Dialysis modality-dependent changes in serum metabolites: accumulation of inosine and hypoxanthine in patients on haemodialysis

Ji-Young Choi¹,⁸*, Yoo Jeong Yoon²,*, Hee-Jeong Choi¹,⁸, Sun-Hee Park¹,⁸, Chan-Duck Kim¹,⁸, In-San Kim⁵, Tae-Hwan Kwon⁷, Jun-Young Do⁶, Sung-Ho Kim⁵, Do Hyun Ryu⁶, Geum-Sook Hwang²,⁷ and Yong-Lim Kim¹,³,⁸

¹Division of Nephrology, Department of Internal Medicine, Kyungpook National University School of Medicine, Daegu, Korea, ²Korea Basic Science Institute, Seoul, Korea, ³Department of Biochemistry and Cell Biology Kyungpook National University School of Medicine, Daegu, Korea, ⁴Division of Nephrology, Department of Internal Medicine, Yeungnam University School of Medicine, Daegu, Korea, ⁵Division of Nephrology, Department of Internal Medicine, Daegu Fatima Hospital, Daegu, Korea, ⁶Department of Chemistry, Sungkyunkwan University, Suwon, Korea, ⁷Graduate School of Analytic Science and Technology, Chungnam National University, Daejeon, Korea and ⁸Clinical Research Center for End Stage Renal Disease in Korea, Daegu, Korea

Correspondence and offprint requests to: Yong-Lim Kim; E-mail: ylkim@knu.ac.kr; Geum-Sook Hwang; E-mail: gshwang@kbsi.re.kr

*These co-first authors contributed equally.

Abstract

**Background.** The body metabolism of patients with end-stage renal disease may be altered in response to long-term dialysis treatment. Moreover, the pattern of serum metabolites could change depending on the type of dialysis modality used. However, dialysis modality-dependent changes in serum metabolites are poorly understood. Our aim was to profile comprehensively serum metabolites by exploiting a novel method of ¹H-NMR-based metabonomics and identify the differences in metabolite patterns in subjects receiving haemodialysis (HD) and peritoneal dialysis (PD).

**Methods.** Anuric and non-diabetic HD patients were matched to PD patients for age, sex and dialysis duration. Accurate concentrations of serum metabolites were determined using the target-profiling procedure, and differences in the levels of metabolites were compared using multivariate analysis.

**Results.** Principal Components Analysis score plots showed that the metabolic patterns could be discriminated by dialysis modalities. Hypoxanthine and inosine were present only with HD, whereas serum xanthine oxidase activity and uric acid levels were not different. In contrast, PD was associated with higher levels of lactate, glucose, maltose, pyruvate, succinate, alanine, and glutamate linked to glucose metabolism and the tri-carboxylic acid cycle. Maltose appeared only in patients using icodextrin solution for PD. Known uraemic retention solutes such as urea, creatinine, myo-inositol and trimethylamine-N-oxide were increased in both dialysis groups.

**Conclusions.** Metabonomics shows apparent differences in the profiles of serum metabolites between HD and PD, which were influenced by dialysis-related processes. Inosine and hypoxanthine are present only in HD patients, which is likely to represent more hypoxic and oxidative stress.

**Keywords:** dialysis modality; haemodialysis; metabonomics; peritoneal dialysis

Introduction

Uraemic syndrome is associated with a complex set of biochemical and pathophysiological changes that still remain poorly understood today. So far, more than 100 different uraemic toxins are known [1]. Clearance of uraemic toxins differs between haemodialysis (HD) and peritoneal dialysis (PD). Clearance of small molecules like urea and creatinine is better with HD, whereas clearance of the medium-sized molecules like β₂-microglobulin is better with PD [2].

Metabonomics is the quantitative measurement of the dynamic multi-parametric metabolic response of a living system in response to biologic perturbation such as pathophysiological or therapeutic stimuli or genetic modification. Metabonomics encompasses the comprehensive and simultaneous profiling of multiple metabolite levels and their systematic and temporal changes. The recent development of new technologies to separate and identify small molecules makes it possible to identify and quantify a number of small-molecule metabolites in complex biological samples [3–5]. ¹H-nuclear magnetic resonance (NMR) spectroscopy is a non-destructive technique that is widely used in chemistry, which provides detailed information on molecular structure, both for pure compounds and in complex mixtures as well as information on absolute or relative concentrations. The successful application of ¹H-NMR spectroscopy to bio-fluids to study metabolic
diseases and toxic processes has now been established, and several novel markers have been discovered for organ-specific toxicity [4–7]. Consistent with this, we recently illustrated the application of metabonomics using kidney tissues and urine to understand the pathophysiology of lithium-induced nephrogenic diabetes insipidus [8].

The feasibility of metabonomics to profile comprehensively low-molecular-weight metabolites in dialysis was suggested [9]. Dialysis-related factors as well as uraemia may affect metabolic patterns in dialysis patients. The enzymatic assay or liquid chromatographic analysis in patients on dialysis has revealed patterns of specifically targeted metabolites like choline, phosphocholine and hypoxanthine [10–12]. However, there are no previous studies showing the comprehensive profiles of serum metabolites in patients on long-term HD and PD compared with data from normal controls. The factors which induce changes in metabolites by dialysis modality, are poorly understood. The understanding of the changes in serum metabolites by dialysis modality is important because whole metabolites with all their interactions rather than a single type of metabolite can affect co-morbidity, particularly in long-term dialysis patients. In this study, the application of 1H-NMR spectroscopy to study the biochemical composition of serum from patients on dialysis was performed to investigate the differences in metabolic patterns according to the dialysis modality.

Materials and methods

Subjects

Patients were recruited from three hospitals in Daegu, South Korea: Kyungpook National University Hospital, Yeungnam University Medical Center and Daegu Fatima Hospital. Ethical approval was obtained from the Institutional Review Board of Kyungpook National University Hospital. Signed informed consent was obtained from all participants prior to study entry. All patients recruited were aged 18 years or older, had been on dialysis for a minimum of 6 months, and had not been hospitalized for 30 days. To avoid the effect of residual renal function and diabetic status on metabolic pattern, anuric (urine volume, <100 mL per day) and non-diabetic patients on dialysis were included. Purposeful recruitment was undertaken to achieve comparable PD and HD groups. The 18 PD patients were recruited first and matched to a HD patient for age, sex and dialysis duration. The 18 normal healthy controls were matched to the dialysis groups for age and sex.

The patients on PD were treated with four exchanges of 2 L of PD solution. The target for PD adequacy was a Kt/V of at least 1.7 per week. Subjects on HD were treated with three 4-h sessions per week. The target for HD adequacy was an equilibrated Kt/V of >1.2 (two-pool). Glucose-based PD fluid (Diancel®, Baxter R&D, Singapore and Safe-balance®, Fresenius Medical Care, Japan) was used in 6 (33.3%) of 18 PD patients, and icodextrin-containing PD fluid (Extraneal®, Baxter R&D, Singapore) was used in 12 (66.7%) patients. Thirteen (72.2%) of 18 HD patients had dialysis performed with a high-flux and five (27.8%) patients with a low-flux dialyser. The dialysate from HD contained 30 mmol/L bicarbonate and 8 mmol/L acetate. Patients with incurable cancer, recent infection, signs of liver cirrhosis, other metabolic disease or diabetes mellitus were excluded. Patients who had changed their dialysis modality within the past 6 months were also excluded.

Sample preparation

Serum samples were obtained after dwelling peritoneal effluent in the morning in PD patients, and before dialysis at the beginning of the week in HD patients. The samples were stored at −80°C until NMR analysis. Before the NMR experiment, frozen serum samples were thawed at room temperature and vortexed. The 250 μL serum samples were mixed with 500 μL saline solution (10% D2O for locking signal, 0.9% sodium azide, pH 7.0) to minimize variations in the pH of the samples. Samples were centrifuged at 13 000 r.p.m. for 10 min, and 600 μL aliquots of supernatant were transferred into 5 mm NMR tubes for analysis.

1H-NMR spectroscopy of serum samples

1H-NMR spectra were acquired using a VNMR 600 MHz NMR spectrometer (Varian Inc., Palo Alto, CA, USA) at 298 K using a triple resonance 5-mm HCN salt-tolerant cold probe. The water suppressed Carr–Purcell–Meiboom–Gill (CPMG) spin-echo pulse sequence (RD-90°–τ–180°–τ–n-ACQ). A total time of T2 relaxation of 60 ms was used to attenuate broad signals from proteins and lipoproteins. For each sample, the 1H-NMR spectrum was collected with 128 transients into 32-K data points using a spectral width of 6720.4 Hz with a relaxation delay of 2.0 s, and an acquisition time of 4.0 s. The free induction decays were weighted by an exponential function with a 0.3 Hz line-broadening factor prior to Fourier transformation. All acquired NMR spectra were phase- and baseline-corrected then referenced to the doublet of lactate at 1.32 p.p.m. NMR signal assignment for serum samples was facilitated by acquisition of two-dimensional (2D) total correlation spectroscopy (TOCSY), heteronuclear multiple-bond correlation (HMBC), heteronuclear single-quantum correlation (HSQC), spiking experiments, and comparisons with values in the literature.

Data processing of the NMR spectra and multivariate pattern recognition

The NMR spectra data between 0.0 and 7.7 p.p.m. were reduced to segments of 0.005 p.p.m. The regions corresponding to water/HOD (6.354–4.27) were removed prior to the normalization and spectra alignment. Glucose (δ 2.49–3.04), maltose (δ 2.51–3.16, 4.70–4.27) and lactate (δ 0.42–0.52, 3.20–3.31) resonance signals were removed for further analysis. The spectral data were then normalized to total spectral area. The data files were imported into MATLAB (R2008a, Mathworks, Inc., 2008), and all spectra were aligned using the correlation optimized warping (COW) method [13]. Signal assignment for representative samples was carried out according to those reported in the literature [14–17]. In addition, the 600 MHz library from Chenomx NMR suite 6.0 was utilized to assign the metabolites in the serum. The resulting datasets were then imported into SIMCA-P version 12.0 (Umetrics, Umeå, Sweden) for multivariate statistical analysis. All imported data were Pareto-scaled for the multivariate data analysis. Then, to discern the presence of inherent similarities of spectral profiles, an unsupervised method, the Principal Components Analysis (PCA), was conducted on the serum samples. The quality of the models was described by R2 and Q2 values. R2 is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and Q2 is defined as the proportion of variance in the data predicted by the model and indicates predictability. The corresponding loading plots, where each point represents a single NMR spectral region segment, were used to identify which spectral variables contributed to the separation of the samples on the score plot.

Measurement of xanthine oxidase

Xanthine oxidase activity was measured in relation to the detection of inosine and hypoxanthine only in the HD group. In addition, serum samples from the uraemia control group (n = 16), who were not on dialysis (MDRD GFR 23.56 ± 10.64 mL/min/1.73 m2), were obtained to determine the effect of uraemia on xanthine oxidase activity as well as on PD or HD dialysate composition per se. Serum samples were centrifuged at 2000 r.p.m. for 15 min at 4°C and stored at −80°C. Frozen serum samples were thawed at room temperature before measurement of xanthine oxidase. The 100-μL serum samples were added to the Xanthine Oxidase Assay Kit (Cayman Chemical Co., MI, USA) and mixed with diluted assay buffer to measure xanthine oxidase activity. The fluorescence, using an excitation wavelength of 520–550 nm and an emission wavelength of 585–595 nm, was monitored.

Statistical analysis

Demographic data are expressed as the mean ± standard deviation (SD) and analysed using one-way analysis of variance (ANOVA), or Kruskal–
Wallis test and chi-square test, or Fischer's exact test, using the SPSS program (SPSS version 14.0, Chicago, IL, USA). Differences between groups for the normalized intensities of $^1$H-NMR spectra were evaluated using the $t$-test, and the raw P-values were adjusted using the false discovery rate method from Benjamini and Yekutieli (2001) with the open-source R statistical data analysis programming environment (Ihaka and Gentleman, 1996, code obtained from Comprehensive R Archive Network) [18,19]. The R software used in this work was a free download from the web site (http://www.r-project.org). One-way ANOVA was done using the Tukey's multiple comparison tests with GraphPad Prism 5.01 software. Statistical significance was determined as a P-value <0.05.

**Results**

**Demographic characteristics of subjects**

The baseline characteristics of the subjects are presented in Table 1. The mean age of the PD and HD groups was $48.1 \pm 16.4$ and $52.0 \pm 17.0$ years, respectively, and that of the normal control group was $48.9 \pm 15.4$ years. Dialysis duration of the PD and HD groups was $99.8 \pm 39.4$ and $94.3 \pm 43.5$ months, respectively. There was no significant difference in the primary renal disease between the dialysis groups. The Kt/V was $>1.7$ per week in PD and per session in HD.

**Metabonomic analysis of serum from PD, HD and control groups**

The serum metabolites in patients on dialysis were investigated using high-resolution $^1$H-NMR spectroscopy coupled with multivariate data analysis (MVDA) to identify metabolic signatures of the dialysis modality and determine a clinical significance for the difference. Figure 1 shows the representative one-dimensional (1D) $^1$H-NMR spectra of serum from control, HD and PD groups. Spectral resonances of metabolites were assigned according to the literature [14–17], and the 600 MHz library from Chenomx NMR suite 6.0 (Chenomx Inc., Edmonton, Canada). The $^1$H-NMR spectra of serum contained a number of metabolites including VLDL/LDL CH$_3$, leucine, isoleucine, valine, VLDL/LDL (CH$_2$)$_n$, lactate, alanine, lipid CH$_2$CH$_2$CO, lipid CH$_2$CH$_2$C = C, acetate, arginine, N-acetylglycoproteins, glutamine, methionine, acetone, acetooacetate, glutamate, pyruvate, succinate, citrate, dimethylamine, creatinine, choline, phosphocholine, trimethylamine-N-oxide (TMAO), myo-inositol, glucose, malto, urea, inosine, tyrosine, t-methylhistidine, phenylalanine, formate, and hypoxanthine.

PCA was initially performed on $^1$H-NMR data obtained in serum from the HD and PD groups (Figure 2). The PCA score plots showed fairly clear differences between the HD and PD groups. In addition, the PD group was divided into two subgroups as shown in Figure 2. According to survey data, PD subgroup A contained 6 patients treated with glucose-based PD fluid, and PD subgroup B contained 12 patients treated with icodextrin-containing PD fluid. Therefore, a separation of the PD group into two subgroups was attributed to treatment with different PD fluids. Further PCA was performed on $^1$H-NMR spectral data excluding the region of $\delta$ 3.54–4.27 (containing the residual peak from the suppressed water resonance), $\delta$ 2.49–3.04 (glucose resonance signal), $\delta$ 2.51–3.16 and 4.70–4.27 (maltose resonance signal), and $\delta$ 0.42–0.52 and 3.20–3.31 (lactate resonance signal) (Figure 3). The PCA score plot showed a clear separation between the control, HD and PD groups, indicating significant metabolic changes in their serum. The control group was very different from both dialysis groups, indicating different metabolic changes in the serum of the dialysis groups compared with the control group. As shown in the PCA and orthogonal partial least-squares (OPLS) score plots, both dialysis groups were mostly distributed in the principal component (PC) 2 and OPLS 2 directions. The PCA score plot showed slightly overlapping separation for the second principal component (PC 2), with partial overlap between the HD and PD groups. To maximize the separation between groups, orthogonal projections to latent structures or orthogonal partial least-squares discriminant analysis (OPLS-DA) model was applied. OPLS score plot showed a clear differentiation between the HD and PD groups. The $Q^2$ and $R^2$ values were calculated for

### Table 1. Baseline patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>PD ($n = 18$)</th>
<th>HD ($n = 18$)</th>
<th>Control ($n = 18$)</th>
<th>P-value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>48.1 $\pm$ 16.4</td>
<td>52.0 $\pm$ 17.0</td>
<td>48.9 $\pm$ 15.4</td>
<td>NS (P = 0.82)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>8/10 (44.4%/55.6%)</td>
<td>6/12 (33.3%/66.7%)</td>
<td>6/12 (33.3%/66.7%)</td>
<td>NS (P = 0.83)</td>
</tr>
<tr>
<td>Dialysis duration (months)</td>
<td>99.8 $\pm$ 39.4</td>
<td>94.3 $\pm$ 43.5</td>
<td>NS (P = 0.63)</td>
<td></td>
</tr>
<tr>
<td>Primary renal disease</td>
<td>CGN 14 (77.8%)</td>
<td>HTN 3 (16.7%)</td>
<td>Unknown 0 (0%)</td>
<td>NS (P = 0.20)</td>
</tr>
<tr>
<td></td>
<td>HTN 3 (16.7%)</td>
<td>SLE 1 (5.6%)</td>
<td>Other 0 (0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other 0 (0%)</td>
<td>Unknown 0 (0%)</td>
<td>Kt/V$^a$ 1.95 $\pm$ 0.35</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± standard deviation. PD, peritoneal dialysis; HD, haemodialysis; CGN, chronic glomerulonephritis; ds, disease; HTN, hypertension; PCKD, polycystic kidney disease; NS, not significant.

$^a$Kt/V per week in PD and per session in HD.  
$^b$PD vs. HD vs. control group.
the models to assess the significance of differentiation. The statistics for the PCA model showed high goodness of fit, with a $R^2$ value of 0.678, and a predictive capability, with a $Q^2$ value of 0.478 (Figure 3A and B). OPLS-DA was used to maximize the separation between the three groups (Figure 3C and D) and showed a clear differentiation. Compared with the PCA model, these OPLS-DA models showed improved predictability with a $Q^2$ value of 0.843.

To identify the metabolites responsible for the differentiation in the PCA score plots between the two groups being compared, PCA loading plots were generated (Figure 4D–F). The PCA score plot showed a clear differentiation between the HD and PD groups (Figure 4A), the control and HD groups (Figure 4B), and the control and PD groups (Figure 4C), which demonstrated a good fit and low predictability, with a $R^2$ value of 0.534, 0.467 and 0.535, respectively, and a $Q^2$ of 0.278, 0.323 and 0.421, respectively.

To investigate the effect of compounds accumulated in biological fluids in uraemia on dialysis methods, we compared the metabolic difference in the serum of the PD and HD groups, and a clear discrimination between these two groups in the PCA score plot (Figure 4A). The loading plots represented which metabolites were quantitatively higher or lower in the two groups. As shown in the PCA loading plot (Figure 4D), the HD group was characterized by elevated levels of VLDL/LDL CH$_3$, VLDL/LDL (CH$_2$)$_n$, lipid CH$_2$CH$_2$CO, 9, lipid CH$_2$CH$_2$C = C; 10, acetate; 11, arginine; 12, N-acetylglycoproteins; 13, glutamine; 14, methionine; 15, acetone; 16, acetoacetate; 17, glutamate; 18, pyruvate; 19, succinate; 20, citrate; 21, dimethylamine; 22, creatinine; 23, choline; 24, phosphocholine; 25, TMAO; 26, myo-inositol; 27, glucose; 28, maltose; 29, urea; 30, inosine; 31, tyrosine; 32, τ-methylhistidine; 33, phenylalanine; 34, hypoxanthine; and 35, formate.

Fig. 1. Representative 600-MHz $^1$H-NMR spectra obtained from serum homogenates of subjects in the control (A), HD (B), and PD (C) groups. 1, VLDL/LDL CH$_3$; 2, leucine; 3, isoleucine; 4, valine; 5, VLDL/LDL (CH$_2$)$_n$; 6, lactate; 7, alanine; 8, lipid CH$_2$CH$_2$CO; 9, lipid CH$_2$CH$_2$C = C; 10, acetate; 11, arginine; 12, N-acetylglycoproteins; 13, glutamine; 14, methionine; 15, acetone; 16, acetoacetate; 17, glutamate; 18, pyruvate; 19, succinate; 20, citrate; 21, dimethylamine; 22, creatinine; 23, choline; 24, phosphocholine; 25, TMAO; 26, myo-inositol; 27, glucose; 28, maltose; 29, urea; 30, inosine; 31, tyrosine; 32, τ-methylhistidine; 33, phenylalanine; 34, hypoxanthine; and 35, formate.
plot (Figure 4E), higher levels of hypoxanthine, inosine, \( \tau \)-methylhistidine, N-acetylglycoproteins, urea, creatinine, myo-inositol, TMAO, dimethylamine, citrate, glutamine, acetooacetate and phenylalanine were observed in the HD group compared with the control group. In addition, levels of formate, tyrosine, choline, acetone, lipid CH\(_2\)CH\(_2\)CO, VLDL/LDL CH\(_3\) and VLDL/LDL (CH\(_2\))\(_n\) were decreased in the HD group compared with the control group.

The PCA score plot from the serum of subjects in the control and PD groups also showed clear differentiation between the two groups (Figure 4C). Several metabolites responsible for differentiation were identified. Of these metabolites (Figure 4F), the levels of \( \tau \)-methylhistidine, phenylalanine, tyrosine, urea, creatinine, myo-inositol, TMAO, dimethylamine, citrate, succinate, pyruvate, glutamate, alanine and N-acetylglycoproteins were elevated in the PD group compared with the control group, while the levels of formate, glutamine, acetone, lipid CH\(_2\)CH\(_2\)CO, VLDL/LDL (CH\(_2\))\(_n\) and VLDL/LDL CH\(_3\) were reduced.

Metabolites responsible for the observed difference between the HD and PD groups are summarized in Table 2, with the relative metabolite concentrations in the control and dialysis groups.

**Xanthine oxidase activity and uric acid levels**

Serum uric acid levels and xanthine oxidase activity were measured to determine the effect of uraemia or deficiency of xanthine oxidase activity, because inosine and hypoxanthine were detected only in the HD group in the NMR data analysis. The uric acid level was significantly higher in the PD (6.1 ± 1.2 mg/dL) and HD (6.9 ± 1.9 mg/dL) groups compared with the normal control group (5.0 ± 1.2 mg/dL) (P < 0.05). The number of patients using medications such as allopurinol or furosemide, which affect serum uric acid levels, was not significantly different among the three groups. Patients taking ACE inhibitors or ARB, which can cause hyperuricaemia, were more prevalent in both dialysis groups compared with the normal control group (P < 0.05). Xanthine oxidase activity (relative light units) was significantly higher in the PD (12.2 ± 3.5), HD (13.0 ± 9.4), and uraemia control group (6.3 ± 1.5) compared with the normal control group (4.5 ± 0.9) (P < 0.05), and was also significantly higher in both dialysis groups compared with the uraemia control group (P < 0.05). However, there was no significant difference between the PD and HD groups (Figure 5).

**Lipid profiles**

Total cholesterol and LDL cholesterol levels were significantly lower in the HD group (142.6 ± 29.9 and 81.9 ± 23.7 mg/dL, respectively) than in the control group (189.9 ± 44.5 and 110.3 ± 37.3 mg/dL, respectively) (P < 0.05). There was no significant difference between the HD and PD groups (163.3 ± 32.8 and 105.3 ± 29.2 mg/dL, respectively). The number of patients using lipid-lowering agents among the three groups was not different. Triglyceride levels showed no significant differences between the three groups. HDL cholesterol was significantly lower in the PD (41.8 ± 10.1 mg/dL) and HD (46.0 ± 10.5 mg/dL) groups than in the control group (56.9 ± 13.3 mg/dL) (P < 0.05). The serum albumin level was also significantly lower in the PD (3.6 ± 0.4 g/dL) and HD (3.7 ± 0.3 g/dL) groups compared with the control group (4.7 ± 0.3 g/dL) (P < 0.05).

**Discussion**

Long-term dialysis treatment in patients with ESRD could change the body’s metabolism. Residual renal function and a diabetic condition could affect the metabolic pattern of patients on dialysis. Therefore, the optimal study population to determine the impact of dialysis on metabolic pattern, anuric and non-diabetic patients, was selected and recruited in this study. Some metabolites have been analysed in patients on dialysis treatment previously [10–12]. However, to the best of our knowledge, a comprehensive profile of serum metabolites and the difference in metabolic pattern according to dialysis modality have not been previously reported, particularly by exploiting \(^1\)H-NMR-based metabonomics. In this study, we hypothesized that serum levels of different metabolites are altered between patients on HD and PD, which may result in different clinical manifestations and outcomes [1].

Differences in the levels of metabolites between subjects treated with HD and PD were compared using multivariate analysis, such as PCA and OPLS-DA, and an accurate concentration of the metabolites in serum was rapidly measured using the target-profiling procedure. The metabolic profile and multivariate pattern recognition approach we used permitted us to observe a broad range of metabolites simultaneously, the concentrations of which could be changed by biologic stimuli or certain disease conditions. Since metabolites can be regulated through a number of metabolic pathways, an investigation of the overall features...
rather than of several select metabolites enabled us to understand the underlying pathophysiological status more comprehensively.

Our study analysed the overall metabolites and the changes in the levels of each metabolite in the serum from control and dialysis patients to identify patterns of metabolites and whether these are affected by the dialysis modality. Metabonomic analysis in this study showed that the pattern of serum metabolites differs between the two dialysis modalities. Although the relatively long-term outcome with PD is comparable with HD, PD may offer a slight advantage to younger, non-diabetic subjects, and HD an advantage to older, diabetic subjects [20]. The different outcomes of each dialysis modality in specific dialysis populations may be attributed to the differences in body metabolism. The metabolic patterns found in the serum in our PD patients were affected by the composition of the PD fluid, like osmotic agents (glucose or icodextrin) and buffer (lactate). The results of the 600 MHz 1H-NMR spectra for lactate, glucose and maltose are more dominant in the PD group than in the HD group. Icodextrin from the peritoneal cavity to the circulation is rapidly metabolized by amylase into small oligosaccharides, especially maltose [21]. In this study, the appearance of maltose in patients using icodextrin solution was confirmed. The metabolites, including pyruvate, succinate, alanine and glutamate, were also higher in the PD group than in the HD or control groups. These metabolites are linked to glucose metabolism and the TCA cycle, which are affected by the high concentrations of glucose in the PD solution. With improved biocompatibility of PD fluids, the metabolic patterns will change.

Inosine and hypoxanthine, which are involved in the purine metabolism pathway, are present in the HD group, but not in the PD or control groups. The exclusive detection of both of these metabolites in the serum of subjects receiving HD has not been reported previously. Xanthine oxidase, the enzyme that catalyses the conversion of hypoxanthine to uric acid, may influence the level of inosine and hypoxanthine. Although serum xanthine oxidase activ-

Fig. 3. PCA score plots as 2D (A) and 3D (B), OPLS score plot 2D (C) and 3D (D) derived from the 1H-NMR spectra of serum homogenate obtained from the control, HD and PD groups, demonstrating clear metabolic differences among groups.
ity and uric acid levels were higher in both dialysis groups than in the normal control group, there was no difference between the two dialysis groups. These findings indicate that the presence of hypoxanthine and inosine in the serum of patients receiving HD is not directly related to deficient xanthine oxidase activity or uraemia per se. Previous studies showed that patients receiving HD are vulnerable to cellular hypoxia and oxidative stress with elevated 8-hydroxy-2′-deoxyguanosine of leucocyte DNA [22–25]. Hypoxanthine is not only an indicator of

Fig. 4. PCA scores (A–C) and loading (D–F) plots derived from the 1H-NMR spectra of serum homogenates obtained from the control, HD and PD groups. $R^2 = 0.467$ and $Q^2 = 0.323$, between the control and HD groups; $R^2 = 0.535$ and $Q^2 = 0.421$, between the control and PD groups; $R^2 = 0.534$ and $Q^2 = 0.278$, between the HD and PD groups. 1, VLDL/LDL CH$_3$; 2, VLDL/LDL (CH$_2$)$_n$; 3, alanine; 4, lipid CH$_3$CH$_2$CO; 5, N-acetylglycoproteins; 6, acetone; 7, acetocetate; 8, pyruvate; 9, glutamine; 10, myo-inositol; 11, choline; 12, phosphocholine; 13, TMAO; 14, creatinine; 15, urea; 16, phenylalanine; 17, succinate; 18, citrate; 19, formate; 20, glutamate; 21, dimethylamine; 22, tyrosine; 23, r-methylhistidine; 24, inosine; and 25, hypoxanthine.
cellular hypoxia but also a pro-oxidant substrate in inducing oxidative stress [22,26]. Inosine accumulates to high levels after tissue ischaemia, and contributes to the maintenance of homeostasis [27,28]. Therefore, the presence of serum hypoxanthine and inosine in the HD group appears to be attributable to potential hypoxia and oxidative stress generated during the HD process.

The increased level of acetoacetate in the HD group is thought to be due to acetate-containing haemodialysate [29]. This finding demonstrates that metabonomic patterns in HD patients are affected by the haemodialysate as they are in PD patients. Glutamine is synthesized from glutamate by the enzyme glutamine synthase. The level of glutamine in this study was highest in the HD group, followed by the control group and, finally, the PD group. The whole body appearance rate for glutamine was higher in the pre-dialysis sample from HD patients than from control subjects [30]. Our finding may reflect altered glutamine metabolism during haemodialysis. However, there was no report for glutamine synthesis in PD patients. Further studies to measure glutamine synthase activity or to compare glutamine synthesis in PD patients must be performed.

Metabolites like urea, creatinine, myo-inositol, TMAO and dimethylamine, which are known to be uraemic toxins or elevated in patients on dialysis, were confirmed in our study. Myo-inositol is known as one of the free, water-soluble, low-molecular-weight uraemic retention solutes [31]. TMAO accumulates in end-stage renal disease and is cleared by dialysis [9,32]. The plasma levels of TMAO correlate with those of urea and creatinine, suggesting that

<table>
<thead>
<tr>
<th>Chemical shift (δ 1H p.p.m.)</th>
<th>Metabolites</th>
<th>HD vs. control</th>
<th>PD vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.36 (m), 0.86 (m)</td>
<td>VLDL/LDL CH₃**</td>
<td>0.811*</td>
<td>0.803*</td>
</tr>
<tr>
<td>1.25 (m)</td>
<td>VLDL/LDL (CH₃)ᵢᵢᵢ**</td>
<td>0.794</td>
<td>0.791*</td>
</tr>
<tr>
<td>3.81 (q), 1.48 (d)</td>
<td>Alanine**</td>
<td>0.827</td>
<td>1.599*</td>
</tr>
<tr>
<td>1.55 (br)</td>
<td>Lipid CH₂CH₂CO**</td>
<td>0.478*</td>
<td>0.377*</td>
</tr>
<tr>
<td>1.91 (s)</td>
<td>Acetate</td>
<td>1.186</td>
<td>1.091</td>
</tr>
<tr>
<td>2.04 (s)</td>
<td>NAC (N-acetylglycoproteins)**</td>
<td>1.152</td>
<td>1.268*</td>
</tr>
<tr>
<td>2.21 (s)</td>
<td>Acetone**</td>
<td>0.260*</td>
<td>0.180*</td>
</tr>
<tr>
<td>2.26 (s), 3.49 (s)</td>
<td>Acetoacetate**</td>
<td>1.726*</td>
<td>1.032</td>
</tr>
<tr>
<td>2.37 (s)</td>
<td>Pyruvate**</td>
<td>0.908</td>
<td>4.888*</td>
</tr>
<tr>
<td>3.76 (m), 2.15 (m), 2.46 (m)</td>
<td>Glutamine**</td>
<td>1.304</td>
<td>0.701*</td>
</tr>
<tr>
<td>3.53 (dd), 4.06 (dd), 3.28 (t), 3.63 (t)</td>
<td>Myo-inositol**</td>
<td>3.163*</td>
<td>7.259*</td>
</tr>
<tr>
<td>3.19 (s), 3.50 (m), 4.07 (m)</td>
<td>Choline**</td>
<td>0.840</td>
<td>0.791*</td>
</tr>
<tr>
<td>3.33 (s), 4.21 (t), 3.61 (t)</td>
<td>Phosphocholine**</td>
<td>0.946</td>
<td>0.541*</td>
</tr>
<tr>
<td>3.27 (s)</td>
<td>Trimethylamine-N-oxide**</td>
<td>2.965*</td>
<td>2.469*</td>
</tr>
<tr>
<td>3.05 (s), 4.05 (s)</td>
<td>Creatinine**</td>
<td>6.144*</td>
<td>5.976*</td>
</tr>
<tr>
<td>5.8 (br)</td>
<td>Urea**</td>
<td>5.205*</td>
<td>9.278*</td>
</tr>
<tr>
<td>3.98 (dd), 7.31 (d), 7.36 (t), 7.42 (t)</td>
<td>Phenylalanine**</td>
<td>2.217*</td>
<td>2.808*</td>
</tr>
<tr>
<td>2.41 (s)</td>
<td>Succinate**</td>
<td>1.537</td>
<td>1.820*</td>
</tr>
<tr>
<td>2.69 (d), 2.54 (d)</td>
<td>Citrate**</td>
<td>1.914*</td>
<td>2.024*</td>
</tr>
<tr>
<td>8.45 (s)</td>
<td>Formate**</td>
<td>0.234*</td>
<td>0.305*</td>
</tr>
<tr>
<td>3.76 (m), 2.06 (m), 2.36 (m)</td>
<td>Glutamate**</td>
<td>1.239</td>
<td>2.438*</td>
</tr>
<tr>
<td>3.72 (s), 2.93 (s)</td>
<td>Dimethylamine**</td>
<td>1.940*</td>
<td>2.267*</td>
</tr>
<tr>
<td>3.87 (m), 2.16 (m), 2.65 (dd), 2.15 (s)</td>
<td>Methionine**</td>
<td>6.420*</td>
<td>4.630*</td>
</tr>
<tr>
<td>3.16 (dd), 3.94 (dd), 6.87 (d), 7.20 (d)</td>
<td>Tyrosine**</td>
<td>0.765</td>
<td>0.968</td>
</tr>
<tr>
<td>7.01 (s), 7.7 (s)</td>
<td>β-Methylhistidine**</td>
<td>2.413*</td>
<td>2.774*</td>
</tr>
<tr>
<td>6.09 (d), 8.22 (s), 8.36 (s), 4.43(t)</td>
<td>Inosine</td>
<td>HD only</td>
<td></td>
</tr>
<tr>
<td>8.19 (d)</td>
<td>Hypoxanthine</td>
<td>HD only</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparison tests. A ratio t-test was performed to assess the statistical significance between HD vs. control group and PD vs. control group.
s, singlet; d, doublet; t, triplet; m, multiples; br, broad.
*Significant difference compared with control (P < 0.05).
**Significant difference compared with control (P < 0.05).

Fig. 5. Xanthine oxidase activity. Xanthine oxidase activity was higher in both PD and HD groups than in the normal and uraemia control groups, and was also higher in the uraemia control group than in the normal control group. Data represent the mean ± standard deviation of relative light units. *P < 0.05 vs. normal control group. †P < 0.05 vs. uraemia control group.
the presence of TMAO is associated with renal failure [33]. Dimethylamine increases in the serum and duodenal contents during uraemia. Increases in the dimethylamine concentration can be attributed to the uraemic state per se, a consequence of uraemia-induced alterations in the bacterial flora of the gastrointestinal tract [34].

Citrate and phenylalanine were higher in both dialysis groups compared with the control group. Citrate is an intermediate of the TCA cycle and transported by the kidney. There were no reports on metabolite pattern analyses of citrate and phenylalanine in dialysis patients. Circumstantial evidence exists for patients with renal dysfunction. The $^1$H-NMR spectroscopy spectra from the urine samples of patients with severe renal tubulointerstitial damage and glomerulonephritis showed inhibition of citrate excretion [35]. Phenylalanine was higher in the rejection group than in a stable group of renal transplant patients [36]. Elevation of citrate and phenylalanine may be associated with uraemia due to deterioration of renal function. The elevated level of these metabolites was confirmed by NMR data analysis.

Lipid metabolites (VLDL/LDL [CH2]n, VLDL/LDL CH3 and lipid CH2CH2CO), choline, phosphocholine and formate were at lower levels in both dialysis groups compared with the control group. Quantification of plasma lipoproteins by proton NMR spectroscopy correlated well with serum lipoprotein concentrations determined by triglyceride and cholesterol measurements [37]. Lower levels of lipid metabolites in the dialysis groups may be due to a poorer nutritional status considering the serum albumin levels. Choline and phosphocholine are associated with the glycerophospholipid metabolism pathway. Previous reports showed increased plasma choline concentrations in HD and PD patients [10,11]. However, the concentrations were slightly decreased in both the PD and HD group in our study, differing from the previous findings.

Our study may be limited by its cross-sectional design and potential selection bias. Anuric patients without diabetes who were recruited to our study were longer-term survivors (mean dialysis duration of over 7 years) among our dialysis patients. Thus, in general, it is likely that metabolic patterns in dialysis patients with residual renal function or with a diabetic condition may be more complex than those seen in this study. In addition, the findings of this study suggest that the current advanced metabonomic technology provides indications of which metabolites or compounds accumulated in biological fluids in uraemia may be affected by the dialysis modality.

In summary, metabolic patterns are apparently different between dialysis types. The composition of the dialysis fluid in PD clearly causes differences in the metabolic pattern. Inosine and hypoxanthine, which were only detected in HD patients, may represent more hypoxic and oxidative stress generated by HD. The results of this study demonstrate that metabonomics has the potential to identify the biocompatibility of PD fluid, a hypoxic state with oxidative stress in HD, and to explore uraemic toxins. Furthermore, this study highlights a non-invasive approach to global metabolic profiling with NMR spectroscopy used as a novel diagnostic technique, and a tool to monitor the dialysis modality in patients with end-stage renal disease.

Acknowledgements. This study was supported by a grant from the Korean Healthcare Technology R&D Project, Ministry for Health, Welfare and Family Affairs, Republic of Korea (A084001), and a grant from the Korean Basic Science Institute (T30613), and the National Research Foundation grant (R01-2007-000-20441-0 and No. 20100019383) funded by the Ministry of Education, Science and Technology (MEST), Korea.

Conflict of interest statement. None declared.

References

Trimestral variations of C-reactive protein, interleukin-6 and tumour necrosis factor-α are similarly associated with survival in haemodialysis patients

Christiaan L. Meuwese1,2, Sunna Snaedal2, Nynke Halbesma1, Peter Stevinvinkel2, Friedo W. Dekker1, Abdul R. Qureshi2, Peter Barany2, Olof Heimburger2, Bengt Lindholm2, Raymond T. Krediet3, Els W. Boeschoten4 and Juan J. Carrero2,5,6

1Department of Clinical Epidemiology, Leiden University Medical Centre, Leiden, The Netherlands, 2Departments of Renal Medicine and Baxter Novum, CLINTEC, Karolinska Institutet, Stockholm, Sweden, 3Department of Nephrology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands, 4Hans Mak Institute, Naarden, The Netherlands, 5Centre for Molecular Medicine and 6Centre for Gender Medicine, Karolinska Institutet, Stockholm, Sweden

Correspondence and offprint requests to: Juan Jesús Carrero; E-mail: juan.jesus.carrero@ki.se

Abstract

Background. The impact of intra-individual changes of inflammatory markers [other than C-reactive protein (CRP)] on mortality in haemodialysis (HD) patients is unknown. We therefore studied survival in relation to trimestral variations of CRP, interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α).

Methods. In 201 prevalent HD patients from the Mapping of Inflammatory Markers in Chronic Kidney Disease cohort, serum CRP, IL-6 and TNF-α were measured 3 months apart and survival was assessed during follow-up. Based on fluctuations along tertiles of distribution, four patterns were defined for each inflammatory marker: stable low, decrease, increase and stable high. Hazard ratios were