Biochemical and Biophysical Research Communications 445 (2014) 412–416

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A metabolomics-based approach for predicting stages of chronic kidney disease

Toshihiro Kobayashi [a,*], Tatsunari Yoshida [b], Tatsuya Fujisawa [c], Yuriko Matsumura [a], Toshihiko Ozawa [a,d], Hiroyuki Yanai [a], Atsuo Iwasawa [a], Toshiaki Kamachi [a], Kouichi Fujiwara [c], Masahiro Kohno [a], Noriaki Tanaka [c,1]

[a] Department of Bioengineering, Graduate School of Bioscience and Biochemistry, Tokyo Institute of Technology, 4259-G1-25 Nagatsuta-cho, Midori-ku, Yokohama 226-8502, Japan
[b] Global Application Development Center, Shimadzu Corporation, 1 Nishinokyo Kuwabara-cho, Nakagyo-ku, Kyoto 604-8511, Japan
[c] Kiyokai Tanaka-Kitanoda Hospital, 707 Kitanoda, Higashi-ku, Sakai 599-8123, Japan
[d] Yokohama College of Pharmacy, 601 Matanocho, Totsuka-ku, Yokohama 245-0066, Japan

ARTICLE INFO

Article history:
Received 4 February 2014
Available online 12 February 2014

Keywords:
CKD
Cystatin C
GFR
LC/MS
OPLS

ABSTRACT

Chronic kidney disease (CKD) is a major epidemiologic problem and a risk factor for cardiovascular events and cerebrovascular accidents. Because CKD shows irreversible progression, early diagnosis is desirable. Renal function can be evaluated by measuring creatinine-based estimated glomerular filtration rate (eGFR). This method, however, has low sensitivity during early phases of CKD. Cystatin C (CysC) may be a more sensitive predictor. Using a metabolomic method, we previously identified metabolites in CKD and hemodialysis patients. To develop a new index of renal hypofunction, plasma samples were collected from volunteers with and without CKD and metabolite concentrations were assayed by quantitative liquid chromatography/mass spectrometry. These results were used to construct a multivariate regression equation for an inverse of CysC-based eGFR, with eGFR and CKD stage calculated from concentrations of blood metabolites. This equation was able to predict CKD stages with 81.3% accuracy (range, 73.9–87.0% during 20 repeats). This procedure may become a novel method of identifying patients with early-stage CKD.

1. Introduction

The kidneys play a critical role in maintaining metabolic homeostasis and in hormone synthesis. Renal hypofunction therefore causes waste products to accumulate and disturbs electrolyte balance, red blood cell synthesis, blood pressure control, and bone health. In addition, renal hypofunction is considered a risk factor for cardiovascular disease [1–3]. Although detecting renal hypofunction as soon as possible is desirable, there are few subjective symptoms of early-phase deterioration of renal function.

Glomerular filtration rate (GFR) is widely used to evaluate renal function clinically. The gold standard method of measuring GFR is inulin clearance, but this method is difficult to perform and time consuming, making it unsuitable for routine clinical use [4]. Therefore, the creatinine-based estimated GFR (eGFR), based on blood concentration of creatinine, age and gender, is more frequently used [5]. Renal function can be categorized into five stages based on the level of eGFR (Table 1), with chronic kidney disease (CKD) defined as stages 3–5 or the presence of kidney damage for over 3 months. Although creatinine concentrations are often affected by muscle mass and exercise, they do not decline during early phases of renal dysfunction. Therefore, creatinine-based eGFR is not optimal for early diagnosis of CKD.

The concentration of cystatin C (CysC), a cysteine protease inhibitor, can be used to estimate the degree of CKD [6]. CysC is a small, 13.3-kDa basic protein, easily filtered by the glomeruli. Following reabsorption in the renal tubules, CysC is catabolized to amino acids. CysC-based eGFR (eGFRcys) was recently shown to be a good marker in patients with early-stage CKD [5] and to improve the associations between eGFR and end-stage renal disease and risk of death [7]. However, blood CysC concentration is also affected by inflammation, high glucocorticoid doses, and thyroid dysfunction, suggesting that

Abbreviations: CKD, chronic kidney disease; GFR, glomerular filtration rate; eGFR, estimated GFR; CysC, cystatin C; LC/MS, liquid chromatography/mass spectrometry; HMDB, human metabolome database; IS, internal standard; OPLS, orthogonal partial least squares; K/DOQI, kidney disease outcomes quality initiative.

* Corresponding author. Fax: +81 45 924 5734.
E-mail address: kobayapi@bio.titech.ac.jp (T. Kobayashi).

[1] Deceased 3 July 2013.

http://dx.doi.org/10.1016/j.bbrc.2014.02.021
0006-291X/

Table 1
Study participants and CKD classification.

Participants	Male	37
	Female	32
Age (years)	Median	68
	Mean (± S.D.)	63.5 ± 18.8
	Range	26–91
eGFR	CKD stage	Number of participants
90>	1	22
60–89	2	8
30–59	3	7
15–29	4	18
15<	5	14

relying on a single parameter to determine the degree of CKD may be unreliable [8].

Biomarkers of renal hypofunction are therefore needed to identify patients with early-stage CKD. Previously, we and other researchers reported that the concentrations of various blood metabolites are altered, along with a decline in renal function, in hemodialysis patients [9–11], outpatients with CKD [11,12], partially nephrectomized rats [9,13,14], and rats with adenine-induced CKD [15,16], suggesting that the concentrations of several of these metabolites can be used to construct a quantitative index of renal function. For example, arginine and tryptophan, not previously regarded as uremic toxins, were altered along with previously identified uremic toxins. In almost all previous studies of patients on hemodialysis or with CKD, renal function was compared with the concentrations of individual blood metabolites. Because blood metabolite concentrations may not be altered in parallel with eGFR, several metabolites should be quantified and combined during screening for CKD. As an extension of our previous study on hemodialysis patients, we assessed whether the concentrations of various blood metabolites could predict CKD stage [10]. The metabolites were measured by quantitative liquid chromatography/mass spectrometry (LC/MS), evaluated by simple linear regression analysis, and combined in multivariate analyses.

2. Materials and methods

2.1. Reagents

Methanol, formic acid, and acetonitrile for LC/MS were purchased from Wako Pure Chemical Industries (Osaka, Japan). The standard reagents, N-α-acetyl-L-arginine, L-kynurenine, N^4-acetylcytidine, N^2, N^2-dimethylguanosine, phenylacetylglutamine, hippuric acid, indoxyl sulfate, and N^6-threonylcarbamoyladenosine, were purchased from commercial sources. ^{15}N-labeled hippuric acid (Cambridge Isotope Laboratories, Andover, MA, USA) was used as internal standard (IS). Each reagent was dissolved in distilled water or dimethylsulfoxide to prepare stock solutions of 1 mg/mL, with each further diluted to 100 μg/mL in distilled water. Working solutions of each reagent, at concentrations of 1000, 500, 250, 100, 50, 10, and 5 ng/mL, were prepared by diluting each 100 μg/mL standard solution with 90% methanol.

2.2. Plasma samples

The study protocol complied with the Declaration of Helsinki and was approved by the Ethics Committees of the Tokyo Institute of Technology and Tanaka-Kitanoda Hospital. Written informed consent was obtained from all participants. Ten milliliters of whole blood were collected from participants using heparinized syringes (Table 1) and centrifuged at 1000 × *g*. Plasma samples were stored at −80 °C until analyzed. Plasma CysC concentration was measured using human cystatin C ELISA kits (RD191009100; BioVendor, Brno, Czech Republic).

For LC/MS analysis, the plasma samples were thawed at room temperature. Proteins and contaminants were removed by mixing 50 μL aliquots of plasma with 450 μL of 500 ng/mL IS in methanol and centrifuging the samples at 13,700 × *g* for 10 min at 4 °C. The supernatants were passed through Millipore Millex-LG (0.20 μm) filters and subjected to LC/MS analysis.

2.3. Identification of unknown peaks

To identify unknown peaks, the samples were analyzed using LC-ion trap-time of flight-mass spectrometry (LCMS-IT-TOF; Shimadzu, Kyoto, Japan), as previously described [15]. Selected peaks [10] were monitored in the single ion monitoring mode, and the exact masses and product ion spectra were searched in the MassBank, HMDB, and METLIN databases. The product ion spectra of identified compounds were compared with those of purified, commercially obtained reagents.

2.4. Quantitative LC/MS analysis

Single quadrupole LC/MS analysis was performed using LCMS-2020 (Shimadzu). Two microliter samples were loaded onto a Cadenza CD-C18 column (150 × 2.0 mm; Imtakt Corporation, Kyoto, Japan) maintained at 35 °C with an OPTI-GUARD Fit ODS precolumn (Tokyo Chemical Industry, Tokyo, Japan). Mobile phase A was 0.05% formic acid, and mobile phase B was acetonitrile. The initial solvent gradient program consisted of 2% B, followed by a linear gradient of 2–65% B from 0 to 10 min, 100% B from 10 to 12 min, and 2% B from 12 to 17 min, making the total cycle time per sample 17 min. The solvent flow rates were set at 0.2 mL/min from 0 to 11.5 min, 0.3 mL/min from 11.5 to 15 min, and 0.2 mL/min from 15 to 17 min. Mass spectra were acquired using an electrospray ionization source, with the ion source and heat block maintained at 300 °C and 400 °C, respectively. The chromatograms were acquired in selected ion monitoring mode; the ions monitored by LC/MS are shown in Table 2. The areas under the chromatogram were determined for each peak, and standard curves were obtained based on the IS mixture, except for a peak derived from an unknown compound; i.e., the positive ion at *m*/*z* 367 with a retention time of 5.21 min.

The concentrations of each metabolite and IS, and the peak areas of the unknown compound, were determined by LabSolutions LCMS 5.60 software (Shimadzu). Data for each metabolite were corrected using IS measurements. When the metabolite concentration in plasma sample was over 1000 ng/mL, it was diluted 2 to 4-fold with 90% methanol and reanalyzed.

Table 2
Targeted compounds in the study.

	Exact mass	MS detection	Experimental *m*/*z*
N-α-acetyl-L-arginine	216.1222	Positive	217
L-Kynurenine	208.0848	Positive	209
N^4-acetylcytidine	285.0961	Positive	286
N^2,N^2-dimethylguanosine	311.1230	Positive	312
Phenylacetylglutamine	264.1110	Positive	265
Hippuric acid	179.0582	Negative	178
Indoxyl sulfate	213.0096	Negative	212
N^6-carbamoylthreonyladenosine	412.1331	Negative	411
UK-2 (unknown metabolite)	366.1433	Positive	367
^{15}N-hippuric acid	180.0552	Negative	179

2.5. Construction of eGFR prediction model

Because the plasma was obtained from Japanese subjects, eGFR was calculated using the equations for Japanese individuals, based on biochemically measured CysC [5]; namely, eGFRcys = (104 × $CysC^{-1.019}$ × 0.996^{age} (×0.929, if female)) −8. CKD stage was defined relative to eGFR, as described [4]. Subjects at each CKD stage were randomized about 2:1, with the former subjects used as the work set and the latter as the test set.

Preliminary analyses showed difficulties in constructing a prediction model for early CKD based on eGFR. We therefore used SIMCA 13 to construct an orthogonal partial least squares (OPLS) regression model for 1/eGFR using data from the work set. The predictive ability of 1/eGFR values was determined by analyzing the test set data using the OPLS regression model. The predicted eGFR was derived from reciprocal transformation of predicted 1/eGFR values and used to classify predictive CKD stage. The accuracy of regression was determined by comparing the CKD stage predicted by the OPLS equation with the CKD stage calculated from biochemical measurements. Each assay was repeated 20 times in a similar manner.

3. Results

3.1. Identification of previously unidentified metabolites

Our earlier study described the detection of three previously unidentified metabolites [10], positive ions at *m*/*z* 367.1433 and *m*/*z* 413.1359 and a negative ion at *m*/*z* 411.1302. The peaks of the positive ion at *m*/*z* 413.1359 and the negative ion at *m*/*z* 411.1302 had the same retention times, suggesting that both may be derived from the same compound. Because the uremic toxin N^6-carbamoylthreonyladenosine had the same *m*/*z* as the positive ion, we hypothesized that the latter would be derived from the same compound [17]. LCMS-IT-TOF analyses of plasma samples and reagent grade N^6-carbamoylthreonyladenosine showed that the positive ion at *m*/*z* 413.1359 and the negative ion at *m*/*z* 411.1302 yielded peaks consistent with each other.

3.2. Simple linear regression

The eGFRcys equation showed that eGFRcys was almost proportional to the inverse of CysC, whereas the linear regression of each metabolite on eGFR did not fit well. Therefore, we measured the reciprocal of eGFRcys, finding that each of the targeted metabolites was positively correlated with 1/eGFR by single regression analyses (Fig. 1). L-kynurenine (R^2 = 0.751), N^4-acetylcytidine (R^2 = 0.829), N^6-carbamoylthreonyladenosine (R^2 = 0.870), and UK-2 (R^2 = 0.903) were especially highly correlated with 1/eGFR.

3.3. Prediction of CKD stage by multivariate analyses

Predicted 1/eGFR values were derived from the OPLS regression equation for 1/eGFR. A typical result is shown in Table 3. The predicted 1/eGFR could be calculated using the equation:

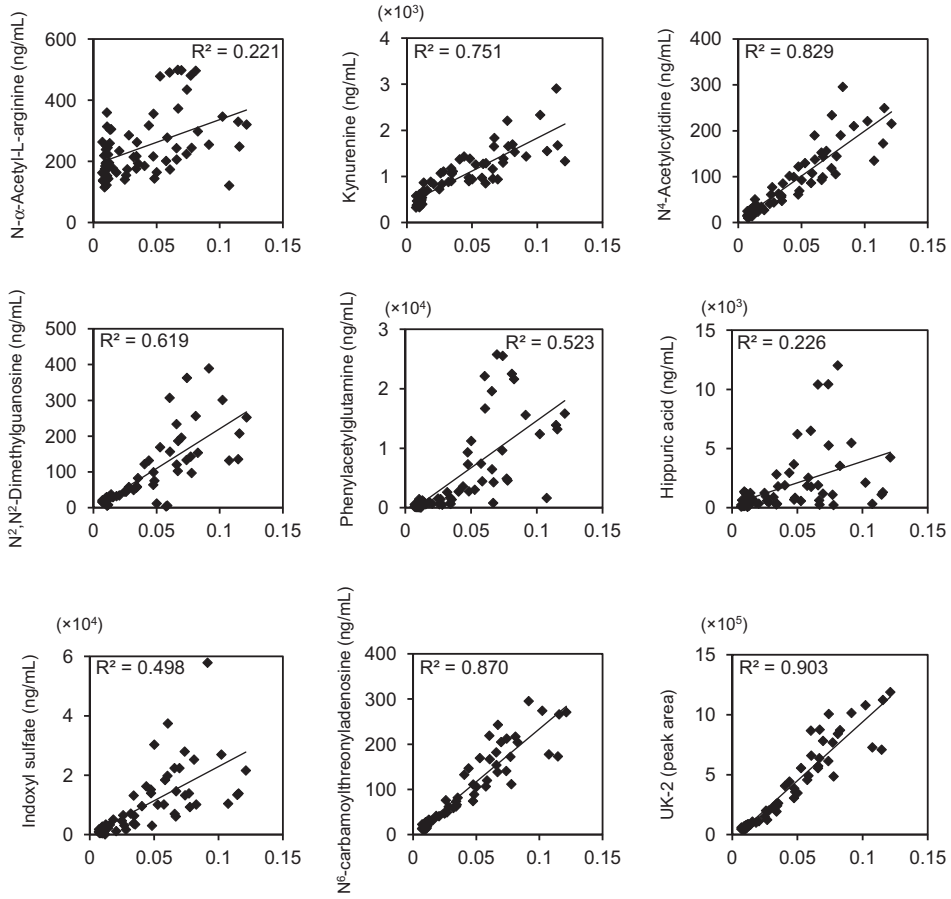

Fig. 1. Simple linear regression of plasma concentration of each metabolite relative to 1/eGFR. The horizontal axes show the reciprocal of eGFR (i.e. 1/eGFR). Except for UK-2, the vertical axes show the concentrations of plasma metabolites (ng/mL); for UK-2, the vertical axis shows the peak area of plasma samples. The correlation coefficient is shown in each graph.

Table 3
Predicted CKD stages using the OPLS regression model. Numbers in italics represent overestimation of CKD stage and numbers in bold represent underestimation of CKD stage, compared with measured CysC-based CKD stage.

Work set			
Stage		eGFR	
Measurement	Predictive	Measurement	Predictive
	1	101.5	128.6
	1	129.9	105.7
	1	132.6	118.5
	1	94.1	121.3
	1	140.5	97.6
	1	96.4	134.0
	1	110.3	104.0
1	*2*	95.6	78.6
	2	92.0	81.8
	1	92.6	165.6
	1	122.0	117.8
	1	113.3	119.7
	1	96.0	94.8
	1	93.8	100.5
	1	117.8	130.0
	2	86.1	81.7
	3	64.5	58.1
2	*3*	77.5	59.5
	2	80.6	73.4
	2	89.0	90.0
	3	31.4	32.2
	3	54.8	49.5
3	3	35.6	34.9
	3	48.8	47.3
	3	38.6	31.2
	4	18.9	19.1
	5	15.2	14.9
	3	29.0	31.7
	4	24.7	20.7
	4	22.8	19.6
4	4	16.5	15.3
	4	17.4	18.7
	4	21.0	24.5
	3	29.4	31.3
	4	15.2	17.9
	4	17.1	17.5
	4	21.1	25.1
	5	13.0	12.5
	5	13.6	14.7
	5	10.9	11.7
	5	8.2	9.7
5	5	12.3	10.8
	5	14.9	11.9
	5	12.1	9.9
	4	15.0	15.8
	5	14.4	14.0
	5	8.7	10.1
Test set			
Stage		eGFR	
Measurement	Predictive	Measurement	Predictive
	1	101.3	108.9
	1	104.2	125.5
	2	106.3	84.6
1	1	142.8	169.2
	1	169.2	109.0
	1	109.0	99.0
	2	103.0	84.1
	2	76.1	74.7
2	2	71.4	70.4
	2	84.5	83.6
3	3	40.2	42.3
	3	37.3	43.0
	5	16.6	13.4
	4	20.0	20.3

Table 3 (*continued*)

Test set			
Stage		eGFR	
Measurement	Predictive	Measurement	Predictive
4	4	20.7	20.8
	3	29.4	31.0
	4	28.4	27.5
	4	12.9	16.3
	5	9.3	13.4
5	5	9.8	9.3
	5	8.7	9.1
	5	13.5	11.1

$$\begin{aligned} 1/\mathrm{eGFR} = {} & -1.18 \times 10^{-5}(\text{N-}\alpha\text{-acetyl-L-arginine}) \\ & + 1.90 \times 10^{-5}(\text{L-kynurenine}) \\ & + 4.51 \times 10^{-5}(\text{N}^4\text{-acetylcytidine}) \\ & - 9.88 \times 10^{-5}(\text{N}^2,\text{N}^2\text{-dimethylguanosine}) \\ & + 2.62 \times 10^{-7}(\text{phenylacetylglutamine}) \\ & + 5.83 \times 10^{-7}(\text{hippuric acid}) \\ & - 2.19 \times 10^{-9}(\text{indoxyl sulfate}) \\ & + 1.06 \times 10^{-4}(\text{N}^6\text{-carbamoylthreonyladenosine}) \\ & + 5.27 \times 10^{-8}(\text{UK-2}) + 3.36 \times 10^{-3}(\times 2,\ \text{if female}) \\ & - 2.98 \times 10^{6}(\text{age}) + 6.75 \times 10^{-3}. \end{aligned}$$

The correlation coefficient between measured 1/eGFR and predicted 1/eGFR was 0.966 (Supplementary Fig. 1).

Subsequent classification of predicted CKD stage according to K/DOQI guidelines showed that the accuracy of this model in predicting CKD stage was 81.3%, even over 20 repeats.

4. Discussion

We attempted to use concentrations of blood metabolites previously identified in hemodialysis patients [10] to predict the stage of CKD, especially early-phase CKD. Previous studies have attempted to identify blood metabolites as markers of renal function using mass spectrometry. These studies, however, compared the concentration of each metabolite with eGFR. To our knowledge, no previous study had evaluated renal function by using LC/MS to measure the concentrations of multiple blood metabolites.

This model overestimated CKD stage in eight samples and underestimated CKD stage in four samples, relative to measured cystatin concentrations. Underestimation is more critical, because it may result in non-identification of patients with CKD. Because CKD in this study was defined as stage 3 and above, using this predictive model for screening may have resulted in the these four subjects, who actually had CKD stage 3, being misdiagnosed as having stage 1 or 2. Although we repeated the trial 20 times, such underestimation was not found.

In the work set, two samples with CKD stage 2 were overestimated as CKD stage 3. However, the predicted value of eGFR itself was almost the same as CysC-based eGFR. Considering that this was a screening test, these subjects could be accurately diagnosed during subsequent complete checkups. Thus, the predictive accuracy of the model described in this study was acceptable. When each sample in the work set was assayed 20 times, while changing the order of the samples, this model predicted the correct CKD stage in 81.3% (range, 73.9–87.0%), suggesting the high accuracy of this model.

Our simple linear regression model showed high correlations between N^6-carbamoylthreonyladenosine and UK-2 concentra-

tions and 1/eGFR. Estimating 1/eGFR from only one metabolite without OPLS analysis would simplify patient diagnosis. We therefore assessed whether these simple regression models could predict CKD stages. However, all of these models, even UK-2, which showed the highest correlation with 1/eGFR, were unable to exceed the predictive accuracy of OPLS (data not shown). This result suggested that multiple metabolites are more predictive of CKD stage than single metabolites.

Quantitative LC/MS analysis of blood metabolites in small volumes of plasma may be a new method of screening for CKD. In this study, 1/eGFR was subjected to OPLS analysis, with CKD stages derived by calculation. Although direct measurements of GFR are preferable, these measurements are difficult and time consuming, and all of our subjects were outpatients. Because measuring GFR was difficult, CysC-based eGFR was measured. To define the optimum factors of the OPLS regression equation, more precise measurements of GFR, i.e., measurements of inulin clearance, are needed in future studies.

In conclusion, the method described in this study, using multivariate analysis of the concentrations of blood metabolites that changed during disease progression, is not limited to CKD. This method may be applicable to predicting the severity of various disorders. Future studies may reveal new disease markers in blood, with mass spectrometry being a powerful tool for analyzing many metabolites quantitatively and simultaneously in small samples.

Conflict of interest

The authors have no conflicts of interest.

Acknowledgments

We thank Mr. Yoshiyuki Murata (Shimadzu) and Ms. Sachiko Yoshinaga (Tokyo Institute of Technology) for secretarial assistance, and Dr. Shigetoshi Yoshikawa (Infocom Corporation) for analytical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.021.

References

[1] R. Vanholder, G. Glorieux, R. De Smet, N. Lameire, European Uremic Toxin Work Group, New insights in uremic toxins, Kidney Int. Suppl. 84 (2003) S6–10.
[2] A.S. Go, G.M. Chertow, D. Fan, C.E. McCulloch, C.Y. Hsu, Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization, N. Engl. J. Med. 351 (2004) 1296–1305.
[3] T. Ninomiya, Y. Kiyohara, M. Kubo, Y. Tanizaki, Y. Doi, K. Okubo, Y. Wakugawa, J. Hata, Y. Oishi, K. Shikata, K. Yonemoto, H. Hirakata, M. Iida, Chronic kidney disease and cardiovascular disease in a general Japanese population: the Hisayama Study, Kidney Int. 68 (2005) 228–236.
[4] A. Levey, K.-U. Eckardt, Y. Tsukamoto, A. Levin, J. Coresh, J. Rossert, D. De Zeeuw, T. Hostetter, N. Lameire, G. Eknoyan, Definition and classification of chronic kidney disease: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO), Kidney Int. 67 (2005) 2089–2100.
[5] M. Horio, E. Imai, Y. Yasuda, T. Watanabe, S. Matsuo, Performance of GFR equations in Japanese subjects, Clin. Exp. Nephrol. 17 (2013) 352–358.
[6] C. Peralta, R. Katz, M. Sarnak, J. Ix, L. Fried, I. De Boer, W. Palmas, D. Siscovick, A. Levey, M. Shlipak, Cystatin C identifies chronic kidney disease patients at higher risk for complications, J. Am. Soc. Nephrol. 22 (2011) 147–155.
[7] M. Shlipak, K. Matsushita, J. Ärnlöv, L. Inker, R. Katz, K. Polkinghorne, D. Rothenbacher, M. Sarnak, B. Astor, J. Coresh, A. Levey, R. Gansevoort, C.K.D.P. Consortium, Cystatin C versus creatinine in determining risk based on kidney function, N. Engl. J. Med. 369 (2013) 932–943.
[8] M. Yashiro, T. Kamata, H. Segawa, Y. Kadoya, T. Murakami, E. Muso, Comparisons of cystatin C with creatinine for evaluation of renal function in chronic kidney disease, Clin. Exp. Nephrol. 13 (2009) 598–604.
[9] K. Saito, S. Fujigaki, M. Heyes, K. Shibata, M. Takemura, H. Fujii, H. Wada, A. Noma, M. Seishima, Mechanism of increases in L-kynurenine and quinolinic acid in renal insufficiency, Am. J. Physiol. Renal Physiol. 279 (2000) F565–572.
[10] E. Sato, M. Kohno, M. Yamamoto, T. Fujisawa, K. Fujiwara, N. Tanaka, Metabolomic analysis of human plasma from haemodialysis patients, Eur. J. Clin. Invest. 41 (2011) 241–255.
[11] T. Niwa, Update of uremic toxin research by mass spectrometry, Mass Spectrom. Rev. 30 (2011) 510–521.
[12] T. Toyohara, Y. Akiyama, T. Suzuki, Y. Takeuchi, E. Mishima, M. Tanemoto, A. Momose, N. Toki, H. Sato, M. Nakayama, A. Hozawa, I. Tsuji, S. Ito, T. Soga, T. Abe, Metabolomic profiling of uremic solutes in CKD patients, Hypertens. Res. 33 (2010) 944–952.
[13] K. Kikuchi, Y. Itoh, R. Tateoka, A. Ezawa, K. Murakami, T. Niwa, Metabolomic search for uremic toxins as indicators of the effect of an oral sorbent AST-120 by liquid chromatography/tandem mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 878 (2010) 2997–3002.
[14] K. Kikuchi, Y. Itoh, R. Tateoka, A. Ezawa, K. Murakami, T. Niwa, Metabolomic analysis of uremic toxins by liquid chromatography/electrospray ionization-tandem mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 878 (2010) 1662–1668.
[15] T. Kobayashi, Y. Matsumura, T. Ozawa, H. Yanai, A. Iwasawa, T. Kamachi, K. Fujiwara, N. Tanaka, M. Kohno, Exploration of novel predictive markers in rat plasma of the early stages of chronic renal failure, Anal. Bioanal. Chem. 406 (2014) 1365–1376, http://dx.doi.org/10.1007/s00216-013-7472-2.
[16] Y.-Y. Zhao, X.-L. Cheng, F. Wei, X.-Y. Xiao, W.-J. Sun, Y. Zhang, R.-C. Lin, Serum metabonomics study of adenine-induced chronic renal failure in rats by ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry, Biomarkers 17 (2012) 48–55.
[17] T. Niwa, Mass spectrometry in the search for uremic toxins, Mass Spectrom. Rev. 16 (1997) 307–332.