08 to 0.33 | 0.004 | 0.003 |
| Proline | log(eGFR) | −1.0 | −1.6 to −0.4 | 0.004 | <0.001 |
| | log(proteinuria) | 0.31 | 0.1 to 0.51 | 0.01 | |
| Tyrosine | log(proteinuria) | 0.18 | 0.05 to 0.31 | 0.01 | 0.02 |
| Valine | log(proteinuria) | 0.32 | 0.12 to 0.52 | 0.01 | 0.04 |
| | $Na^+$ | −0.13 | −0.24 to −0.02 | 0.04 | |

All models included age, sex, diabetes, weight, eGFR, proteinuria, serum $HCO_3^-$, serum $Na^+$, serum albumin, and serum CRP as covariates. eGFR, estimated GFR; CRP, C-reactive protein; ADMA, asymmetric dimethylarginine; EAA, sum of essential amino acids; NEAA, sum of nonessential amino acids; Arg, arginine; Cit, citrulline; Orn, ornithine; Val, valine; Gly, glycine; SDMA, symmetric dimethylarginine; OH-Kyn, hydroxykynurenine; Trp, tryptophan.

restriction, HD patients had lower concentrations of AA and EAA and a lower nutritional state. After dialysis, losses of AAs in the dialysate have been reported to be as high as 6–8 g, and plasma AA levels have been reported to be reduced by 20%–40% (12). As a consequence, results obtained in HD could not be clearly associated with reaching the ultimate stage of the disease.

Limiting the analysis to non-HD patients and studying eGFR as a continuous variable, we observed significant associations between CKD severity and plasma or urinary AA and amine profiles. Previous studies performed in patients with CKD stages 3–5 showed that reducing kidney function was associated with increasing plasma concentrations of citrulline (2,10,13). Citrulline is mainly produced in the liver through the urea cycle and is further catabolized into arginine in renal cells (Supplemental Figure 2). In the present study, we observed that the increase in plasma citrulline concentrations occurred when eGFR reached values below 45 ml/min per 1.73 $m^2$. In addition, the relative presence of arginine compared with citrulline (Arg/Cit) was progressively lower in more advanced CKD stages, suggesting a lower activity of argininosuccinate synthase (Enzyme Commission [EC] number 6.3.4.5) and/or argininosuccinate lyase (EC4.3.2.1). In an animal model, CKD induced a reduction in the renal uptake of citrulline in the abundance of these enzymes (14). We also found that reduced kidney function was associated with a greater urinary citrulline excretion, confirming that increased citrullinemia was not related to retention but rather, had a metabolic origin. A causal relationship between renal loss and altered

**Table 5. Plasma ratios of amino acids and amines in nondialyzed and dialyzed CKD patients**

| Variables | CKD2–3 | CKD4–5 | Hemodialysis | *P* Value |
|---|---|---|---|---|
| EAA/NEAA | 0.68±0.16 | 0.60±0.12 | 0.55±0.14 | <0.01[a] |
| Orn/Arg | 0.62±0.23 | 0.77±0.30 | 0.93±0.46 | 0.01[a] |
| Arg/Cit | 2.83±1.31 | 1.60±0.53 | 1.09±0.44 | <0.001[a] |
| Cit/Orn | 0.71±0.27 | 0.96±0.33 | 1.24±0.41 | <0.001[a] |
| Pro/Orn | 4.14±1.14 | 3.96±1.48 | 5.26±2.16 | 0.01[a] |
| Tyr/Phe | 1.05±0.23 | 0.90±0.23 | 0.73±0.17 | <0.001[a] |
| Val/Gly | 1.29±0.68 | 1.19±0.46 | 1.11±0.67 | 0.58 |
| Ser/Gly | 0.47±0.13 | 0.45±0.11 | 0.40±0.12 | 0.12 |
| Ala/BCAA | 0.78±0.30 | 0.91±0.29 | 0.80±0.30 | 0.25 |
| ADMA/SDMA | 0.73±0.46 | 1.36±1.01 | 2.95±1.65 | <0.001[a] |
| Arg/ADMA | 193.6±115.7 | 115.8±73.1 | 41.3±20.5 | <0.001[a] |
| OH-Kyn/Trp | 0.023±0.020 | 0.082±0.072 | 0.262±0.094 | <0.001[a] |

All values are mean ± SD. EAA, sum of essential amino acids; NEAA, sum of nonessential amino acids; Orn, ornithine; Arg, arginine; Cit, citrulline; Pro, proline; Tyr, tyrosine; Phe, phenylalanine; Val, valine; Gly, glycine; Ser, serine; Ala, alanine; BCAA, branched chain amino acid; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; OH-Kyn, hydroxykynurenine; Trp, tryptophan.
[a]$P<0.05$.

citrulline metabolism is supported by experimental results in mice showing that partial nephrectomy induced citrullinuria (15), possibly because of overload and saturation of tubular transporters. Interestingly, we also found that, independent of CKD severity, diabetic patients had higher plasma citrulline concentrations. This finding is in line with the results from a diabetic rat model, in which citrullinemia increased early in the course of the disease, although the mechanism remains unclear (16).

In our population, we observed a significant increase in plasma ornithine concentrations in patients with CKD4–5 compared with CKD2–3. Ornithine concentrations are increased in the plasma of uremic patients compared with controls (2,17), but they do not seem to be correlated with the degree of kidney function (9). Ornithine is an intermediate of the urea cycle, during which it is converted into citrulline (Supplemental Figure 2). The relative increase in plasma citrulline (increasing Cit/Orn) in more severe CKD indicates an activation of ornithine catabolism directed to citrulline synthesis and possibly, proline synthesis. This hypothesis is suggested by the increased prolinemia and Pro/Orn found in HD patients and the association between proline excretion and eGFR. These results suggest an increased proline synthesis from accumulating ornithine, which would be compensated by urinary excretion in the early stages of the disease. Tubular reuptake of proline depends on a low-affinity system shared with glycine and a specific system with high affinity (18). We did not observe changes in glycinuria suggesting that the high-affinity transporter SLC6A20 could be involved.

We observed a gradual increase in plasma ADMA concentrations and a decrease of its urinary excretion in patients with more severe CKD. Reduced GFR probably explained the lower excretion and participated in ADMA accumulation, hereby confirming its classification as a uremic retention solute (19,20). Other mechanisms have also been observed *in vivo*, such as an increased activity in protein arginine methyltransferases responsible for ADMA synthesis and a reduced catabolism by dimethylargininase (EC3.5.3.18) (21). We also showed that plasma ADMA concentrations were directly associated with serum sodium levels. This association could be causal, since ADMA infusion has been shown to induce sodium retention (22) and would further support the link between ADMA and hypertension, glomerulosclerosis, and CKD progression (22,23). ADMA also acts on vascular function by inhibiting NO synthase. We found that the relative presence of arginine, estimated by Arg/ADMA, was progressively reduced in patients with more severe CKD. A low ratio could inhibit NO production (24) and explain the NO deficiency observed in early CKD patients with normal arginine levels (25).

In this study, plasma concentrations of tryptophan progressively declined with reducing renal function, and this decline was not explained by urinary losses. Tryptophan catabolism by indoleamine 2,3-dioxygenase (EC1.13.11.52) has been shown to increase with CKD severity (26,27). In our patients, we also measured plasma hydroxykynurenine and confirmed that this pathway was upregulated, although microbial production of indoles from tryptophan could also be involved (28). In contrast, plasma phenylalanine concentrations were independent of kidney function but increased with the degree of inflammation and acidosis. Interestingly, plasma phenylalanine concentration has been shown to be increased in inflammatory diseases, including uremia (29). Additionally, CKD is expected to directly affect phenylalanine metabolism through the availability of phenylalanine 4-hydroxylase (EC1.14.16.1), a renal and hepatic enzyme responsible for converting phenylalanine into tyrosine (30). In our population, the relative presence of tyrosine (Tyr/Phe ratio) was gradually lower in more evolved CKD stages, and we found a tendency to declining tyrosine concentrations with CKD severity. A similar pattern was expected for the renal glycine hydroxymethyltransferase (EC2.1.2.1), which converts glycine to serine; however, variations in Ser/Gly were less clear cut, and changes in plasma serine were only observable in HD patients. Still, these results suggest that metabolic disturbances and reduced synthesis rates induced by altered renal function could be clinically relevant and possibly lead to AA deficiencies.

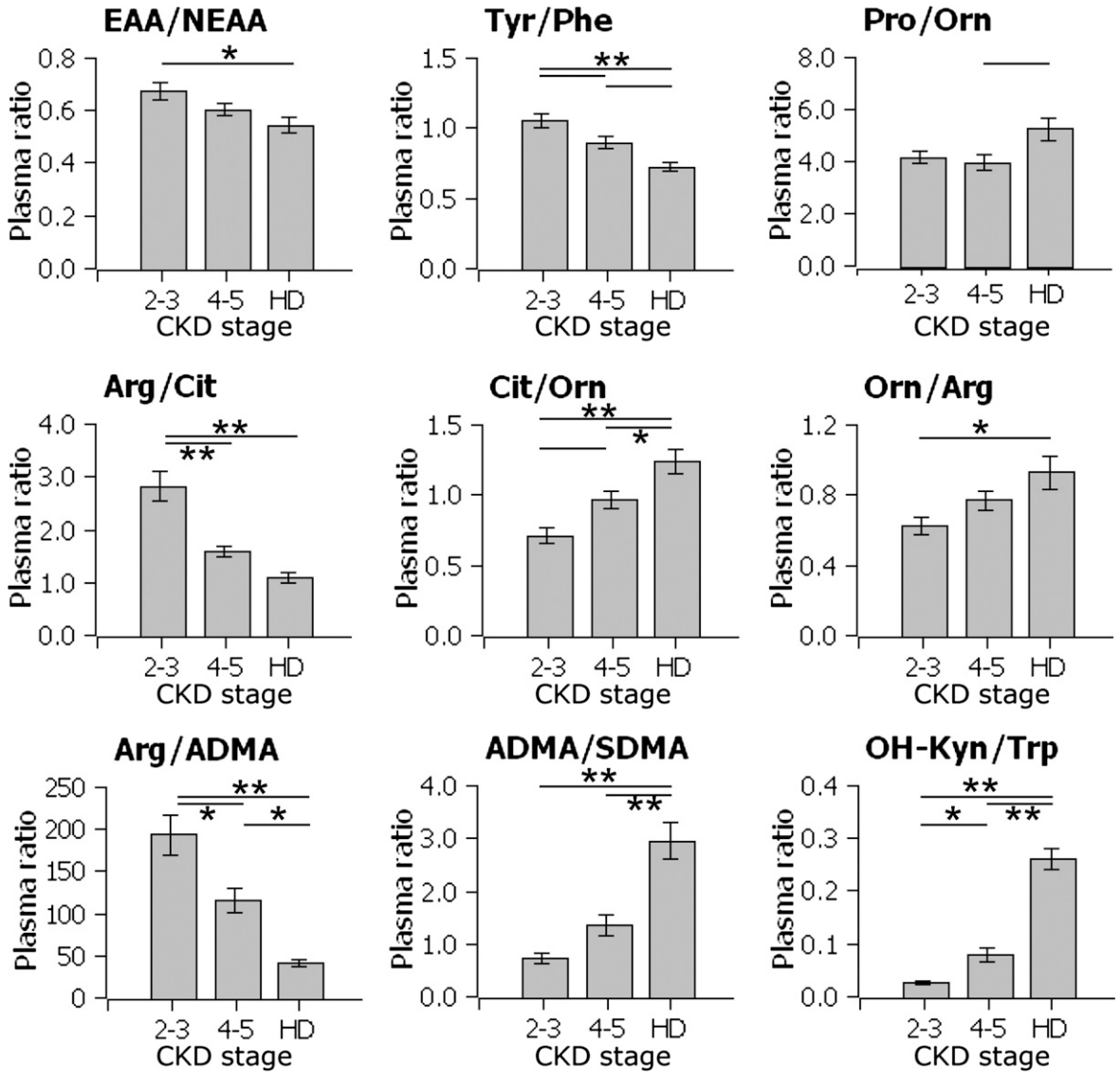


**Figure 2.** | **Plasma ratios in CKD stages 2 and 3, CKD stages 4 and 5, and hemodialysis (HD) patients.** Bars are mean ± SEM. Lines represent significant differences after adjusting for multiple comparisons (simple line, $P<0.05$; *$P<0.01$; **$P<0.001$). ADMA, asymmetric dimethylarginine; Arg, arginine; Cit, citrulline; EAA, essential amino acid; NEAA, nonessential amino acid; OH-Kyn, hydroxykynurenine; Orn, ornithine; Phe, phenylalanine; Pro, proline; SDMA, symmetric dimethylarginine; Trp, tryptophan; Tyr, tyrosine.

Our study has some limitations that should be considered. First, protein intake of patients was not assessed, whereas there was some evidence of a higher risk of malnutrition in those patients with more advanced CKD (lower plasma albumin levels and lower EAA/NEAA and Val/Gly ratios). In multivariate models, however, eGFR was a better predictor of plasma AA levels than albuminemia, suggesting that observations were not explained by malnutrition only. Second, fasting could not be ensured, but the timing of blood sampling did not affect result. Third, it was questioned whether the greater importance of proteinuria in severe CKD could have influenced the results of urinary AA excretion. Although urine contains protease activity, proteolysis is essentially completed after voiding (31). Unspecific proteolytic cleavage of urinary proteins *in vivo* may have induced unspecific free AA production. Additionally, results from multivariate analyses confirmed the presence of significant associations with other factors, including eGFR. Also, it is noteworthy that citrulline excretion increased with proteinuria but was not a constitutive element of proteins.

Using the latest urinary and plasma metabolomic approaches, we observed significant associations between disease severity and AA and amine metabolism in CKD patients. We identified situations suggesting an overproduction occurring at a systemic level (Cit) and situations of urinary retention (ADMA) and urinary excretion (proline). These data add to our understanding of the CKD-associated modifications observed in AA metabolism and may be of relevance for clinical evaluation of these patients, particularly in regard to metabolic and nutritional aspects.

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**Disclosures**

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