

## ORIGINAL ARTICLE

# Metabolomic profiling of uremic solutes in CKD patients

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Early detection and accurate monitoring of patients with chronic kidney disease (CKD) is likely to improve care and decrease the risk of cardiovascular and cerebrovascular diseases. As a new diagnostic tool, we examined the retention of uremic solutes as a simpler, more accurate method to assess renal function. To achieve this, we comprehensively evaluated these solutes in CKD patients. By capillary electrophoresis with mass spectrometry, we found 22 cations and 30 anions that accumulated significantly as the estimated glomerular filtration rate (eGFR) decreased. These compounds included 9 cations and 27 anions that were newly identified in this study. In contrast, we also found 7 cations (2 new) and 5 anions (all new) that decrease significantly as eGFR declines. We evaluated each substance for its suitability to detect early CKD stage. Compounds that are highly correlated with eGFR and whose plasma concentration changed in a manner approximated by the first-degree equation are excellent candidates for detecting CKD and identifying uremic toxins that might aggravate kidney function in the early stage of CKD. These results identify a number of uremic compounds, many of which are novel and which predict worsening renal function. These compounds provide diagnostic information and may be targets for therapies designed to treat the complications of CKD patients.

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**Keywords:** biomarker; CE-MS; CKD; uremic toxin

## INTRODUCTION

Chronic kidney disease (CKD) is an important public health problem and determination of the glomerular filtration rate (GFR) has been essential for the evaluation of CKD patients.<sup>1–3</sup> Early detection of impairment of renal function allows treatment to prevent further deterioration and complications. With the progression of CKD, various uremic toxins accumulate, subsequently causing renal damage and hypertension.<sup>4,5</sup> Recently, we revealed that many compounds accumulate during renal failure, and that the kidney-specific organic anion transporter SLCO4C1 excretes uremic toxins, resulting in the reduction of blood pressure and renal inflammation.<sup>6</sup> To generalize these results for clinical use, it is necessary to examine the accumulation of uremic solutes precisely. Therefore, the aims of this study were (1) to identify new uremic retention solutes that accumulate in CKD patients comprehensively by capillary electrophoresis with mass spectrometry (CE-MS) and (2) to determine substances that are more sensitive for detecting early renal damage than serum creatinine (Cr) or cystatin C.

## MATERIALS AND METHODS

### Study population

The study population comprised 41 CKD patients who visited the outpatient clinic of Tohoku University Hospital between April 2007 and December 2008.

The study protocol complied with the Declaration of Helsinki and was approved by the Committees on the Ethics of Human Research of Tohoku University. Informed consent was obtained from each participant. Cystatin C was measured using a BNII nephelometer (Dade Behring, currently Siemens Healthcare Diagnostics, Deerfield, IL, USA) and a particle-enhanced immunonephelometric assay (N Latex cystatin C; Siemens Healthcare Diagnostics). Biochemical analyses were carried out using routine methods at the Department of Clinical Chemistry at Tohoku University Hospital.

### CE-MS measurement for metabolome analysis

A comprehensive and quantitative analysis of charged metabolites by CE-MS was performed.<sup>6–10</sup> CKD patient's plasma (50  $\mu$ l) was immediately plunged into methanol (450  $\mu$ l) containing internal standards (20  $\mu$ M each of methionine sulfone; Wako, Osaka, Japan) for cations, MES (Dojindo, Kumamoto, Japan) and CSA (D-camphol-10-sulfonic acid; Wako). Deionized water (200  $\mu$ l) and chloroform (500  $\mu$ l) were then added, and the mixture was thoroughly mixed. The solution was centrifuged at 4600 $\times$ g for 5 min at 4°C, and the upper aqueous layer was centrifugally filtered through a Millipore 5000 Da cutoff filter (Millipore, Billerica, MA, USA) to remove proteins. The filtrate was lyophilized and dissolved in 25  $\mu$ l of Milli-Q water containing reference compounds (200  $\mu$ M each of 3-aminopyrrolidine; Sigma-Aldrich, St Louis, MO, USA) and trimesate (Wako) before CE-TOFMS (capillary electrophoresis with electrospray ionization time-of-flight mass spectrometry) analysis. All CE-TOFMS experiments were performed using the Agilent CE capillary

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**Table 1** Baseline characteristics of 41 CKD patients

Age	62.8 ± 14.6 (27–84)
Sex (M/F)	16/25
Cystatin C (mg per 100 ml)	2.29 ± 0.90 (0.96–4.11)
Creatinine (mg per 100 ml)	2.64 ± 1.60 (0.8–7.4)
eGFR (ml min <sup>-1</sup> per 1.73 m <sup>2</sup> )	24.4 ± 13.9 (5.9–57.0)
Urine protein (ggCr <sup>-1</sup> )	1.58 ± 1.64 (0.00–7.07)
Uric acid (mg per 100 ml)	7.68 ± 1.57 (4.4–9.8)
Hemoglobin (g per 100 ml)	11.28 ± 2.06 (7.1–17.5)
Height (cm)	158.0 ± 7.57 (140.1–175)
Weight (kg)	57.2 ± 10.0 (42–72.5)
Systolic blood pressure (mm Hg)	129.07 ± 14.21 (107–160)
Diastolic blood pressure (mm Hg)	76.29 ± 11.66 (53–110)
<i>Cause of CKD</i>	
Alport syndrome	1 (2.4%)
Analgesic-induced nephropathy	1 (2.4%)
ANCA-related nephropathy	1 (2.4%)
Aortitis syndrome	1 (2.4%)
Bilateral renal infarction	1 (2.4%)
Diabetic nephropathy	8 (19.5%)
Focal segmental glomerulosclerosis	3 (7.3%)
Glomerulosclerosis	4 (9.7%)
Hydronephrosis	1 (2.4%)
IgA nephropathy	9 (21.9%)
Lipoprotein glomerulopathy	1 (2.4%)
Lupus nephropathy	2 (4.8%)
Membrane proliferative glomerulonephritis	1 (2.4%)
Membranous nephropathy	2 (4.8%)
Polycystic kidney	1 (2.4%)
Renal tuberculosis	1 (2.4%)
Sarcoidosis	1 (2.4%)
Unknown	2 (4.8%)
<i>Baseline disease</i>	
Cardiovascular disease	10 (24.3%)
Diabetes mellitus	12 (29.2%)
Hypertension	40 (97.5%)
Hyperlipidemia	16 (39.0%)
Smoking	11 (26.8%)
Body mass index (> 30 kg m <sup>-2</sup> )	1 (2.4%)
<i>Drug</i>	
Allopurinol	12 (29.2%)
Angiotensin receptor blocker	30 (73.1%)
AST-120	5 (12.1%)
Erythropoietin	6 (14.6%)
Statin	8 (19.5%)

Abbreviations: ANCA, antineutrophil cytoplasmic autoantibody; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate.

electrophoresis system (Agilent Technologies, Waldbronn, Germany), the Agilent G3250AA LC/MSD TOF system (Agilent Technologies, Palo Alto, CA, USA), the Agilent 1100 series binary high performance liquid chromatography pump, the G1603A Agilent CE-MS adapter and the G1607A Agilent CE-ESI-MS sprayer kit. For data acquisition, we used G2201AA Agilent ChemStation software for CE and Analyst QS for Agilent TOFMS software.<sup>9</sup>

### Data analysis

GFR was estimated using the simplified prediction equation derived from the modified version described in the Modification of Diet in Renal Disease Study as proposed by the Japanese Society of Nephrology:<sup>11</sup> eGFR = 194 × [age]<sup>-0.287</sup> × [serum creatinine (mg per 100 ml)]<sup>-1.094</sup> × [0.739 if female]. All values are expressed as

**Table 2** The 42 cationic compounds detected by CE-MS that negatively correlated with eGFR

Compound	Spearman's correlation coefficients	P-value	Reference
Creatinine	-0.965	<0.001	5,6
SDMA	-0.946	<0.001	5,14
Guanidinosuccinate	-0.933	<0.001	5,6,15
Citrulline	-0.801	<0.001	6,16,17
1-Methyladenosine	-0.772	<0.001	5,18
<i>N</i> -Acetylglucosamine	-0.751	<0.001	
γ-Butyrobetaine	-0.734	<0.001	
Ophthalmate	-0.687	<0.001	
3-Methylhistidin	-0.669	<0.001	6,16,17
Hydroxyproline	-0.666	<0.001	19
Trimethylamine <i>N</i> -oxide	-0.665	<0.001	6,20
Allantoin	-0.653	<0.001	6,21
ADMA	-0.614	<0.001	5,6,14
<i>N</i> -ε-Acetylysine	-0.602	<0.001	
Kynurenine	-0.579	<0.001	5,22
Cytosine	-0.570	<0.001	
Indole-3-acetate	-0.475	0.002	5,23
Hypotaurine	-0.447	0.004	
<i>N,N</i> -Dimethylglycine	-0.428	0.006	6,24
7-Methylguanine	-0.406	0.010	
Methionine sulfoxide	-0.405	0.010	6 <sup>a</sup>
Asn	-0.337	0.033	
Cysteine-glutathione disulphide	-0.279	0.077	
Gln	-0.273	0.083	
Arg	-0.271	0.087	
His	-0.267	0.091	
Ethanolamine phosphate	-0.261	0.098	
Gly	-0.258	0.102	
Piperazine	-0.253	0.110	
β-Ala	-0.243	0.124	
Ornithine	-0.192	0.244	17
Homoarginine;	-0.183	0.248	
N6-N6-N6-trimethyllysine			
Sarcosine	-0.182	0.250	
Pipecolate	-0.122	0.439	6 <sup>b</sup>
<i>O</i> -Acetylcarnitine	-0.118	0.453	
Pro	-0.113	0.474	
Butanoate	-0.108	0.491	
5-Methyltetrahydrofolate	-0.088	0.576	
Ala	-0.070	0.656	
5-Oxoproline	-0.032	0.837	
Taurine	-0.024	0.879	17
Ser	-0.0003	0.998	

Abbreviations: ADMA, asymmetric dimethylarginine; CE-MS, capillary electrophoresis with mass spectrometry; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; SDMA, symmetric dimethylarginine.

These compounds are lined in an ascending order of Spearman's correlation coefficients.

A value of  $P < 0.05$  is accepted as indicating significant correlation with eGFR (22 cations).

Shaded compounds have not been reported before. Methionine sulfoxide was increased in CKD patients, although it decreased in rat renal failure. Pipecolate was increased in rat renal failure; however, it was not increased significantly in CKD patients.

<sup>a</sup>Decreased.

<sup>b</sup>Increased.

mean ± s.d. The statistical analysis was performed using statistical software SPSS 11 version 11 (Chicago, IL, USA). Spearman's rank correlation was calculated to assess the correlation between eGFR and the plasma concentrations of the compounds. A value of  $P < 0.05$  was accepted as indicating statistical significance.

To estimate the log likelihood, we used PROC GENMOD in SAS software version 9 (SAS Institute, Cary, NC, USA). We assumed that the compounds might fit either the first-degree or second-degree model. The first-degree model

**Table 3** The 24 cationic compounds detected by CE-MS that are positively correlated with eGFR (that is, the plasma concentrations of these compounds are decreased as eGFR declines)

Compound	Spearman's correlation		Reference
	coefficients	P-value	
Trp	0.668	<0.001	6,16
Val	0.478	0.002	17
Tyr	0.453	0.004	6,16,17
2-Aminobutyrate	0.453	0.004	
Guanidoacetate	0.441	0.005	6,15
Glu	0.422	0.007	
Leu	0.386	0.014	17
Asp	0.262	0.097	
Hypoxanthine	0.259	0.101	
Ile	0.225	0.153	
Lys	0.219	0.166	
Glycerophosphorylcholine	0.211	0.182	
Guanosine	0.207	0.190	
1-Methyl-2-pyrrolidone	0.207	0.190	
Carnitine	0.202	0.201	6 <sup>a</sup>
Inosine	0.193	0.223	
Urocanate	0.181	0.251	
1-Methylnicotinamide	0.165	0.294	
Thr	0.152	0.336	
Met	0.121	0.444	
Betaine	0.098	0.535	
Creatine	0.084	0.593	
Phe	0.058	0.712	
$\alpha$ -Amino adipate	0.045	0.775	

Abbreviations: CE-MS, capillary electrophoresis with mass spectrometry; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate. These compounds are lined in descending order of Spearman's correlation coefficients. A value of  $P < 0.05$  is accepted as indicating significant correlation with eGFR (seven cations). Shaded compounds have not been reported before. Carnitine was decreased in rat renal failure; however, it was not increased significantly in CKD patients.  
<sup>a</sup>Decreased.

**Table 4** The 38 anionic compounds detected by CE-MS that are negatively correlated with eGFR (that is, the plasma concentrations of these compounds are increased as eGFR declines)

Compound	Spearman's correlation		Reference
	coefficients	P-value	
Isethionate	-0.929	<0.001	
Gluconate	-0.929	<0.001	
trans-Aconitate	-0.869	<0.001	6
Pimelate	-0.847	<0.001	
3-Indoxyl sulfate	-0.834	<0.001	5,6,26
isocitrate	-0.825	<0.001	
N-Acetyl- $\beta$ -alanine	-0.805	<0.001	
N-Acetylglutamate	-0.782	<0.001	27
Sebacate	-0.751	<0.001	
4-Oxopentanoate	-0.725	<0.001	
cis-Aconitate	-0.719	<0.001	
Homovanillate	-0.711	<0.001	
Adipate	-0.677	<0.001	
Citramalate	-0.670	<0.001	
2-Isopropylmalate	-0.669	<0.001	
Threonate	-0.631	<0.001	
Hippurate	-0.608	<0.001	5,28
N-Acetylaspartate	-0.594	<0.001	
4-Hydroxy-3-methoxymandelate	-0.572	<0.001	

**Table 4** Continued

Compound	Spearman's correlation		Reference
	coefficients	P-value	
Oxamate	-0.559	<0.001	
Glutarate	-0.465	0.003	
Azelate	-0.463	0.003	
Phthalate	-0.456	0.003	
Citrate	-0.454	0.004	29
Malonate	-0.440	0.005	
Citraconate	0.401	0.011	
Quinate	-0.392	0.013	
Succinate	-0.362	0.021	
Cysteine S-sulfate	-0.343	0.030	
4-Hydroxy-3-methoxybenzoate	-0.322	0.041	
Glycerophosphate	-0.284	0.072	
2-Hydroxyoctarate	-0.208	0.189	
Malate	-0.157	0.321	29
2-Hydroxypentanoate	-0.139	0.379	6 <sup>a</sup>
Heptanoate	-0.128	0.416	
Laurate	-0.100	0.527	
5-Oxoproline	-0.067	0.671	
Mucate	-0.064	0.686	

Abbreviations: CE-MS, capillary electrophoresis with mass spectrometry; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate. These compounds are lined in ascending order of Spearman's correlation coefficients. A value of  $P < 0.05$  is accepted as indicating significant correlation with eGFR (30 anions). Shaded compounds have not been reported before. Although 2-hydroxypentanoate was increased in rat renal failure, it was not increased significantly in CKD patients.  
<sup>a</sup>Increased.

**Table 5** The 13 anionic compounds detected by CE-MS that are positively correlated with eGFR (that is, the plasma concentrations of these compounds are decreased as eGFR declines)

Compound	Spearman's correlation		Reference
	coefficients	P-value	
4-Methyl-2-oxopentanoate	0.673	<0.001	
2-Oxoisopentanoate	0.645	<0.001	
Lactate	0.349	0.027	
Octanoate	0.348	0.027	
2-Oxoglutarate	0.331	0.036	
Pyruvate	0.305	0.053	
2-Hydroxyisobutyrate,	0.226	0.152	
2-hydroxybutyrate			
Fumarate	0.155	0.327	
3-Hydroxybutyrate	0.094	0.553	
Pelargonate	0.063	0.689	
Pentanoate, 3-methylbutanoate	0.021	0.892	
Decanoate	0.015	0.923	
Hexanoate	0.011	0.946	6 <sup>a</sup>

Abbreviations: CE-MS, capillary electrophoresis with mass spectrometry; eGFR, estimated glomerular filtration rate. These compounds are lined in ascending order of Spearman's correlation coefficients. A value of  $P < 0.05$  is accepted as indicating significant correlation with eGFR (five anions). Shaded compounds have not been reported before.  
<sup>a</sup>Increased.

fits the compound concentrations in plasma of CKD patients  $C_p = \beta_1(eGFR) + \alpha$ , and the second-degree model fits  $C_p = \beta_1(eGFR)^2 + \beta_2(eGFR) + \alpha$ . The  $\beta_1$  and  $\beta_2$  are calculated using regression models. To estimate the suitability of both the first-degree and second-degree models, the appropriateness of the addition of  $eGFR^2$  as an independent variable was examined by comparing the log likelihood ratios of the models.<sup>12,13</sup> The difference between the log likelihood ratios is used for testing the significance of the contribution of  $eGFR^2$  to

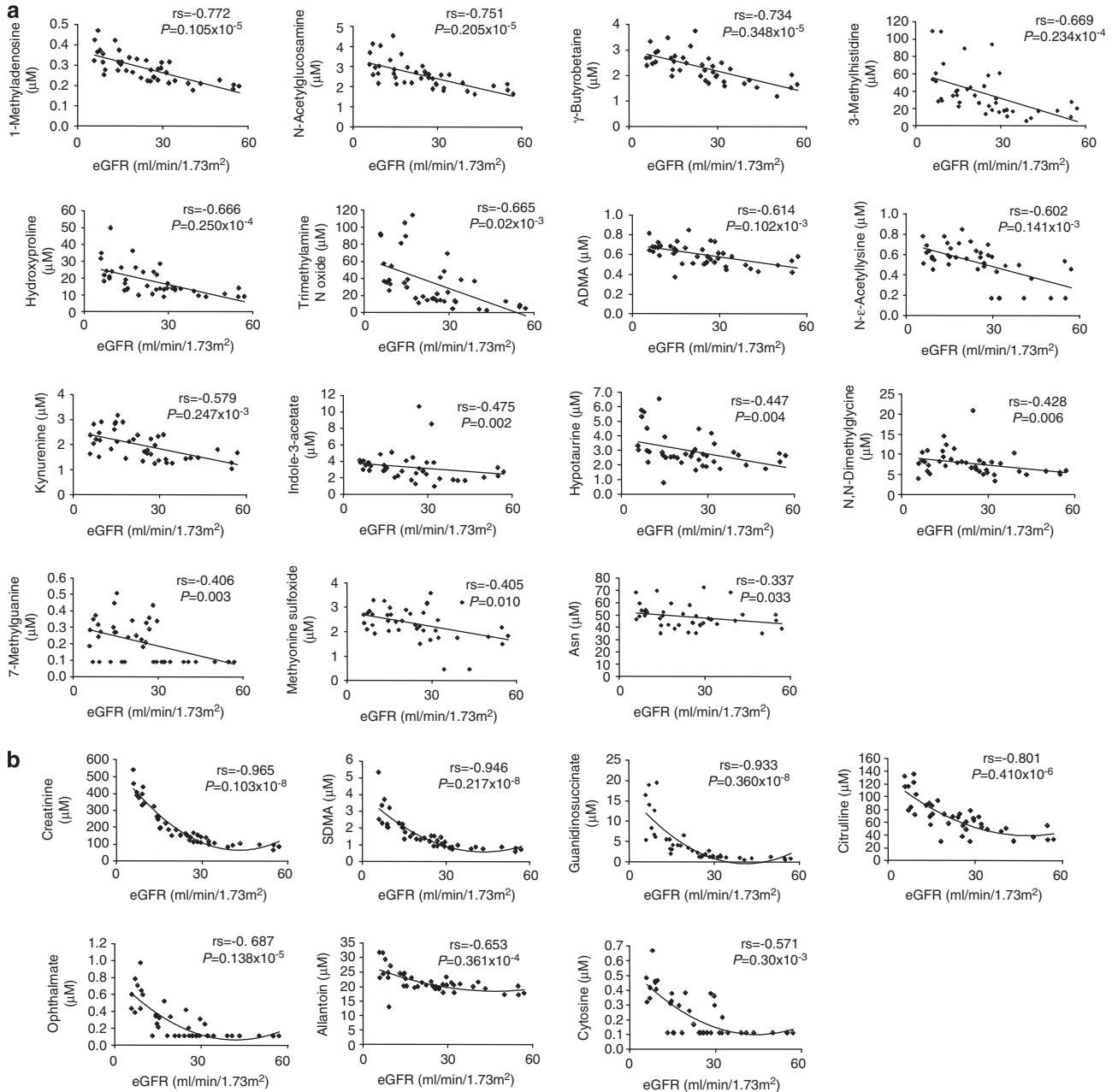
the model.<sup>13</sup> This value follows  $\chi^2$  distribution with 1 d.f. If the calculated difference is  $>3.84$ , we consider that the compound is more likely to fit the second-degree equation. To compare the plasma concentration change between the first-degree and second-degree models in the same graph, we converted Cp to Cpsd by calculating Z-scores:  $Cpsd = (Cp - \text{mean of } Cp) / \text{s.d. of } Cp$ . Cpsd means how many folds of s.d. the plasma concentration separates from the mean value.

## RESULTS

### Study population and CE-MS analysis

The characteristics of the patients are in Table 1. Using CE-MS, 312 cationic and 193 anionic compounds, whose molecular weights from 73.0 to 460.1 *m/z* were detectable, and 65 cationic and 52

anionic compounds were identified in CKD patients (Tables 2–5). To find candidates that might be useful in detecting CKD, we analyzed the relationship between the compound concentrations and eGFR. As in Table 2, 42 cationic compounds were negatively correlated with eGFR (that is, the plasma concentrations of these compounds increased as eGFR declined), and the 22 cationic compounds were significant ( $P < 0.05$ ). Among 22 compounds, 13 compounds were already reported as uremic retention solutes.<sup>5,14–24</sup> On the other hand, other nine cationic compounds, *N*-acetylglucosamine,  $\gamma$ -butyrobetaine, ophthalmate, *N*- $\epsilon$ -acetyllysine, cytosine, hypotaurine, 7-methylguanidine, methionine sulfoxide and Asn, have not been reported to be accumulated (shaded in Table 2).



**Figure 1** (a, b) The relation between eGFR and plasma concentration of 22 cationic compounds that accumulated significantly as eGFR declined is shown. (a) Compounds that are categorized in the first-degree equation group. (b) Compounds that are categorized in the second-degree equation group. (c, d) The relation between eGFR and plasma concentration of seven cationic compounds that accumulated significantly as eGFR declined is shown. (c) Compounds that are categorized in the first-degree equation group. (d) Compounds that are categorized in the second-degree equation group.

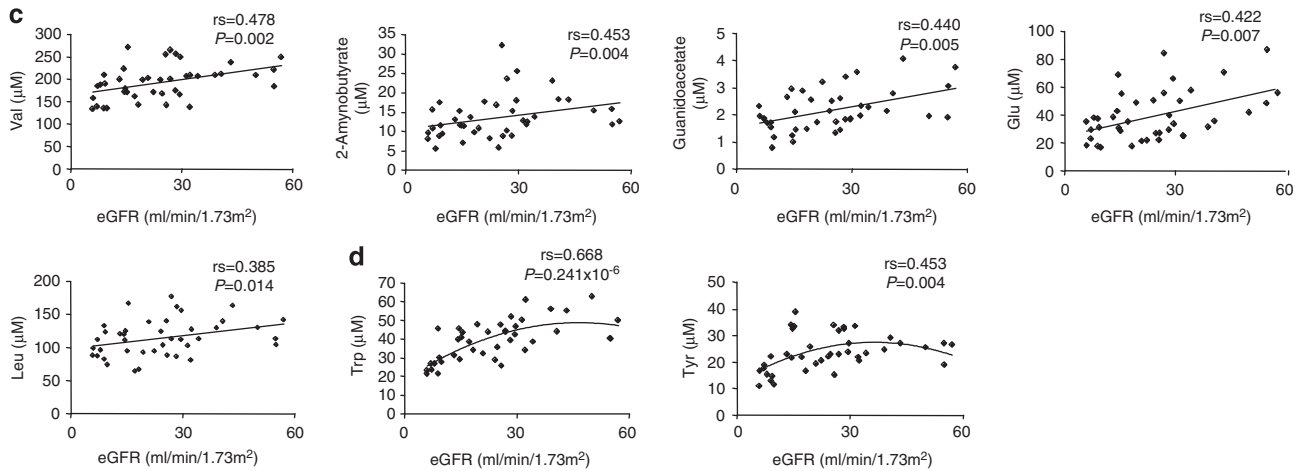


Figure 1 Continued.

In Table 3, 24 cationic compounds were positively correlated with eGFR (that is, the plasma concentrations of these compounds were decreased as eGFR declined). Among these, seven cationic compounds, Trp, Val, Tyr, 2-aminobutyrate, guanidoacetate, Glu and Leu, were significantly correlated with eGFR. Five of these were previously reported,<sup>16,17,25</sup> but 2-aminobutyrate and Glu have not been known as compounds that decreased (shaded in Table 3).

In Table 4, 38 anionic compounds that negatively correlated with eGFR are shown. Among these, 30 anionic compounds were significantly correlated with eGFR.

Five anionic compounds, 3-indoxyl sulfate, *N*-acetylglutamate, *trans*-aconitate, hippurate and citrate, were already reported to increase in renal failure.<sup>5,6,26–29</sup> On the other hand, 25 other compounds, isethionate, gluconate, pimelate, isocitrate, *N*-acetyl- $\beta$ -alanine, sebacate, 4-oxopentanoate, *cis*-aconitate, homovanillate, adipate, citramalate, 2-isopropylmalate, threonate, *N*-acetylaspartate, 4-hydroxy-3-methoxymandelate, oxamate, glutarate, azelate, phthalate, malonate, citraconate, quinate, succinate, cysteine *S*-sulfate and 4-hydroxy-3-methoxybenzoate, were clarified as new uremic retention anions (shaded in Table 4). In Table 5, 13 cationic compounds that positively correlated with eGFR are shown. Among these, five compounds, 4-methyl-2-oxopentanoate, 2-oxoisopentanoate, lactate, octanoate and 2-oxoglutarate, were significantly correlated with eGFR and the plasma concentrations of these five anions have not been reported to be decreased as eGFR declines (shaded in Table 5). These data identified various uremic solutes that correlated with the change of eGFR in CKD patients.

#### Comparison of manner of change during renal damage

We next examined whether the identified uremic retention solutes would be better markers for detecting early stage of CKD than Cr or cystatin C by fitting the first-degree or the second-degree equation models.<sup>13</sup> Figures 1a and b show the relationship between eGFR and the plasma concentration of 22 cationic compounds accumulating significantly as eGFR declines. Among these, 15 compounds, 1-methyladenosine, *N*-acetylglucosamine,  $\gamma$ -butyrobetaine, 3-methylhistidine, hydroxyproline, trimethylamine *N*-oxide, asymmetric dimethylarginine, *N*- $\epsilon$ -acetyllysine, kynurenine, indole-3-acetate, hypotaurine, *N,N*-dimethylglycine, 7-methylguanine, methionine sulfoxide and Asn, were approximated and categorized in the first-degree equation group (Figure 1a). This means that the concentrations of these compounds increased linearly from eGFR 60 to 10 ml min<sup>-1</sup> per

1.73 m<sup>2</sup>. On the other hand, seven compounds, Cr, symmetric dimethylarginine, guanidinosuccinate, citrulline, ophthalmate, allantoin and cytosine, were categorized in the second-degree equation group (Figure 1b). Figures 1c and d show the positive relation between eGFR and the plasma concentration of seven cationic compounds. Val, 2-aminobutyrate, guanidoacetate, Glu and Leu were categorized and approximated in the first-degree equation group (Figure 1c). This means that the concentration of these compounds decreased linearly from eGFR 60 to 10 ml min<sup>-1</sup> per 1.73 m<sup>2</sup>. On the other hand, Trp and Tyr were categorized in the second-degree equation group (Figure 1d).

Figures 2a and b show the relation between eGFR and the plasma concentration of 30 anionic compounds, which accumulated significantly as eGFR declined. Sebacate, *cis*-aconitate, homovanillate, adipate, citramalate, 2-isopropylmalate, *N*-acetylaspartate, 4-hydroxy-3-methoxymandelate, oxamate, glutarate, azelate, phthalate, citrate, malonate, citraconate, quinate, succinate, cysteine *S*-sulfate and 4-hydroxy-3-methoxybenzoate were categorized in the group approximated by the first-degree equation and negatively correlated with eGFR (Figure 2a). On the other hand, isethionate, gluconate, *trans*-aconitate, pimelate, 3-indoxyl sulfate, isocitrate, *N*-acetyl- $\beta$ -alanine, *N*-acetylglutamate, 4-oxopentanoate, threonate and hippurate were categorized in the second-degree equation group (Figure 2b).

Figures 2c and d show the relation between eGFR and the plasma concentration of five anionic compounds that decreased significantly as eGFR declined. Thus, 4-methyl-2-oxopentanoate, 2-oxoisopentanoate, lactate and octanoate were approximated and categorized in the first-degree equation group (Figure 2c). On the other hand, 2-oxoglutarate was categorized in the second-degree equation group (Figure 2d).

We further analyzed the distribution of the compounds that sensitively represented the decline of eGFR in the early stage of CKD. Figure 3 shows the relationship between eGFR and the converted plasma concentration values of the compounds. The concentration of Cr and cystatin C approximated by the second-degree equation started to increase from eGFR 30 ml min<sup>-1</sup> per 1.73 m<sup>2</sup> with an exponential curve. On the other hand, when we chose compounds that significantly correlated with eGFR ( $r_s > 0.7$ ) and approximated by the first-degree equation, the concentration of 1-methyladenosine, *N*-acetylglucosamine,  $\gamma$ -butyrobetaine, sebacate, *cis*-aconitate and homovanillate began to increase from eGFR 60 ml min<sup>-1</sup> per 1.73 m<sup>2</sup> in a linear correlation.

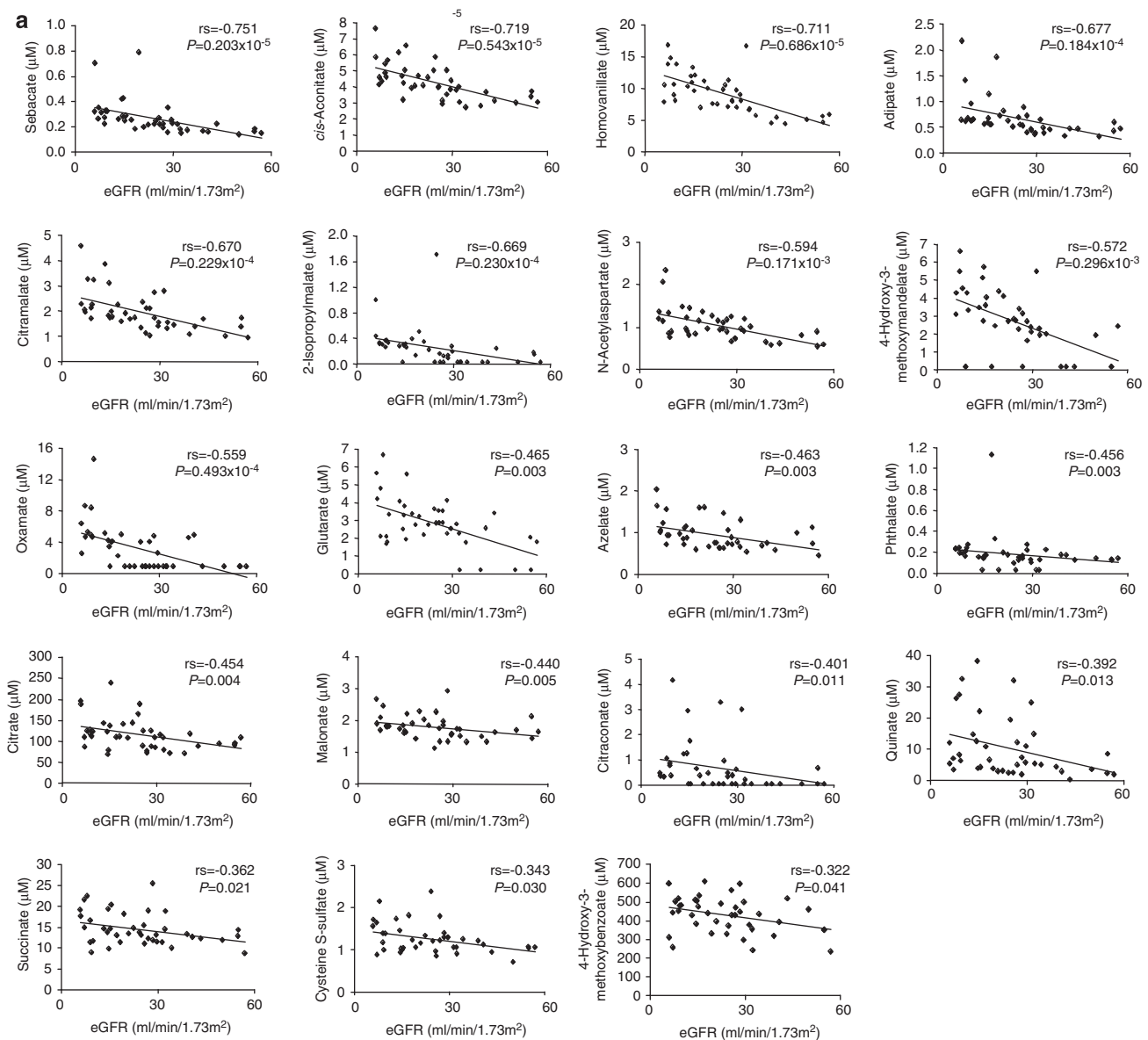


## DISCUSSION

The accumulation of uremic toxins leads to difficulty in controlling blood pressure and impaired renal function and worsens the prognosis.<sup>5,14,30</sup> So far, more than 110 organic compounds have been identified as uremic retention solutes.<sup>4,5</sup> We found 22 cations and 30 anions that accumulated significantly as the eGFR decreases. These compounds included 9 cations and 27 anions that were newly identified in this study. We also found 7 cations and 5 anions, including 2 newly found cations and 5 anions, that decreased significantly as eGFR declined. Thus, knowledge of the retained compounds in CKD could serve as markers for detecting the progression of CKD.

In clinical practice, the plasma Cr concentration is inversely related to the GFR and used for estimating GFR. However, the serum Cr level

does not increase until the GFR has moderately decreased and this is referred to as a 'creatinine-blind GFR area' (40–70 ml min<sup>-1</sup> per 1.73 m<sup>2</sup>).<sup>31</sup> Recently, cystatin C has been a better marker of kidney function.<sup>32</sup> However, analysis of the data with receiver operating characteristic curves did not show any difference in diagnostic accuracy between cystatin C and Cockcroft and Gault results.<sup>32</sup> In this study we propose that a number of compounds, all of which have a high correlation coefficients with eGFR ( $r_s > 0.7$ ) and whose plasma manner of concentration change is approximated by the first-degree equation increase or decrease in a linear manner from the early stage of CKD, could be better markers for detecting early change of GFR. On the basis of these criteria, 1-methyladenosine, *N*-acetylglucosamine,  $\gamma$ -butyrobetaine, sebacate, *cis*-aconitate and homovanillate could be candidates as markers for detecting the



**Figure 2** (a, b) The relation between eGFR and plasma concentration of 30 anionic compounds that accumulated significantly as eGFR declined is shown. (a) Compounds that are categorized in the first-degree equation group. (b) Compounds that are categorized in the second-degree equation group. (c, d) The relation between eGFR and plasma concentration of five anionic compounds that accumulated significantly as eGFR declined is shown. (c) Compounds that are categorized in the first-degree equation group. (d) Compounds that are categorized in the second-degree equation group.

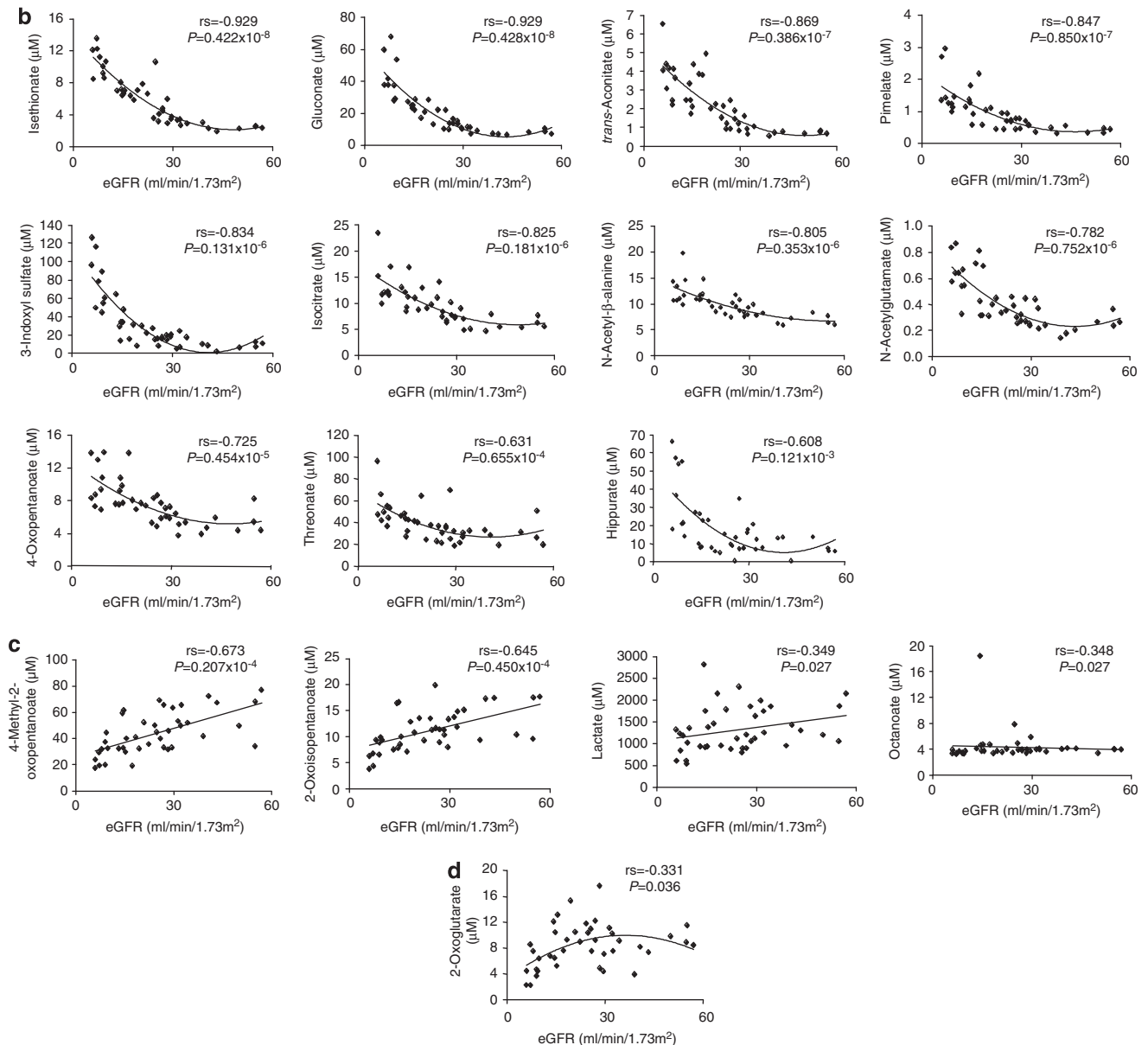


Figure 2 Continued.

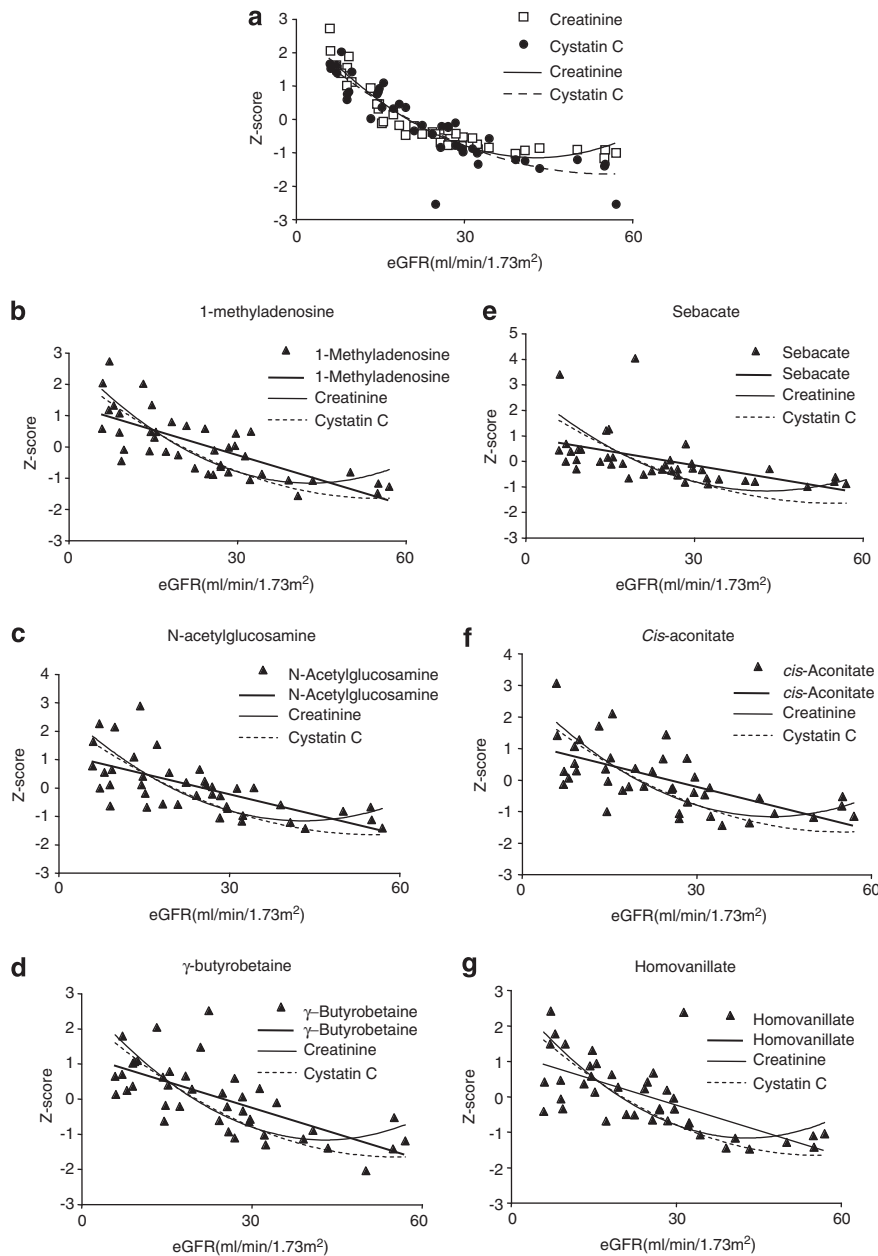
early stage of renal damage in CKD patients (Figure 3). In addition, the serum level of 1-methyladenosine has been reported as cancer marker in the patients.<sup>33</sup>

The most important limitation of our analysis is that we did not examine the association of these compounds with age, gender, body weight, cigarette smoking, nutrition status or inflammation. We could not analyze any effects of prescribed drugs or causative diseases because the number of patients was too small to evaluate.

We also found no significant difference between patients with immunoglobulin A nephropathy or diabetic nephropathy and without the diseases. Further experiment should be needed to clarify the effect of drugs. In addition, we used eGFR to estimate the renal function instead of directly measuring GFR by measuring the clearance of exogenous substances such as inulin. We also need to clarify the kinetics, toxicity and metabolic pathway of the compounds. Among the candidate of early marker for CKD patients, 1-methyladenosine is

known to accumulate in CKD patients because of lowering of excretion in urine.<sup>18</sup> However, the exact accumulation mechanisms of the other candidates have not been so far known. Further investigations classifying these compounds according to their pathophysiological importance will be a clue for preserving renal function in CKD patients.

We also detected species differences. As reported,<sup>6</sup> the concentrations of anionic compounds (indoxyl sulfate and *trans*-aconitate) were increased in both rat renal failure and CKD patients. On the other hand, increases in 4-acetylbutyrate, hexanoate, 2-hydroxypentanoate and argininosuccinate in rat renal failure were not detected in CKD patients. Among the cationic compounds, the concentrations of asymmetric dimethylarginine, creatinine, guanidinosuccinate, citrulline, 3-methylhistidine, *N,N*-dimethylglycine, allantoin and trimethylamine *N*-oxide were increased in both rat renal failure and in CKD patients. On the other hand, no increases in  $\alpha$ -amino adipate and



**Figure 3** The relationship between eGFR and Z-scores of creatinine (Cr), cystatin C and new uremic marker candidates. (a) Relationship between eGFR and Z-score of creatinine and cystatin C (second-degree group). Approximate curves are shown in solid line (Cr) and dashed line (cystatin C). (b–d) Relationship between eGFR and Z-score of cationic compounds, 1-methyladenosine (b), *N*-acetylglucosamine (c) and  $\gamma$ -butyrobetaine (d). (e–g) Relationship between eGFR and Z-score of anionic compounds, sebacate (e), *cis*-aconitate (f) and homovanillate (g).

pipicolate were detected in CKD patients. Furthermore, the concentrations of guanidinoacetate, Trp and Tyr were decreased in both rat renal failure and CKD patients, but carnitine, desethylatrazine and methionine sulfoxide were decreased only in rat renal failure. On the contrary, methionine sulfoxide was increased only in CKD patients. These data suggest careful interpretation of data from rodent experiments into humans.

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