Metabolomic Identification of Predictive and Early Biomarkers of Cisplatin-induced Acute Kidney Injury in Adult Head and Neck Cancer Patients

Yong Jin Lim[1], Steven Xiu[1], M Kuruvilla[1], Eric Winquist[1], Stephen Welch[1], Morgan Black[1], Lauren Faught[1], Jasmine Lee[2], Michael (Guest Editor) Rieder[3], Tom Blydt-Hansen[4], Michael Zappitelli[2], and Brad Urquhart[1]

[1]Western University Schulich School of Medicine & Dentistry
[2]SickKids Research Institute
[3]University of Western Ontario
[4]British Columbia Children's Hospital

September 14, 2022

Abstract

Aim: Cisplatin causes acute kidney injury (AKI) in approximately one-third of patients. Serum creatinine and urinary output are poor markers of cisplatin-induced (AKI). Metabolomics was utilized to identify predictive or early diagnostic biomarkers of cisplatin-induced AKI. Methods: Thirty-one adult head and neck cancer patients receiving cisplatin (dose [?] 70 mg m2 -1) were recruited for metabolomics analysis. Urine and serum samples were collected prior to cisplatin (pre), 24-48 hours after cisplatin (24-48h), and 5-14 days (post) after cisplatin. Based on serum creatinine concentrations measured at the post timepoint, 11/31 patients were classified with clinical AKI. Untargeted metabolomics was performed using liquid chromatography-mass spectrometry. Results: Metabolic discrimination was observed between "AKI" patients and "no AKI" patients at all timepoints. Urinary glycine, hippuric acid sulfate, 3-hydroxydecanedioc acid, and suberate were significantly different between AKI patients and no AKI patients prior to cisplatin infusion. Urinary glycine and hippuric acid sulfate were lower (-2.22-fold and -8.85-fold), whereas 3-hydroxydecanedioc acid and suberate were higher (3.62-fold and 1.91-fold) in AKI patients relative to no AKI patients. Several urine and serum metabolites were found to be altered 24-48 hours following cisplatin infusion, particularly metabolites involved with mitochondrial energetics. Conclusion: We propose glycine, hippuric acid sulfate, 3-hydroxydecanedioc acid, and suberate as predictive biomarkers of predisposition to cisplatin-induced AKI. Metabolites indicative of mitochondrial dysfunction may serve as early markers of subclinical AKI.

TITLE: Metabolomic Identification of Predictive and Early Biomarkers of Cisplatin-induced Acute Kidney Injury in Adult Head and Neck Cancer Patients

RUNNING TITLE: Metabolomics of cisplatin-induced AKI

AUTHORS

Yong Jin Lim[1](ylim33@uwo.ca)

Steven G. Xiu[1](sxiu3@uwo.ca)

M. Sara Kuruvilla[2](sara.kuruvilla@lhsc.on.ca)

Eric Winquist[2](eric.winquist@lhsc.on.ca)

Stephen Welch[2](stephen.welch@lhsc.on.ca)

Morgan Black[2](morgan.black@lhsc.on.ca)

Lauren N. Faught[1](lauren.faught@icloud.com)

Jasmine Lee[3] (jasmine.lee@sickkids.ca)

Michael J. Rieder[1,4,5](mrieder@uwo.ca)

Tom D. Blydt-Hansen[6](tom.blydthansen@cw.bc.ca)

Michael Zappitelli[3](michael.zappitelli@sickkids.ca)

Bradley L. Urquhart[1,7](brad.urquhart@schulich.uwo.ca)

AFFILIATIONS

[1]Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, Western University, London, ON, Canada

[2]Division of Medical Oncology, Department of Oncology, Schulich School of Medicine and Dentistry, Western University, London, ON, Canada

[3]Division of Nephrology, Department of Pediatrics, Hospital for Sick Children, Toronto, ON, Canada

[4]Department of Paediatrics, Schulich School of Medicine and Dentistry, Western University, London, ON, Canada,

[5]Division of Clinical Pharmacology, Department of Medicine, Schulich School of Medicine and Dentistry, Western University, Canada

[6]Division of Nephrology, Department of Pediatrics, British Columbia Children's Hospital, Vancouver, BC, Canada

[7]Division of Nephrology, Department of Medicine, Schulich School of Medicine & Dentistry, Western University, London, ON, Canada

The authors confirm that Bradley L. Urquhart is the PI for this paper. M. Sara Kuruvilla and Eric Winquist had direct clinical responsibility for patients in this study.

CORRESPONDENCE

Bradley L. Urquhart, PhD

1151 Richmond Street

Medical Sciences Building Room 216

London, ON, N6A 5C1

E-mail: Brad.Urquhart@schulich.uwo.ca

Phone: (519) 661-3756

Fax: (519) 661-3827

KEYWORDS: Metabolomics, biomarker, cisplatin, acute kidney injury

Word count : 3985 (Introduction, Methods, Results, Discussion)

Table count : 2

Figure count : 6

What is already known about this subject:

Nephrotoxicity is a dose-limiting complication of cisplatin treatment, resulting in acute kidney injury (AKI) in approximately a third of patients receiving cisplatin

Serum creatinine and urinary output are poor markers of kidney damage, only detectable after severe functional impairment of kidneys

What this study adds:

- Patients who develop clinical AKI following cisplatin infusion exhibit metabolic differences compared to patients who do not
- We reveal glycine, hippuric acid sulfate, 3-hydroxydecanedioc acid, and suberate as potential predictive markers of predisposition to cisplatin-induced AKI
- Identification of several urine and serum metabolites exhibiting early alterations after cisplatin infusion are potential markers of subclinical nephrotoxicity

ABSTRACT

Aim: Cisplatin causes acute kidney injury (AKI) in approximately one-third of patients. Serum creatinine and urinary output are poor markers of cisplatin-induced (AKI). Metabolomics was utilized to identify predictive or early diagnostic biomarkers of cisplatin-induced AKI.

Methods: Thirty-one adult head and neck cancer patients receiving cisplatin (dose [?] 70 mg $m^{2\ -1}$) were recruited for metabolomics analysis. Urine and serum samples were collected prior to cisplatin (pre), 24-48 hours after cisplatin (24-48h), and 5-14 days (post) after cisplatin. Based on serum creatinine concentrations measured at the post timepoint, 11/31 patients were classified with clinical AKI. Untargeted metabolomics was performed using liquid chromatography-mass spectrometry.

Results: Metabolic discrimination was observed between "AKI" patients and "no AKI" patients at all timepoints. Urinary glycine, hippuric acid sulfate, 3-hydroxydecanedioc acid, and suberate were significantly different between AKI patients and no AKI patients prior to cisplatin infusion. Urinary glycine and hippuric acid sulfate were lower (-2.22-fold and -8.85-fold), whereas 3-hydroxydecanedioc acid and suberate were higher (3.62-fold and 1.91-fold) in AKI patients relative to no AKI patients. Several urine and serum metabolites were found to be altered 24-48 hours following cisplatin infusion, particularly metabolites involved with mitochondrial energetics.

Conclusion: We propose glycine, hippuric acid sulfate, 3-hydroxydecanedioc acid, and suberate as predictive biomarkers of predisposition to cisplatin-induced AKI. Metabolites indicative of mitochondrial dysfunction may serve as early markers of subclinical AKI.

1. INTRODUCTION

Cisplatin is an effective chemotherapeutic agent widely used for the treatment of a variety of malignancies, including head and neck, testicular, ovarian, cervical, and bladder cancers. Cisplatin is primarily eliminated by the kidneys through tubular secretion and glomerular filtration, and consequently accumulates in the kidneys to cause kidney injury. Cisplatin-induced nephrotoxicity presents as acute kidney injury (AKI) in approximately one-third of patients receiving cisplatin. AKI is characterized as a rapid decline in kidney function and has been associated with increased risk for chronic kidney disease, major cardiovascular events, and mortality. Clinical diagnosis of AKI is based on increases in serum creatinine (SCr) concentrations or a decrease in urine output. However, serum creatinine and decreased urine output are markers of functional impairment, only manifesting after significant kidney injury and impairment of glomerular filtration. Biomarkers for earlier detection or prediction of cisplatin-induced nephrotoxicity are needed to guide cisplatin therapy, improve AKI prognosis, and allow for development of nephroprotective interventions.

Novel markers for the early detection of AKI are currently under investigation, including neutrophil gelatinase-associated lipocalin, kidney injury molecule-1, cystatin C, tissue inhibitor of metalloproteinase 2, and insulin-like growth factor binding protein 7. However, these markers are not necessarily specific to

AKI, do not allow for discrimination of AKI etiology, and do not predict a patient's predisposition to developing cisplatin-induced nephrotoxicity. There is consensus that a combination of kidney function or damage markers should be utilized to not only diagnose AKI, but to also discriminate AKI etiology, assess severity, and evaluate the prognosis of AKI.

In this study, we utilized untargeted metabolomics to analyze urine and serum samples from a cohort of adult head and neck cancer patients. We aimed to identify both early diagnostic markers of cisplatin-induced AKI, as well as predictive markers of patient predisposition to cisplatin-induced AKI. Although untargeted metabolomics has been used in rodent models of cisplatin-induced AKI, to our knowledge, our study is the first to use untargeted metabolomics in a cohort of patients receiving cisplatin therapy.

2. METHODS

2.1 Study participants and sample collection

Participants were recruited as part of a pilot for the ACCENT (**A C** anadian study of **C** isplatin m**E** tabolomics and **N** ephro**T** oxicity) study, an ongoing Canada-wide, multi-center initiative to identify serum and urine metabolites for the prediction or early diagnosis of cisplatin-induced nephrotoxicity. The ACCENT study is being conducted in accordance with the Medical Association Declaration of Helsinki. After receiving written informed consent, 31 adult patients were recruited at the London Regional Cancer Program (Victoria Hospital, London, ON, Canada) between 2018-2020. Ethics approval for this study was obtained from the Health Sciences Research Ethics Board at the University of Western Ontario. The cohort included adult patients (>18 years of age) initiating cisplatin treatment (dose [?] 70 mg $m^{2\ -1}$) for head and neck cancer. Exclusion criteria were chronic kidney disease (GFR $<$ 60 ml min^{-1}) at baseline, previous exposure to cisplatin/other nephrotoxic drugs in the 2 weeks leading up to cisplatin treatment, radiotherapy within 1 month prior to the study, or previous hematopoietic stem cell transplant. Patient demographic information (**Table 1**) was recorded upon enrollment. Urine and blood samples were collected from patients prior to ("pre"), 24–48 hours after ("24-48h"), and 5-14 days following ("post") each cisplatin infusion, and laboratory results/patient data were collected for each sample collection timepoint. Samples were stored at -80°C prior to analysis. A detailed outline of the ACCENT study has been previously published.

2.2 AKI classification

Patients were classified as "no AKI" or "AKI" based on the Kidney Disease Improving Global Outcomes (KDIGO) guidelines, which defines AKI as a [?] 1.5 times increase in serum creatinine (SCr) versus baseline or as an increase in SCr [?] 26.5 μmol L^{-1}. Serum creatinine concentrations measured at the post timepoint were compared to those measured at the pre timepoint to classify AKI.

2.3 Sample preparation for untargeted metabolomics

For untargeted metabolomics analysis, serum and urine samples were thawed on ice. Ice-cold acetonitrile (ACN) containing internal standards chlorpropamide (5 μM), atenolol-d7 (1.8 μM), was used for protein precipitation of urine and serum samples as described previously.

2.4 Phenylisothiocyanate and 3-nitrophenylhydrazine derivatization of serum and urine samples

Derivatization was employed to increase the coverage of the metabolites captured by metabolomics analysis. Phenylisothiocyanate (PITC) was used to derivatize amino acids, amino acid derivatives, biogenic amines, glucose/hexose, lipids, and acylcarnitines, whereas 3-nitrophenylhydrazine (3-NPH) was used to derivatize keto- and carboxyl-containing compounds. The protocols for PITC and 3-NPH derivatization were adapted from previously published work by Zheng et. al.

2.5 Chromatography and mass spectrometry

Metabolites were separated by reverse-phase liquid chromatography using a Waters ACQUITY UPLC HSS T3 column (1.8 μm particle size, 2.1 mm x 100 mm) and subsequent by time-of-flight mass spectrometry on

a Waters Xevo-G2S QTof mass spectrometer. Parameters for liquid chromatography and mass spectrometer sample acquisition can be found in the supplementary information (**Table S1, S2**). LC-MS sample injection order was randomized, and pooled samples were injected every six sample injections.

2.6 Data Processing

Waters raw data files generated from the metabolomics analysis were converted to mzData files using the convert.waters.raw R package. Using the isotopologue parameter optimization (IPO) R package, the quality control pooled sample injections were used to find the optimal peak processing parameters, retention time corrections and grouping parameters. Parameters generated from IPO were used for XCMS processing of metabolomics data. The CAMERA package was applied to XCMS processed features to annotate possible isotopes and adducts. The resulting data was subsequently normalized to internal standards, and features with >30% relative standard deviation (rsd) within quality control injections were excluded from analysis. Urine features were normalized to their corresponding urinary creatinine and internal standard signals, to account for differences in urine concentration. Using the CAMERA package, features were grouped based on Pearson correlation coefficients and retention time into "pcgroups". Within each pcgroup, only the feature with the highest mean raw intensity was kept for further data analysis. Duplicate features found in both the untargeted and derivatized experiments were removed from the derivatized dataset before analysis. The raw intensity values of all features were log transformed using MetaboAnalyst 5.0, to remove heteroscedasticity and correct for skewed data distribution. Any 0 values during log transformation were treated as 1/5 of the minimum intensity values of each feature. Log transformed feature intensity values were used for all analyses unless stated otherwise.

2.7 Statistical Analysis

The EZInfo 3.0 software (Umetrics, Umeå, Sweden) was used to perform multivariate analysis on the metabolomics dataset. Data was centered, pareto scaled and subsequently analyzed by principal component analysis (PCA), an unsupervised approach to visualize the metabolic differences between no AKI and AKI patients at each timepoint. Orthogonal partial least squares discriminant analysis (OPLS-DA), a supervised discriminatory analysis, was used for the pairwise discrimination of no AKI and AKI patients at each timepoint. For each OPLS-DA, metabolites were ranked by their variable importance in projection (VIP) values and features with VIP values [?] 1 were considered to have discriminatory value in discriminating between no AKI and AKI. This VIP filtering was repeated until OPLS-DA model statistics (R2 and Q2 values) were maximized to select for the most important features to annotate. The final optimized OPLS-DA model was used to generate a list of features to identify, using a VIP value threshold of [?] 1 and correlation (p(corr)) values less than -0.4 and greater than 0.4.

Features determined to have discriminatory value were analyzed by two-way ANOVA with Benjamini-Hochberg false discovery rate (FDR) correction. Individual features that were found to be significantly different by AKI classification following two-way ANOVA and FDR correction were further analyzed by pairwise t-tests comparing no AKI and AKI patients at each timepoint, with p-values adjusted for multiple comparisons using Bonferroni correction. To find metabolites altered over time, serum and urine features were analyzed by one-way ANOVA with FDR correction, followed by Tukey's test for metabolites significant by one-way ANOVA after FDR correction. $p<0.05$ was considered as significantly significant for all univariate data analysis.

Receiver operating characteristic (ROC) curves were generated and area under the ROC (AUROC) values were calculated using MetaboAnalyst 5.0.

2.8 Putative Metabolite Annotation and Identification

The m/z and fragmentation spectra of features were cross referenced with online metabolite databases Human Metabolome Database (HMDB), METLIN, or MassBank for putative metabolite annotation. Analytical standards were purchased where available and analyzed in parallel with biological samples to achieve a level 1 identification based on the Chemical Analysis Working Group (CAWG) guidelines on metabolomics

reporting standards.

Derivatized features were identified by cross-referencing m/z and retention time with a panel of derivatized metabolites previously generated in-house by the derivatization of the mass spectrometry library of standards (MSMLS) kit, purchased from IROA technologies (Sea Girt, NJ, USA).

3. RESULTS

3.1 Patient demographics

Thirty-one patients were recruited with the majority male (29/31) and all patients were Caucasian. Twenty patients (64.5%) were classified as no AKI and 11 patients (35.5%) were classified as AKI based on serum creatinine concentrations measured at the post timepoint (**Table 1**). Baseline demographics including age, BMI, baseline SCr/eGFR, and cigarette packs/day were nearly identical between no AKI and AKI patients (**Table 1**). Out of the 11 patients in the AKI group, seven patients had stage 2 AKI (2.0-2.9 times increase in SCr compared to baseline) and 4 patients had stage 1 AKI (1.5-1.9 times increase in SCr compared to baseline).

3.2 Metabolomic profiling of no AKI vs. AKI patients

Following XCMS processing of chromatographic data collected from untargeted metabolomics, exclusion of features with high variability in pooled quality control samples, and removal of potential adducts/isotopes, 758 serum features and 484 urine features were included for subsequent multivariate analysis. The derivatized metabolomics dataset was similarly processed, without the removal of potential adducts or isotopes. Additionally, any duplicate features detected in both the derivatized and untargeted datasets were removed. Ultimately, a total of 975 serum features and 2355 urine features remained after processing of derivatized data. After cross referencing the derivatized features with a library of 263 derivatized small molecule standards, 35 serum and 117 urine features were matched bym/z and retention time with the library of standards.

To select the features most important in discriminating between no AKI and AKI patients at each timepoint, OPLS-DA models were sequentially generated, each time excluding features with VIP values < 1. This sequential exclusion of features was repeated until model statistics of OPLS-DA models were maximized. With the remaining features, PCA score plots were generated to visualize the metabolic differences between no AKI and AKI patients at each timepoint. Score plots of urine samples showed moderate separation at the pre (**Figure 1A**) and 24-48h timepoints (**Figure 1B**), and clear separation at the post timepoint (**Figure 1C**). In serum, strong separation was observed in both the pre (**Figure 2A**) and post (**Figure 2C**) timepoints, with moderate separation observed at the 24-48h timepoint (**Figure 2B**). Corresponding OPLS-DA models comparing no AKI and AKI patients at each timepoint mirrored the visual separation observed in the PCA score plots, with a high degree of fit (R2Y) and predictive ability (Q2Y) at the post timepoint for both urine and serum (**Figure 1F, 2F**), as well as the pre timepoint for serum (**Figure 2D**). Moderate model statistics were observed for the 24-48h timepoint for both urine and serum (**Figure 1E, 2E**), as well as the pre timepoint for urine (**Figure 1D**). Features with $0.4 < \text{p(corr)} < -0.4$ and VIP > 1 in the OPLS-DA models were considered as important discriminators of AKI and were thus followed up for identification. Identified metabolites that were significantly different between no AKI and AKI groups by two-way ANOVA are summarized in **Table 2** .

3.3 Predictive and early urinary markers of cisplatin-induced acute kidney injury

In urine, glycine, 3-hydroxydecanedioc acid, hippuric acid sulfate, and suberate were found to be significantly different between no AKI and AKI patients prior to cisplatin infusion (**Figure 3**). Levels of hippuric acid sulfate were 8.85-fold and 9.08-fold lower in the AKI group compared to the no AKI group at the pre and 24-48h timepoints, respectively (**Table 2** , **Figure 3C, 3E**). 3-hydroxydecanedioc acid and suberate were significantly higher in AKI patients compared to no AKI patients at the pre timepoint (3.62-fold and 1.91-fold, respectively) and trended towards being higher at the 24-48h timepoint (1.98-fold and 1.82-fold, respectively), though the difference was not significant (**Table 2** , **Figure 3B, 3D**). Finally, glycine levels were 2.22-fold and 2.55-fold lower in AKI patients relative to no AKI patients at the pre and post timepoints,

respectively (**Table 2, Figure 3A**). Diagnostic performance of the markers was assessed by calculation of AUROC. AUROC values [?] 0.7 are generally considered to be acceptable discrimination. Though serum creatinine was an excellent discriminatory marker of no AKI vs. AKI at the post timepoint (AUROC of 0.947), it performed poorly (AUROC < 0.7) at the pre and 24-48h timepoints (**Figure 3F**). Glycine, 3-hydroxydecanedioc acid, hippuric acid sulfate, and suberate all had AUROC > 0.7 at the pre timepoint, with suberate exhibiting the strongest performance (AUROC > 0.8). These metabolites, with the exception of glycine, also had AUROC > 0.7 at the 24-48h timepoint, with hippuric acid sulfate and suberate possessing AUROC > 0.8.

3.4 Late urinary markers of cisplatin-induced acute kidney injury

Aminoisobutanoate (—4.69-fold), betaine (—2.67-fold), glutamine (—1.98-fold), glycolate (—2.71-fold), histidine (—2.63-fold), indole-3-acetate (—4.03-fold), serine (—2.80-fold), taurine (—2.56-fold), threonine (—2.90), and tyramine (—2.87-fold) were all significantly lower at the post timepoint in the AKI group relative to the no AKI group (**Table 2** , **Figure 4**). With the exception of aminoisobutanoate, all of the aforementioned metabolites exhibited AUROC [?] 0.8 at the post timepoint, with AUROC of glycolate and tyramine being greater than 0.9 (**Figure 4H).** Though not statistically significant, betaine, indole-3-acetate, and threonine trended lower in AKI patients compared to no AKI patients at the pre timepoint with AUROC values [?] 0.7 (**Figure 4B, 4F, 4I**). Similarly, urine levels of aminoisobutanoate, indole-3-acetate, and tyramine trended lower in AKI patients at the 24-48h timepoint with AUROC [?] 0.7, though the difference was not statistically significant (**Figure 4A, 4F, 4J**).

3.5 Late serum markers of cisplatin-induced acute kidney injury

Serum metabolites that were significantly different between AKI and no AKI were predominantly elevated in the AKI group (**Figure 5**), with the exception of glutamine, which was 1.55-fold lower in AKI patients at the post timepoint (**Table 2** , **Figure 5B**). Serum levels of creatinine (—1.81-fold), linolenic acid (—1.97-fold), octenoylcarnitine (—2.09-fold), octanoylcarnitine (—2.46-fold), indoxyl sulfate (—2.87-fold), and N,N,N-trimethyl-L-alanyl-L-proline betaine (TMAP, —1.87-fold) were significantly higher in AKI patients at the post timepoint compared to no AKI patients (**Table 2, Figure 5A, 5C-5G**). AUROC values for all serum metabolites at the post timepoint were greater than 0.7, with octenoylcarnitine and TMAP having AUROC > 0.8, and creatinine, octanoylcarnitine, and indoxyl sulfate AUROC values > 0.9 (**Figure 5H**). Additionally, the AUROC values of ocanoylcarnitine at the pre timepoint and indoxyl sulfate and TMAP at the 24-48h timepoint were greater than 0.7, though the differences were not statistically significant (**Figure 5H**).

3.6 Alterations of urine and serum metabolites over time in patients without clinical acute kidney injury

Urine and serum samples from the no AKI group were analyzed via one-way ANOVA to investigate the effects of cisplatin treatment independent of AKI. Following cisplatin infusion, metabolites primarily followed one of two trends: 1) an initial increase or decrease 24-48 hours after cisplatin, and a subsequent return to baseline at the post timepoint; 2) an initial increase or decrease at the 24-48h timepoint that was sustained at the post timepoint. In urine, L-acetylcarnitine (—3.64-fold), L-carnitine (—8.91-fold), 3-hydroxydecanedioc acid (—2.42-fold), malate (—3.56-fold), pyruvate (—4.87-fold), and valerylcarnitine (—4.76-fold), followed the first trend, where metabolite levels were significantly increased 24-48h following cisplatin treatment and returned to baseline levels by the post timepoint (**Figure 6A, 6B, 6E, 6F, 6H**). Serum levels of L-arginine (—1.96-fold), L-carnitine (—1.40-fold), proline (—1.63-fold), TMAP (—4.14-fold), and valerylcarnitine (—1.79-fold) followed the same pattern, with a significant increase at 24-48h, and a return to baseline at the post timepoint (**Figure 6I, 6K, 6N, 6O, 6P**). Conversely, levels of urine indole-3-acetate (—1.82-fold) and serum cortisol (—5.18-fold) were significantly lower 24-48h following cisplatin treatment, with a subsequent return to baseline by the post timepoint (**Figure 6D, 6J**). Urinary succinate and serum 4-hydroxycinammic acid and phenylalanine followed the second trend, where metabolite levels were significantly altered 24-48h following cisplatin infusion (—1.88-fold for succinate; —1.56-fold and —1.43-fold for

4-hydroxycinammic acid and phenylalanine, respectively), with the alterations being sustained at the post timepoint (**Figure 6G, 6L, 6M**). Similar metabolite alterations were observed over time for patients in the AKI group (**Figure S1**).

4. DISCUSSION

Untargeted metabolomics was employed in urine and serum samples of adult head and neck cancer patients receiving cisplatin for the identification of predictive or early biomarkers of cisplatin-induced AKI. This allowed for the identification of important metabolites that are early or predictive biomarkers of cisplatin AKI. Future metabolomics studies in larger adult and pediatric cohorts recruited as part of the ACCENT study will employ fully quantitative metabolite analysis. Metabolomics has been used to investigate cisplatin-induced acute kidney injury in the past but has predominantly been utilized in rodent models. To our knowledge, this study is the first metabolomic investigation of cisplatin-induced AKI in human patients, providing insight into the metabolic differences present between patients who present with clinical AKI upon cisplatin infusion and those who do not, in addition to highlighting the early metabolic alterations induced by cisplatin.

Four urinary biomarkers were identified as predictive markers of clinical AKI: glycine, 3-hydroxydecanedioc acid, hippuric acid sulfate, and suberate. All four metabolites were significantly different between the no AKI and AKI groups at the pre timepoint with fold changes of -2.2-fold, 3.62-fold, 8.85-fold, and 1.91-fold, respectively, in AKI patients relative to no AKI patients (**Table 2**).

Glycine is an amino acid component of the potent antioxidant molecule glutathione and has been associated with beneficial effects in reducing oxidative stress. Alterations in glycine levels have been observed previously in a mouse model of ischemia-reperfusion AKI, where glycine levels were decreased in kidney and heart tissues following ischemic AKI. A metabolomic investigation of urine samples from combat casualties also revealed that lower levels of glycine were associated with need for renal replacement therapy, and glycine levels were higher in patients with moderate to severe AKI compared to mild AKI. In both cases, it was suggested that decreases in glycine levels were associated with upregulation of glutathione production under oxidative stress. Furthermore, glycine was shown to protect against cisplatin nephrotoxicity and ischemia reperfusion renal injury *in vivo* when administered to rats before cisplatin treatment or ischemic insult. Cisplatin is well documented to cause mitochondrial dysfunction and oxidative stress, and availability of glycine may be an important factor in antioxidant defense against cisplatin-induced oxidative stress.

3-hydroxydecanedioc acid and suberate are dicarboxylic acids that have been associated with fatty acid β-oxidation disorders. Increased urinary excretion of 3-hydroxydecanedioc acid and suberate have been used to diagnose medium-chain acyl-CoA dehydrogenase deficiency (MCAD) and indicates a block in fatty acid oxidation. Dysfunctional mitochondrial fatty acid oxidation is believed to be a crucial mechanism in cisplatin-induced AKI. Cisplatin has previously been shown to inhibit mitochondrial fatty acid β-oxidation by deactivating PPAR-α, a crucial nuclear receptor in the regulation β-oxidation. An accumulation of intracellular acyl-CoAs due to disorders of fatty acid β-oxidation is associated with lipotoxicity and detrimental to mitochondrial function. Additionally, serum levels of acylcarnitines octanoylcarnitine and octenoylcarnitine were significantly higher at the post timepoint in AKI patients relative to the no AKI group and showed increased trends in the pre and 24-48h timepoints, though the differences were not significant (**Figure 5D, 5E**). Elevation of serum acylcarnitines is also a marker of dysfunction in fatty acid β-oxidation. Taken together, the elevation of urinary 3-hydroxydecanedioc acid, urinary suberate, and serum acylcarnitines in AKI patients suggest a lower capacity for fatty acid oxidation in patients who develop clinical AKI following cisplatin therapy. An underlying diminished capability for fatty acid oxidation may leave these patients more susceptible towards cisplatin-induced mitochondrial dysfunction and accumulation of toxic lipid compounds.

Hippuric acid sulfate was identified to potentially be both a predictive and early diagnostic marker of cisplatin-induced AKI. Hippuric acid sulfate is not well studied, and very few articles have been published regarding this metabolite. Hippuric acid sulfate is a sulfated derivative of hippuric acid, a uremic toxin that accumulates in chronic kidney disease (CKD). Hippuric acid is derived from the conversion of dietary

polyphenols into benzoic acid by the gut microbiome, followed by conjugation with glycine by hepatic or renal glycine-N-acyltransferase. Though hippuric acid has been implicated in both CKD and AKI, hippuric acid sulfate has yet to be implicated with kidney disease.

Cisplatin is well known to be nephrotoxic, manifesting as AKI in approximately one third of patients. It is likely that patients who don't develop AKI are able to withstand the nephrotoxic insult mediated by cisplatin. To evaluate the metabolic response to cisplatin in patients that don't progress to AKI, we evaluated metabolic alterations in no AKI patients over the three timepoints of this study. This analysis revealed cisplatin induces early metabolic changes in both the urine and serum even in patients who don't progress to AKI (**Figure 6**). Many of the metabolites found to be altered at the 24-48h were intermediates of the citric acid cycle or associated with fatty acid oxidation, further emphasizing the central role of mitochondrial dysfunction in cisplatin-induced nephrotoxicity. Of special interest was TMAP, a dipeptide biomarker of reduced kidney function in CKD, which was elevated in both no AKI and AKI patients at the 24-48h timepoint but only remained elevated at the post timepoint in patients with clinical AKI (**Figure 5G**). These findings are in accordance with the concept of subclinical AKI induced by cisplatin whereby there is an increase in AKI biomarkers without presentation of clinical AKI. In other words, subclinical AKI is kidney damage without substantial loss of function. Though there has been some work highlighting potential prognostic benefits of using markers of subclinical AKI, the clinical relevance of subclinical AKI is unclear. Further investigation of these early subclinical markers of AKI may provide further insight into the mechanisms of cisplatin nephrotoxicity.

One strength of our study was the high degree of similarity in baseline patient demographics such as age, BMI, ethnicity, and baseline SCr/eGFR between the AKI and no AKI groups, minimizing interindividual variability that could potentially confound metabolic profiling (**Table 1**). Furthermore, the collection of three separate timepoints allowed for comprehensive metabolic profiling of patients prior to and shortly after cisplatin infusion, as well as upon establishment of clinical AKI (or lack thereof).

There were some limitations to this study. Firstly, the sample size for our study was relatively small, ranging from 11 in the AKI group and 20 in the no AKI group. Despite this small sample size, a number of key metabolic alterations were characterized. Future metabolomics studies with larger discovery cohorts may help extract more distinct and robust differences between AKI and no AKI patients. Furthermore, the incidence of head and neck cancer is 2-4-fold higher in men compared to women, and this disparity was reflected in our cohort. As only 2 out of 31 patients were female, sex differences could not be investigated in our analysis. Similarly, all 31 patients in this study were Caucasian which limits the generalizability of our findings to other ethnicities.

Though serum creatinine remains the principal biomarker in AKI diagnosis, cisplatin nephrotoxicity occurs prior to the detection of elevated serum creatinine. Accordingly, there is a need for biomarkers capable of early diagnosis of AKI or prediction of AKI onset prior to cisplatin therapy. In this study, we identified glycine, 3-hydroxydecanedioc acid, hippuric acid sulfate, and suberate as potential predictive markers of clinical cisplatin-induced AKI. Additionally, we provided insight into early metabolic alterations following cisplatin infusion. Further investigations are necessary to validate the applicability and clinical utility of these proposed biomarkers. Future metabolomics studies are planned in large discovery and validation cohorts to further investigate the metabolic effects of cisplatin and elucidate the underlying metabolic differences between patients who present with clinical AKI and patients who do not.

AUTHOR CONTRIBUTIONS

B.L.U., M.Z., and T.D.B. designed the study. S.K., E.W., S.W., M.B., J.L., M.J.R. and L.N.F. were responsible for research coordination and sample collection. Y.J.L. and S.G.X. performed the research and data analysis. Y.J.L. drafted the manuscript. B.L.U. edited the manuscript for final submission. All authors have reviewed and approved the submitted manuscript.

ACKNOWLEDGEMENTS

The authors are grateful to the support from the nurses and staff at the London Regional Cancer Program for support of this study.

FUNDING INFORMATION

This study was funded by a project grant from the Canadian Institutes of Health Research and an Accelerator grant from Western University.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY

Data collected in this study are available upon request from the corresponding author Bradley L. Urquhart.

ORCID

Bradley L. Urquhart: 0000-0001-8324-1472

REFERENCES

TABLES

Table 1. Baseline demographics of study population. Patient serum creatinine (SCr) levels at the post timepoint were used to classify patients as no AKI (n=20) or AKI (n=11) according to KDIGO guidelines. Patient sex is presented as a ratio of males to females (M/F). Ethnicity is given as a ratio of Caucasian, Black, Asian, Indigenous, and Other (C/B/A/I/O). Demographic characteristics of age, body mass index (BMI), baseline SCr, baseline eGFR, and smoking are presented as mean ± standard deviation. Statistical differences between no AKI and AKI groups were determined using unpaired t-tests for each characteristic, with *p<0.05. Groups were not statistically different from each other for any of the baseline characteristics.

	Sex (M/F)	Age (years)	Body mass index (kg/m^2)	Ethnicity (C/B/A/I/O)	Baseline SCr (μmol/L)	Baseline eGFR (mL/min/1.73m^2)	Smoking (packs/day)
No AKI (n=20)	19/2	59.5 (7.1)	26.7 (5.2)	21/0/0/0/0	69.2 (13.4)	101.9 (9.0)	0.8 (0.6)
AKI (n=11)	10/0	61.7 (3.8)	26.7 (6.0)	10/0/0/0/0	63.2 (10.4)	104.3 (6.8)	0.7 (0.6)

Table 2. Summary of metabolite alterations in AKI patients compared to no AKI patients. Fold change was calculated using mean raw intensity values for each metabolite. All observed *m/z* values were <5 ppm mass error relative to the theoretical *m/z* .

Metabolite Identity	ID Level	Chemical Formula	Observed m/z	t_R (min)	Sample Type	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Cha in AKI Patien vs. No AKI Patien
						Pre	Pre	Pre	24-48 hours	24-48 hours	24-48 hours	Post	Post
						Direction	Fold	p	Direction	Fold	p	Direction	Fold
Aminoisobutanoate (NPH)		C4H9NO2 + C6H5N3O [M-H]	237.0987	1.44	Serum	-	-	-	-	-	-	-	-
					Urine	—	2.08	0.7735	—	3.58	0.1717	—	4.69
Betaine	1	C5H11NO2 [M+H]	118.0863	0.62	Serum	—	1.08	1.0000	—	1.22	0.1569	—	1.12
					Urine	—	1.21	0.5552	—	1.79	0.1653	—	2.67
Creatinine	1	C4H7N3O [M+H]	114.0664	0.61	Serum	—	1.14	0.5215	—	1.16	0.4374	—	1.81
					Urine	-	-	-	-	-	-	-	-
Glutamine (fragment)	1	C5H7NO3 [M+H]	130.0499	0.56	Serum	—	1.32	0.4544	—	1.13	1.0000	—	1.55
					Urine	—	1.38	0.6298	—	1.17	0.3192	—	1.98
Glycine (PITC)	1	C2H5NO2 + C7H5NS [M+H]	211.0542	1.94	Serum	—	1.09	1	—	1.45	0.4264	—	1.51
					Urine	—	2.22	0.0412	—	1.99	0.1871	—	2.55
Glyocolate (NPH)	1	C2H4O3 + C6H5N3O [M-H]	210.0514	1.74	Serum	—	1.04	1.0000	—	1.23	0.7735	—	1.47
					Urine	—	1.59	0.0829	—	1.27	0.2142	—	2.71
Hippuric acid sulfate	2	C9H9NO6S [M-H]	258.0072	1.36	Serum	-	-	-	-	-	-	-	-
					Urine	—	8.85	0.0437	—	9.08	0.0071	—	1.82
Histidine	1	C6H9N3O2 [M-H]	154.0616	0.53	Serum	-	-	-	-	-	-	-	-
					Urine	—	1.44	0.2450	—	1.48	0.0418	—	2.63

Metabolite Identity	ID Level	Chemical Formula	Observed m/z	t_R (min)	Sample Type	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Cha in AKI Patien vs. No AKI Patien
3-Hydroxydecanedioic acid	1	C10H18O5 + C6H5N3O [M-H]	417.1075	1.76	Serum	-	-	-	-	-	-	-	-
					Urine	—	3.62	0.0305	—	1.98	0.0984	—	1.90
Indole-3-acetate (NPH)	1	C10H9NO2 + C6H5N3O [M-H]	309.0987	2.55	Serum	-	-	-	-	-	-	-	-
					Urine	—	1.77	0.0549	—	1.91	0.1226	—	4.03
Indoxyl sulfate	1	C8H7NO4S [M-H]	212.0017	1.87	Serum	—	1.16	1.0000	—	1.68	0.3071	—	2.87
					Urine	—	1.75	0.3584	—	1.54	0.5244	—	1.11
Linolenic acid	3	C18H30O2 [M+H]	279.2319	4.36	Serum	—	1.60	0.3398	—	1.40	1.0000	—	1.97
					Urine	-	-	-	-	-	-	-	-
L-Octanoylcarnitine	1	C15H29NO4 [M+Na]	310.2015	2.00	Serum	—	1.29	0.5105	—	1.14	0.5053	—	2.46
					Urine	—	1.31	0.2154	—	1.08	1.000	—	1.79
2-Octenoylcarnitine	2	C15H27NO4 [M+H]	286.2016	1.88	Serum	—	1.40	0.4940	—	1.18	0.6974	—	2.09
					Urine	—	1.10	0.9346	—	1.07	1.0000	—	2.47
Serine (PITC)	1	C3H7NO3 + C7H5NS [M+H]	241.0647	1.86	Serum	-	-	-	-	-	-	-	-
					Urine	—	1.39	0.2089	—	1.37	0.6135	—	2.80
Suberate (NPH)	1	C8H14O4 + C6H5N3O [M-H]	443.1676	2.59	Serum	-	-	-	-	-	-	-	-
					Urine	—	1.91	0.0395	—	1.82	0.0673	—	1.05
Taurine	1	C2H7NO3S [M-H]	124.006	0.57	Serum	-	-	-	-	-	-	-	-
					Urine	—	1.16	1.0000	—	1.07	0.5806	—	2.56

Metabolite Identity	ID Level	Chemical Formula	Observed m/z	t_R (min)	Sample Type	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Change in AKI Patients vs. No AKI Patients	Cha in AKI Pa- tien vs. No AKI Pa- tien
Threonine (PITC)	1	C4H9NO3 + C7H5NS [M+H]	255.0803	1.86	Serum	-	-	-	-	-	-	-	-
					Urine	—	1.03	0.2927	—	1.35	0.7733	—	2.90
TMAP	1	C11H20N2O3 [M+H]	229.1554	1.15	Serum	—	1.58	1.0000	—	1.30	0.1353	—	1.87
					Urine	—	1.23	0.9216	—	1.11	1.000	—	1.27
Tyramine (PITC)	1	C8H11NO + C7H5NS [M+H]	273.1061	2.52	Serum	-	-	-	-	-	-	-	-
					Urine	—	1.05	1.0000	—	1.45	0.0521	—	2.87

FIGURE LEGENDS

Figure 1 . Principle component analysis (A-C) and orthogonal parti al least squares discriminant analysis (D-F) scores plots comparing urine samples from no AKI (black) and AKI (red) patients at each timepoint: (A, D) pre, (B, E) 24-48h, and (C, F) post. Model statistics for OPLS-DA were as follows: (D) pre: R2Y = 0.784, Q2Y = 0.389 (E) 24-48h: R2Y = 0.752, Q2Y = 0.384 (F) post: R2Y = 0.969, Q2Y = 0.700. n=18-20 for no AKI patients and n=10-11 for AKI patients.

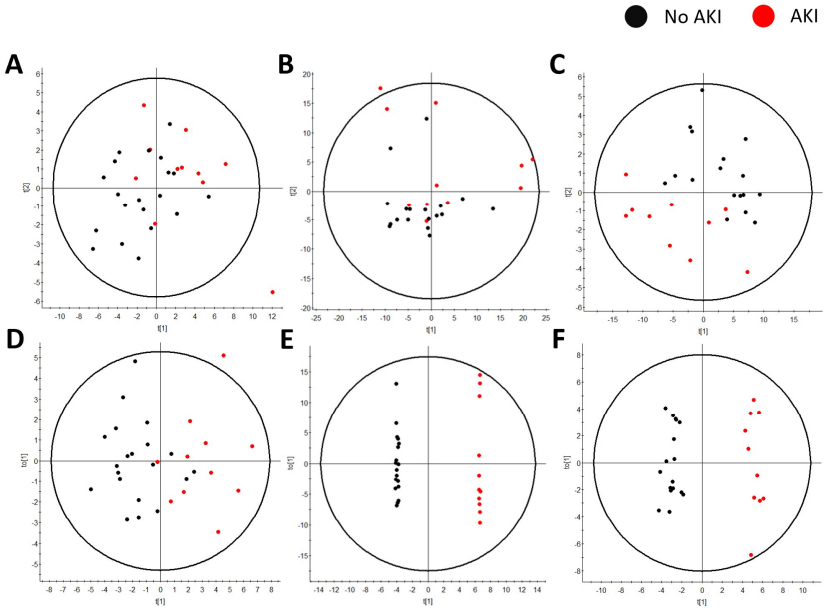
Figure 2. Principle component analysis (A-C) and orthogonal partial least squares discriminant analysis (D-F) scores plots comparing serum samples from no AKI (black) and AKI (red) patients at each timepoint: (A, D) pre, (B, E) 24-48h, and (C, F) post. Model statistics for OPLS-DA were as follows: (D) pre: R2Y = 0.991, Q2Y = 0.668 (E) 24-48h: R2Y = 0.776, Q2Y = 0.317 (F) post: R2Y = 0.995, Q2Y = 0.813. n=18-20 for no AKI patients and n=10-11 for AKI patients.

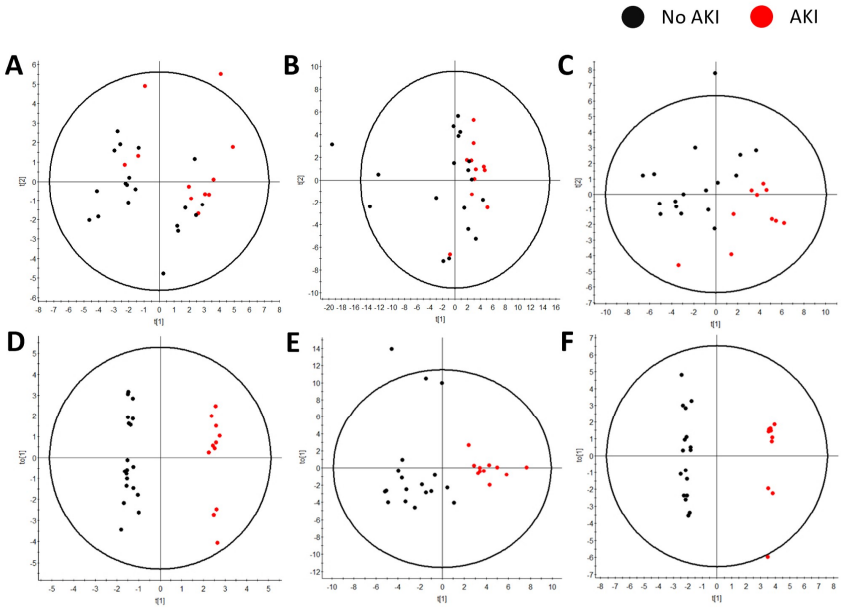
Figure 3 . (**A-D**) Log transformed relative intensity of urine features significantly different between no AKI and AKI patients at the pre timepoint. Data are presented as box and whisker plots, where boxes represent the median and interquartile range (IQR), and whiskers represent 1.5x IQR. **(F)** Diagnostic performance of urine metabolites as assessed by the area under the receiver operating curve. Statistical analysis was performed using two-way ANOVA, *p<0.05, **p<0.01 compared to no AKI patients at each timepoint. n=18-20 for no AKI patients and n=10-11 for AKI patients.

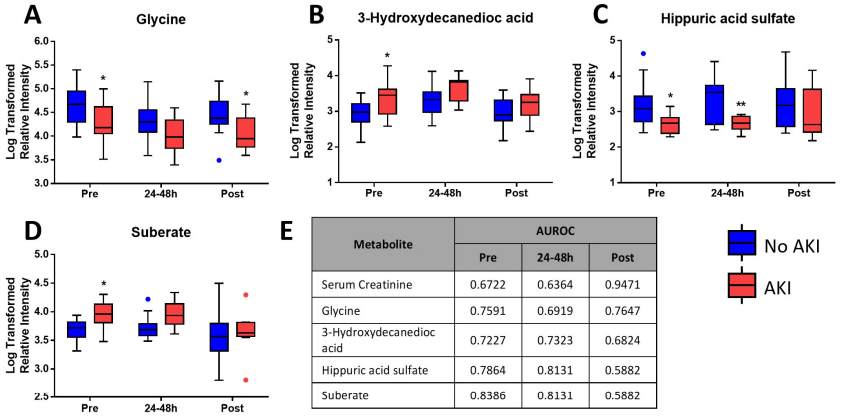
Figure 4. (**A-J**) Log transformed relative intensity of urine features significantly different between no AKI and AKI patients at the post timepoint. Data are presented as box and whisker plots, where boxes represent the median and interquartile range (IQR), and whiskers represent 1.5x IQR. **(K)** Diagnostic performance of urine metabolites as assessed by the area under the receiver operating curve. Statistical analysis was performed using two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001 compared to no AKI patients at each timepoint. n=18-20 for no AKI patients and n=10-11 for AKI patients.

Figure 5. (**A-G**) Log transformed relative intensity of serum features significantly different between no AKI and AKI patients at the post timepoint. Data are presented as box and whisker plots, where boxes represent the median and interquartile range (IQR), and whiskers represent 1.5x IQR. **(H)** Diagnostic performance of urine metabolites as assessed by the area under the receiver operating curve. Statistical analysis was performed using two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001 compared to no AKI patients at each timepoint. n=18-20 for no AKI patients and n=10-11 for AKI patients.

Figure 6. Log transformed relative intensity of urine and serum features significantly altered over time in no AKI patients. Data are presented as box and whisker plots, where boxes represent the median and interquartile range (IQR), and whiskers represent 1.5x IQR. Statistical analysis was performed using one-way ANOVA, *p<0.05, **p<0.01, ***p<0.001 compared to saline control at each timepoint, n=18-20.

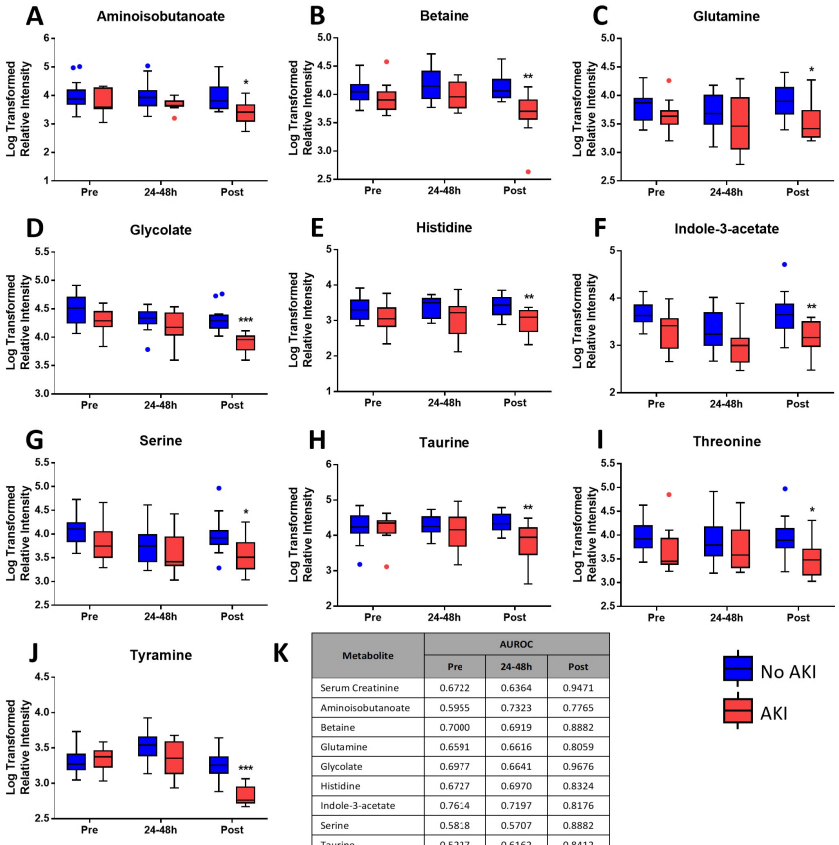






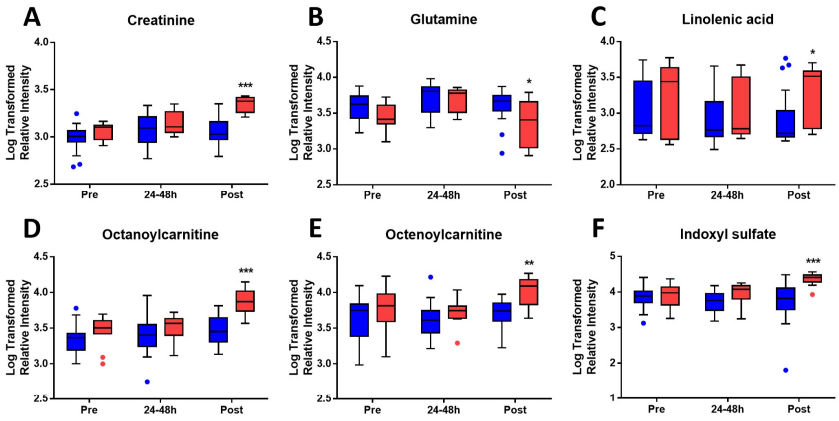
E

Metabolite	AUROC		
	Pre	24-48h	Post
Serum Creatinine	0.6722	0.6364	0.9471
Glycine	0.7591	0.6919	0.7647
3-Hydroxydecanedioc acid	0.7227	0.7323	0.6824
Hippuric acid sulfate	0.7864	0.8131	0.5882
Suberate	0.8386	0.8131	0.5882



K

Metabolite	AUROC		
	Pre	24-48h	Post
Serum Creatinine	0.6722	0.6364	0.9471
Aminoisobutanoate	0.5955	0.7323	0.7765
Betaine	0.7000	0.6919	0.8882
Glutamine	0.6591	0.6616	0.8059
Glycolate	0.6977	0.6641	0.9676
Histidine	0.6727	0.6970	0.8324
Indole-3-acetate	0.7614	0.7197	0.8176
Serine	0.5818	0.5707	0.8882
Taurine	0.5227	0.6162	0.8412
Threonine	0.7318	0.6313	0.8118
Tyramine	0.5636	0.7071	0.9618



H

Metabolite	AUROC		
	Pre	24-48h	Post
Serum Creatinine	0.6722	0.6364	0.9471
Glutamine	0.6746	0.6111	0.7412
Linolenic acid	0.5789	0.6061	0.7765
Octanoylcarnitine	0.7010	0.6667	0.9471
Octenoylcarnitine	0.6268	0.6919	0.8529
Indoxyl sulfate	0.5837	0.7677	0.9118
TMAP	0.5789	0.7121	0.8471