Serum metabolomic profiles from patients with acute kidney injury: A pilot study

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ARTICLE INFO

ABSTRACT

Low sensitivity of current clinical markers (serum creatinine and blood urea nitrogen (BUN)) in early stages of the development of acute kidney injury (AKI) limits their utility. Rapid LC/MS-based metabolic profiling of serum demonstrated in a pilot study that metabolomics could provide novel indicators of AKI. Metabolic profiles of serum samples from seventeen hospitalized patients with newly diagnosed AKI were compared with the profiles of serum from age-matched subjects with normal kidney function. Increases in acylcarnitines and amino acids (methionine, homocysteine, pyroglutamate, asymmetric dimethylarginine (ADMA), and phenylalanine) and a reduction in serum levels of arginine and several lysophosphatidyl cholines were observed in patients with AKI compared to healthy subjects. Increases in homocysteine, ADMA and pyroglutamate have been recognized as biomarkers of cardiovascular and renal disease, and acylcarnitines represent biomarkers of defective fatty acid oxidation. The results of this pilot study demonstrate the utility of metabolomics in the discovery of novel serum biomarkers that can facilitate the diagnosis and determine prognosis of AKI in hospitalized patients.

Published by Elsevier B.V.

1. Introduction

The term acute kidney injury (AKI) is currently recognized as the preferred nomenclature for the complex clinical syndrome formerly known as acute renal failure (ARF). The use of this new terminology better defines the broad spectrum of this disease, which includes patients with a transient elevation of serum creatinine to individuals that ultimately require dialysis [1]. The acute decline in kidney function is often secondary to an injury that causes functional or structural changes in the kidneys. Transient azotemia represents one-third of all causes of AKI in hospitalized patients, and is independently correlated with a significantly increased risk of death [2]. Previous epidemiological studies have demonstrated a wide variation in etiologies and risk factors for AKI and confirmed

that this disease is associated with increased morbidity and mortality, especially when dialysis treatment is required [3]. Recent clinical studies have validated the use of Risk-Injury-Failure-Loss-End Stage Renal Disease (RIFLE) criteria to establish the presence or absence of clinical AKI in a given patient population. In addition, in various epidemiological studies RIFLE criteria based on changes in serum creatinine have been used to study possible associations between the severity of clinical AKI and clinical outcomes in patients with hospital-acquired AKI [4]. However, a recent study based on creatinine kinetics questions the validity of defining AKI on the basis of fractional/percentage increases in serum creatinine, rather than absolute serum creatinine increments, particularly in patients with underlying chronic kidney disease (CKD) [5]. Serum creatinine concentrations are dependent upon age, gender, muscle mass, medication, and hydration status and may not be altered until a significant amount of kidney function is lost [6]. Therefore, measurement of only the serum creatinine as the gold standard marker of kidney function carries the risk of missing an important therapeutic window because of the time lag between the inciting insult and the diagnostic elevation of creatinine. More sensitive candidate protein biomarkers of acute kidney injury have been proposed, and in some cases, their preclinical performance has been assessed in the early diagnosis and stratification of AKI [7].

Metabolic profiling [8,9] combined with chemometrics analysis has the ability to measure global alterations in metabolism

within tissues or biofluids that precede conventional biochemical and pathological changes. This has contributed to the emergence of metabolic profiling as a promising scientific platform for prediction of drug safety or disease progression stage. Pattern recognition and expert systems have been developed to allow analysis of complex metabolomics data sets, including use of a training set that consists of samples from specimens exhibiting a specific toxicity or pathology and a control group in order to generate a defined classification scheme. Once a model of the metabolomic data is generated and spectral biomarker(s) are determined, standard analytical techniques are used to identify the biomarker(s). In this manner, spectral biomarkers and the associated metabolites that define differences between groups can be identified. In all, metabolic profiling permits identification of biomarkers or patterns of biomarker changes related to disease in biofluid samples such as urine and blood that can be collected with relative ease.

In this pilot study, non-targeted metabolic profiles of serum samples obtained from seventeen hospitalized patients with newly diagnosed AKI were compared with the metabolic profile of serum samples obtained from healthy volunteers with normal kidney function. The aim of this pilot study was to demonstrate that the systemic metabolic alterations associated with AKI could be evaluated using a rapid metabolomics screening technique. As such, the identification of serum biomarkers could provide useful information not only about early diagnosis of AKI, but also could help elucidate novel serum biomarkers associated with prognosis after an AKI episode in hospitalized patients.

2. Material and methods

2.1. Patient population and sample collection

Venous samples were obtained from 17 patients with newly diagnosed AKI at the Central Arkansas Veterans Healthcare System (CAVHS) and from 17 healthy subjects.

2.1.1. Inclusion/exclusion criteria for AKI patients

Inclusion criteria for AKI patients included: women of childbearing age with a negative pregnancy test, male and females 18 years of age or older, and serum creatinine less than 5.0 mg/dL at time of enrollment. Exclusion criteria for the study included: (1) any elevation in serum creatinine greater than or equal to 5.0 mg/dL over the previous 12 month period, (2) anuria, (3) pregnancy, (4) organ transplant recipient, (5) presence of obstructive uropathy which was ruled out by performing a renal ultrasound, and (6) inability to give informed consent. Acute kidney injury was defined by a serum creatinine level that had increased 50% or more from previous baseline measures. The AKI diagnosis was made in the 17 patients by detecting an increase in serum creatinine of more than 0.3 mg/dL over baseline or at least a 50% increase of serum creatinine over baseline at the time of the renal consult.

2.1.2. Sample collection

For each patient, one serum sample was collected in 5 mL Vacuette tubes (Greiner Bio-One NA, Monroe, NC) at the time that renal service was consulted for evaluation of acute kidney injury. Each serum sample was processed for metabolic profiling. Serum was prepared by centrifugation at $2000 \times g$ (Beckman) for 10 min at 4 °C. The research protocol for collection of these samples was approved by the CAVHS and the University of Arkansas for Medical Sciences Institutional Review Boards (IRB), and blind analysis of these de-identified serum samples was also approved by the Institutional Review Board of the FDA's Research Involving Human Subjects Committee (RIHSC). All samples were stored at −80 °C until analysis.

2.2. Sample preparation

Serum (100 μL aliquot) was mixed with 300 μL methanol and incubated at −20 °C for 20 min and then centrifuged at 13,000 rpm for 12 min at 4 °C to precipitate proteins. The supernatant (300 μL) was then transferred into clean tubes and evaporated to dryness using a SpeedVac concentrator (Thermo Scientific, Waltham, MA). The samples were reconstituted in 200 μL 95:5 water/acetonitrile, vortexed for 2 min and kept at 4 °C for 20 min. The resulting solution was then centrifuged at 13,000 rpm for 12 min at 4 °C. The supernatant was transferred to autosampler vials for LC/MS analysis. The same sample preparation methods were applied to the serum samples obtained from healthy individuals with normal renal function as well as to the samples obtained from standard reference material (SRM) 909b from the National Institute of Standards and Technology (NIST).

2.3. Global profiling using UPLC/QTof-MS

The analysis order of serum samples from the 17 AKI patients and the serum samples from 17 healthy subjects with normal kidney function was randomized. A 3 μL aliquot of serum supernatant was introduced into a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA) equipped with a Waters bridged ethyl hybrid (BEH) C8 column (2.1 mm × 10 cm, 1.7 μm) held at 40 °C. The UPLC mobile phases consisted of 0.1% formic acid in water (solution A) and 0.1% formic acid in acetonitrile (solution B). While maintaining a constant flow rate of 0.4 mL/min, the metabolites were eluted using linear gradients of 2–80% B from 0 to 15 min and 80–98% B from 15 to 17 min. The final gradient composition was held constant for 2 min followed by a return to 2% B at 19.1 min.

The mass spectrometric data were collected with a Waters Q-Tof Premier mass spectrometer (Waters, Milford, MA) operated in positive and negative ionization electrospray modes as reported previously [10,11]. Briefly, MS^E analysis was performed on a Q-Tof mass spectrometer set up with 5 eV for low collision energy and a ramp collision energy file from 20 to 30 eV. Full scan mode from m/z 100 to 900 and from 0 to 22 min was used for data collection in both positive ion and negative ion modes.

Raw UPLC/MS data were analyzed using Micromass MarkerLynx XS Application Version 4.1 (Waters, Milford, MA) with extended statistical tools. The same parameter settings for peak extraction from the raw data were used as previously reported [10,11]. The resulting dataset was analyzed by unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA). The Student's T-test (p-value < 0.05) was calculated to determine significantly altered metabolites in AKI patients compared with the healthy control group with normal kidney function. In order to detect biomarkers that are closely associated with clinical creatinine data and that could potentially serve as biomarkers for AKI, all of the metabolites identified were subjected to Pearson's rank correlation using Statistica Version 9.0 (StatSoft, Tulsa, OK). Compounds detected by MS were confirmed by mass accuracy, isotope distribution pattern, fragmentation mass spectrum analysis; and some metabolites were also confirmed by comparison with authentic standards.

3. Results

3.1. Changes in renal function

All 17 patients were diagnosed with AKI based on RIFLE criteria including a significant change in the serum creatinine levels when compared to baseline values [4,5]. Although metabolomic

analysis was performed on only one single serum sample obtained from every patient at the time of a renal consult, the renal function of each patient was followed for several days during hospitalization after the diagnosis of AKI. These records (data not shown) confirmed that their renal function was affected for several days. In addition, examination of urine sediment in a random urine sample obtained the day of the renal consult in every patient showed the presence of granular casts and renal tubular epithelial cells consistent with the presence of a sustained form of acute tubular injury such as acute tubular necrosis. Table 1 lists the clinical information for all of the 17 AKI patients including clinical chemistry values, as well as their disease history and any associated co-morbidities as diagnosed by the attending physician or by previous medical histories. Of these 17 AKI patients, five required dialysis; nine had a previous history of diabetes; eleven patients had a history of coronary artery disease; twelve had a history of hypertension; ten patients had a history of hyperlipidemia; and four patients had a history of congestive heart failure. Serum creatinine levels at the time of enrollment were increased in the range of 1.6–10 times over their baseline levels. The mortality of the AKI patients in the study was 47% (eight out of 17 patients) after a follow up of one year following the episode of AKI. The average serum creatinine level of the 17 age-matched healthy subjects with normal renal function was 0.86 mg/dL, which was much lower than that from the AKI group (4.21 mg/dL). Six of the healthy volunteers had history of hypertension (HTN); no other co-morbid conditions were observed in the healthy control group with normal kidney function.

3.2. Serum profile analysis

Rapid serum profiling was conducted to screen for significant changes in serum metabolites from 17 AKI patients and compared with the metabolic profile of 17 subjects with normal renal function. Fig. 1 displays examples of total ion chromatograms (TICs) of sera in positive mode from a healthy subject (Fig. 1A) and from a patient with AKI (Fig. 1B). Based on visual observation alone, a group of peaks between retention times 4–6 min are present in the serum sample from a patient with AKI but absent in the healthy sample. The mass spectra (data not shown) of the chromatographic peaks (in the box of Fig. 1B) had the same pattern exhibiting a 44 Da mass difference between m/z peaks, which is characteristic of polyethylene glycol (PEG). The presence of PEG might be associated with drugs prescribed to the patients. Alternately, the source of PEG may be from the blood collection tubes as demonstrated previously [12]. In the control samples from healthy volunteers with normal kidney function, a group of peaks between 11 and 13 min were present and identified as lysophosphatidyl choline (LysoPC). Several LysoPCs had a lower concentration in the AKI patients relative to the levels of healthy samples.

Metabolite information, including intensity and retention time, were extracted from raw LC/MS data using MarkerLynx software. Partial least square discriminant analysis (PLS-DA) was applied to study the global changes in serum metabolites. PLS-DA was performed in order to obtain a maximum separation among classes, and to determine which metabolites best distinguished the groups. Fig. 2A shows the scores plot from PLS-DA of the LC/MS data from positive ionization mode with the goodness of fit parameters $R^2X[1] = 0.3572$ and $R^2X[2] = 0.1707$. Each point in Fig. 2A represents one sample. The patient samples are clearly separated from healthy samples and NIST samples along t[1]. Seven NIST samples (utilized as QC samples), randomly analyzed during the whole sample analysis, were tightly clustered and showed no correlation with injection order. The tight clustering of the QC samples indicated that little instrumental variation occurred during the sample run. In this study, there are no observable separations based on gender or race between the subjects in patient and healthy groups.

Table 1
Demographics of the patient in study group.

Patient ID	Dialysis	Baseline creatinine	Creatinine at enrollment (mg/dL)	T. bili (mg/dL)	AST (U/L)	ALT (U/L)	LDH	ALKP (U/L)	CHF	DM	HTN	CAD	HLP	PVD	Anemia	Liver disease	Death
P1	Yes	1.4	3.4	1	85	N/A	N/A	112	Yes	Yes	Yes	Yes	No	No	Yes	No	Yes
P2	Yes	2.8	4.4	0.4	17	6	N/A	81	No	Yes	Yes	Yes	Yes	No	No	No	Yes
P3	Yes	1.1	1.6	1.3	267	144	1968	53	No	No	Yes	Yes	No	Yes	No	No	No
P4	No	0.8	3.4	N/A	N/A	N/A	160	403	No	No	Yes	No	No	Yes	No	No	No
P5	Yes	0.7	1.4	12.6	>8000	3718	N/A	174	No	No	No	No	No	No	No	No	Yes
P6	No	1.3	2.7	N/A	32	21	N/A	N/A	No	No	No	Yes	Yes	No	No	Yes	No
P7	No	1.3	3.6	N/A	N/A	N/A	N/A	N/A	No	No	Yes	Yes	Yes	Yes	No	No	Yes
P8	No	1.9	3	N/A	N/A	N/A	N/A	N/A	No	No	Yes	Yes	No	Yes	No	No	No
P9	No	1	2.1	N/A	63	24	N/A	N/A	No	Yes	No	No	Yes	Yes	No	Yes	No
P10	No	1.38	3.1	0.3	162	32	N/A	N/A	No	Yes	Yes	No	No	No	No	No	Yes
P11	No	1.3	12.5	N/A	N/A	N/A	N/A	N/A	No	No	No	No	No	No	Yes	Yes	No
P12	No	1.3	4.4	5.8	164	65	N/A	N/A	No	No	No	No	Yes	No	No	Yes	Yes
P13	No	0.9	4.5	N/A	51	33	N/A	N/A	No	No	No	No	No	No	No	No	Yes
P14	No	1.3	5.1	N/A	412	61	511	N/A	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
P15	No	1.2	4.9	N/A	N/A	N/A	N/A	N/A	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No
P16	Yes	1.8	4.5	N/A	N/A	N/A	N/A	N/A	Yes	Yes	Yes	Yes	Yes	No	Yes	No	No
P17	No	2.38	7	N/A	N/A	N/A	N/A	N/A	Yes	Yes	Yes	Yes	Yes	No	Yes	No	No

T. bili: total bilirubin; AST: aspartate transaminase; ALT: alanine transaminase, LDH: lactate dehydrogenase, ALKP: alkaline phosphatase, CHF: congestive heart failure; DM: diabetes; HTN: hypertension; CAD: coronary artery disease; HLP: hyperlipidemia (history of having high cholesterol or triglycerides levels not necessarily that these levels were high on the day the sample was taken); PVD: peripheral vascular disease.
Reference range of creatinine 0.6–1.3 mg/dL; total bilirubin: 0.0–1.1 mg/dL; AST 15–37 U/L; ALT 11–63 U/L; LDH: 100–190 U/L; and ALKP: 31–126 U/L.
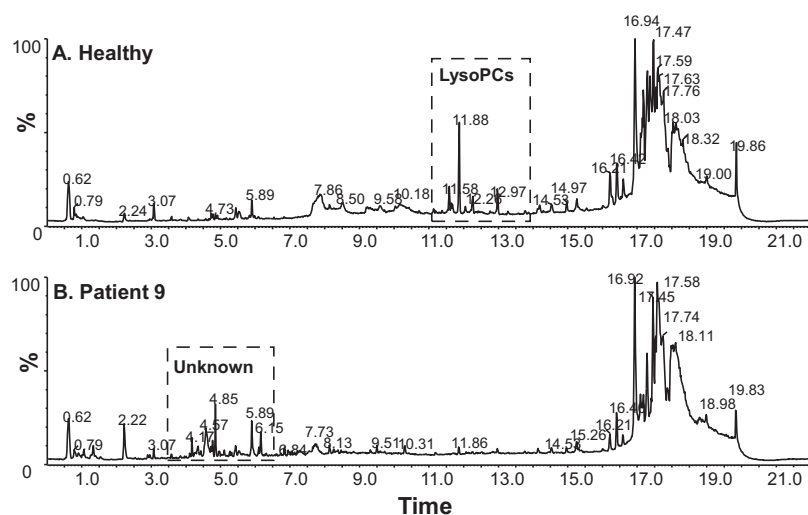N/A: not available.

Fig. 1. Total ion chromatograms (TICs) of serum in positive mode from (A) a healthy subject sample and (B) one patient sample.

The S-loadings plot from PLS-DA analysis (Fig. 2B) shows the weight of each individual ion feature towards the group separations of AKI patients and healthy subjects. Each ion feature represented by a dot equates to a single retention time and m/z pair. The ions located furthest from the zero line of the vertical axis are most responsible for the group separations. Therefore, the focus was towards the identification of those ion features that best separated the different groups. Some identified ion features are labeled in Fig. 2B. Metabolites that demonstrated the most statistically significant increase in AKI patients when compared to healthy individuals with normal renal function included: phenylalanine, acetylcarnitine, creatinine, homocysteine and other unknown PEG compounds. Another statistically significant change observed in AKI patients when compared to healthy controls was a reduction in serum LysoPC levels.

The changes in ions detected in UPLC/MS from PLS-DA are summarized in Table 2, which also provides fold changes in serum metabolites in AKI patients when compared with healthy subjects and p-values from a T-test analysis. Only the compounds that were altered in the same manner (Table S1) in terms of intensities (reflection of serum levels) in the AKI patients versus healthy subjects were considered as potential AKI biomarkers. Thus, the possibility of the detected metabolite abnormalities being due to other complications such as liver disease or chronic illnesses was minimized.

Amino acids, including homocysteine, pyroglutamate, dimethylarginine, methionine and phenylalanine, were significantly

Fig. 2. (A) Scores plot from a PLS-DA of the LC/MS data from positive ionization mode to indicate good model fit. The $t[1]$ and $t[2]$ values represent the scores of each sample in principal component 1 and 2, respectively. The cumulative fitness $R^2X[1]$ and $R^2X[2]$ of the model are 0.3572 and 0.1707, respectively. (B) S-loadings plot from a PLS-DA, which shows the weight of each individual ion feature towards the group separations of patients and healthy subjects.

Table 2
Altered principal ions of patients compared with healthy subject samples.

Metabolite	p-Value	Fold change
Acetylcarnitine	0.015	1.46
Octanoylcarnitine	0.010	1.95
Decenoylcarnitine	0.038	1.61
Decanoylcarnitine	0.035	1.62
Phenylalanine	3.25E−04	2.13
Creatinine	2.93E−09	3.94
Dimethylarginine[a]	4.07E−10	91.08
Homocysteine	1.29E−07	3.54
Methionine	0.006	2.29
Pyroglutamate	1.90E−10	8.52
Arginine	0.013	0.62
Tryptophan	1.45E−05	0.56
LysoPC(14:0)	1.18E−10	0.10
LysoPC(16:0)	9.57E−14	0.28
LysoPC(16:1)	1.22E−07	0.17
LysoPC(18:0)	2.34E−12	0.25
LysoPC(18:1)	3.43E−09	0.27
LysoPC(18:2)	3.73E−09	0.13
PEG POLYMER	NA	Only present in patient sample

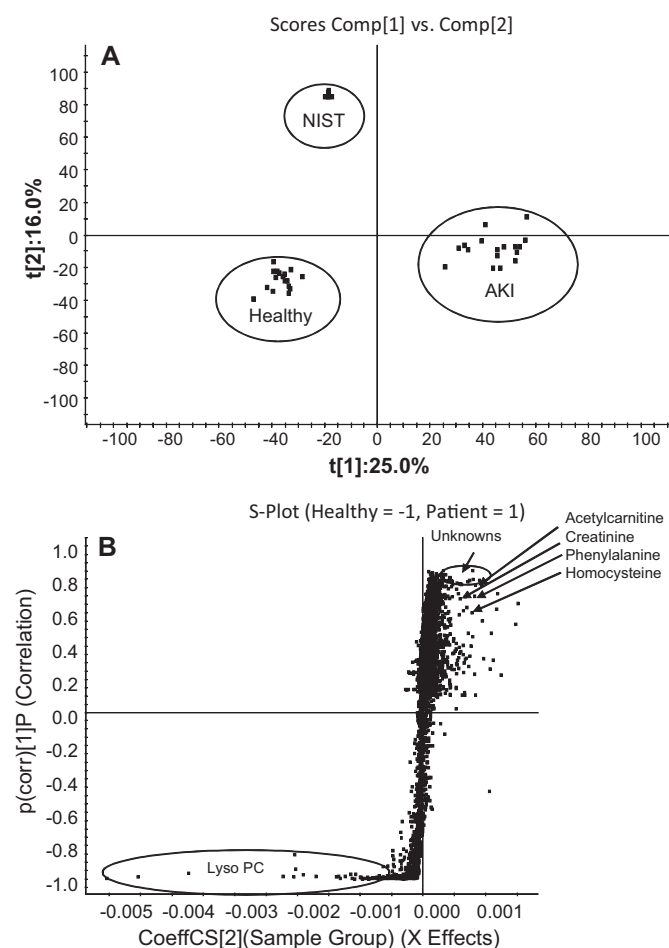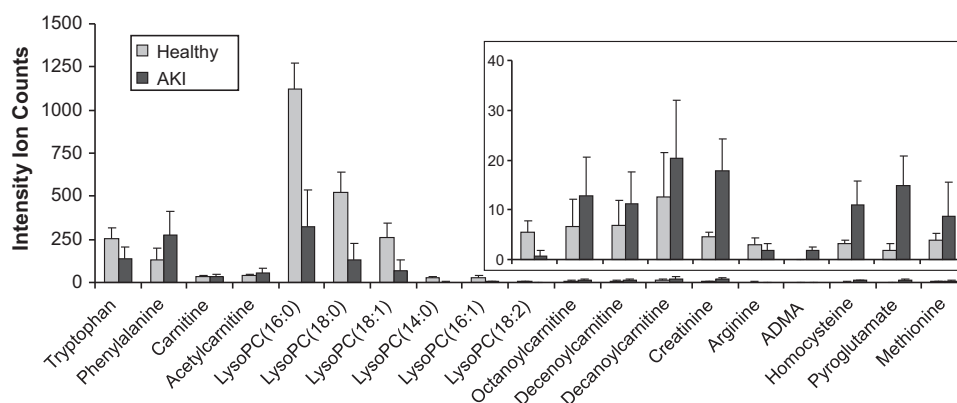[a] Dimethylarginine is detected as asymmetric dimethylarginine (ADMA).

Fig. 3. Bar graphs of metabolites that were altered in AKI patients to a statistically significant extent ($p < 0.05$). The boxed figure is enlarged for the 10 low abundant metabolites. ADMA is asymmetric dimethylarginine.

increased in the serum from AKI patients. Acylcarnitines (including acetylcarnitine, octanoylcarnitine, decenoylcarnitine and decanoylcarnitine) were significantly increased in the serum from AKI patients. Dimethylarginine was identified as asymmetric dimethylarginine (ADMA) based on a neutral loss of $(CH_3)_2NH$ from the molecular ion in the fragmentation mass spectrum (data not shown). This neutral loss can differentiate ADMA from symmetric dimethylarginine. Serum creatinine, a traditional clinical biomarker of renal function, was detected over 3 times higher in AKI patients when compared to the serum from the healthy subjects. Fig. 3 shows bar graphs of the detected metabolite levels in serum samples from healthy and AKI patient samples. The intensity levels for acylcarnitines, creatinine, phenylalanine, homocysteine and pyroglutamate were significantly increased in AKI patients, while LysoPCs were significantly decreased (p-value < 0.05). Pearson correlation analysis showed that homocysteine had the highest positive correlation (0.84, p-value < 0.001) with clinical chemistry creatinine levels in the serum from both healthy subjects and AKI patients. There were non-correlations of the altered metabolites with the other co-morbidities.

4. Discussion

This metabolomics pilot study performed in AKI patients and healthy volunteers is the first to report significant differences in the levels of serum metabolites such as increased serum acylcarnitine levels in a small cohort of hospitalized patients with newly diagnosed AKI. The significant changes in metabolites between the two populations suggest the presence of metabolic abnormalities in the AKI group. Some of these metabolic abnormalities have been previously described in serum and kidney tissue samples obtained from various animal models of AKI [13–15]. In addition, this LC/MS-based metabolomic study correlated the intensity level of the serum metabolites with the clinical chemistry data including serum creatinine. For example, the classically obtained clinical data (Table 1), showed the average serum creatinine levels for the 17 patients with AKI was 4.21 mg/dL ($\pm$2.55) at enrollment. From the LC/MS data (Supplemental Table 1), the average creatinine intensity of the 17 patients was 3.94 times that of the healthy sample with normal renal function. From the clinical chemistry data, the average creatinine level of the AKI patients was 4.87 times (4.21/0.86 = 4.87) that of the healthy sample. These data are very close to the concentrations seen with the LC/MS method considering reasonable sample loss during sample processing as well as the routine difference between clinical laboratories. Based on the comparison of the clinical biomarkers data with the LC/MS results, it is reasonable to assume that LC/MS can quantitatively evaluate

serum metabolite changes that are reflective of the standard clinical measurements.

Serum LysoPCs levels were significantly reduced (Table 2 and Fig. 3) in AKI patients. Patients with chronic kidney disease are well known to develop premature atherosclerosis and increased incidence of cardiovascular morbidity and mortality [16,17]. The clinical data in Table 1 shows that a significant proportion of patients with AKI had either hypertension or prior history of hyperlipidemia. A potential explanation for the observed reduced serum levels of LysoPCs in AKI patients could be related to a possible reduction in lecithin:cholesterol acyl transferase (LCAT) enzyme activity (an enzyme that converts unesterified cholesterol and phosphatidylcholine (PC) to esterified cholesterol and lysophosphatidylcholine). Plasma LCAT activity and its concentration have been reported to be markedly reduced in end-stage-renal-disease (ESRD) patients [18,19]. It has been reported also that LCAT deficiency causes phosphatidylcholine levels to increase, while LysoPCs levels are reduced [20]. Further, the serum lipid profile can be influenced by age, genetic disorders of lipid metabolism, severity of diseases and lipid-altering drugs such as statins, fibrates, rapamycin and others. It is interesting to note that all six LysoPCs reported in this pilot study had statistically significant negative correlations ($r < -0.52$, $p < 0.001$) with death, meaning that lower levels of LysoPCs could be correlated with worse outcome in AKI patients. Since it is difficult to distinguish the reasons for the altered lipid profile in AKI patients, our results will require further validation in future studies that can include a larger sample of AKI patients.

Homocysteine, ADMA, and pyroglutamate, compounds involved in the oxidative stress pathway (Fig. 4) were significantly increased by approximately 3.5-, 91.08- and 8.5-fold, respectively, in AKI patients when compared to samples obtained from healthy volunteers with normal kidney function. Glutathione (GSH), a tri-peptide containing glutamic acid, cysteine and glycine, plays an important role in immunomodulation, amino acid metabolism and most importantly as an antioxidant to prevent damage to cellular component caused by reactive oxygen species. Loss of a terminal methyl group from methionine produces homocysteine, which contains a free thiol group and can be converted to cysteine and further be synthesized to glutathione. Increases in methionine could be caused by high levels of homocysteine in AKI patients since homocysteine can also be converted back to methionine by remethylation via methionine synthase or via betaine:homocysteine methyltransferase (BHMT) [21,22]. In this study, the blood methionine levels had a statistically significant correlation with mortality ($r = 0.56$, $p < 0.001$). Hyperhomocysteinaemia is regarded as an independent risk factor and indicator [23,24] for cardiovascular events and is associated with various human diseases such as chronic kidney
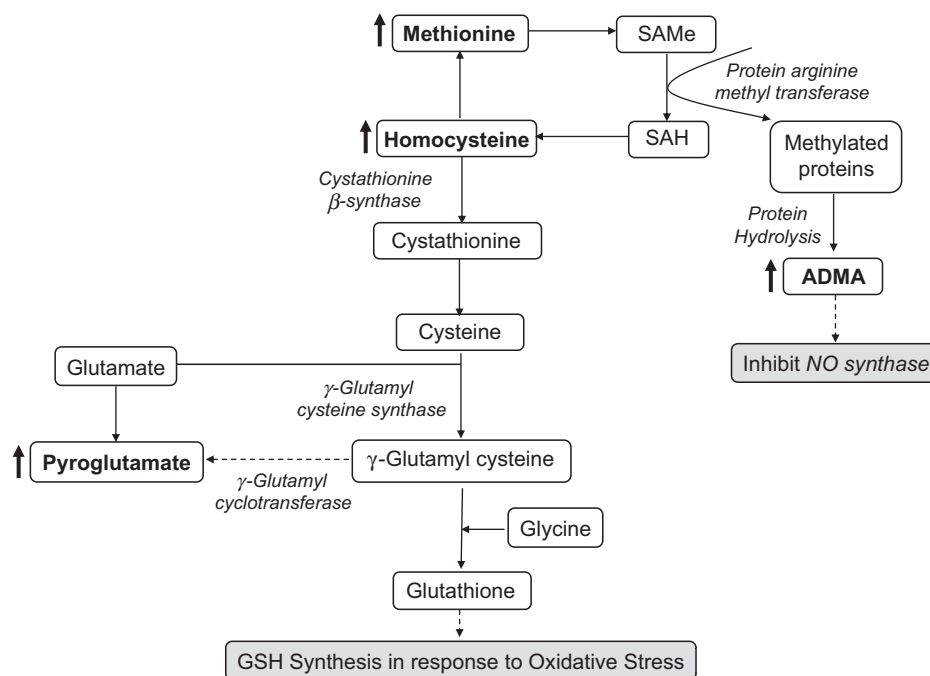
Fig. 4. Metabolic pathway changes in AKI.

disease. Similar to the results presented within, homocysteine levels were found elevated in patients with diabetes and cardio-vascular disease. Mechanisms linking homocysteinaemia and poor cardiovascular outcome are not well understood, but could involve the presence of increased oxidative stress related to increased levels of oxidized low-density lipoprotein. However, lowering homocysteine levels with vitamins has not shown to reduce the risk of heart disease in dialysis patients [25]. Further, increases in ADMA (generated from arginine methylation in protein where the methyl is derived from the methyl donor S-adenosylmethionine [26], an intermediate in the metabolism of homocysteine) in AKI patients were also linked with elevated levels of homocysteine. Elevations of ADMA levels in serum have been reported in patients with ESRD and chronic kidney disease [27,28]. In the present pilot study, higher serum levels of homocysteine and ADMA had high correlations ($r = 0.89$ and 0.86, respectively; $p < 0.05$) with higher serum creatinine levels in AKI patients. The results from this pilot study underscore the important role of kidney in the regulation of homocysteine concentrations in the circulation [29,30]. In the kidney, homocysteine is metabolized primarily through the trans-sulfuration pathway, in which the cystathionine β-synthase (CBS) enzyme regulates the rate-limiting step by condensing homocysteine with serine to form cystathionine. A recent study using an AKI model of ischemia-reperfusion injury demonstrated a significant reduction in mRNA, protein levels and enzyme activity of kidney CBS via decreased Sp1 transcriptional activity [31]. Therefore it is quite possible that CBS expression could be also reduced in kidney tissue of our AKI patients and this metabolic abnormality could explain the observed high serum levels of homocysteine detected by this metabolomics study in AKI patients.

In addition to homocysteine and ADMA, serum pyroglutamate levels, a component of the GSH pathway derived from cysteine, were also elevated. Pyroglutamic acidaemia has been reported as a cause of high anion gap metabolic acidosis in kidney failure patients [32,33] and/or in the setting of acetaminophen use [32,34]. We speculate that depletion of glutathione caused by oxidative stress can stimulate the production of γ-glutamyl cysteine from cysteine and glutamate (Fig. 4). The resulting high level of γ-glutamyl

cysteine can be converted to pyroglutamate by γ-glutamyl cyclo-transferase. While some causes of elevations in pyroglutamate have been described above, an exact cause is difficult to ascertain as pyroglutamic acidaemia is a multifactorial process [34]. Further, it is possible that acute kidney injury could cause diminished excretion of homocysteine and pyroglumate resulting in accumulation of these compounds in the serum. ADMA and oxidative stress has been reported to be associated with the progression of renal disease [35]. Therefore, persistent high serum levels of homocysteine, ADMA, and pyroglutamate might be good indicators for increased oxidative stress and could be considered potential biomarkers of progression of AKI to chronic kidney disease.

Acylcarnitines (including acetyl carnitine and other three medium-chain carnitines) were also significantly increased in the serum from AKI patients. Fatty acids transported into renal epithelial cells are used for energy production through fatty acid β-oxidation primarily in mitochondria and peroxisomes. Fatty acyl groups are transported through the carnitine carrier system to cross the membrane into mitochondria for the reaction. During acute kidney injury, there is a significantly reduced expression and activity of mitochondrial and peroxisomal fatty acid oxidation enzymes in kidney tissue [36,37]. This is the first LC/MS-based metabolomics study to show marked elevations in serum acylcarnitines concentrations in hospitalized patients with newly diagnosed AKI. A previous study using a radiochemical enzymatic assay had shown that total carnitine, free carnitine, short-chain and long-chain acyl-carnitine concentrations were also increased in plasma samples obtained from patients with acute kidney injury when compared with healthy subjects [38]. In addition, another study [39] demonstrated increased levels of serum acylcarnitines in patients with CKD as well as in hemodialysis (HD) patients. In that study, free serum carnitine levels were not reduced in CKD patients, but were significantly reduced in HD patients. Altogether these results support the notion of a reduced activity of fatty acid oxidation enzymes during acute kidney injury, a metabolic abnormality previously described in kidney tissue obtained from various animal models of acute kidney injury [13–15]. These lipid abnormalities could explain the increased mortality associated with the development of acute kidney injury in hospitalized patients.

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This pilot study had limitations since it was a small single center study of adult patients who developed AKI in a hospital setting. The results will need to be further validated in a larger population, including adults with confounding variables and co-morbid conditions that normally accumulate with increasing age. Nevertheless, the results illustrate how a rapid serum profiling method successfully employed in AKI patients can yield useful novel information about serum biomarkers of AKI.

5. Conclusion

Metabolomics data detected increases in blood levels of homocysteine, ADMA and pyroglutamate, which are commonly recognized as major risk factors for having both increased incidence of cardiovascular and kidney disease. Furthermore, metabolomic analysis has discovered acylcarnitines as potential biomarkers of AKI. In summary, this study demonstrated that metabolomics is a powerful tool that can provide potentially useful medical information and has a high potential to identify new mechanistic insights that can predict outcome once AKI has developed.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.02.042.

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