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Removal of uremic retention products by hemodialysis is coupled with indiscriminate loss of vital metabolites

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ABSTRACT

Background: Although dialysis ameliorates uremia and fluid and electrolytes disorders, annual mortality rate remains high in dialysis population reflecting its shortcoming in replacing renal function. Unlike the normal kidney, dialysis causes dramatic shifts in volume and composition of body fluids and indiscriminate removal of vital solutes. Present study was undertaken to determine the impact of hemodialysis on plasma metabolites in end-stage renal disease (ESRD) patients.

Methods: 80 hemodialysed patients and 80 age/gender-matched healthy controls were enrolled in the study. Using ultra performance liquid chromatography-high-definition mass spectrometry, we measured plasma metabolites before, during, and after hemodialysis procedure and in blood entering and leaving the dialysis filter.

Results: Principal component analysis revealed significant difference in concentration of 214 metabolites between healthy control and ESRD patients' pre-dialysis plasma (126 increased and 88 reduced in ESRD group). Comparison of post-dialysis with pre-dialysis data revealed significant changes in the 362 metabolites. Among ESI⁺ metabolites 195 decreased and 55 increased and among ESI⁻ metabolites 82 decreased and 30 increased following hemodialysis. Single blood passage through the dialyzer caused significant changes in 323 metabolites. Comparison of ESRD patients' post-hemodialysis with healthy subjects' data revealed marked differences in metabolic profiles. We identified 55 of the 362 differential metabolites including well known uremic toxins, waste products and vital biological compounds.

Conclusion: In addition to uremic toxins and waste products hemodialysis removes large number of identified and as-yet un-identified metabolites. Depletion of vital biological compounds by dialysis may contribute to the high morbidity and annual mortality rate in this population.

1. Introduction

Pandemics of type-2 diabetes and hypertension over the past 2 decades have resulted in a dramatic rise in prevalence of chronic kidney disease (CKD) and expanded use of dialysis modalities for renal replacement therapy worldwide. Although chronic dialysis modalities are highly effective in ameliorating uremia and fluid and electrolytes disorders, overall annual mortality rate remains high in dialysis-dependent population. This reflects the shortcomings of dialysis modalities in replacing normal kidney functions. Under normal conditions via continuous excretion of metabolic waste products and excess fluid

and electrolytes, kidney plays a central part in maintaining homeostasis of the volume and composition of body fluids and blood pressure. In contrast, volume and composition of body fluids and arterial pressure undergo dramatic shifts during the inter- and intra-dialytic periods. These events can lead to adverse consequences. For instance intra-dialytic and post-dialytic hypotension which is a common complication of hemodialysis is associated with numerous adverse consequences including cerebral atrophy [1] and increased mortality rate [2]. In addition rapid removal of urea and other osmotically active substances together with delayed osmotic equilibrium of the intracellular fluid with extracellular fluid compartment contributes to intradialytic hypotension and neuro-

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logical disorders [3]. In an attempt to achieve a greater uremia control, fluid and electrolyte homeostasis while simultaneously minimizing the magnitude of the inter- and intra-dialytic changes in hemodynamics and body fluid composition, several clinical trials have explored the effect of longer and more frequent dialysis regimens on clinical outcomes in end-stage renal disease (ESRD) patients. The ADEMEX trial which had enrolled 965 peritoneal dialysis patients, compared a 2-year, high intensity dialysis protocol ($Kt/V = 2.0$, creatinine clearance = 60 L/week) with a standard dialysis protocol. The study revealed no difference in patient survival, suggesting that more intensive elimination of urea and other water-soluble small molecular weight substances is not beneficial [4]. Similarly, no difference in annual mortality rate was observed in the HEMO trial which had enrolled 1846 chronic hemodialysis patients randomized to either high-intensity ($Kt/V = 1.45$ and urea reduction ratio = 75%) or standard-intensity ($Kt/V = 1.05$ or urea reduction ratio = 65%) hemodialysis treatment [5]. Likewise the FHN trial [6] which compared the outcomes in a group of ESRD patients randomized to high hemodialysis intensity (6 dialysis sessions/week), with those in a group of patients undergoing standard hemodialysis therapy (3 dialysis sessions/week) showed no significant difference in cognition, serum albumin or requirement for erythropoiesis-stimulating agents. Moreover, despite significant increase in Kt/V between the two groups (2.57 in conventional vs. 3.6 in intensive group) patients randomized to high intensity hemodialysis had a higher annual mortality rate than those assigned to conventional dialysis. Finally the ACTIVE (A Clinical Trial of Intensive Dialysis) trial in which participants were randomly assigned to 24 or more hours versus 12 to 15 h of hemodialysis/week and followed for up for 12 months revealed no significant reduction in mortality among patients enrolled in the extended dialysis program [7]. Taken together these observations suggest that the high morbidity and annual mortality rate in ESRD populations maintained on dialysis modalities are not exclusively due to inadequate control of uremia, fluid and electrolytes disorders or the magnitude of dialysis-induced hemodynamic stress, and disequilibrium in body fluid compartments. Thus other factors must also contribute to high morbidity and annual mortality rate in this population.

It should be noted that in addition to removing excess fluids, electrolytes and metabolic waste products dialysis modalities can indiscriminately remove biologically important circulating small molecular weight compounds. This is unlike the normal kidney in which the vital molecules present in the glomerular filtrate are reabsorbed by proximal tubules. Therefore, inevitable losses of biologically important small molecules may, in part, contribute to high morbidity and annual mortality rate in dialysis-dependent patients and account for the reported lack of benefit or worsened outcomes with increased dialysis intensity [4–6]. While much is known about the efficacy of dialysis modalities in the management of CKD associated fluid, electrolyte disorders and clearance of the uremic retention metabolites, little attention has been paid to the indiscriminate loss of biologically important compounds and its potential contribution to the high morbidity and annual mortality rate in this population. Metabolomics has been widely applied to disease diagnosis [8–10], drug discovery [11–15], toxicity evaluation [16–18] and disease action mechanism [19–21]. Ultra performance liquid chromatography-high-definition mass spectrometry (UPLC-HDMS) has been widely used for metabolomics due to its enhanced analytic speed and sensitivity [22–25]. Using UPLC-HDMS in ESI^+ and ESI^- modes to measure plasma metabolites before, during, and after hemodialysis procedure, the present study was undertaken to determine the effect of hemodialysis on blood metabolome in patients with end-stage renal disease.

2. Methods

2.1. Patients and controls

80 stable patients (45 men and 35 women, age: 53.7 ± 15.3 years) with ESRD and 80 age- and gender-matched healthy control persons were enrolled in the study. The ESRD patients were maintained on 3.5-h hemodialysis sessions 3 times per week for an average of 4 years at the Affiliated Hospital of Shanxi Institute of Traditional Chinese

Xi'an, China. Healthy controls were excluded if they had any of the following conditions: cardiovascular disease, diabetes, hypertension, kidney dysfunction or use of regular medications. The clinical and demographic features of the study population are provided in Table 1.

Polysulfone dialyzers and the dialysis solution containing Na 140 mmol/L, K 2.0 mmol/L, Ca 1.50 mmol/L, Mg 0.5 mmol/L, Cl 107.0 mmol/L, HCO_3^- 34.0 mmol/L, CH_3COO^- 5.0 mmol/L were used in all dialysis sessions. Dialysate flow rate and blood flow rate were 500 ml/min and 260 ml/min respectively. Heparin was used for systemic anticoagulation during hemodialysis procedure. Blood was drawn from the arterial line immediately before hemodialysis, 30 min after the onset of dialysis and at the end of the hemodialysis procedure. In addition, samples were taken from blood entering and leaving the dialyzer at 10 min. Dialysate effluent was collected at 10 min, 30 min and immediately before the end of hemodialysis session. Blood samples were immediately centrifuged at $3000 \times g$ for 10 min at 4 °C and plasma was separated and stored at -80 °C. The study was conducted in accordance with the Helsinki declaration and approved by the institutions' ethics committees. Signed informed consent was obtained from all patients prior to their inclusion in the study.

2.2. Biochemical assays

Plasma creatinine (PCr), blood urea nitrogen (BUN), total cholesterol, LDL-C, HDL-C, albumin, total protein, uric acid and triglyceride were analyzed by Olympus AU640 automatic analyzer. White blood cell, hemoglobin, blood platelet were determined by HF-3800 Routine blood analyzer. The measurements for each of the sample for biochemical parameters were replicated 3 times.

2.3. Metabolomics analysis

Plasma for metabolomics analysis was prepared as described previously [26–29] and processed using Waters Acquity™ Ultra Performance LC system equipped with a Waters Xevo™ G2-S QT of MS. Chromatographic separation,

Table 1

Summary of clinical and demographic baseline characteristics of patients with CKD and health control subjects in this study.

	Healthy controls	ESRD patients
Number	80	80
Age (years)	49.1 ± 14.8	53.7 ± 15.3
Women (%)	65	44
Pre-dialysis body weight (kg)	NA	61.0 ± 12.5
Post-dialysis body weight (kg)	NA	58.1 ± 12.2
Body weight change (kg)	NA	2.95 ± 1.21
Pre-dialysis SBP (mm Hg)	NA	146.6 ± 18.9
Pre-dialysis DBP (mm Hg)	NA	$85.6 \pm 12.8^*$
Post-dialysis SBP (mm Hg)	NA	$139.3 \pm 16.0^{##}$
Post-dialysis DBP (mm Hg)	NA	$81.1 \pm 7.5^{##}$
eGFR (ml/min/1.73m ²)	108.72 ± 37.77	0.00***
BUN (mg/dl)	12.16 ± 3.31	$68.82 \pm 25.88^{***}$
Plasma creatinine (mg/dl)	0.68 ± 0.14	$12.03 \pm 3.53^{***}$
Uric acid (mg/dl)	5.52 ± 1.59	$7.45 \pm 1.77^{***}$
Triglycerides (mg/dl)	4.39 ± 1.42	$5.26 \pm 1.84^{**}$
Total cholesterol (mg/dl)	12.63 ± 3.66	11.79 ± 2.82
LDL-C (mg/dl)	6.49 ± 2.18	7.14 ± 2.68
HDL-C (mg/dl)	5.35 ± 1.70	$4.03 \pm 1.09^{**}$
Total protein (g/dl)	7.27 ± 0.33	$6.6 \pm 0.73^{***}$
Albumin (g/dl)	4.73 ± 0.42	$3.9 \pm 0.39^{***}$
White blood cell ($\times 10^9/L$)	5.62 ± 1.16	5.28 ± 1.69
Hemoglobin (g/dl)	13.8 ± 1.7	$9.5 \pm 1.8^{***}$
Blood platelet ($\times 10^9/L$)	214.25 ± 53.18	$172.35 \pm 62.51^{***}$

Data are expressed as the means $\pm$ standard deviation.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$ compared with health control group.

$P < 0.01$ compared with pre-dialysis.

mass spectrometry and data processing were conducted as described in detail in the Supplementary information.

2.4. Statistical analysis

Fold change was obtained by calculating average mass response ratio between post-hemodialysis (3.5 h) vs pre-hemodialysis, post-hemodialysis (3.5 h) vs control, pre-hemodialysis vs control or the blood leaving vs the entering dialysis filter (10 min). Fold changes were calculated by Metaboanalysis 3.0. Student's *t*-test and Mann-Whitney test were performed using SPSS 19.0. Variable importance in the projection (VIP) was obtained from the partial least squares-discriminant analysis (PLS-DA) model. VIP was used to rank the contribution of each variable based on the PLS-DA model, and the variables with $VIP > 1.0$ were considered relevant for time point discrimination. *P* values < 0.05 were considered significant. Based on the Hochberg-Benjamini method, the *P* values from Student's *t*-test were further adjusted by FDR.

3. Results

3.1. General data

Data are shown in Table 1. Compared with the healthy controls, the pre-hemodialysis ESRD patients exhibited significant increase in BUN, PCr, uric acid, and significant reductions in red blood cell count, platelet count and blood hemoglobin concentrations. The post-hemodialysis ESRD patients exhibited reduction of body weight and arterial blood pressure.

3.2. Metabolomic data in ESRD versus control group

Principal component analysis (PCA) performed on the plasma metabolites revealed a clear separation between the pre-dialysis plasma from ESRD patients compared to the plasma from healthy controls (Fig. 1A). In addition, a supervised PLS-DA structure built to compare the metabolic profile of ESRD patients' pre-hemodialysis plasma with healthy subjects' plasma, demonstrated distinctly separate profiles (Fig. S1A). In fact, comparison of the ESRD patients' pre-dialysis data with those found in the control group revealed significant changes in the plasma level of 214 metabolites, which were identified according to the previous publications [30–33]. They were selected by using the VIP values (> 1.5) from PLS-DA. The plasma levels of 126 metabolites were significantly increased and 88 metabolites were significantly decreased in ESRD patients, which were consistent with previous publications [34–39].

3.3. Effect of dialysis procedure on plasma metabolome

Plasma metabolites data determined by PCA and PLS-DA analysis at four time points during the course of hemodialysis procedure are shown in Figs. 1B and S1B. The metabolic profile of the arterial blood obtained at 10 min after the onset of hemodialysis showed no significant separation compared to the pre-hemodialysis profile and the profile obtained at 30 min showed a modest intergroup separation on the scores plot compared with the pre-hemodialysis profile. However, metabolic profile obtained immediately after hemodialysis showed a clear separation on the score plots from that observed immediately before hemodialysis. In fact, comparing the post-dialysis with pre-dialysis data we found significant changes in the blood level of 362 metabolites (250 in ESI^+ mode and 112 in ESI^- mode). They were selected by using the VIP values (> 1.5) from PLS-DA. Among the differential metabolites in the ESI^+ mode, plasma levels of 195 metabolites decreased and 55 metabolites increased following hemodialysis procedure. Among the differential metabolites in the ESI^- mode plasma levels of 82 differential metabolites were decreased and 30 were increased.

Figs. 1C and S1C display the PCA and PLS-DA data from healthy subjects and post-hemodialysis data from ESRD patients. A clear intergroup separation in plasma metabolites was observed between the two groups. The plasma levels of 81 metabolites were higher and 280 metabolites were lower in the post-dialysis plasma from ESRD patients compared with the healthy controls. Taken together these findings demonstrate that hemodialysis procedure does not normalize and induces additional changes to the metabolic profile of ESRD patients.

3.4. Effect of hemodialysis on identified metabolites

Using the previously reported methods [40–44] we identified 55 of the 362 differential metabolites according to their molecular size, MS^E fragments and i-FIT values comparing with the authentic standards or literatures and data base resources (Table 2). Fig. 1D depicts the ratio of the average plasma levels of the identified metabolites pre-hemodialysis versus the control on a binary logarithmic scale. Most polar small molecules were elevated in ESRD patients, including 34 that reached statistical significance ($P < 0.05$). Meanwhile, most lipid analytes were also slightly increased in ESRD patients relative to controls, including 13 that reached statistical significance. Fig. 1E depicts the ratio of the average plasma levels of the identified metabolites pre-hemodialysis versus the post-hemodialysis on a binary logarithmic scale. As expected, plasma level of

Fig. 1. (A) PCA scores plot of pre-hemodialysis ESRD patients and healthy control. (B) PCA scores plot of hemodialysed patients at different time points during hemodialysis. (C) PCA scores plot of post-hemodialysis (3.5 h) ESRD patients and healthy control. (D) Differential effects of ESRD on polar versus lipid metabolites. ESRD versus controls. Each data point represents one metabolite. The y-axis shows the ratio of the metabolite average level in pre-hemodialysis versus the metabolite average level in control on a binary logarithmic scale. (E) Differential effects of hemodialysis on polar versus lipid metabolites. Each data point represents one metabolite. The y-axis shows the ratio of the metabolite average level in post-hemodialysis (3.5 h) versus the metabolite average level in pre-hemodialysis on a binary logarithmic scale. The following annotations apply for (D) and (E): The x-axis shows the number of metabolites. Metabolites are ordered along the x-axis from lowest to highest ratio. Red data points signify metabolites in which the difference between pre- and post-hemodialysis metabolite level reached statistical significance ($P < 0.05$).

Table 2
Differentially expressed metabolites between the post-hemodialysis (3.5 h), pre-hemodialysis and control.

Metabolites	Post-hemodialysis (3.5 h) vs pre-hemodialysis				Post-hemodialysis (3.5 h) vs control		Pre-hemodialysis vs control	
	ESI	VIP ^a	FC ^b	p ^{c,d}	FC ^b	p ^c	FC ^b	p ^c
LPC(18:1)	+	2.32	1.18E-02	p < 0.001	1.47E-02	p < 0.001	1.25E + 00	p < 0.01
PE(40:4)	−	2.09	1.22E-02	p < 0.001	2.59E-02	p < 0.001	2.12E + 00	p < 0.05
Sphinganine	+	4.56	1.15E-02	p < 0.001	1.85E-02	p < 0.001	1.60E + 00	p < 0.001
LPC(16:0)	+	45.28	2.54E-04	p < 0.001	3.59E-04	p < 0.001	1.41E + 00	p < 0.001
PE(42:4)	+	2.72	5.09E-03	p < 0.001	7.47E-03	p < 0.001	1.47E + 00	p < 0.001
DG(38:8)	+	2.40	2.95E-01	p < 0.001	4.66E-01	p < 0.001	1.58E + 00	p < 0.001
PC(36:4)	+	55.42	5.11E-03	p < 0.001	4.87E-03	p < 0.001	9.52E-01	p = 0.403
PC(33:2)	−	2.09	6.30E-02	p < 0.001	5.92E-02	p < 0.001	9.40E-01	p = 0.729
PC(38:5)	+	24.00	9.78E-03	p < 0.001	8.04E-03	p < 0.001	8.21E-01	p < 0.001
CPA(18:1)	+	1.64	1.78E-02	p < 0.001	2.58E-02	p < 0.001	1.45E + 00	p < 0.001
DG(38:3)	+	3.29	8.11E-02	p < 0.001	4.47E-02	p < 0.001	5.51E-01	p < 0.001
PC(32:0)	+	4.43	1.36E-01	p < 0.001	2.20E-01	p < 0.001	1.62E + 00	p < 0.001
PC(38:6)	+	5.14	1.44E-01	p < 0.001	2.05E-01	p < 0.001	1.42E + 00	p < 0.001
PC(28:2)	+	3.42	3.31E-01	p < 0.001	4.24E-01	p < 0.001	1.28E + 00	p < 0.001
PC(34:3)	+	13.97	2.95E-01	p < 0.001	3.34E-01	p < 0.001	1.13E + 00	p = 0.260
PE(38:4)	+	6.95	4.48E-01	p < 0.001	4.67E-01	p < 0.001	1.04E + 00	p = 0.361
PC(44:6)	−	2.05	7.92E + 00	p < 0.001	1.30E + 01	p < 0.001	1.64E + 00	p < 0.01
Trimethylamine-N-oxide	+	2.82	2.18E-03	p < 0.001	3.27E-03	p < 0.001	1.50E + 00	p < 0.001
Indoxyl sulfate	−	1.95	1.99E-01	p < 0.001	2.89E + 00	p = 0.868	1.45E + 01	p < 0.05
p-Cresol sulfate	−	1.82	8.12E-02	p < 0.001	2.77E + 00	p = 0.460	3.41E + 01	p < 0.001
p-Cresol glucuronide	+	3.14	1.26E-01	p < 0.001	1.69E-01	p < 0.001	1.35E + 00	p < 0.001
Uric acid	+	1.91	3.21E-01	p < 0.001	5.01E-01	p < 0.001	1.56E + 00	p < 0.001
Hippuric acid	+	2.21	2.90E-01	p < 0.001	4.32E-01	p < 0.001	1.49E + 00	p < 0.001
L-Serine	+	2.40	5.53E-02	p < 0.001	7.69E-02	p < 0.001	1.39E + 00	p < 0.001
L-Phenylalanine	−	1.98	6.28E-03	p < 0.001	7.80E-02	p < 0.001	1.24E + 01	p < 0.01
Homocysteine	+	2.77	1.81E-02	p < 0.001	3.60E-02	p < 0.001	1.99E + 00	p < 0.001
L-Homoserine	+	2.32	3.10E-01	p < 0.001	4.36E + 01	p = 0.485	1.41E + 00	p < 0.001
L-Tryptophan	+	1.75	1.93E-01	p < 0.001	3.42E-01	p < 0.001	1.77E + 00	p < 0.001
L-Alanine	+	2.53	4.21E-01	p < 0.001	5.46E-01	p < 0.05	1.30E + 00	p < 0.001
L-Arginine	+	1.63	3.65E-01	p < 0.001	4.56E + 01	p < 0.01	1.25E + 00	p < 0.001
Asparagine	+	1.87	4.92E-01	p < 0.001	4.53E-01	p < 0.001	9.19E-01	p = 0.249
Proline	+	1.62	5.02E + 00	p < 0.001	6.51E + 00	p < 0.001	1.30E + 00	p < 0.001
Glycerophosphocholine	+	1.76	1.43E-03	p < 0.001	2.04E-03	p < 0.001	1.43E + 00	p < 0.001
3-Hydroxytetradecanoic acid	+	6.78	2.05E-02	p < 0.001	4.67E-02	p < 0.001	2.28E + 00	p < 0.001
Ethyl glucuronide	+	1.89	7.31E-04	p < 0.001	9.00E-04	p < 0.001	1.23E + 00	p < 0.001
5-Androstetriol	−	2.06	6.02E-03	p < 0.001	2.41E-03	p < 0.001	4.00E-01	p < 0.001
L-cystathionine	+	2.47	1.00E-03	p < 0.001	1.28E-03	p < 0.001	1.28E + 00	p < 0.001
N-Acetylserotonin sulfate	−	2.04	3.85E-02	p < 0.001	1.87E-01	p < 0.001	4.86E + 00	p < 0.001
Tryptophanol	+	7.42	5.40E-03	p < 0.001	7.98E-03	p < 0.001	1.48E + 00	p < 0.001
Vaccenyl carnitine	+	1.99	3.18E-02	p < 0.001	4.86E-02	p < 0.001	1.53E + 00	p < 0.001
3-Dehydroxycarnitine	+	11.03	2.12E-02	p < 0.001	3.99E-02	p < 0.001	1.88E + 00	p < 0.001
12-Ketodeoxycholic acid	+	2.04	1.91E + 01	p < 0.001	5.36E + 00	p < 0.001	2.80E-01	p < 0.001
Tryptamine	+	4.47	1.51E + 00	p < 0.001	3.21E + 00	p < 0.001	2.13E + 00	p < 0.001
Dihydrothymine	−	2.16	2.07E-01	p < 0.001	1.63E-01	p < 0.001	7.90E-01	p = 0.547
Imidazoleacetic acid	+	2.38	1.82E + 00	p < 0.001	2.32E + 00	p < 0.001	1.27E + 00	p < 0.001
L-Carnitine	+	5.83	2.25E + 00	p < 0.001	3.81E + 00	p < 0.001	1.69E + 00	p < 0.001
Vitamin e	+	2.9	3.39E + 00	p < 0.001	3.49E + 00	p < 0.001	1.03E + 00	p = 0.467
Creatine	+	5.79	2.68E + 00	p < 0.001	3.64E + 00	p < 0.001	1.36E + 00	p < 0.001
Guanidoacetic acid	+	5.5	3.49E + 00	p < 0.001	5.54E + 00	p < 0.001	1.59E + 00	p < 0.001
Ne,Ne dimethyllysine	+	1.9	5.33E + 00	p < 0.001	9.60E + 00	p < 0.001	1.80E + 00	p < 0.001
Ketoleucine	+	3.22	4.34E + 00	p < 0.001	7.84E + 00	p < 0.001	1.81E + 00	p < 0.001
Dimethylbenzimidazole	+	12.45	6.05E + 00	p < 0.001	1.13E + 01	p < 0.001	1.87E + 00	p < 0.001
3-Mercaptopyruvic acid	+	2.85	6.35E + 00	p < 0.001	7.25E + 00	p < 0.001	1.14E + 00	p < 0.001
Sebacinic acid	−	2.01	3.34E + 01	p < 0.001	1.53E + 02	p < 0.001	4.59E + 00	p = 0.709
Dimethylbenzimidazole	−	2.19	6.05E + 00	p < 0.001	1.13E + 01	p < 0.001	1.87E + 00	p = 0.551

LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; DG, diacylglycerol; PC, phosphatidylcholine; CPA, cyclic phosphatidic acid.

^a Variable importance in the projection (VIP) was obtained from the PLS-DA model.

^b Fold change was obtained by calculating average mass response ratio between post-hemodialysis (3.5 h) vs pre-hemodialysis, post-hemodialysis (3.5 h) vs control or pre-hemodialysis vs control.

^c The p value was calculated from Student's t-test.

^d All the metabolites were discriminant (Student's t-test p < 0.05), with an FDR of 5%.

most polar small molecules decreased significantly with hemodialysis. In addition, plasma content of most lipid analytes decreased significantly.

To further understand the changes of the identified metabolites during hemodialysis, a clustering heatmap was constructed to visualize their changes at four time points. Fig. 2 shows clear separation of the pre-dialysis abundance of the 55 identified metabolites from their post-hemodialysis values. However, no significant changes were detected at 10 min. The relative contents of the identified lipids, amino acids and uremic toxins between pre-hemodialysis and post-

hemodialysis is shown in Fig. 3. Lipid compounds including LPC(16:0), LPC(18:1), PE(40:4), PE(38:4), PE(42:4), Sphinganine, CPA(18:1), DG(38:8), PC(36:4), PC(33:2), PC(38:5), DG(38:3), PC(32:0), PC(38:6), PC(28:2), PC(34:3), and PC(44:6) decreased significantly. Likewise, uremic toxins and waste products including trimethylamine-N-oxide, indoxyl sulfate, p-cresol sulfate, p-cresol glucuronide, uric acid and hippuric acid as well as amino acids including L-serine, L-phenylalanine, homocysteine, L-homoserine, L-trypto-

Fig. 2. Heat maps of the differential metabolites between pre- and post-hemodialysis (3.5 h) at 4 time points. The color of each section denotes the concentration value of each metabolite calculated by peak area method.

phan, L-alanine, L-arginine, asparagine decreased significantly. But proline level increased significantly.

Comparison of the levels of the 55 identified metabolites between the ESRD patients and healthy control group revealed significant elevation of the uremic toxins and most lipid compounds in ESRD patients (Fig. 1A and Table 2). Hemodialysis resulted in significant reduction of plasma levels of the measured lipid compounds, uremic toxins and amino acids in ESRD patients. The changing trends in the level of most measured metabolites between the pre- and post-dialysis were consistent with their appearance in the effluent dialysate collected at 10 min (Table S1). Comparison of the levels of the 55 identified metabolites in the post-dialysis plasma from ESRD group with those found in the healthy control group revealed significant reduction of the uremic toxins, most amino acids and most lipid compounds in ESRD patients (Fig. 1C and Table 2). However, proline level increased significantly.

3.5. Effect of single passage of blood through dialyzer

Comparison of the plasma concentrations of metabolites in blood entering and leaving the dialysis filter at 10 min revealed significant change in concentrations of 323 metabolites (201 metabolites in ESI⁺ mode and 122 in ESI⁻ mode). The observed changes included significant reductions in 128 ESI⁺ metabolites and 81 ESI⁻ metabolites. We identified 37 of 323 plasma metabolites whose concentrations were significantly altered with single passage through dialysis filter (Table 3). As shown in Table 3, single passage of blood

through the dialyzer resulted in significant removal of not only the well-known uremic toxins and waste products such as indoxyl, *p*-cresol sulfate and uric acid but many other compounds including amino acids such as L-phenylalanine, L-glutamine, and L-alanine as well as lipid derivatives. In addition, we measured the level of BUN and PCr upon single passage of blood through the dialyzer at 10 min. The levels of BUN and PCr in blood entering the dialyzer were 25.35 ± 16.52 mg/dl and 8.76 ± 1.09 mg/dl respectively. The BUN and PCr levels in the blood leaving the dialyzer were 9.88 ± 5.04 mg/dl and 3.96 ± 0.92 mg/dl respectively. The BUN and PCr decreased by 61.0% and 54.8% respectively. The magnitude of fall in plasma concentrations of most measured uremic toxic and amino acids upon single passage of blood through the dialyzer at 10 min was close to those of BUN and PCr (Table 3: indoxyl, 0.29; uric acid, 0.15; L-glutamine, 0.75; L-phenylalanine, 0.42 folds respectively) reflecting indiscriminate removal of harmful and useful metabolites by dialysis procedure.

4. Discussion

In confirmation of earlier studies, the metabolic profile of our ESRD patients' plasma obtained immediately before hemodialysis was markedly different from that found in the healthy control group [45–47]. Several factors contribute to profound changes in the composition of the body fluids in patients with advanced chronic kidney disease. These include: I- Retention of the metabolic waste products of the endogenous and exogenous compounds which are

Fig. 3. Box-and-whisker plots showing significant differential metabolite changes in plasma between pre-hemodialysis and post-hemodialysis (3.5 h). (A) Lipids changes (B) Uremic toxins changes (C) Amino acid changes. Boxes show interquartile ranges, lines show medians and whiskers extend to extreme data points within 1.5 times interquartile range. Diamond represented metabolite intensity in plasma samples. *** $p < 0.001$ compared with the pre-hemodialysis group.

Table 3

Effect of single passage of blood through the dialysis filter on plasma concentrations of identified metabolites 10 min after the onset of hemodialysis procedure.

Metabolites	ESI	Metabolite levels in blood exiting vs entering dialyzer at 10 min				
		VIP ^a	FC ^b	P values ^c	FDR ^d	AUC
Tryptophanol	+	1.37	0.72	3.54E-03	3.85E-03	0.82
Adrenic acid	—	1.52	0.94	3.50E-01	3.60E-01	0.64
Leukotriene B4	—	1.36	1.67	2.30E-07	1.06E-06	1.00
Inosine	—	1.36	1.67	9.58E-08	8.87E-07	1.00
Oxadipic acid	—	1.35	0.24	1.93E-08	7.16E-07	1.00
Urothion	+	1.68	0.51	9.46E-04	1.09E-03	0.93
Dodecanoylcarnitine	+	1.38	0.27	3.93E-07	1.32E-06	0.99
9,10-DiHODE	+	1.37	0.23	3.15E-06	6.47E-06	0.98
L-Octanoylcarnitine	+	1.36	0.05	1.57E-04	2.00E-04	0.95
Ergosterol	+	1.34	4.37	6.15E-07	1.90E-06	1.00
PE(30:0)	+	1.53	0.64	2.67E-05	4.48E-05	0.98
PE(34:4)	+	1.44	0.08	1.02E-02	1.08E-02	0.94
PC(33:1)	+	1.43	0.32	3.95E-08	4.87E-07	1.00
PE(42:2)	+	1.41	4.82	3.23E-08	5.98E-07	1.00
MG(20:3)	+	1.39	4.01	7.58E-07	2.16E-06	1.00
PC(44:5)	+	1.37	0.58	1.32E-04	1.74E-04	0.98
PE(46:6)	+	1.36	4.99	1.54E-07	9.49E-07	1.00
TG(56:9)	+	1.36	4.00	1.16E-07	8.61E-07	0.99
TG(53:0)	+	1.35	5.70	3.49E-07	1.29E-06	1.00
PE(38:7)	+	1.34	0.71	9.82E-07	2.60E-06	1.00
LPA(18:0)	—	1.55	0.78	1.27E-05	2.25E-05	0.97
DG(39:0)	—	1.51	0.30	1.55E-06	3.82E-06	1.00
PC(38:3)	—	1.44	0.49	4.04E-05	6.22E-05	0.9
MG(18:0)	—	1.42	20.43	3.98E-05	6.39E-05	0.99
CPA(18:0)	—	1.42	2.40	1.90E-07	1.01E-06	1.00
PE(34:4)	—	1.42	0.39	1.06E-05	1.97E-05	0.99
DG(35:0)	—	1.40	1.46	1.79E-06	4.15E-06	0.99
PC(34:3)	—	1.38	0.41	3.40E-06	6.62E-06	0.97
DG(36:3)	—	1.37	3.19	2.27E-06	4.94E-06	0.98
Indoxyl	+	1.33	0.29	4.65E-04	5.73E-04	0.97
p-Cresol sulfate	—	1.46	0.15	9.26E-05	1.37E-04	0.95
Uric acid	—	1.51	0.15	1.11E-04	1.57E-04	0.99
L-Phenylalanine	+	1.35	0.42	5.33E-04	6.37E-04	0.93
L-Glutamine	+	1.31	0.75	7.35E-01	7.35E-01	0.75
L-Arginine	+	1.35	4.24	2.35E-07	9.67E-07	1.00
L-Alanine	—	1.36	0.12	3.24E-03	3.64E-03	0.91

LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; DG, diacylglycerol; PC, phosphatidylcholine; CPA, cyclic phosphatidic acid; MG, monoacylglycerol; TG, triacylglycerol; LPA, lysophosphatidic acid.

^a Variable importance in the projection (VIP) was obtained from the PLS-DA model.

^b Fold change was obtained by calculating the average mass response ratio between the blood leaving and the entering dialysis filter (10 min).

^c The p value was calculated from Student's t-test.

^d FDR value was obtained from the adjusted p value of FDR correction by Benjamini-Hochberg method.

normally excreted in the urine by the kidney, II- Dietary restrictions imposed to limit fluid overload, electrolyte disorders, acid-base abnormalities, hypertension, and vascular calcification, III- Profound transformation of the intestinal microbiome from a symbiotic to a dysbiotic state [48,49] leading to production, absorption, and retention of numerous gut-derived uremic toxins including P-cresol sulfates, indoxyl sulfate, trimethylamine-N-oxide among others [50,51]. The microbial dysbiosis in CKD is driven by profound changes in the biochemical and biophysical milieu of the gastrointestinal tract occasioned by the influx of urea and uric acid in the intestinal tract and commonly prescribed medications and dietary restrictions [52,53], IV-disruption of the intestinal epithelial barrier structure and function [54] enabling influx of noxious luminal contents of intestine to the systemic circulation. V- Oxidative stress and inflammation which are common features of CKD [55,56] and profoundly alter the metabolic profile by promoting formation of oxidized and myeloperoxidized products. VI- CKD-induced insulin resistance, secondary hyperparathyroidism and other endocrine disorders which alter the metabolic profile in this population, VII-CKD-induced down-regulation and impaired activities of lipoprotein lipase and hepatic lipase coupled with elevated adipocytes intracellular lipase [57–59] which result in significant elevation of serum triglycerides, phospholipids, and free fatty acids concentrations, and finally VIII- CKD-associated catabolic state which by promoting breakdown and limiting biosynthesis of

proteins [60,61] lead to release of amino acid in body fluids and their removal by hemodialysis in ESRD patients [62,63].

In addition to removing metabolic waste products and excess fluids and electrolytes, dialysis modalities indiscriminately remove all small molecular weight solutes including amino acids, organic compounds, vitamins and other important molecules. Moreover, via activation of immune system, exposure of blood to the extracorporeal circuit can intensify inflammation and oxidative stress and production of harmful products. Depletion of biologically important products and recurrent activation of inflammatory response can contribute to the high morbidity and annual mortality rate of ESRD patients maintained on chronic dialysis. Comparison of the metabolic profile of our ESRD patients' post-dialysis with the pre-dialysis plasma revealed significant changes in concentrations of 362 metabolites. Concentration of the majority of the altered metabolites (277) decreased after hemodialysis procedure reflecting their removal by dialysis. This was confirmed by the fall in plasma concentration of those solutes upon single passage of blood through the dialyzer. We identified 55 known molecules of the 362 metabolites whose plasma concentration were significantly altered by hemodialysis. Besides the water-soluble molecules, hemodialysis resulted in significant fall in plasma concentration of most lipid compounds. This is, in part, due to systemic anticoagulation with heparin which is used to prevent blood coagulation in the extracorporeal system. Heparin triggers activation and release of tissue-bound lipoprotein lipase and he-

patic lipase which catalyze hydrolysis and clearance of circulating triglycerides and phospholipids [64].

The pre-dialysis plasma concentrations of uremic toxins which were much higher in the ESRD patients than in the healthy subjects significantly declined after hemodialysis. Likewise hemodialysis resulted in the fall in plasma concentration of all measured lipid metabolites except PC(44:6). In addition, in confirmation of a previous study by Chuang et al., hemodialysis resulted in significant reduction of plasma concentration of all identified amino acids except proline [62]. Taken together, the present data reveals that removal of uremic toxins and waste products by hemodialysis is accompanied by removal of large numbers of identified and as-yet un-identified metabolites. Depletion of these biological compounds can contribute to the catabolic state and high morbidity and annual mortality rate in this population. In fact, depletion of amino acids due to protein-energy wasting and increased catabolic state and their removal by hemodialysis can contribute to high morbidity and annual mortality rate in ESRD population [60,61].

While plasma concentration of most metabolites declined, plasma levels of several metabolites including 13 identified compounds rose after hemodialysis. Among the identified metabolites plasma concentrations of PC(44:6), proline, imidazoleacetic acid, L-carnitine, vitamin E, creatine, guanidoacetic acid, Ne,Ne dimethyllysine, ketoleucine, dimethylbenzimidazole, 3-mercaptopyruvic acid, sebacic acid, and dimethylbenzimidazole significantly increased in our ESRD patients after dialysis. The rise in concentration of these metabolites reflects their enhanced endogenous release or production and to a lesser extent their reduced volume distribution occasioned by fluid removal with ultrafiltration.

In conclusion, the present data reveals that removal of uremic toxins and waste products by hemodialysis is accompanied by removal of large numbers of identified and as-yet un-identified metabolites. Depletion of these biological compounds may contribute to the high morbidity and annual mortality rate in this population.

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Competing financial interests

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clinbiochem.2017.09.012>.

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