



## Use of serum and urine metabolome analysis for the detection of metabolic changes in patients with stage 1-2 chronic kidney disease

Kaori Hayashi<sup>1</sup>, Hiroyuki Sasamura<sup>1\*</sup>, Takako Hishiki<sup>2</sup>, Makoto Suematsu<sup>2</sup>, Satsuki Ikeda<sup>3</sup>, Tomoyoshi Soga<sup>3</sup>, Hiroshi Itoh<sup>1</sup>

<sup>1</sup> Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan

<sup>2</sup> Department of Biochemistry, School of Medicine, Keio University, Tokyo, Japan

<sup>3</sup> Institute for Advanced Biosciences, Keio University, Yamagata, Japan

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### ABSTRACT

**Background:** Chronic kidney disease (CKD) is a major health problem throughout the world, and understanding the pathological condition of CKD has become increasingly important. The recent development of advanced metabolomic assay techniques now allows the human metabolic condition to be evaluated sensitively and comprehensively.

**Objectives:** The aim of this study was to use metabolomic analysis to perform a preliminary survey of metabolic changes occurring in patients with stage 1-2 CKD.

**Patients and Methods:** Serum and urine metabolomic profiles of 15 patients with stage 1-2 CKD were analyzed using our previously reported capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) systems, and compared to 7 healthy volunteers.

**Results:** The CE-TOFMS systems in three different modes for cation, anion, and nucleotide analyses detected multiple metabolites in serum and urine samples. In cation analysis mode, several increases in nonessential amino acids were identified in patients with stage 1-2 CKD, similar to those reported for end-stage renal disease (ESRD). Free-radical scavengers carnosine and hypotaurine were decreased in the urine, whereas serum hypotaurine and taurine were increased, consistent with changes in renal and/or systemic oxidative stress. Moreover, the cardiotoxin hypoxanthine was markedly increased in the serum, whereas serum and urine adenosine and urine guanine were decreased, suggesting changes in purine nucleotide metabolism which could affect cardiovascular prognosis. Changes in other unidentified metabolites were also detected.

**Conclusions:** These results suggest that multiple changes in the metabolism are already detectable in stage 1-2 CKD using metabolome analysis. Further studies on these metabolic changes may result in new strategies to prevent cardiovascular events and progression to ESRD in patients with CKD.

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#### ► Implication for health policy/practice/research/medical education:

The results of this study suggest that multiple changes in the metabolism are already detectable in stage 1-2 CKD, and these changes may be detected using metabolome analysis.

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\* Corresponding author at: Hiroyuki Sasamura, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, 160-8582, Tokyo, Japan. Tel: +81-35363-3796, Fax: +81-33359-2745.

E-mail: [sasamura@a8.keio.jp](mailto:sasamura@a8.keio.jp)

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## 1. Background

It has been reported that the prevalence of chronic kidney disease (CKD) has increased to over 10% (1), and is now

a major health problem throughout the world. A number of co-morbidities including cardiovascular diseases are associated with CKD and prognosis is poor, with many patients experiencing disease progression (2). CKD is not only a strong risk factor for cardiovascular diseases (3, 4), but also the precursor of end stage renal failure (ESRD, also known as CKD stage 5), which needs renal replacement therapy such as dialysis. Previous studies using ion-exchange chromatography or HPLC have identified several changes in amino acid metabolism in patients with ESRD (5-8), but a survey of metabolomic changes has not been reported. Metabolomics is a discipline dedicated to the global study of metabolites, their dynamics, composition, interactions, and responses to interventions or to changes in their environment (9). The recent developments of advanced metabolomic assay techniques now allow the human metabolic condition to be evaluated sensitively and comprehensively. Metabolomics has already been reported to be effective for the discovery of biomarkers for disease diagnosis, such as cancer (10, 11) and cardiovascular diseases (12), but its effectiveness for the assessment of renal physiology and kidney disease is still uncertain. Metabolomics may be a useful tool for analyzing the condition of CKD, because CKD is recognized to be a disease affecting multiple biochemical pathways, and thus may cause multiple changes in systemic metabolism. Moreover, CKD is a strong risk factor for cardiovascular disease, which is highly correlated with metabolic changes. Finally, blood and urine examinations are non-invasive procedures, and due to the fact that they identify changes in both systemic and renal metabolism, may

be particularly useful for the non-invasive assessment of patients with CKD.

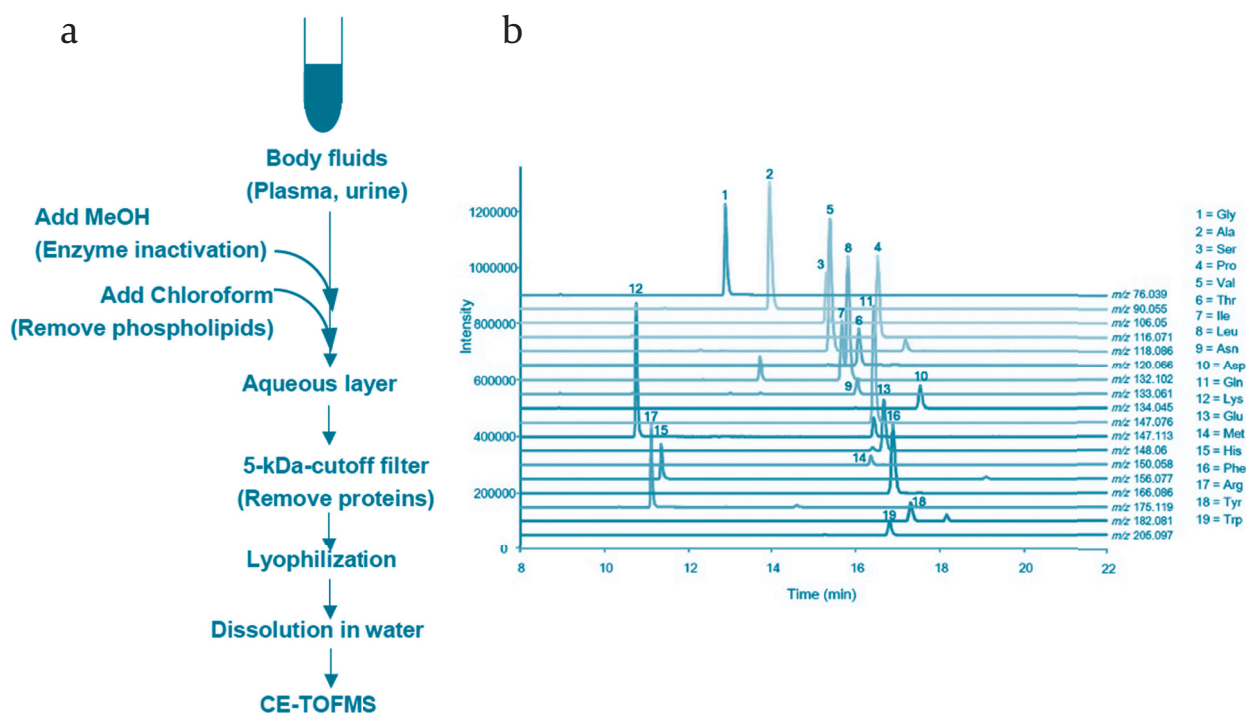
## 2. Objectives

Because of the potential importance of understanding metabolic changes in patients with CKD, the aim of this study was to examine the serum and urine metabolites of patients with stage 1-2 CKD, using our recently developed CE-TOFMS system (13), and to compare the results to healthy volunteers, in order to see if metabolic changes can be detected at an early stage in CKD.

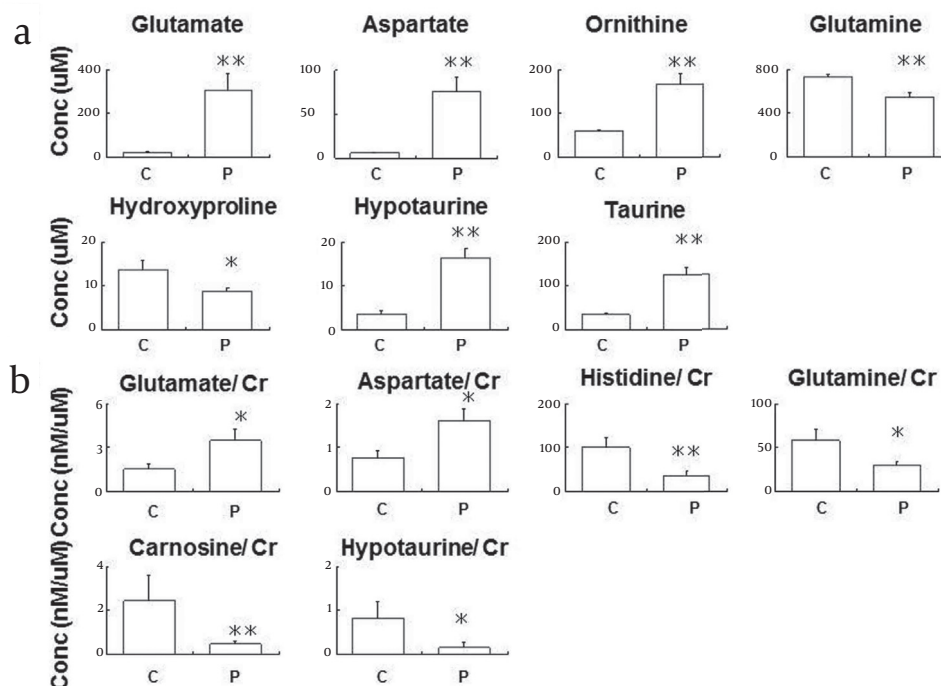
## 3. Materials and Methods

### 3.1. Patient recruitment and sample collection

This study followed the ethical standards of the Helsinki Declaration and was approved by the Ethics Committee of Keio University. Informed consent was obtained from each participant. A total of fifteen patients with stage 1-2 CKD who were admitted to Keio University Hospital, Tokyo, Japan from January 2008 to March 2009 for kidney biopsy were enrolled in this study. Seven healthy volunteers (5 male and 2 female, with no medical problems including urine abnormalities) participated in this study as controls. CKD was defined according to the criteria of the KDIGO group based on the K/DOQI clinical practice guidelines for CKD (3). In accordance with these criteria, stage 1 CKD was defined as CKD with normal or increased GFR ( $\geq 90$  mL/min/1.73 m<sup>2</sup>), and stage 2 CKD was defined as CKD with a mild decrease in GFR (60-89 mL/min/1.73 m<sup>2</sup>).



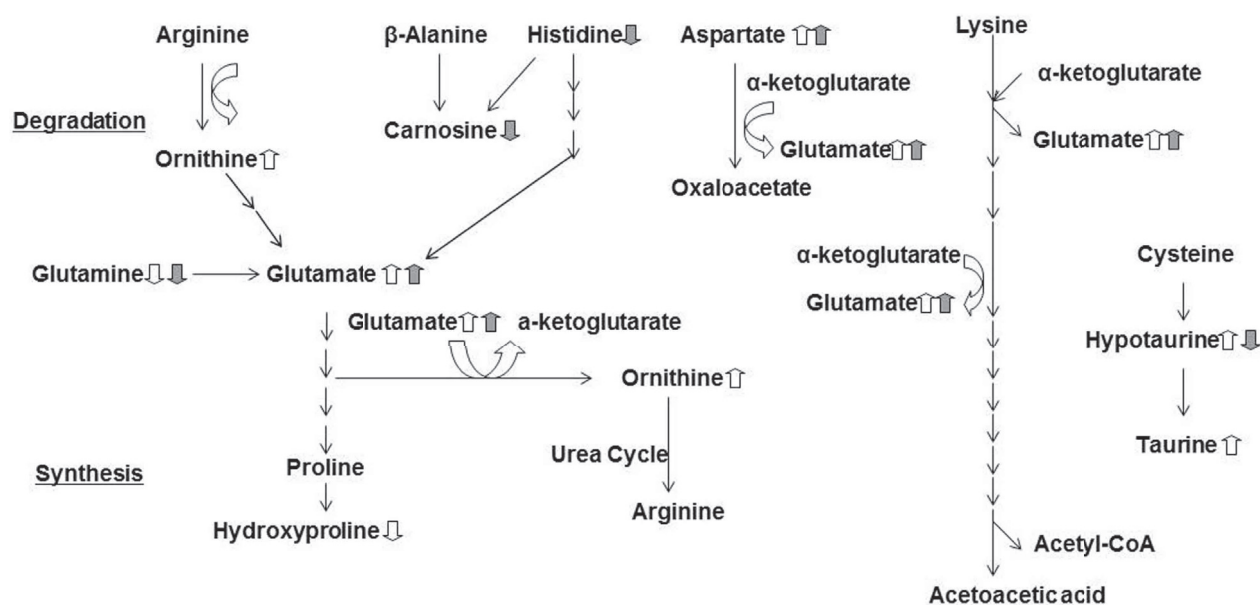
**Figure 1.** (a) Schematic representation of metabolite extraction method prior to CE-TOFMS analysis. (b) Representative electropherogram of CE-TOFMS.



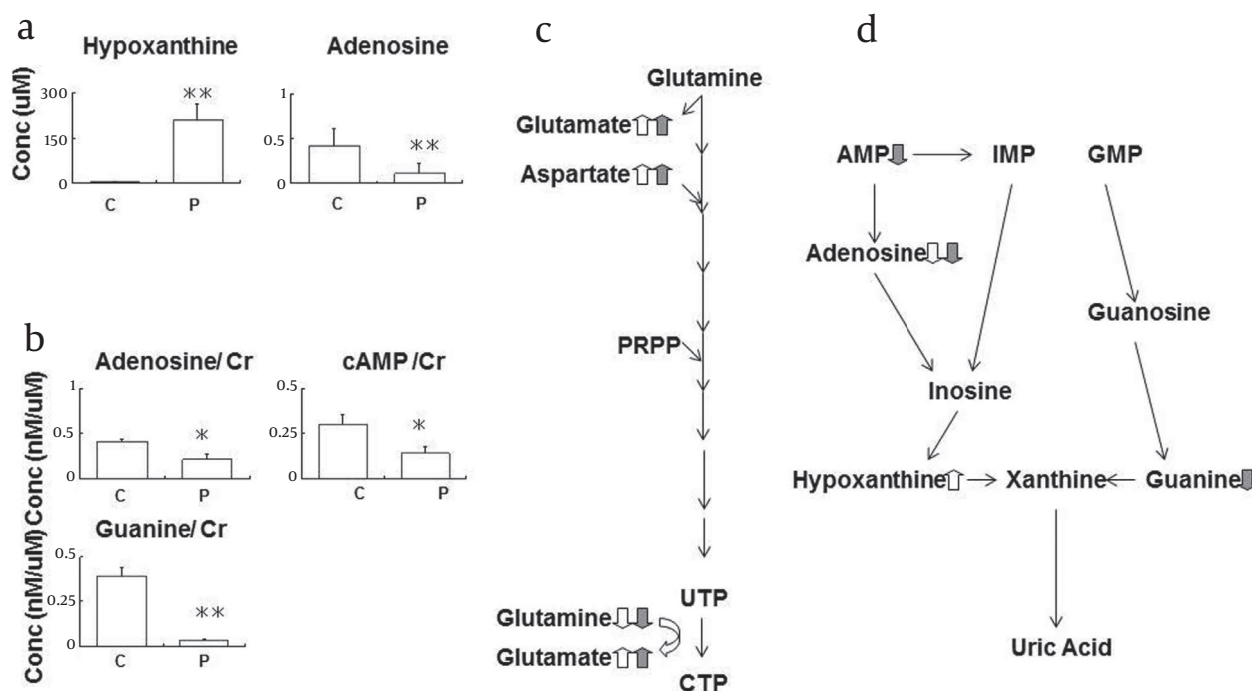
**Figure 2.** Changes in amino acid metabolites in (a) serum and (b) urine of patients with stage 1-2 CKD. C: Control, P: CKD patients. \*, \*\*:  $p < 0.05$ ,  $p < 0.01$  vs. controls.

2) (14). Because these patients were candidates for renal biopsy, the presence of proteinuria ( $\geq 0.5$  g/g creatinine) had been checked on at least 3 separate occasions during a period of over 3 months. Moreover, the kidney biopsies were checked in all the patients to confirm that there were no false positive or false negatives in the diagnosis of CKD. Blood and urine samples for metabolomic studies were obtained after an overnight fast. The protocol

for urine collection was to void the bladder at bedtime, then to obtain a mid-stream sample from the first morning sample. Samples were then centrifuged without delay at 3000 rpm for 10 min at 4 °C, and the supernatant was stored at -80 °C until extraction and assay. Values of serum chemistries were obtained using standard hospital laboratory techniques, and GFR was calculated from the age and serum creatinine concentrations.



**Figure 3.** Observed metabolite changes in the serum and urine of patients with stage 1-2 CKD mapped onto the pathways involved in amino acid degradation and synthesis. Open arrows represent significant increases or decreases in serum samples, and closed arrows represent significant changes in urine samples.



**Figure 4.** Changes in nucleic acid metabolites in (a) serum and (b) urine of patients with stage 1-2 CKD. Observed metabolite changes mapped onto the pathways involved in (c) pyrimidine nucleotide synthesis and (d) purine nucleotide degradation. Open arrows represent significant increases or decreases in serum samples, and closed arrows represent significant changes in urine samples. \*, \*\*:  $p < 0.05$ ,  $p < 0.01$  vs. controls.

### 3.2. Metabolites Extraction

Serum or urine samples (100  $\mu$ l) were added to methanol (900  $\mu$ l) containing internal standards (20  $\mu$ M each of methionine sulfone, MES, and D-Camphol-10-sulfonic acid) and mixed to inactive enzymes. After adding deionized water (400  $\mu$ l) and chloroform (1 ml), the solution was centrifuged at 4600 g for 5 min at 4  $^{\circ}$ C and the 300  $\mu$ l upper aqueous layer was filtered through a Millipore 5-kDa cutoff centrifuge filter to remove proteins. The filtrate was lyophilized and dissolved in 50  $\mu$ l of Milli-Q water containing reference compounds (200  $\mu$ l each of 3-aminopyrrolidine and trimesate) prior to capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) analysis. Samples from patients and controls were prepared and quantified simultaneously to avoid inter-assay variations (Figure 1).

### 3.3. CE-TOFMS Conditions for Cationic Metabolite Analysis

The instrumentation and measurement conditions of CE-TOFMS are described elsewhere (13, 15, 16). Separations were carried out in a fused silica capillary (50  $\mu$ m inner diameter x 100 cm total length) filled with 1M formate as the electrolyte. Approximately 3  $\mu$ l of sample solution were injected at 50 mbar for 3 sec, and 30 kV of Voltage was applied. The capillary was maintained at 20  $^{\circ}$ C, and the sample tray was cooled below 5  $^{\circ}$ C. Methanol water (50 % v/v) containing 0.1  $\mu$ M Hexakis (2,2-difluorothoxy) phosphazene was delivered as the sheath liquid at 10  $\mu$ l/min.

### 3.4. CE-TOFMS Conditions for Anionic Metabolite Analysis

A cationic polymer-coated COSMO (+) capillary was used as the separation capillary. A 50mM ammonium acetate solution (pH 8.5) was used as electrolyte solution for CE separation. Sample solution (30  $\mu$ l) was injected at 50 mbar for 30 s and -30 kV of voltage was applied. Ammonium acetate (5 mM) in 50 % methanol-water (v/v) containing 0.1  $\mu$ M Hexakis (2, 2-difluorothoxy) phosphazene was delivered as the sheath liquid at 10  $\mu$ l/min.

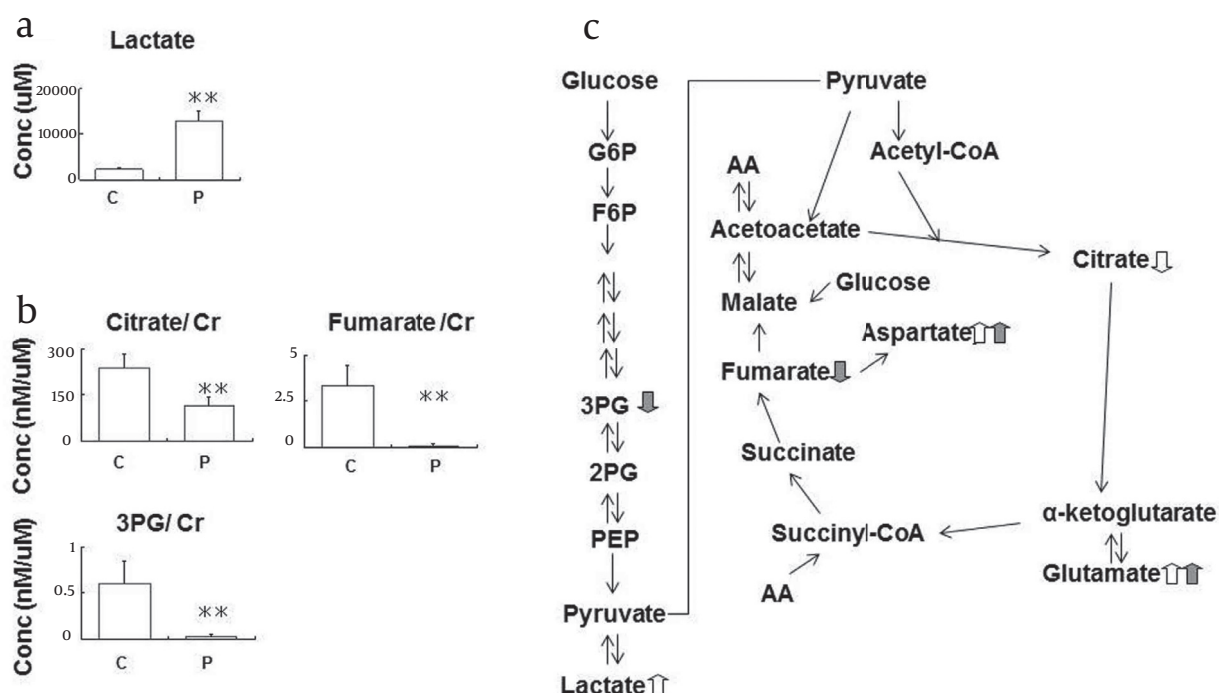
### 3.5. CE-TOFMS Conditions for Nucleotide-related Metabolite Analysis

Separations were carried out in a fused silica capillary filled with 50 mM ammonium acetate (pH 7.5). ESI-TOFMS was operated in the negative ion mode, and the capillary voltage was set at 3500 V. A flow rate of heated dry nitrogen gas (heater temperature 300  $^{\circ}$ C) was maintained at 7 l/min. Other conditions were identical to those used in anionic metabolite analysis.

### 3.6. Data processing and statistical analysis

Raw data were analyzed with our proprietary software named MasterHands-1.0.6.16 and JDAMP-128, as described previously (13, 15). For each urine sample, the measured metabolite concentrations were normalized using concentration of creatinine to obtain the amount of metabolite contained (nmol) per creatinine level ( $\mu$ mol) of each sample. Statistical comparisons were made by





**Figure 5.** Changes in carbohydrate metabolites in (a) serum and (b) urine of patients with stage 1-2 CKD. (c) Observed metabolite changes mapped onto carbohydrate metabolic pathways. Open arrows represent significant increases or decreases in serum samples, and closed arrows represent significant changes in urine samples. C: Control, P: CKD patients. \*\*:  $p < 0.01$  vs. controls.

Mann-Whitney's U-test. P values < 0.05 were considered to be statistically significant.

## 4. Results

#### 4.1. Baseline characteristics

Serum and urine metabolite profiles were compared between 15 patients with stage 1-2 CKD and 7 healthy volunteers. The baseline characteristics of the patients and controls are shown in *Table 1*. The two groups were similar in age, gender, BMI, and blood pressure. Estimated GFR (eGFR) in patients with stage 1-2 CKD showed a significant decrease ( $74.84 \pm 4.30$  ml/min/1.73m<sup>2</sup>,  $p < 0.01$ ), which was compatible with the definition of stage 1-2 CKD patients, where stage 1 is defined as renal function of 90 ml/min/1.73 m<sup>2</sup> or greater, and stage 2 as 60-89 ml/min/1.73 m<sup>2</sup> respectively. Blood urea nitrogen and creatinine levels were not significantly different between the two groups. 24-h urinary protein excretion was increased in the patient group, whereas the value in controls was below the detectable threshold.

#### 4.2. Changes in amino acid metabolites in the serum and urine of patients with stage 1-2 CKD

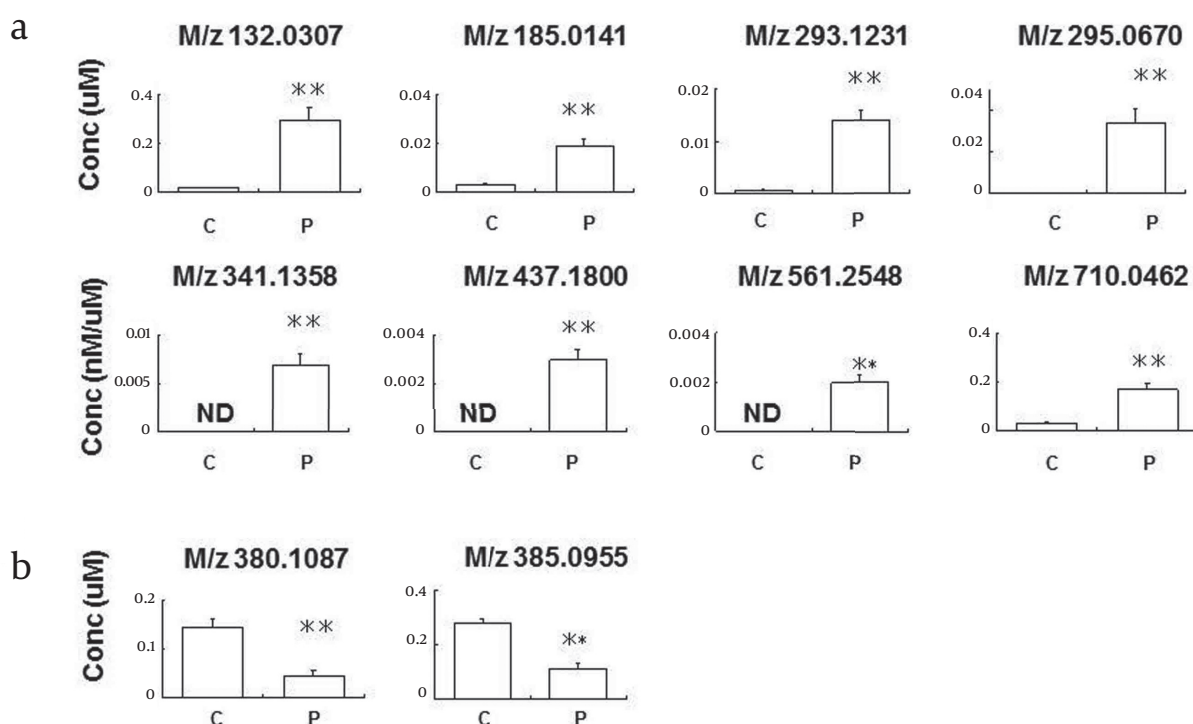
The CE-TOFMS systems in three different modes for cation, anion, and nucleotide analyses detected multiple metabolites, in serum and urine samples. In cation analysis mode, various changes in amino acid metabolites were found in the serum and urine of patients with

early stage CKD compared to healthy volunteers (Figure 2, 3). Several nonessential amino acids, in particular glutamate, aspartate, and ornithine were significantly increased in the serum (glutamate: from  $22.7 \pm 2.7$  to  $294.7 \pm 78.8$ ; aspartate: from  $5.7 \pm 0.5$  to  $76.2 \pm 16.2$ ; ornithine: from  $59.4 \pm 3.1$  to  $167.1 \pm 24.4$   $\mu\text{M}$ ,  $p < 0.01$ ). Similar increases were found in the urine for glutamate and aspartate. In contrast, the essential amino acid histidine was decreased in the urine (from  $100.6 \pm 24.2$  to  $36.6 \pm 9.1$   $\text{nM}/\mu\text{M}$ ,  $p < 0.01$ ). Both serum and urine glutamine were significantly decreased, suggesting a possible change in the equilibrium for glutamine-glutamate conversion by glutamine synthetase. Hydroxyproline was significantly

**Table 1.** Clinical characteristics of patients included in the study

Variable	Control (No. = 7)	CKD patients (No. = 15)
<b>Age</b>	30.9 ± 5.0	43.7 ± 4.2
<b>Sex</b> (male/female)	5/2	10/5
<b>Body Mass Index</b> (kg/m <sup>2</sup> )	20.4 ± 0.6	21.7 ± 0.9
<b>Systolic BP</b> (mmHg)	112 ± 4	121 ± 3
<b>Diastolic BP</b> (mmHg)	73 ± 4	75 ± 3
<b>Blood urea nitrogen</b> (mg/dl)	12.4 ± 1.0	14.3 ± 1.0
<b>Serum creatinine</b> (mg/dl)	0.74 ± 0.05	0.83 ± 0.03
<b>eGFR</b> (ml/min/1.73m <sup>2</sup> )	101.71 ± 4.88	74.84 ± 4.30 <sup>a</sup>
<b>Serum uric acid</b> (mg/dl)	5.4 ± 0.4	6.1 ± 0.4
<b>Urine protein</b> (g/day)	below threshold	1.35 ± 0.32

<sup>a</sup> p < 0.01 vs. controls



**Figure 6.** Changes in unidentified metabolites which were (a) increased or (b) decreased in the serum of patients with stage 1-2 CKD. C: Control, P: CKD patients. M/z: mass-to-charge ratio of the metabolite. ND: not detectable. \*\*:  $p < 0.01$  vs. controls.

decreased in the serum (from  $13.8 \pm 2.0$  to  $8.7 \pm 0.8 \mu\text{M}$ ,  $p < 0.05$ ). Several changes were also found in oxidative stress-related amino acids. In particular, carnosine and hypotaurine were significantly lower in the urine of CKD patients, whereas hypotaurine and taurine were elevated in the serum.

#### 4.3. Changes in nucleic acid metabolites in the serum and urine of patients with stage 1-2 CKD

In cation and anion analysis mode, multiple changes in nucleic acid metabolites were observed in the serum and urine of patients with early stage CKD (Figure 4). In particular, hypoxanthine in the serum was markedly elevated (from  $4.0 \pm 0.7$  to  $209.8 \pm 53.3 \mu\text{M}$ ,  $p < 0.01$ ), whereas adenosine was decreased in both the serum and urine of patients.

#### 4.4. Changes in carbohydrate metabolites in the serum and urine of patients with stage 1-2 CKD

Several changes in carbohydrate metabolites were also observed in the serum and urine of patients with CKD (Figure 5). Serum lactate increased from  $2331 \pm 422$  to  $12903 \pm 2273 \mu\text{M}$  ( $p < 0.01$ ), whereas urine citrate, fumarate and 3-phosphoglycerate were decreased compared to controls.

#### 4.5. Changes in unidentified metabolites in the serum of patients with stage 1-2 CKD

The metabolome analysis revealed that serum levels of

several novel unidentified metabolites were also markedly increased (Figure 6a) or decreased (Figure 6b) in the patients with CKD compared to controls.

## 5. Discussion

There have been several reports about serum amino acid patterns in advanced (stage 5) CKD, also known as end-stage renal disease (ESRD) (5-8). In general, the essential amino acid levels are decreased, while the nonessential amino acids are either within the normal range or increased, so the ratio of essential to nonessential amino acids is decreased in ESRD. It has been assumed that these changes are due to low protein intake, deficiency of excretory and metabolic functions of the diseased kidneys, toxic effect of uremia and, in dialysis patients, loss of protein and amino acids by the dialytic procedure (6, 7). The results of this study were compatible with the previous reports on patients with ESRD, and suggest that the changes in amino acid metabolism were already detectable at an early stage of CKD. It is interesting that these changes were seen even without marked renal insufficiency, suggesting that changes in amino acid metabolism are an early event in the course of CKD, and do not require the presence of uremia. Interestingly, not all nonessential amino acids were increased. In particular, glutamine was decreased in both the serum and urine, whereas glutamate was increased, suggesting possible changes in the conversion equilibrium of these two amino acids in these patients. We also found evidence for changes in oxidative stress in early stage CKD. In particular, the free-radical scavengers carnosine

and hypotaurine were decreased in the urine of patients, but conversely hypotaurine and taurine were increased in the serum. We speculate that these free-radical scavengers may have been decreased in the urine because of increased oxidative stress in the kidney, and this was counteracted by increases in the serum.

An important advantage of this method is that multiple metabolic pathways could be analyzed simultaneously using the three modes of electropherogram analysis. Regarding nucleic acid metabolites, serum and urine adenosine and urine guanine were decreased and serum hypoxanthine increased in the patient group, suggesting the possibility that degradation of purine nucleotide was elevated in these patients with stage 1-2 CKD. Interestingly, hypoxanthine was markedly increased in the serum of patients, to about 50 times the level of controls. Previous report in patients on dialysis showed that plasma concentrations of hypoxanthine and uric acid were increased in patients with ESRD (17). In this study hypoxanthine was already increased in patients with stage 1-2 CKD, even though serum uric acid was unchanged. These results may be important because hypoxanthine may act as a cardiotoxin (18), possibly by causing mitochondrial damage through increased oxidative stress (19). A recent report also suggested that hypoxanthine accumulation in xanthine oxidoreductase depletion mice caused progression of renal interstitial fibrosis, also by an oxidative stress-related mechanism (20). These results suggest the hypothesis that increased hypoxanthine may be one reason for the increased incidence of cardiovascular disease in patients with CKD (3, 4), as well as a potential risk factor for progression of renal disease.

Concerning carbohydrate metabolism, we found that serum lactate was increased, but other TCA cycle metabolites, such as citrate and fumarate, were decreased, suggesting that changes in glucose metabolism may also be evident from an early stage in CKD. An important advantage of metabolome analysis is the potential to identify new and unidentified metabolites which could have important pathophysiological functions. In our studies, we found that several novel unidentified metabolites were significantly increased in the serum of patients with CKD, whereas others were decreased. At present, the molecular structures of these metabolites are unknown. It is possible that these unidentified products may have novel pathophysiological functions, or may be new disease markers for renal injury. We are therefore planning further extended studies to examine these possibilities. One caveat of this study is that the patients with stage 1-2 CKD in our study were all candidates for renal biopsy, and may not be representative of the general population of stage 1-2 CKD. Thus, the possibility that these changes specifically appeared in proteinuric kidney diseases, but may not seen in early stage CKD without proteinuria, cannot be completely ruled out. Moreover, CKD of various etiologies were considered together in the patient group, because we were unable to discover a clear correlation

between specific etiologies and their metabolomic profiles. Based on our current findings, further studies are warranted for comparisons between different renal diseases. In summary, the results of this study suggest that metabolic analysis may be used for detecting changes in amino acid, nucleic acid, and carbohydrate metabolites in the serum and urine of patients with early stage CKD, as well as for detecting unidentified metabolites which may have novel functions. Understanding these changes may be important for developing new strategies to prevent cardiovascular events and progression to ESRD in patients with CKD.

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None declared.

## Conflict of interest

The authors declare that they have no conflicts of interests related to this study.

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