Identification of Early Diagnostic and Predictive Biomarkers of Cisplatin-Induced Acute Kidney Injury Using Metabolomics

Yong Jin Lim, Western University

Supervisor: Urquhart, Bradley L., The University of Western Ontario
A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Physiology and Pharmacology
© Yong Jin Lim 2023

Follow this and additional works at: https://ir.lib.uwo.ca/etd

Part of the Pharmacology, Toxicology and Environmental Health Commons

Recommended Citation

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlswadmin@uwo.ca.
Abstract

Cisplatin is an effective chemotherapeutic agent used for the treatment of a wide variety of solid tumors and hematologic cancers. Despite its effectiveness, cisplatin is associated with several toxic effects to non-cancerous tissues. Nephrotoxicity is the main dose-limiting toxicity in cisplatin therapy, manifesting as acute kidney injury (AKI) in approximately one-third of patients receiving cisplatin. AKI is defined as an abrupt decline in kidney function and is associated with several short- and long-term adverse outcomes, including chronic kidney disease, cardiovascular disease, and mortality. AKI is currently diagnosed by increased serum creatinine (SCr) concentrations. However, SCr is a marker of functional impairment of the kidneys and is only elevated after significant kidney injury. Novel biomarkers are necessary for early diagnosis of cisplatin-induced AKI and to identify high risk patients prior to cisplatin therapy. Untargeted metabolomics was utilized to characterize the metabolic alterations induced by cisplatin in a mouse model of cisplatin-induced nephrotoxicity, an adult cohort of head and neck cancer patients receiving cisplatin, and a cohort of pediatric cancer patients receiving cisplatin. In our mouse model, we identified 26 metabolites that show early alterations following cisplatin administration, prior to elevations in plasma creatinine and histological evidence of kidney injury. Many of these metabolites were indicative of mitochondrial dysfunction or gut-derived metabolites. In the metabolomic investigations of adult and pediatric cancer patients, patients who developed AKI (AKI group) after cisplatin therapy were compared with patients who did not develop AKI (no AKI group) to identify metabolites that discriminate between AKI and no AKI patients. The central role of mitochondria in cisplatin nephrotoxicity was further reinforced in the adult cohort where we identified markers of fatty acid β-oxidation as predictive markers of cisplatin-induced AKI. Metabolomic investigation of the pediatric cohort revealed metabolites involved with de novo NAD+ synthesis were consistently elevated in the urine of AKI patients compared to no AKI patients. Metabolites identified as early diagnostic markers or predictive markers must be further validated in larger patient cohorts. Collectively, these results should be used to guide future targeted metabolomics investigations and experiments to test therapeutic interventions against cisplatin-induced AKI.
Keywords

Metabolomics, pharmacometabolomics, cisplatin, acute kidney injury, nephrotoxicity, biomarkers, liquid chromatography, mass spectrometry
Summary for Lay Audience

Cisplatin is an effective and commonly used drug to treat cancer. However, cisplatin has several toxic side effects. One of the main toxic effects of cisplatin is kidney toxicity. Kidney toxicity caused by cisplatin can lead to acute kidney injury (AKI) in one-third of patients receiving cisplatin. AKI is a sudden decline in kidney function and puts patients at greater risk of developing long-term consequences such as long-term kidney dysfunction, heart disease, and death. AKI is diagnosed by measuring creatinine levels in the blood. However, creatinine levels are only elevated in the blood after significant kidney injury has already occurred which usually takes almost a week after patients receive cisplatin. For prevention of AKI, new biological markers are necessary for earlier diagnosis and to identify patients at high risk of developing AKI. Metabolomics is an analytical technique used to take a snapshot of all the biochemical reactions occurring in the body at a given time; it does so by measuring the levels of metabolites, which are by-products of the biochemical reactions happening in the body. Evaluating changes in metabolite levels provides indications as to which metabolic reactions are altered/disrupted. We used metabolomics in mice treated with cisplatin to investigate which metabolites were changed early after cisplatin treatment; we found 26 metabolites that were changed before elevations in blood creatinine levels. Many of these metabolites were associated with dysfunctional mitochondria (powerhouse of the cell). Metabolomics was also used to compare patients who received cisplatin and developed AKI vs. those who did not develop AKI. The goal was to find metabolites that could predict which patients would develop AKI after receiving cisplatin. In adult patients, metabolites linked to mitochondrial breakdown of fats were found to predict which patients would go on to develop AKI. In children, metabolites involved in the synthesis of an important energy metabolite called NAD+ were consistently elevated in the urine of patients with AKI. These markers of AKI must be validated in larger groups of patients to confirm their utility. These findings can help guide future research to develop protective measures against kidney damage caused by cisplatin.
Co-Authorship Statement

Chapter 3:

B.L.U. and Y.J.L. designed the study. Y.J.L. performed the experiments and data analysis. E.D.H. and N.C.T. assisted with animal experiments. T.J.V. provided guidance with data analysis. A.H. developed and implemented kidney injury scoring and 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.

Chapter 4:

B.L.U., M.Z., and T.D.B. designed the study. M.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.

Chapter 5:

B.L.U., M.Z., and T.D.B. designed the study. Y.J.L. performed the experiments and data analysis.
Acknowledgments

I would like to express my deepest gratitude to Dr. Brad Urquhart for his guidance, mentorship, and support throughout my graduate studies. Brad has been a fantastic role model as a scientist, mentor, and person, and it has truly been a privilege to have him as my supervisor. From day one, Brad has been available and happy to provide consultation despite his busy schedule. Brad has always treated me with kindness, understanding, and empathy, and his positivity and unwavering support has helped me immensely during times where I felt unsure or overwhelmed. Brad leads by example to foster a positive and welcoming work environment, and his positive attitude undoubtedly trickles down to the rest of the members of his lab. I have always admired how Brad makes time to check in with his students and makes an effort to connect with his trainees on a personal level. Brad, I will genuinely miss our chats about sports, food, and life in general. I cannot thank you enough for all that you have done for me. I am not eloquent enough to truly express the impact that you have had on me, so I will just end with this: You are the GOAT.

As I reflect on my time as a graduate student, I am truly grateful to have had the support and companionship of my lab friends. Emily, thank you for showing me the ropes when I first started in the lab; you set a wonderful example and I am thankful for your patience, kindness, and willingness to help. To the GG members Jay and Nick, thank you for the countless laughs and memories we have shared in and out of the lab. Jay, thanks for sharing in my nerdy interests and love of memes. Nick, I appreciate your big personality and ability to bring the lab together. With that hippocampus and interested mind, I know you will make a fantastic clinician. Rachel, I admire your consistency, tenacity, and dedication to research. You have always been the constant in the bullpen, and your steadiness has been a source of comfort through the turbulence of grad school. You inspire me to work harder, and I have no doubt you will succeed in whatever you put your mind to. To the midnight mallard Adrien “Adgemoney” “Gus-Star” Augustus Eric RaoPeters and Steven “frosted-tips” Xiu (triple tap), thank you for teaching me the language of the youth and keeping me young at heart. I am thankful that we bonded over our mutual love of soccer and food and will always fondly remember our shenanigans on the soccer pitch as you two attempted to coach me. Nicole, thanks for the laughs we have shared over the years. I’ve seen more pictures of Rex than anything else in my life, and his image will forever be burned in my brain. May you find someone else to inadvertently match clothes with
after I am gone. Last but not least, Aaron, thank you for using your ancient wisdom to provide 
me with guidance and advice. I am grateful that you are always willing to listen to my problems 
and always down to chat about random things. I really enjoyed our morning chats, walks home 
after work, and the occasional game of catch. Thank you all for making my time in the lab 
enjoyable through the ups and downs of research.

I would also like to thank Dr. Nica Borradaile for her kindness and mentorship, as well as the 
past and present members of the Borradaile lab for being an integral part of the lab family. To 
Dr. Tom Velenosi, thank you for taking the time to provide me with assistance and advice, 
long after your departure from the lab. Additionally, I would like to express my gratitude to 
the members of my advisory committee, Dr. Lakshman Gunaratnam, Dr. Rommel Tirona, and 
Dr. Tim Regnault, for their invaluable support and guidance in shaping my research. Thank 
you also to Dr. Michael Zappitelli, Dr. Tom Blydt-Hansen, Dr. Kelly McMahon, and the rest 
of the ABLE/ACCENT investigators and coordinators for their hard work in coordinating and 
conducting patient recruitment and sample collection.

Finally, a huge thank you to my family and friends who have supported and encouraged me 
throughout my graduate studies. I especially want to thank my parents who have sacrificed so 
much on my behalf; I could not have done this without your love and support.
Table of Contents

Abstract ............................................................................................................................... ii
Summary for Lay Audience ............................................................................................... iv
Co-Authorship Statement ................................................................................................. v
Acknowledgments ............................................................................................................. vi
Table of Contents ............................................................................................................. viii
List of Tables ................................................................................................................... xiii
List of Figures .................................................................................................................. xiv
List of Appendices .......................................................................................................... xvii
List of Abbreviations ..................................................................................................... xviii
Chapter 1 ............................................................................................................................. 1

  1 Introduction ................................................................................................................ 1
    1.1 Acute Kidney Injury ............................................................................................... 1
      1.1.1 Definition and Diagnosis of Acute Kidney Injury ...................................... 1
      1.1.2 Epidemiology, Etiology, and Outcomes of AKI ......................................... 3
    1.2 Cisplatin ............................................................................................................ 9
      1.2.1 Cisplatin Chemotherapy and Mechanisms of Action ................................. 9
      1.2.2 Pharmacokinetics of Cisplatin .................................................................. 10
      1.2.3 Adverse Effects of Cisplatin Therapy ....................................................... 11
      1.2.4 Cisplatin Nephrotoxicity ........................................................................... 16
    1.3 Metabolomics ........................................................................................................ 35
      1.3.1 Overview of Metabolomics......................................................................... 35
      1.3.2 Analytical Techniques for Metabolomics .................................................... 36
      1.3.3 Data Processing and Analysis of LC-MS Metabolomics Datasets.............. 36
      1.3.4 Pharmacometabolomics ............................................................................ 38
4.3.6 Alterations Of Urine and Serum Metabolites Over Time in Patients Without Clinical Acute Kidney Injury .................................................... 142

4.4 Discussion ........................................................................................................... 144

4.5 Conclusion .......................................................................................................... 147

4.6 References ........................................................................................................... 149

Chapter 5 ......................................................................................................................... 154

5 Metabolomic Investigation of Cisplatin-induced Acute Kidney Injury in Pediatric Cancer Patients ................................................................. 154

5.1 Introduction ......................................................................................................... 155

5.2 Materials and Methods ........................................................................................ 156

5.2.1 Chemicals and Reagents .............................................................................. 156

5.2.2 Study Participants and Sample Collection .................................................... 157

5.2.3 Classification of AKI .................................................................................... 157

5.2.4 Sample Preparation for Untargeted Metabolomics .................................. 158

5.2.5 Chemical Derivatization of Patient Serum and Urine Samples ............... 158

5.2.6 Chromatography and Mass Spectrometry ................................................ 158

5.2.7 Processing of LC-MS Chromatographic Data .......................................... 159

5.2.8 Statistical Analysis ....................................................................................... 159

5.2.9 Putative Annotation and Identity Confirmation of Metabolites ............ 160

5.3 Results ................................................................................................................. 161

5.3.1 Patient Demographics ............................................................................... 161

5.3.2 Metabolic Profiling of No AKI vs. AKI Patients .................................... 161

5.3.3 Discriminatory Metabolites for AKI in Serum ...................................... 165

5.3.4 Discriminatory Metabolites for AKI in Urine ......................................... 169

5.3.5 Diagnostic Performance of Biomarkers in Classification of No AKI and AKI Patients ................................................................. 169

5.3.6 Investigation of Sex Differences in Metabolic Alterations ..................... 177
5.3.7 Stratification by Patient Age ................................................................. 178

5.4 Discussion ............................................................................................. 184

5.5 Conclusion ............................................................................................. 187

5.6 References ............................................................................................. 189

Chapter 6 .......................................................................................................... 194

6 Discussion and Conclusions ...................................................................... 194

6.1 Discussion and Significance of Research .............................................. 194

6.2 Chapter 3 ............................................................................................... 195

6.2.1 Summary of Chapter 3 ................................................................. 195

6.2.2 Chapter 3 Limitations and Future Directions ............................... 196

6.3 Chapter 4 ............................................................................................... 197

6.3.1 Summary of Chapter 4 ................................................................. 197

6.3.2 Chapter 4 Limitations and Future Directions ............................... 199

6.4 Chapter 5 ............................................................................................... 199

6.4.1 Summary of Chapter 5 ................................................................. 199

6.4.2 Chapter 5 Limitations and Future Directions ............................... 201

6.5 Conclusions ............................................................................................ 202

6.6 References ............................................................................................. 205

Appendices ...................................................................................................... 210

Appendix A: Ethics Approvals ................................................................. 210

Appendix B: Supplementary Information for Chapter 3 ......................... 212

Appendix C: Supplementary Information for Chapter 4 ......................... 217

Appendix D: Supplementary Information for Chapter 5 ......................... 221

Curriculum Vitae .......................................................................................... 228
List of Tables

Table 1-1 KDIGO classification of AKI................................................................. 2

Table 1-2 Causes of acute kidney injury.............................................................. 5

Table 3-1 Summary of metabolite alterations over time in plasma, urine, and kidney of C57BL/6 mice treated with cisplatin compared to C57BL/6 mice treated with saline. .......... 92

Table 3-2 Summary of metabolite alterations over time in plasma, urine, and kidney of FVB/N mice treated with cisplatin compared to FVB/N mice treated with saline............... 94

Table 4-1 Baseline demographics of study population...................................... 129

Table 4-2 Summary of metabolite alterations in AKI patients compared to no AKI patients. ............................................................................................................................................... 133

Table 5-1 Baseline demographics of study population...................................... 163

Table 5-2 Summary of metabolite alterations in AKI patients compared to no AKI patients. ............................................................................................................................................... 166

Table 5-3 Diagnostic performance of individual serum metabolites for classification between no AKI and AKI patients at each timepoint, as assessed by the area under the receiver operating curve (AUC) and corresponding confidence intervals (CI). ...................... 172

Table 5-4 Diagnostic performance of individual urine metabolites for classification between no AKI and AKI patients at each timepoint, as assessed by the area under the receiver operating curve (AUC) and corresponding confidence intervals (CI). ...................... 173

Table 6-1 Summary of proposed early and predictive metabolite markers of AKI......... 204
List of Figures

Figure 1.1 Chemical structure of cisplatin................................................................. 10

Figure 1.2 Overview of proposed mechanisms of cisplatin accumulation, biotransformation, and cell toxicity in renal tubular epithelial cells......................................................... 18

Figure 3.1 Assessment of renal injury in untreated, saline-treated, and cisplatin-treated C57BL/6 mice and FVB/N mice............................................................................................. 85

Figure 3.2 Orthogonal partial least squares discriminant analysis scores plots comparing plasma, urine, and kidney samples from saline-treated and cisplatin-treated C57BL/6 mice at each timepoint........................................................................................................................ 87

Figure 3.3 Orthogonal partial least squares discriminant analysis scores plots comparing plasma, urine, and kidney samples from saline-treated and cisplatin-treated FVB/N mice at each timepoint........................................................................................................................ 88

Figure 3.4 Time course cluster analysis of plasma, urine, and kidney features that were significantly altered by cisplatin treatment in C57BL/6 mice.............................................. 90

Figure 3.5 Time course cluster analysis of plasma, urine, and kidney features that were significantly altered by cisplatin treatment in FVB/N mice.............................................. 91

Figure 3.6 Log transformed relative intensity of features significantly affected by cisplatin treatment in plasma samples from C57BL/6 mice................................................................. 97

Figure 3.7 Log transformed relative intensity of features significantly affected by cisplatin treatment in plasma samples from FVB/N mice................................................................. 98

Figure 3.8 Log transformed relative intensity of features significantly affected by cisplatin treatment in urine samples from C57BL/6 mice................................................................. 100

Figure 3.9 Log transformed relative intensity of features significantly affected by cisplatin treatment in urine samples from FVB/N mice................................................................. 101
Figure 3.10 Log transformed relative intensity of features significantly affected by cisplatin treatment in kidney samples from C57BL/6 mice. ............................................................... 103

Figure 3.11 Log transformed relative intensity of features significantly affected by cisplatin treatment in kidney samples from FVB/N mice. ............................................................... 104

Figure 3.12 Receiver operating characteristic (ROC) analysis of early plasma biomarkers in C57BL/6 mice and FVB/N mice................................................................. 107

Figure 3.13 Receiver operating characteristic (ROC) analysis of early urine biomarkers in C57BL/6 mice and FVB/N mice................................................................. 108

Figure 4.1 Principle component analysis and orthogonal partial least squares discriminant analysis scores plots comparing urine samples from no AKI and AKI patients at each timepoint. .............................................................................................................................. 131

Figure 4.2 Principle component analysis and orthogonal partial least squares discriminant analysis scores plots comparing serum samples from no AKI and AKI patients at each timepoint. .............................................................................................................................. 132

Figure 4.3 Log transformed relative intensity of urine features significantly different between no AKI and AKI patients at the pre timepoint. ................................................................. 136

Figure 4.4 Log transformed relative intensity of urine features significantly different between no AKI and AKI patients at the post timepoint. ................................................................. 138

Figure 4.5 Log transformed relative intensity of serum features significantly different between no AKI and AKI patients at the post timepoint. ................................................................. 141

Figure 4.6 Log transformed relative intensity of urine and serum features significantly altered over time in no AKI patients ................................................................. 143

Figure 5.1 Orthogonal partial least squares discriminant analysis scores plots comparing serum and urine samples from no AKI and AKI patients at the pre, post, and discharge timepoints ................................................................. 164
Figure 5.2 Log transformed relative intensity of serum features significantly different between no AKI and AKI patients. ................................................................. 168

Figure 5.3 Log transformed relative intensity of urine features significantly different between no AKI and AKI patients. ................................................................. 171

Figure 5.4 Multivariate receiver operating characteristic (ROC) curves of serum metabolites and corresponding area under the curve and confidence intervals at the pre, post, and discharge timepoints. ............................................................................................................ 175

Figure 5.5 Multivariate receiver operating characteristic (ROC) curves of urinary metabolites and corresponding area under the curve and confidence intervals at the pre, post, and discharge timepoints. ............................................................................................................ 176

Figure 5.6 Orthogonal partial least squares discriminant analysis scores plots comparing serum and urine samples from no AKI and AKI patients under the age of 3 at the pre, post, and discharge timepoints. ................................................................. 179

Figure 5.7 Orthogonal partial least squares discriminant analysis scores plots comparing serum and urine samples from no AKI and AKI patients over the age of 3 at the pre, post, and discharge timepoints. ................................................................. 180

Figure 5.8 Log transformed relative intensity of urine features in no AKI and AKI patients under the age of 3. ................................................................................. 181

Figure 5.9 Multivariate receiver operating characteristic (ROC) curves of urinary metabolites and corresponding area under the curve and confidence intervals at the pre, post, and discharge timepoints. ................................................................................. 183
List of Appendices

Appendix A: Ethics Approvals ............................................................................................. 210
Appendix B: Supplementary Information for Chapter 3 ...................................................... 212
Appendix C: Supplementary Information for Chapter 4 ...................................................... 217
Appendix D: Supplementary Information for Chapter 5 ...................................................... 221
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-NPH</td>
<td>3-nitrophenylhydrazine</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>ABLE</td>
<td>Applying biomarkers to long-term effects in child and adolescent cancer treatment</td>
</tr>
<tr>
<td>ACCENT</td>
<td>A Canadian study of cisplatin metabolomics and nephrotoxicity</td>
</tr>
<tr>
<td>ACMSD</td>
<td>Aminocarboxymuconate semialdehyde decarboxylase</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>AP</td>
<td>Aminopeptidase N</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating transcription factor 6</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia and Rad3 related protein</td>
</tr>
<tr>
<td>AUROC</td>
<td>Area under the receiving operating characteristic curve</td>
</tr>
<tr>
<td>AV</td>
<td>Acute visit</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>BiP</td>
<td>Binding immunoglobulin protein</td>
</tr>
<tr>
<td>CHK</td>
<td>Checkpoint kinase</td>
</tr>
<tr>
<td>CHOP</td>
<td>CCAAT/enhancer-binding protein homologous protein</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CisPN</td>
<td>Cisplatin-induced peripheral neuropathy</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage-associated molecular patterns</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
</tr>
<tr>
<td>EIC</td>
<td>Extracted ion chromatogram</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Eukaryotic initiator factor 2α</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated protein degradation</td>
</tr>
<tr>
<td>ESKD</td>
<td>End-stage kidney disease</td>
</tr>
<tr>
<td>FAO</td>
<td>Fatty acid β-oxidation</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>GGT</td>
<td>Gamma glutamyl transpeptidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GRP78</td>
<td>78-kDa glucose-regulated protein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic interaction liquid chromatography</td>
</tr>
<tr>
<td>HMDB</td>
<td>Human metabolome database</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IGFBP7</td>
<td>Insulin-like growth factor-binding protein 7</td>
</tr>
<tr>
<td>IPO</td>
<td>Isotopologue parameter optimization</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>IRE1α</td>
<td>Inositol-requiring enzyme 1α</td>
</tr>
<tr>
<td>IRF3</td>
<td>Interferon regulatory factor 3</td>
</tr>
<tr>
<td>KDIGO</td>
<td>Kidney disease improving global outcomes</td>
</tr>
<tr>
<td>KIM-1</td>
<td>Kidney injury molecule-1</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MATE1</td>
<td>Multi-drug and toxin extrusion protein 1</td>
</tr>
<tr>
<td>MCAD</td>
<td>Medium-chain acyl-coa dehydrogenase deficiency</td>
</tr>
<tr>
<td>miR-709</td>
<td>MicroRNA-709</td>
</tr>
<tr>
<td>MLKL</td>
<td>Mixed lineage kinase domain-like protein</td>
</tr>
<tr>
<td>MRP2</td>
<td>Multidrug resistance-associated protein 2</td>
</tr>
<tr>
<td>MSMLS</td>
<td>Mass Spectrometry Metabolite Library of Standards</td>
</tr>
<tr>
<td>MT-CO1</td>
<td>Mitochondrial-encoded cytochrome c oxidase subunit 1</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>MyD88</td>
<td>The myeloid differentiation primary response 88</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NGAL</td>
<td>Neutrophil-gelatinase associated lipocalin</td>
</tr>
<tr>
<td>NK-1</td>
<td>Neurokinin-1</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NLR family pyrin domain containing 3</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
</tr>
<tr>
<td>NRF1</td>
<td>Nuclear respiratory factor 1</td>
</tr>
<tr>
<td>NRF2</td>
<td>Nuclear factor-erythroid-2 p45-related factor 2</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>OAT</td>
<td>Organic anion transporter</td>
</tr>
</tbody>
</table>
OATP  Organic anion transporting polypeptide
OCT   Organic cation transporter 2
OCTN2 Organic cation/carnitine transporter 2
OPLS-DA Orthogonal partial least squares discriminant analysis
OR    Odds ratio
PAMPs Pathogen-associated molecular patterns
PARP1 Poly [ADP-ribose] polymerase 1
PCA   Principal component analysis
PERK  Protein kinase R-like ER kinase
PGC-1α Peroxisome proliferator-activated receptor γ coactivator 1 α
PITC  Phenylisothiocyanate
PUMA  p53-upregulated modulator of apoptosis
QC    Quality control
q-ToF Quadrupole time of flight
RIP   Receptor-interacting protein kinase
ROC   Receiver operating characteristic
ROS   Reactive oxygen species
RPLC  Reverse-phase liquid chromatography
RR    Risk ratio
RRT   Renal replacement therapy
rsd   Relative standard deviation
SCr   Serum creatinine
SH    Thiol
SNP   Single nucleotide polymorphism
TauT  Taurine transporter
TCA   Tricarboxylic acid
TFAM  Mitochondrial transcriptional factor A
TFEB  Transcription factor EB
TIC   Total ion current chromatogram
TIMP2 Tissue inhibitor of metalloproteinase 2
TLR   Toll-like receptor
TNF-α Tumor necrosis factor α
Treg  Regulatory T cells
TRIF  Toll/IL-1R domain-containing adaptor-inducing interferon-β
UO    Urine output
UPLC-MS Ultra performance liquid chromatography-mass spectrometry
UPR   Unfolded protein response
VIP   Variable importance in projection
XBP1  X-box binding protein 1
Chapter 1

1 Introduction

1.1 Acute Kidney Injury

1.1.1 Definition and Diagnosis of Acute Kidney Injury

Acute kidney injury (AKI) is a broad clinical syndrome described as an abrupt decline in kidney function that occurs over the course of hours or days\(^1\). The currently accepted definition of AKI comes from the 2012 Kidney Disease: Improving Global Outcomes (KDIGO) guidelines, which is based on an increase in serum creatinine (SCr) or a decrease in urine output\(^1\). AKI is defined as an increase in SCr \(\geq 1.5\) times baseline within 7 days, an increase in SCr of \(\geq 26.5\) μmol/L within 48 hours, or a reduction in urine output (UO) < 0.5 mL/kg/h for more than 6 hours (Table 1.1)\(^1\). Additionally, AKI is stratified based on severity from stage 1 to stage 3, from least severe to most severe (Table 1.1)\(^1\). If SCr and UO classify a patient into different stages, the patient is classified using the criteria (either SCr or UO) that classifies them into the most severe stage\(^1\). The current understanding of AKI has significantly advanced since the KDIGO guidelines were published in 2012. As such, KDIGO is currently working to update the AKI clinical practice guidelines to incorporate new information into clinical practice\(^2\).

Though current definitions of AKI are based on SCr and UO, these markers have several limitations in AKI diagnosis. SCr and UO are used as surrogates for glomerular filtration rate (GFR)\(^3\); in other words, these markers measure impairment of kidney function and are delayed responses that only manifest after significant kidney injury has occurred\(^4\). SCr starts to increase several days following injury, by which point approximately 50% of GFR has already been lost\(^5\). Furthermore, SCr is a non-specific marker of AKI, as SCr levels are influenced by numerous factors including age, sex, race, muscle mass, fluid therapy, medications, and diet\(^1,5,6\). UO can also be confounded by changes in hydration status, fluid therapy, and diuretics\(^3,5\). For successful mitigation and management of AKI, the onset must be diagnosed as early as possible, and patients at high risk for developing AKI must be identified prior to kidney damage\(^4\).
**Table 1-1 KDIGO classification of AKI.**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Serum Creatinine</th>
<th>Urine Output</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>1.5–1.9 times baseline OR ≥ 26.5 μmol/L increase</td>
<td>&lt; 0.5 ml/kg/h for 6-12 hours</td>
</tr>
<tr>
<td>Stage 2</td>
<td>2.0–2.9 times baseline</td>
<td>&lt; 0.5 ml/kg/h for ≥ 12 hours</td>
</tr>
<tr>
<td>Stage 3</td>
<td>3.0 times baseline OR ≥353.6 μmol/L increase OR initiation of renal replacement therapy OR in patients &lt;18 years, decrease in estimated glomerular filtration rate (eGFR) to &lt; 35 ml/min/1.73 m²</td>
<td>&lt;0.3 ml/kg/h for ≥24 h OR anuria ≥12 h</td>
</tr>
</tbody>
</table>
1.1.2 Epidemiology, Etiology, and Outcomes of AKI

1.1.2.1 Epidemiology of AKI

When studying epidemiology, it is important to consider that AKI is a complex and multifaceted syndrome that arises in numerous clinical scenarios and encompasses various etiologies and patient-specific risk factors\(^7\). Risk factors for AKI are categorized into modifiable and non-modifiable risk factors. Non-modifiable risk factors include age (very young or elderly), male sex, chronic kidney disease (CKD), diabetes, liver disease, cardiovascular disease, and sepsis. Modifiable risk factors for AKI include hypovolemia, hypotension, anemia, and use of nephrotoxic drugs. Due to the complicated nature of AKI and heterogeneous definitions historically used for the diagnosis of AKI, the reported incidence of AKI is highly variable\(^7,8\). A systematic review of large cohort studies was conducted for reports published from 2004 to 2012, including a total of 312 studies (n=49,147,878)\(^8\). Of the 312 studies, 154 studies either used KDIGO-equivalent AKI definitions or were reclassified using the KDIGO criteria (n=3,585,911). This meta-analysis reported that the pooled incidence rates of AKI in patients hospitalized with acute illness was approximately 22% in adults and 34% in children when classification was conducted using the KDIGO definition of AKI. Pooled incidence rates were variable depending on the clinical setting, with pooled incidence rates being highest in intensive care unit (ICU) patients and following cardiac surgery (32% and 24%, respectively). Reported incidence of AKI in each individual study included in the meta-analysis was highly variable and wide-ranging; regardless, it is evident that AKI is common and affects a significant proportion of hospitalized patients. Recent multi-national prospective observational studies have focused on characterizing the incidence and outcomes of AKI in critically ill neonatal (AWAKEN study) and pediatric patients (AWARE study)\(^9,10\). The incidence of AKI in both studies was comparable, with an incidence of 29.9% of patients in the AWAKEN study and 26.9% in the AWARE study.

Incidence of AKI has been rising, but it is unclear how much of this increase in AKI incidence is an actual increase in disease incidence as opposed to increased diagnosis due to the introduction of standardized definitions of AKI, increased sensitivity in administrative coding of AKI, and/or increased overall awareness of AKI\(^11,12\). Increasing
incidence of comorbidities, such as CKD, cardiovascular disease, and sepsis, higher frequency of invasive procedures, and increased use of nephrotoxic medications may contribute to the observed rises in AKI incidence.

1.1.2.2 Etiology of AKI

There are numerous potential causes of AKI, which have traditionally been broadly classified into three categories: pre-renal, intrinsic, and post-renal causes. Pre-renal AKI arises as a result of renal hypoperfusion and a subsequent decrease in GFR. Renal hypoperfusion can arise in conditions of hypovolemia, impaired cardiac function, systemic vasodilation, and increased vascular resistance. Intrinsic or intrarenal AKI is further sub-categorized to damage to four kidney structures: tubules, glomeruli, interstitium, and intra-renal blood vessels. Post-renal AKI occurs following obstruction to urinary flow, increasing intra-tubular pressure and subsequently decreasing GFR. If the obstruction precedes the bladder and is unilateral, the impaired drainage may not present as AKI, as the unaffected kidney can often compensate for loss in function in the obstructed kidney; exceptions include patients with pre-existing renal insufficiency or patients with a single kidney. Specific causes and conditions leading to pre-renal, intrinsic, and post-renal AKI are summarized in Table 1.2. Although the classification of AKI into the categories of pre-renal, intrinsic, and post-renal offers a straightforward and convenient view of AKI, it is important to note that AKI is multi-faceted and can develop due to a combination of pathological conditions.
Table 1-2 Causes of acute kidney injury\textsuperscript{13–15,17–20}.

<table>
<thead>
<tr>
<th>Category of AKI</th>
<th>Description</th>
<th>Condition Leading to AKI</th>
<th>Causes of Condition</th>
</tr>
</thead>
</table>
| Pre-renal       | Arises from impaired perfusion of the kidneys and resultant decrease in GFR | Hypovolemia | • Hemorrhage  
• Excessive vomiting/diarrhea  
• Excessive diuresis  
• Burns  |
|                 |             | Impaired cardiac function | • Congestive heart failure  
• Cardiac tamponade  
• Massive pulmonary embolism  
• Myocardial infarction  |
|                 |             | Systemic vasodilation     | • Septic shock  
• Angiotensin II receptor blockers and angiotensin converting enzyme inhibitors  
• Anaphylaxis  
• Cirrhosis  |
| Renal vasoconstriction |             |                          | • Hypercalcemia  
• Drugs causing renal vasoconstriction (e.g., NSAIDs, cyclosporin or tacrolimus)  
• Hepatorenal syndrome  |
| Intrinsic/Intrarenal | Damage to tubules, glomeruli, interstitium, and blood vessels of the kidneys | Tubular damage | • Renal ischemia  
• Nephrotoxic medications (e.g., aminoglycosides, cisplatin)  
• Endogenous nephrotoxins (e.g., hemoglobin and myoglobin)  |
|                 |             | Glomerular damage         | • Post-infectious glomerulonephritis  
• IgA nephropathy  
• Lupus nephritis  
• Anti-glomerular basement membrane disease  
• Wegener’s granulomatosis  
• Microscopic polyangiitis  |
|                 |             | Interstitial damage       | • Bacterial and viral infections  
• Medications (e.g., antibiotics, NSAIDs)  |
|                 |             | Vascular damage           | • Large vessels:  
Renal artery stenosis  
Microvasculature:  
Thrombotic microangiopathies  
Vasculitis  |
| Post-renal      | Obstruction of urinary flow causing increased intra-tubular pressure and decreased GFR | Intrarenal obstruction | • Kidney stones  
• Papillary necrosis  |
|                 |             | Extrarenal obstruction    | • Blockage in bladder outlet or ureter due to benign prostatic hypertrophy, cancer (prostate, gynecologic), kidney stones  |
1.1.2.3 Outcomes of AKI

The current body of evidence indicates that AKI is an important factor in the long-term prognosis of patients, as AKI has been associated with numerous adverse short- and long-term clinical outcomes. Short-term outcomes of AKI include increased in-hospital mortality, increased length of stay, prolonged mechanical ventilation, and increased costs in both pediatric and adult patients\textsuperscript{10,21–24}. Long-term outcomes of AKI include kidney events such as recurrence of AKI and progression to CKD, cardiovascular events, and mortality. This section will further discuss the long-term adverse outcomes of AKI.

1.1.2.3.1 Kidney Events

Recurrent AKI is a common event in hospitalized patients with AKI complications. A retrospective study of 11,683 adult patients who developed AKI during hospitalization reported that 25% of patients were re-hospitalized with recurrent AKI within 12 months of discharge\textsuperscript{25}. Patients that were re-hospitalized with recurrent AKI exhibited higher 1-year mortality rate compared to patients without recurrent AKI (35% vs. 18%)\textsuperscript{25}. A separate retrospective study of 38,659 hospitalized adult patients who experienced AKI reported a similar AKI recurrence rate of 28.6% and showed that patients with recurrent AKI had a 66% increased risk of death compared to patients without recurrent AKI\textsuperscript{26}. Recurrent AKI has also been associated with increased risk of CKD, with each episode of AKI (up to three episodes) doubling the risk of stage 4 CKD in a cohort of diabetic patients\textsuperscript{27}. In children receiving multiple cardiac surgeries, AKI was more prevalent after the second surgery for patients who had AKI after the first surgery, with an adjusted odds ratio (OR) of 2.89\textsuperscript{28}.

Several studies have demonstrated that patients with prior AKI have increased risk of developing CKD or end-stage kidney disease (ESKD). CKD is stratified into five stages of severity, based on decreasing estimated GFR (eGFR) values: stage 1) normal/high (eGFR \(\geq 90\) mL/min/1.73 m\(^2\)); stage 2) mildly decreased (eGFR 60–89 mL/min/1.73 m\(^2\)); stage 3a) mildly to moderately decreased (eGFR 45–59 mL/min/1.73 m\(^2\)); stage 3b) moderately to severely decreased (eGFR 30–44 mL/min/1.73 m\(^2\)), stage 4) severely decreased (eGFR 15–29 mL/min/1.73 m\(^2\)), stage 5) kidney failure/ESKD (eGFR < 15 mL/min/1.73 m\(^2\))\textsuperscript{29}. A systematic review was conducted using 10 adult cohort studies investigating the association
between AKI and CKD\textsuperscript{30}. Stage 3 CKD was used as the threshold to define the CKD outcome in 6 of the studies, stage 4 CKD was used in 2 studies, and a 25\% decrease in GFR was used in 2 studies\textsuperscript{30}. AKI was associated with increased risk of CKD in all included studies. When all 10 studies were pooled, overall incidence of CKD was higher in patients with AKI compared to patients without AKI (17.76 vs. 7.59 cases per 100 person-years), and pooled adjusted risk of CKD was 2.67-fold higher in patients with AKI compared to patients without AKI\textsuperscript{30}. Three of the studies included in the meta-analysis explored CKD risk based on the stages of AKI and reported increasing risk of CKD based on AKI severity (hazard ratio (HR) of 2.32, 4.00, 12.12 for stages 1, 2, and 3 respectively); the authors noted that this finding was not statistically significant, as there were only three studies included in this analysis\textsuperscript{30}. In nine adult cohort studies investigating the association between AKI and ESKD, patients with AKI were at significantly higher risk of developing ESKD compared to patients without AKI (pooled adjusted HR of 4.81; 0.47 versus 0.08 cases per 100 person-year). Risk of ESKD was also found to increase with AKI severity\textsuperscript{30}. Compared to adults, the long-term adverse outcomes associated with AKI in children are not well characterized. However, a growing body of evidence in pediatric patients in the ICU, receiving cardiac surgery, suffering serious infection, and in oncology settings has shown the association between pediatric AKI and CKD\textsuperscript{31}.

1.1.2.3.2 Cardiovascular Events

AKI has also been linked to an increased risk of cardiovascular events. A systematic review published in 2011 characterized cardiovascular outcomes among patients with and without contrast-induced AKI following coronary angiography\textsuperscript{32}. Fourteen studies were included in the meta-analysis, and cardiovascular outcomes were defined as: cardiovascular mortality, myocardial infarction, target vessel reocclusion or need for revascularization, stroke, heart failure, or any combination of those. Patients with AKI had a greater risk of cardiovascular outcomes in all 14 studies individually, as well as after pooling of the studies. It is important to note that six of the studies included in this meta-analysis reported risk estimates that were not adjusted for confounding variables. In another systematic review of 25 studies (published between 2003–2016) involving 254,408 adult patients, AKI was associated with increased risk of cardiovascular mortality (pooled risk ratio (RR)
of 1.86), major cardiovascular events (RR of 1.38), congestive heart failure (pooled RR of 1.58), acute myocardial infarction (pooled RR of 1.40), and stroke (pooled RR of 1.15). Furthermore, the risk of heart failure increased with increasing severity of AKI\cite{33}. A 2018 retrospective study in a cohort of 146,941 hospitalized adults in Northern California showed that patients with AKI were at higher risk of developing the composite outcome of hospitalization for heart failure, acute coronary syndrome, peripheral arterial disease, and ischemic stroke compared to patients without AKI (adjusted HR of 1.18)\cite{34}. Most of this increased risk was driven by the risk of heart failure (adjusted HR 1.44). Patients with AKI during hospitalization were also more likely to develop hypertension two years after hospitalization in a retrospective cohort study of 43,611 hospitalized adults\cite{35}. 730 days after discharge, 46% of patients with AKI during hospitalization had hypertension (>140/90 mmHg) versus 41% for patients without AKI. The association between AKI and risk of elevated BP was strongest 180 days after discharge (adjusted OR 1.40) and decreased with time (adjusted OR 1.22 at 730 days). AKI severity was also associated with higher risk of hypertension. Two recently published retrospective cohort studies also observed increased risk of hypertension in critically ill pediatric patients with AKI and pediatric patients that required dialysis to treat their AKI\cite{36,37}. However, in a systematic review of long-term consequences of AKI after pediatric cardiac surgery, three out of four studies investigating hypertension as an outcome found no association between AKI and hypertension\cite{38}. One study found an association at 12 months following the operation, but not at the subsequent follow-up times.

1.1.2.3.3 Mortality

The association between an episode of AKI and all-cause mortality has been well established in the literature. A meta-analysis investigating the incidence of AKI reported the overall pooled rate of AKI-associated mortality was 23%; when patients were divided into adults and children, the AKI-associated mortality rates were 23.9% and 13.8%, respectively\cite{8}. The pooled OR for all-cause mortality was also higher in patients with AKI vs. patients without and increased based on AKI severity, but the reported ORs were not adjusted for confounding variables. In a systematic review of 70 studies (n=1,817,999) reporting on the long-term risk of mortality associated with AKI in adults, incidence of
mortality was higher in patients with AKI compared to patients without (13.19 versus 7.26 deaths per 100 person-years), and the pooled adjusted HR for mortality was 1.80\textsuperscript{30}. Patients were further divided into subgroups based on the following clinical settings: angiography, cardiovascular surgery, general hospital setting, and intensive care unit (ICU)\textsuperscript{30}. Risk of mortality following an episode of AKI was highest in patients undergoing angiography (adjusted HR of 3.07), followed by cardiovascular surgery (adjusted HR of 1.75), ICU setting (adjusted HR of 1.47), and general hospitalization (adjusted HR of 1.41)\textsuperscript{30}. The risk of death also increased based on severity of AKI, both in the full cohort and after division into subgroups based on clinical setting\textsuperscript{30}. Recent studies have further reported the association between AKI and long-term mortality in pediatric patients in various clinical settings\textsuperscript{9,10,36,39,40}.

1.2 Cisplatin

1.2.1 Cisplatin Chemotherapy and Mechanisms of Action

Cisplatin is an effective chemotherapeutic agent widely used for the treatment of a variety of cancers in both adult and pediatric patients. Since the Food and Drug Administration (FDA) approved cisplatin for the treatment of testicular cancer in 1978, cisplatin has been a mainstay in cancer chemotherapy despite ongoing investigations for alternate platinum-based antineoplastic agents\textsuperscript{41,42}. In adult cancer patients, cisplatin is used for the treatment of testicular, ovarian, bladder, cervical, lung, esophageal, and head/neck cancer\textsuperscript{41,43–48}. Cisplatin is particularly effective in the treatment of testicular cancer, demonstrating overall remission rates exceeding 90\%\textsuperscript{49}. In pediatric cancer patients, cisplatin is used to treat neuroblastomas, osteosarcomas, germ cell tumours, hepatoblastomas, and central nervous system tumours such as medulloblastomas\textsuperscript{50}. Cisplatin is given as monotherapy or combination therapy in conjunction with other chemotherapeutic agents. Most cisplatin treatment protocols consist of multiple cycles of chemotherapy, where cisplatin is administered intravenously at a dose of 50-100 mg/m\textsuperscript{2}/cycle and each cycle is typically 3-4 weeks in length\textsuperscript{51–55}. 
The cisplatin molecule is composed of a platinum atom complexed to two chlorine and two ammonia groups in a *cis* arrangement (*Figure 1.1*)\(^5\). Cisplatin enters cells through passive diffusion across the plasma membrane and through facilitated transport mediated by the copper transporter CTR1\(^5\). Upon entering the cell, cisplatin is activated intracellularly as one or both chloride groups are displaced by a water molecule, forming a potent and highly reactive electrophile\(^5\). Following this activation, cisplatin can bind various nucleophilic sites in the cell, including purine nucleotides, and peptides/proteins with sulfur-containing amino acids (cysteine and methionine). Cisplatin covalently binds to purine DNA nucleotides at the N7 position of the imidazole ring to form intra- and inter-strand cross-linkages, preventing DNA replication and cell division, inducing DNA damage, and ultimately leading to cell apoptosis\(^4\)\(^2\),\(^5\)\(^9\)–\(^6\)\(^1\). In addition to DNA damage, cisplatin further contributes to apoptotic signaling by inducing oxidative stress, disrupting calcium homeostasis, and inducing endoplasmic reticulum (ER) stress\(^6\)\(^0\)–\(^6\)\(^2\).

![Chemical structure of cisplatin.](image)

### 1.2.2 Pharmacokinetics of Cisplatin

Cisplatin rapidly and irreversibly binds to plasma proteins, such as albumin, transferrin, and gamma globulin\(^6\)\(^3\). Approximately 90% of platinum is protein-bound at the end of a 24-hour infusion, 2 hours after a 3-hour infusion, and 3 hours after a rapid infusion of cisplatin\(^6\)\(^4\). As such, the pharmacokinetics of cisplatin is often studied in terms of total plasma platinum, or free, unbound platinum. The pharmacokinetic parameters of cisplatin can vary widely depending on whether total or free platinum is studied. The pharmacokinetic parameters also vary based on infusion duration, sampling timepoints, and pharmacokinetic models used\(^6\)\(^5\). Measurement of total platinum typically shows bi- or tri-exponential decay, with an initial rapid decay associated with distribution followed by
a slow, prolonged terminal decay\textsuperscript{63–67}. Half-life in the initial phase ranges from 13 – 49 minutes and may be dependent on the duration of infusion\textsuperscript{63–67}. One study of rapid and prolonged infusions of cisplatin reported that half-life of total platinum was 13 min for rapid infusion of cisplatin, 40 minutes for 2 to 3-hour infusions, and 220 minutes for 24-hour infusions\textsuperscript{64}. Terminal half-life in the slow decay phase is in the order of days, ranging from 30 hours to over 200 hours\textsuperscript{63–67}. For unbound platinum, decay is bi-exponential for rapid or shorter infusion times, but mono-exponential in 24-hour infusions\textsuperscript{64,67}. For the initial decay phase, half-life of unbound platinum is comparable to total platinum, but the terminal phase is shorter for unbound platinum compared to total platinum\textsuperscript{64,67}.

The volume of distribution of cisplatin is highly variable but is generally reported to be large\textsuperscript{63,64,66,67}. Autopsies performed in patients who received cisplatin up to 6 months antemortem revealed that platinum can persist as long as 180 days after cessation of cisplatin therapy\textsuperscript{68}. Platinum concentrations were highest in the liver, prostate, and kidney, lower in bladder, muscle, testicle, pancreas, and spleen and lowest in the bowels, adrenal glands, heart, lungs, cerebrum, and cerebellum\textsuperscript{68}. Cisplatin is primarily cleared by the kidneys, through glomerular filtration and secretion. Using radio-labeled cisplatin to monitor excretion of cisplatin in the urine, it was demonstrated that 15–27\% of total platinum is excreted within the first 6 hours, 18–34\% after 24 hours, and 27–45\% after 5 days\textsuperscript{68}.

### 1.2.3 Adverse Effects of Cisplatin Therapy

Although cisplatin is widely used and effective, cisplatin therapy is often limited by adverse effects in non-cancerous tissues. Use of cisplatin is associated with numerous toxic effects, including ototoxicity, neurotoxicity, nausea and vomiting, and nephrotoxicity. These adverse effects can affect patient quality of life, result in chemotherapy dose reduction, and may necessitate delays or even cessation of chemotherapy. Cisplatin nephrotoxicity is the main focus of this dissertation and will be covered separately in section 1.2.4.
1.2.3.1 Ototoxicity

The incidence of ototoxicity following cisplatin treatment is estimated to be approximately 48% in adult patients, and up to 45-60% in pediatric patients. Ototoxicity can present as unilateral or bilateral, can be cochlear or vestibular in nature, and manifests as tinnitus, ear pain, problems in balance, and hearing loss. Hearing loss typically starts at the high-frequency range, and in the later stages can affect hearing in the lower frequency ranges. Hearing loss has a detrimental effect on patient quality of life, especially in children, where hearing loss can affect educational and social development, independence, and emotional well-being. In adults, high cumulative dose of cisplatin is the most commonly reported risk factor for ototoxicity. In children, cumulative cisplatin dose, younger age at treatment, and male sex have been reported as risk factors for ototoxicity.

Cisplatin enters the inner ear through the blood-labyrinth barrier, a specialized capillary network found in the stria vascularis. The stria vascularis is a constituent of the lateral wall of the scala media (otherwise known as the cochlear duct). Cisplatin has been shown to accumulate in inner and outer hair cells, spiral ganglion cells, and vascular striatal cells of the cochlea. Transporters CTR1 and organic cation transporter 2 (OCT2) are thought to play a role in the trafficking of cisplatin into the endolymph of the scala media or the uptake of cisplatin into hair cells, as pharmacological inhibition, or genetic knockout of CTR1 and OCT2 confers protection against cisplatin-induced ototoxicity. In vivo studies have also confirmed the expression of CTR1 and OCT2 in the cochlea, but the expression profiles in hair cells and various support cells of the cochlea have been inconsistent. Passive diffusion is also believed to play a role in the uptake of cisplatin into the various cell types of the cochlea. Further investigations are necessary to determine the uptake and trafficking mechanisms of cisplatin in the cochlea.

Cisplatin is believed to mediate its ototoxic effects through DNA damage leading to apoptosis, induction of reactive oxygen species (ROS) and depletion of antioxidant defense mechanisms, and proinflammatory signaling. DNA damage induced by cisplatin activates ataxia telangiectasia mutated (ATM), a protein kinase recruited to sites of DNA double-strand breaks, which in turn activates p53. p53 is well known to be a mediator of apoptosis, leading to the activation of the intrinsic pathway of apoptosis. Genetic ablation
or pharmacological inhibition of p53 prevented the loss of hair cells and preserved hearing in vivo\textsuperscript{90}. NADPH oxidase 3 (NOX3) is believed to be a major contributor to ROS production in the inner ear, with mRNA expression of NOX3 reported to be \textgtr 50-fold greater in the inner ear compared to any other tissue\textsuperscript{91}. NOX3 expression is up-regulated by cisplatin, and knockdown of NOX3 has been shown to protect against cisplatin-induced ototoxicity\textsuperscript{92}. Rodent studies of cisplatin-induced ototoxicity have also shown reduced cochlear expression and activity of antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase\textsuperscript{93,94}. Mitophagy is responsible for degrading damaged mitochondria and plays an important role in mitochondrial turnover. Inhibition of mitophagy decreased cell viability in HEI-OC1 mouse auditory cells treated with cisplatin, exacerbated mitochondrial dysfunction, and increased p-JNK associated apoptosis, suggesting a protective role of mitophagy against cisplatin-induced ototoxicity\textsuperscript{95}. Furthermore, enhancement of mitophagy was able to improve cell survival in cells exposed to cisplatin\textsuperscript{96}. Additionally, cisplatin increases the production of proinflammatory cytokines, such as tumour necrosis factor-\(\alpha\), interleukin-1\(\beta\) and interleukin-6 in cochlear cells\textsuperscript{97,98}. Suppression of inflammation in the cochlea has been shown to protect against ototoxicity\textsuperscript{97,98}.

1.2.3.2 Neurotoxicity

Cisplatin-induced neurotoxicity typically affects the peripheral nervous system. Cisplatin-induced peripheral neuropathy (CisPN) manifests in a “stocking-and-glove” distribution (feet/hands) as paresthesia, dysesthesia, pain/burning sensation, decreased vibratory sensitivity, and loss of tendon reflexes (e.g., ankle jerk reflex)\textsuperscript{99,100}. Prolonged exposure may lead to more severe symptoms, such as loss of proprioception and ataxic gait. The “coasting” effect is often observed in patients receiving platinum-based chemotherapy, where neuropathic symptoms can progress for months after cessation of chemotherapy\textsuperscript{101}. Recovery from cisplatin neurotoxicity is slow and potentially irreversible, with patients exhibiting symptoms years after cessation of chemotherapy\textsuperscript{102,103}. A meta-analysis of the incidence of chemotherapy-induced peripheral neuropathy estimated the incidence of peripheral neuropathy to be 42.2\% in patients receiving cisplatin-based chemotherapy regimens\textsuperscript{104}. CisPN is associated with increasing cumulative dose of cisplatin. Neuropathy
typically develops after cumulative doses of 250-350 mg/m², and nearly all patients develop neuropathic symptoms at a cumulative dose of 500-600 mg/m².\textsuperscript{101}

The dorsal root ganglion (DRG) is the primary site of cisplatin-induced neurotoxicity. Cells of the DRG are susceptible to cisplatin toxicity as they are not protected by the blood brain barrier and express organic cation transporter 2 (OCT2) and CTR1, transporters responsible for uptake of cisplatin.\textsuperscript{105–108} It is worth noting that expression of OCT2 and CTR1 are variable among subpopulations of neurons and non-neuronal cells in the DRG.\textsuperscript{109–112} Mechanisms of CisPN consist of nuclear DNA damage, induction of ROS, damage to mitochondrial DNA mediating alterations to mitochondrial function, and activation of the immune system and release of pro-inflammatory cytokines. DRG neurons accumulate high levels of platinum-DNA adducts following cisplatin treatment, causing DNA damage and leading to apoptosis.\textsuperscript{113–115} Mice deficient in nucleotide excision repair showed accelerated accumulation of cisplatin cross-linkages in the DRG and exhibited earlier onset of peripheral neuropathy symptoms, reinforcing the role of platinum-DNA adduct formation in CisPN.\textsuperscript{116} The mitochondria are another major target in the pathophysiology of CisPN. Cisplatin forms DNA adducts with mitochondrial DNA (mtDNA), inhibits mtDNA replication, mtDNA transcription, and alters mitochondrial morphology in cultured DRG.\textsuperscript{117} A separate study showed that the treatment of cultured DRG neurons with cisplatin induced apoptosis, reduced axon length, reduced the number of functioning mitochondria, caused morphological abnormalities in mitochondria, and decreased mitochondrial membrane potential.\textsuperscript{118} DRG sensory neurons exposed to cisplatin showed increased ROS production and mitochondrial stress as evidenced by reduced oxygen consumption rate, basal respiration and maximal respiratory capacity, ATP-linked respiration, and increased proton leak.\textsuperscript{119} Cisplatin also caused increased cytosolic concentrations of Ca\textsuperscript{2+} and reduced mitochondrial Ca\textsuperscript{2+} concentrations, which may result in calpain release and contribute to axonal degeneration.\textsuperscript{119} Inflammation is believed to be a key mediator in the peripheral neuropathy of platinum-based chemotherapy. In vivo studies using anti-inflammatory agents were able to alleviate cisplatin mediated neuropathy, confirming the importance of inflammation in CisPN.\textsuperscript{120,121} The non-neuronal glial cells of the peripheral nervous system, such as the satellite glial cells, contribute to
the release of pro-inflammatory cytokines following chemotherapy and play a role in modulating excitability of sensory neurons\textsuperscript{122,123}.

\subsection*{1.2.3.3 Gastrointestinal Toxicity}
Chemotherapy is associated with numerous gastrointestinal (GI) symptoms, including nausea, vomiting, diarrhea, mucositis, and constipation. Without anti-emetic prophylaxis, it is estimated that up to 80-90\% of patients receiving chemotherapy may experience nausea and vomiting. Even with anti-emetic prophylaxis, incidence of chemotherapy-induced nausea and vomiting may be 50-60\%\textsuperscript{124,125}. Chemotherapy-induced nausea and vomiting is biphasic and considered acute presentation if symptoms occur within 24 hours of chemotherapy and delayed presentation if symptoms begin at least 24 hours after chemotherapy. Emetogenic drugs are classified into four categories of increasing likelihood for causing emesis in the absence of prophylactic anti-emetics (minimal; <10\%, low; 10-30\%, moderate; 30-90\%, high; >90\%)\textsuperscript{126}. Cisplatin is categorized as “highly emetogenic” as it causes nausea and vomiting in >90\% of patients in the absence of prophylaxis\textsuperscript{126}.

Cisplatin induces acute vomiting by enhancing serotonin (5-HT) and substance P release from enterochromaffin cells in the GI tract and the brainstem. 5-HT binds to 5-HT3 receptors on vagal afferents to stimulate the vomiting reflex. These vagal afferents terminate at the nucleus tractus solitarius and area postrema, which relay information to the vomiting center in the medulla oblongata\textsuperscript{127}. The acute phase of cisplatin-induced emesis is driven primarily by the serotoninergic system, as 5-HT3 receptor antagonists are effective in inhibiting emesis in the acute phase. Historically, first generation 5-HT3 receptors antagonists such as ondansetron were ineffective in preventing emesis in the delayed phase\textsuperscript{128}. Delayed emesis is traditionally thought to be driven by the neuropeptide substance P and neurokinin-1 (NK-1) receptors, as NK-1 receptor antagonists are effective at preventing emesis in the delayed phase\textsuperscript{127}. However, recent findings suggest that 5-HT and substance P are important emetic neurotransmitters during both phases, rather than acting predominantly in a single phase\textsuperscript{128–130}. The second generation 5-HT3 receptor antagonist palonosetron has been effective in suppressing both the acute and delayed
phases, and combination of palonosetron and the NK1 receptor antagonist netupitant was more effective in the acute phase than palonosetron alone. Cisplatin also mediates gastrointestinal toxicity through direct toxicity to intestinal mucosal cells. *In vivo* studies have shown cisplatin to cause histological damage and alterations in intestinal structures (loss of crypts, villi desquamation and degeneration, reduced villi length, and immune cell infiltration), increase oxidative stress, deplete antioxidant defense mechanisms, induce mtDNA damage and mitochondrial dysfunction, decrease activity of brush border enzymes, and lead to apoptosis of intestinal epithelial cells. Administration of antioxidant compounds protected against the effects of cisplatin, once again demonstrating the crucial role of oxidative stress in the toxic mechanism of cisplatin. Cisplatin induces intestinal cytokine production, potentially by enhancing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mediated inflammation. Cisplatin induced the hyper-activation of poly [ADP-ribose] polymerase 1 (PARP1), leading to depletion of nicotinamide adenine dinucleotide (NAD+) and decreased activity of the NAD+-dependent deacetylase SIRT1. Decreased SIRT1 activity resulted in the loss of SIRT1-mediated NF-κB p65 deacetylation, and consequently activated NF-κB p65. NF-κB is well documented to upregulate the expression of pro-inflammatory cytokines. Cisplatin was also shown to activate the NLR family pyrin domain containing 3 (NLRP3) inflammasome, a crucial component of the innate immune system that can contribute to inflammatory disorders when dysregulated.

### 1.2.4 Cisplatin Nephrotoxicity

#### 1.2.4.1 Presentation of Cisplatin Nephrotoxicity and Risk Factors

Cisplatin nephrotoxicity manifests clinically in several ways, such as acute kidney injury, renal wasting of electrolytes such as magnesium, potassium, phosphate, calcium, and sodium, defects in renal concentration, anemia, polyuria, and transient proteinuria. Acute kidney injury is a common and serious manifestation of nephrotoxicity, affecting roughly one-third of patients receiving cisplatin chemotherapy. Incidence of cisplatin-induced AKI may be higher in pediatric patients, with a recent prospective study of 159 children
reporting 46% of participants developed AKI during cisplatin chemotherapy. Electrolyte disturbances are also very common with cisplatin therapy; of the electrolyte disturbances, hypomagnesemia is the most common with reported incidence rates ranging from 56-90%\(^\text{141}\). Hyponatremia (43-59%), hypokalemia (27%), hypophosphatemia (10-77%), and hypocalcemia (6-20%) also commonly present in cisplatin-induced nephrotoxicity\(^\text{141}\). Cisplatin-induced AKI is often non-oliguric\(^\text{41}\); therefore, cisplatin-induced AKI is generally diagnosed solely based on increased SCr concentrations.

The risk factors for cisplatin-induced AKI are not well defined, and studies investigating risk factors have typically been conducted in relatively small studies. Previously reported risk factors of cisplatin-induced AKI in adults include high dose, number of cycles, cardiac disease, hypertension, higher baseline creatinine, smoking, old age, female sex, African American race, hypoalbuminemia, and increased hydration and magnesium replacement requirements\(^\text{141–149}\). In children, high dose, younger (≤3 years of age) and older age (≥15 years of age), short infusion duration, higher peak plasma platinum levels, and high body mass index have been reported as potential risk factors of cisplatin-induced AKI\(^\text{50,150–155}\).

1.2.4.2 Mechanisms of Cisplatin Nephrotoxicity

1.2.4.2.1 Cisplatin Accumulation, Uptake, and Biotransformation in the Kidney

Cisplatin is primarily cleared by the kidneys, through both tubular secretion and glomerular filtration\(^\text{41}\). Renal excretion of cisplatin, coupled with the innate tendency of the kidneys to concentrate its filtrate, leads to the accumulation of cisplatin in renal tubule cells\(^\text{156}\). Proximal tubule cells are major sites of accumulation and are particularly prone to cisplatin-mediated injury (\textbf{Figure 1}). In addition to DNA, mitochondria have more recently emerged as another critical target of cisplatin toxicity\(^\text{41}\). Sensitivity of cells to cisplatin-induced cell death has been correlated to the density of mitochondria\(^\text{157}\), and proximal tubule cells possess high mitochondrial density relative to the rest of the kidney\(^\text{41}\). The accumulation of cisplatin and abundance of mitochondria within proximal tubule cells may explain their increased sensitivity to cisplatin-mediated injury.
Figure 1.2 Overview of proposed mechanisms of cisplatin accumulation, biotransformation, and cell toxicity in renal tubular epithelial cells. Image was created using BioRender.com.
The uptake of cisplatin into renal tubular epithelial cells and subsequent biotransformation are depicted and summarized in **Figure 1.2**. Two basolateral membrane transporters have been established as facilitators of cisplatin uptake into kidney cells: CTR1 and OCT2. Inhibition of CTR1 and OCT2 *in vitro* and *in vivo* is associated with decreased uptake of cisplatin and reduced cisplatin-induced toxicity. The rs316019 single nucleotide polymorphism (SNP) on chromosome 6 (808G>T) is associated with decreased OCT2 transport and has been shown to confer protection against cisplatin-induced nephrotoxicity. Passive diffusion also plays a role in entry of cisplatin into the tubular epithelial cells. It has been suggested that cisplatin undergoes biotransformation to a more potent nephrotoxin upon entry into renal tubular epithelial cells. Through the actions of glutathione-S-transferase (GST), cisplatin is glutathionylated in circulation or within the kidneys themselves. As these glutathione-cisplatin conjugates are effluxed into the renal tubular lumen, they are cleaved by apical membrane-bound gamma glutamyl transpeptidases (GGT) into cysteinyl-glycine-conjugates. Aminopeptidase N (AP), also found on the tubular apical membrane, further metabolizes cysteinyl-glycine-conjugates to cysteine-conjugates. The cysteine-conjugates may subsequently be transported back into the renal tubular epithelial cell, where they are metabolized once more to a highly reactive thiol (SH) conjugate by cysteine-S-conjugate beta lyases. These reactive thiol-cisplatin conjugates have been shown to be more nephrotoxic than the parent cisplatin compound. It has recently been demonstrated that basolateral organic anion transporters 1 and 3 (OAT1/3) also contribute to cisplatin-induced nephrotoxicity. Mice deficient in Oat1 and Oat3 were protected from cisplatin-mediated kidney injury compared to wildtype mice. The authors demonstrated that OAT1/3 facilitate the uptake of a cisplatin-N-acetylcysteine (NAC) conjugate into proximal tubular cells. Cisplatin-NAC conjugate compounds can be deacetylated into the aforementioned cysteine-cisplatin conjugate, and subsequently metabolized to toxic reactive thiols.

Efflux of cisplatin into the urine is mediated by the transporters multi-drug and toxin extrusion protein 1 (MATE1) and multidrug resistance-associated protein 2 (MRP2). MATE1 is a proton antiporter on the apical membrane of tubular epithelial cells that mediates the efflux of organic cations in the kidneys. Cisplatin has been demonstrated *in vitro* to be a substrate of MATE1 which mediates cisplatin efflux out of proximal tubule...
epithelial cells\textsuperscript{168}. MATE1 deficient mice exhibit greater accumulation of cisplatin in the plasma and kidneys and increased kidney injury compared to wildtype mice\textsuperscript{169}. Similar results have been observed following pharmacological inhibition of MATE1 using ondansetron\textsuperscript{170}. MRP2 is also found on the apical membrane and primarily transports organic anions, including drug conjugates (particularly glutathione conjugates). A study using Mrp2 deficient mice showed greater renal and hepatic accumulation of platinum and increased proximal tubule injury in Mrp2 deficient mice compared to wildtype\textsuperscript{171}.

\subsection*{1.2.4.2.2 Cisplatin-mediated DNA Damage in Nephrotoxicity}

Cisplatin has been shown to bind DNA in proximal tubule cells, causing structural alterations, damaging DNA, and leading to activation of DNA damage repair mechanisms\textsuperscript{172–174}. The sensor proteins ATM and ataxia telangiectasia and Rad3 related protein (ATR) are recruited to the site of DNA damage and activated\textsuperscript{175}. Upon activation, ATM and ATR phosphorylate downstream targets to amplify the DNA damage response, including checkpoint kinase 1 and 2 (CHK1 and CHK2)\textsuperscript{175}. CHK1 and CHK2 phosphorylate downstream mediators of DNA repair, cell cycle arrest, and apoptosis\textsuperscript{175}. One of the downstream targets of CHK1 and CHK2 is the tumour suppressor p53. Activation of p53 has been shown to induce mitochondrial outer membrane permeabilization by up-regulating transcription of pro-apoptotic proteins (such as p53-upregulated modulator of apoptosis, PUMA) and leading to accumulation and oligomerization of BAK and BAX on the outer mitochondrial membrane\textsuperscript{174,176}. Permeabilization of the outer mitochondrial membrane facilitates the release of pro-apoptotic factors from the mitochondria, leads to activation of caspases, and subsequently results in apoptosis\textsuperscript{176}. In both cultured proximal tubule cells and mouse kidneys, cisplatin has been shown to activate ATR and CHK2, leading to p53 phosphorylation\textsuperscript{174}. Inhibition of ATR or CHK2 \textit{in vitro} was able to attenuate p53 activation and apoptosis\textsuperscript{174}. In contrast, a study using proximal tubule specific deletion of Atr in mice showed that mice deficient in proximal tubule Atr were more sensitive to cisplatin-induced nephrotoxicity, exhibiting greater functional impairment, DNA damage and apoptosis compared to wildtype mice\textsuperscript{177}. A potential explanation for this discrepancy may be that the role of ATR-mediated DNA damage response may be dependent on the extent of DNA damage. The ATR-mediated
DNA damage response may facilitate DNA repair in situations of non-lethal DNA damage, but in severe DNA damage, may result in apoptosis instead\textsuperscript{176}.

**1.2.4.2.3 Role of Oxidative Stress in Nephrotoxicity**

Oxidative stress is believed to play a crucial role in the nephrotoxicity of cisplatin. Many \textit{in vitro} and pre-clinical \textit{in vivo} studies have shown increased generation of ROS following cisplatin administration. Cisplatin depletes thiol containing antioxidants such as glutathione and reduces expression and activity of antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione S-transferase\textsuperscript{178–180}. Cisplatin is also associated with mitochondrial dysfunction, leading to excess mitochondrial ROS production. A number of \textit{in vitro} and pre-clinical \textit{in vivo} studies have previously demonstrated increased ROS production specifically in the mitochondria\textsuperscript{181–184}. Mitochondrial injury is one of the main mechanisms of cisplatin-induced nephrotoxicity and will be discussed in further detail in the next section. Additionally, cisplatin induces ROS producing enzymes, such as NADPH oxidase 2 and 4 (NOX2 and NOX4) and interacts with cytochrome P450 (CYP) 2E1 to increase ROS production\textsuperscript{185}. mRNA expression of NOX2 and NOX4 were increased in mouse kidneys as early as 24 hours after cisplatin treatment and coincided with leukocyte infiltration in the kidneys\textsuperscript{186}. The NOX2 isoform is expressed in phagocytes and coupled with the coinciding observation of infiltration of immune cells, the authors proposed a potential inflammation-mediated secondary wave of ROS generation\textsuperscript{186,187}. In HK-2 cells and in mice, knockdown of NOX4 protected against cisplatin-induced nephrotoxicity and reduced the level of inflammatory cytokines\textsuperscript{188}. Cisplatin has also been shown to downregulate nuclear factor-erythroid-2 p45-related factor-2 (NRF2), a transcription factor that controls the transcription of antioxidant genes by binding to the antioxidant-response element (ARE)\textsuperscript{189,190}.

**1.2.4.2.4 Cisplatin and Mitochondrial Dysfunction**

Mitochondria have emerged as a major target of cisplatin toxicity in the kidneys. As mentioned previously, proximal tubule cells contain high concentrations of mitochondria to facilitate transport of electrolytes; the high density of mitochondria in proximal tubule
cells is believed to be one of the reasons for the sensitivity of proximal tubule cells to cisplatin-mediated damage. Cisplatin has been shown to induce morphological alterations in mitochondria in cultured proximal tubule cells as well as in mouse kidneys. Morphological changes include reduction of mitochondrial mass, disruption of mitochondrial cristae, mitochondrial swelling, and mitochondrial fragmentation. Cisplatin has been shown to accumulate in mitochondria, and preferentially bind mitochondrial DNA (mtDNA) compared to nuclear DNA. mtDNA is more susceptible to damage due to lack of histones and proximity to sites of ROS generation. Cisplatin has been shown to reduce mtDNA content in mouse kidneys and increase markers of mtDNA damage in HK-2 cells. In A549 cells (adenocarcinomic alveolar basal epithelial cells), cisplatin decreased the mRNA and protein expression of mitochondrial-encoded cytochrome c oxidase subunit 1 (MT-CO1) and increased ROS production. Carboplatin did not significantly reduce expression of MT-CO1 and did not induce ROS, suggesting that inhibition of mtDNA transcription is an important contributor to the mitochondrial toxicity of cisplatin.

Mitochondrial dysfunction is observed following cisplatin treatment in vitro and in mouse models. Cisplatin reduces the expression and activity of mitochondrial electron transport complexes and has been shown to decrease mitochondrial membrane potential. In cultured mouse proximal tubular epithelial cells, cisplatin decreases mitochondrial membrane potential, oxygen consumption rate, mtDNA copy number, and increases ROS production. These findings were corroborated in kidneys collected from mice treated with cisplatin, with the addition of decreased complex I activity and cytochrome b expression. The mitochondrial dysfunction induced by cisplatin was mediated by microRNA-709 (miR-709), a microRNA found to be elevated in renal tubule cells exposed with cisplatin and in renal biopsies taken from AKI patients. miR-709 was found to contribute to mitochondrial dysfunction by inhibiting mitochondrial transcriptional factor A (TFAM), a transcription factor that plays a crucial role in biosynthesis of mtDNA, mitochondrial biogenesis and overall mitochondrial function. Further in line with dysregulation of mitochondrial biogenesis, cisplatin has been demonstrated to downregulate peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1α), a key regulator of mitochondrial biogenesis. Active PGC-1α translocates into
the nucleus to activate nuclear respiratory factor 1 (NRF1) and NRF2, which in turn promote the transcription of nuclear-coded genes for mitochondrial electron transport chain components and activates expression of TFAM\textsuperscript{200,201}.

Mitochondrial biogenesis and mitophagy (mitochondria specific autophagy) work in conjunction to maintain mitochondrial turnover and homeostasis. While biogenesis is responsible for generation of new mitochondria, mitophagy is responsible for eliminating damaged or dysfunctional mitochondria. Recent studies have demonstrated that inhibition of mitophagy exacerbates cisplatin-induced mitochondrial dysfunction. HK-2 cells treated with cisplatin exhibited increased mitophagy. Inhibition of autophagy resulted in increased sensitivity to cisplatin cytotoxicity and exacerbated cisplatin-induced mitochondrial dysfunction, increasing ROS production, reducing mitochondrial membrane potential, and further reducing ATP production\textsuperscript{202}. On the contrary, pharmacological enhancement of mitophagy decreased cisplatin-mediated apoptosis and improved mitochondrial function\textsuperscript{202}. These findings were consistent in mouse kidneys, where cisplatin treatment induced mitophagy and enhancement of mitophagy was associated with protection against nephrotoxicity\textsuperscript{203}. Furthermore, knockout of Parkin and PTEN-induced kinase 1, important contributors in the priming of mitochondria for mitophagy, aggravated cisplatin nephrotoxicity\textsuperscript{203}. However, other studies have shown that cisplatin inhibits mitophagy, and decreases expression of transcription factor EB (TFEB), an important regulator of lysosomal biogenesis and mitophagy\textsuperscript{204,205}. Further research needs to be conducted to elucidate the effects of cisplatin on mitophagy, but current evidence indicates the importance of mitophagy in cisplatin nephrotoxicity.

1.2.4.2.5 Cisplatin-induced Endoplasmic Reticulum Stress

Endoplasmic reticulum (ER) stress is characterized by the accumulation of unfolded/misfolded proteins inside of the ER lumen. The unfolded protein response (UPR) is a signaling pathway that can reprogram gene transcription, protein translation, and protein modification to relieve the load of unfolded or misfolded proteins and re-establish ER homeostasis. However, in situations of prolonged ER stress, the UPR can lead to apoptosis. There are three transmembrane sensors of ER stress: inositol-requiring enzyme 1α (IRE1α), protein kinase R-like ER kinase (PERK), and activating transcription factor 1α (ATF1α).
Under normal conditions, the stress sensors are inactivated through physical interaction with 78-kDa glucose-regulated protein (GRP78), also known as binding immunoglobulin protein (BiP). During ER stress, GRP78 dissociates from the ER stress sensors, leading to their activation and subsequent activation of the UPR. Once activated, PERK phosphorylates eukaryotic initiator factor 2α (eIF2α), which initiates the translation of ATF4. ATF4 is a transcription factor that among multiple functions, induces expression of CCAAT/enhancer-binding protein homologous protein (CHOP), a proapoptotic factor. Activation of IRE1α leads to the mRNA splicing of the transcript encoding X-box binding protein 1 (XBP1), allowing for its expression. XBP1 upregulates UPR-associated genes that encode ER chaperones, folding enzymes, and components of ER-associated protein degradation (ERAD), a process where misfolded proteins in the ER are transported back into the cytosol and polyubiquitinated to be degraded.

Cisplatin has been demonstrated to induce ER stress in mouse kidney tissue, inducing morphological abnormalities in the ER of proximal tubular epithelial cells, upregulating phosphorylated-PERK, and increasing expression of GRP78, PERK, IRE1α, ATF6, and their downstream targets ATF4, XBP1, and CHOP. The cisplatin-mediated induction of ER stress was further confirmed in HK-2 cells. A separate study corroborated the presence of ER stress markers following cisplatin treatment, in both cultured mouse proximal tubule cells and mouse kidney tissue. Cisplatin has also been shown to activate caspase-12, which is localized to the ER and activated in ER stress conditions. Caspase-12 activates caspase-9 independent of cytochrome-c release and leads to subsequent activation of caspase-3 and apoptosis.

1.2.4.2.6 Cisplatin-mediated Inflammation in Nephrotoxicity

The association between cisplatin and inflammation is well documented, with in vitro studies and animal models demonstrating that the inflammatory response plays an important role in cisplatin-induced nephrotoxicity. Current evidence has implicated the importance of toll-like receptor (TLR)-mediated signaling in cisplatin-induced inflammation. TLRs are transmembrane pattern recognition receptors that facilitate host surveillance of pathogenic and endogenous molecular signatures. TLRs are composed of three components; an intracellular C-terminal toll/interleukin-1 receptor domain that
mediates signal transduction upon receptor interaction with activating ligands, a central helix domain that spans the plasma membrane, and an ectodomain that extends into extracellular space and interacts with a broad range of ligands. TLRs recognize and respond to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). While PAMPs are expressed on pathogens, DAMPs are endogenous molecules released in scenarios of cell stress, damage, and death, and can be present in both the presence and absence of pathogenic infection. DAMPs include extracellular matrix components, components of the plasma membrane, various intracellular proteins, and molecules released by damaged or fragmented organelles. Upon activation of TLRs, two distinct signaling pathways are initiated. The myeloid differentiation primary response 88 (MyD88)-dependent pathway is common to all TLRs except for TLR3 and leads to upregulation of mitogen-activated protein kinases (MAPKs) such as JNK and p38, activation of pro-inflammatory transcription factors such as AP-1 and NF-κB, and transcription of pro-inflammatory cytokines. TLR3 and TLR4 signal through a toll/IL-1R domain-containing adaptor-inducing interferon-β-mediated pathway, which leads to activation of interferon regulatory factor 3, a transcription factor that regulates expression of type 1 interferons176,214,215.

TLR4 signaling is required for the full manifestation of the cisplatin-mediated inflammatory response, and the full presentation of cisplatin nephrotoxicity. TLR4 deficient mice are less susceptible to cisplatin-induced nephrotoxicity, showing less functional impairment and histological injury, and decreased leukocyte infiltration after cisplatin administration216. Cisplatin-treated mice showed strong upregulation in the expression of cytokines, chemokines, and adhesion molecules in the kidneys compared to saline-treated controls. The upregulation of cytokines TNF-α, IL-10, and IL-18, chemokines MCP-1, KC, and IP-10, and adhesion molecule ICAM-1, was dependent on TLR4, as gene expression was not increased in TLR4 deficient mice. Cisplatin treatment increased concentrations of TNF-α, IL-6, IL-10, and IL-1β in the serum of wildtype mice, but concentrations were lower in the TLR4 deficient mice. Furthermore, cisplatin increased concentrations of TNF-α, IL-6, MCP-1, KC, and IP-10 in the kidneys, and TNF-α, IL-6, IL-2, MCP-1, KC, IP-10, and RANTES in the urine. In the TLR4 deficient mice, these changes were absent. Activation of MAPKs p38 and JNK were also dependent on TLR4,
as activity of p38 and JNK were shown to be significantly lower in cisplatin treated TLR4 deficient mice compared to wildtype\textsuperscript{216}. A separate study of TLR4 knockout mice confirmed the importance of TLR4 in the inflammatory response following cisplatin treatment; cisplatin increased serum concentrations of many proinflammatory cytokines in wildtype mice, which was alleviated in TLR4 knockout mice\textsuperscript{217}. Interestingly, recent studies have proposed that cisplatin may bind directly to TLR4 to activate TLR4 signaling\textsuperscript{218,219}.

Contrary to TLR4, TLR2 and TLR9 are reported to be protective against cisplatin nephrotoxicity. Knockout of TLR2 in mice was shown to exacerbate impairment of kidney function, histological injury, and infiltration of immune cells compared to wildtype mice. Cisplatin was found to decrease expression of autophagy-related genes in the kidneys, which was exacerbated further in TLR2 deficient mice. These results were confirmed in cultured renal tubular cells isolated from TLR2 deficient mice\textsuperscript{217}. The protective effect of TLR2 was observed in another study utilizing TLR2 deficient mice, where TLR2 deficient mice exhibited impaired autophagy when given cisplatin, compared to wildtype mice. Inhibition of autophagy was also evident in TLR2 knockdown in cultured renal tubular epithelial cells\textsuperscript{220}. The authors also demonstrated potential crosstalk between TLR2 and the PI3K/Akt pathway which promotes cell survival, proliferation, and growth. Inhibition of TLR2 decreased activation of the PI3K/Akt pathway, whereas activation of TLR2 increased activation\textsuperscript{220}. The protective role of autophagy against cisplatin-induced nephrotoxicity has previously been established in mice and cultured renal tubular epithelial cells, and induction of autophagy by TLR2 may mediate its protective role in cisplatin nephrotoxicity\textsuperscript{221–223}. Like TLR2, TLR9 deficient mice show increased kidney injury upon treatment with cisplatin. TLR9 knockout mice additionally exhibited decreased recruitment of regulatory T cells (Treg) in the kidneys compared to wildtype mice. Treg cells act to suppress the immune response. The authors found that Treg cells maintained their functionality in the absence of TLR9 but had reduced expression of adhesion molecules important in trafficking of Treg cells into the kidneys. TLR9 is therefore proposed to mediate protection against cisplatin nephrotoxicity by regulating the recruitment of Treg cells to the kidney\textsuperscript{224}. 
As previously described, cisplatin induces the expression of a wide array of cytokines and chemokines; among these, tumor necrosis factor α (TNF-α) is an important mediator of cisplatin nephrotoxicity. Renal expression of TNF-α is increased in cisplatin-treated mice and inhibition of TNF-α attenuates cisplatin-induced renal impairment and kidney injury. TNF-α binds to TNF receptors TNFR1 and TNFR2 to mediate inflammation and apoptosis. TNFR1 and TNFR2 signaling leads to activation of NF-κB and MAPK, stimulating transcription of pro-inflammatory cytokines and pro-survival genes. TNFR1 activation can further exacerbate cytotoxicity and inflammation by its ability to induce cell death in the form of apoptosis (extrinsic pathway) and necroptosis\textsuperscript{225,226}. Necroptotic tubular cell death results in DAMP release from dying cells, leading to increased TLR signaling and exacerbation of the inflammatory response\textsuperscript{226}. Cisplatin is reported to facilitate necroptosis both through TNF-α dependent and independent pathways, as cells deficient in TNFR1 show complete mitigation of TNF-α induced necroptosis, but only a partial alleviation of cisplatin-induced necroptosis\textsuperscript{227}. Although a combination of the cytokines TNF-α, TNF-related weak inducer of apoptosis, and IFN-γ was demonstrated to induce necroptosis in cultured proximal tubule cells, cisplatin-induced necroptosis was not mitigated by neutralization of TNF-α or blockage of cytokine secretion, further supporting the notion that cisplatin induces necroptosis both through cytokine and non-cytokine mediated pathways\textsuperscript{228}. TNFR1 forms a complex with receptor-interacting protein kinase (RIP) 1 and RIP3, and mixed lineage kinase domain-like protein (MLKL), to initiate necroptosis. Cisplatin-treated mice deficient in RIP3 and MLKL show a reduced upregulation in inflammatory cytokine expression compared to wildtype mice treated with cisplatin, indicating that necroptosis enhances the inflammatory response associated with cisplatin\textsuperscript{228}. Thus, the pathogenesis of cisplatin-induced nephrotoxicity may involve a positive feedback loop between necroptosis and necroptosis-triggered inflammation\textsuperscript{228}.

1.2.4.3 Therapeutic Interventions Against Cisplatin-Induced AKI

Currently, management of cisplatin-induced AKI involves careful monitoring of patients to ensure maintenance of hydration status, serum electrolyte levels, and urine output. If necessary, dose adjustments may be made, or even discontinuation of cisplatin may be necessary\textsuperscript{229–231}. Aggressive hydration is maintained with saline infusion prior to and
following cisplatin administration, to minimize the duration of cisplatin exposure in the kidneys. Use of diuretics such as mannitol has become standard in the management of cisplatin-induced AKI to further increase urine output, minimize transit time of cisplatin in the tubules, and enhance cisplatin excretion, but the benefits of diuretic use are controversial\textsuperscript{229,232}. Electrolyte disturbances are managed with appropriate supplementation of electrolytes. Hypomagnesemia is especially common in cisplatin-induced AKI, and careful monitoring is required to ensure proper magnesium levels are maintained\textsuperscript{229,230}. Despite the prevention strategies employed, cisplatin-induced AKI is still common; there is a need for more effective therapeutic interventions against cisplatin-induced AKI.

With advances in our understanding of the molecular mechanisms of cisplatin-induced nephrotoxicity, several potential pathways have emerged as targets for nephroprotective interventions. Though many studies have investigated potential therapeutic agents to alleviate cisplatin-induced nephrotoxicity, there is currently no therapy for the prevention or treatment of AKI. Various pharmacological, molecular, and genetic interventions against cisplatin-induced nephrotoxicity have been investigated in pre-clinical models; these approaches target the molecular pathways involved in mediating cisplatin-induced nephrotoxicity\textsuperscript{176,231}. Based on the current knowledge of the nephrotoxic mechanisms of cisplatin, studies have aimed to reduce cisplatin uptake and biotransformation, inhibit oxidative stress by inhibiting ROS production and supplementation of antioxidant compounds, inhibit cell death by targeting apoptotic or necroptotic pathways, enhance mitochondrial biogenesis and metabolism, induce autophagy/mitophagy, and alleviate inflammation\textsuperscript{176,231}. Although these therapeutic approaches show promise in mitigating nephrotoxicity in the pre-clinical setting, it is important to recognize that many of the nephrotoxic mechanisms of cisplatin overlap with its antineoplastic mechanisms. It is therefore critical that renoprotective interventions are designed to target only the kidneys or work via a mechanism that prevents cytotoxicity only in non-cancerous tissues. Additionally, pre-clinical studies should evaluate the effects of therapeutic interventions in tumour-bearing animals to verify protection against nephrotoxicity without compromising tumour killing activity.
1.2.4.4 Biomarkers of AKI

As discussed in section 1.1.1, the current biomarkers for AKI diagnosis are suboptimal. SCr has low specificity and low sensitivity and cannot diagnose AKI until there is a functional decrease in kidney function. Even a mild impairment of kidney function, as indicated by small increases SCr, are associated with serious clinical outcomes; it is therefore imperative to identify high risk patients before kidney injury occurs and to diagnose kidney injury as early as possible for interventions to be effective. AKI biomarkers fall into three categories: 1) **filtration markers** that inform about changes in glomerular filtration (e.g., SCr and cystatin c); 2) **damage markers** that are released by the damaged kidneys or upregulated in response to injury (e.g., kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin); 3) **stress markers** that indicate a state of cell stress, which may progress to further damage or resolve (e.g., tissue inhibitor of metalloproteinase 2 and insulin-like growth factor-binding protein 7). This section will focus on kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), tissue inhibitor of metalloproteinase 2 (TIMP-2) and insulin-like growth factor-binding protein 7 (IGFBP7), as they are some of the most well-characterized biomarkers in animal and pre-clinical studies.

KIM-1 is a transmembrane glycoprotein that is expressed in the proximal tubule in response to tubular injury. In the setting of kidney injury, KIM-1 is involved with the phagocytotic removal of apoptotic cells and necrotic tissue fragments and cell repair processes following cell injury. Initial rodent studies demonstrated that KIM-1 exhibits minimal expression in healthy kidneys, but KIM-1 expression is significantly upregulated following ischemic or drug-induced kidney injury. Expression of KIM-1 was further confirmed in kidney biopsies taken from patients with confirmed acute tubular necrosis, and in the urine of patients with ischemic acute tubular necrosis. In the setting of AKI, KIM-1 acute overexpression and shedding of the KIM-1 extracellular domain leads to increased levels of KIM-1 in blood and urine. KIM-1 is elevated in the early stages of kidney injury, prior to the rise in SCr. Though KIM-1 has been extensively studied, the diagnostic performance of KIM-1 as a biomarker of AKI is variable. A systematic review of 14 studies of adult patients in various hospital settings (e.g., post-cardiac surgery, ICU,
general hospital, coronary angiography, etc.) illustrated the variability of KIM-1 performance as a diagnostic marker\textsuperscript{236}. Studies included in the systematic review reported the sensitivity of KIM-1 to range between 0.40–1.00, and specificity to range between 0.52–1. The area under the receiving operating characteristic curve (AUROC) is used to summarize overall diagnostic performance, where an AUROC value of 0.50 indicates that the diagnostic performance is no better than chance alone, and an AUROC of 1 represents perfect binary classification. Reported AUROC values for KIM-1 ranged from 0.35–0.95. Pooled sensitivity and specificity for the 14 included studies were 0.74 and 0.84, respectively, and summary AUROC was 0.62\textsuperscript{236}. The timepoint of KIM-1 measurement varied widely in these studies, which may partly explain the variability in diagnostic performance. In a prospective study of 150 septic patients, diagnostic performance was highly variable based on timing of KIM-1 measurement, showing the importance of the timepoint at which KIM-1 is measured\textsuperscript{237}. Fewer studies have investigated the utility of KIM-1 specifically in cisplatin-induced AKI. Reported AUROC values for KIM-1 24 hours after cisplatin infusion range from 0.78–0.94, indicating moderate to excellent performance; however, sample sizes were small in these studies and more work is necessary to assess the utility of KIM-1 in cisplatin-induced AKI\textsuperscript{238–240}. Very few studies have explored KIM-1 in pediatric patients treated with cisplatin. In a study of 64 children receiving platinum-based chemotherapy or methotrexate, the performance of KIM-1 was lowest 24 hours after cisplatin (AUROC of 0.71), and highest after 1 week (AUROC of 0.82)\textsuperscript{241}. A pan-Canadian prospective study of 159 children receiving cisplatin chemotherapy showed that the AUROC for discrimination of AKI ranged from 0.48–0.75 depending on the timepoint of sample collection and the cisplatin cycle (early cycle or late)\textsuperscript{242}.

NGAL is a protein secreted by active neutrophils, but also expressed in a number of tissues, including the kidneys. NGAL plays an anti-microbial role in the innate immune system by preventing bacteria from obtaining iron and is involved with regulation of iron in the body\textsuperscript{243}. Initial rodent studies showed a marked induction of renal mRNA and protein expression of NGAL following ischemic insult as early as 3 hours following ischemic injury, and NGAL was detectable in the urine within 2–3 hours of ischemic insult\textsuperscript{244}. These findings were further confirmed in a mouse model of cisplatin nephrotoxicity\textsuperscript{245}. Though
initially believed to originate from the proximal tubule, it is now known that NGAL is upregulated in the distal portions of the nephron upon kidney injury, such as the ascending loop of Henle and collecting duct. Normally, NGAL is almost completely reabsorbed by endocytosis in the proximal tubule; however, upon proximal tubular damage, reabsorption is impaired. Upregulation and impairment of reabsorption leads to excretion of NGAL in the urine\textsuperscript{243,246}. The first study to assess NGAL as a predictor of AKI was conducted in a cohort of children undergoing cardiopulmonary bypass. In this study, urine NGAL measured 2 hours after the procedure was reported to predict AKI with an AUROC of 0.998. Since then, many studies have investigated the predictive performance of NGAL in both single- and multi-center studies in cardiac surgery and ICU settings, to variable results\textsuperscript{247}. Part of the variability in results may be attributed to the non-renal release of NGAL that occurs in conditions of systemic inflammation; studies have shown that NGAL levels are elevated after cardiac surgery in the absence of postoperative AKI, as well as in septic patients without renal impairment\textsuperscript{247}. A recent systematic review and meta-analysis was conducted using 110 studies reporting on the assessment of AKI biomarkers in adult patients in the hospital setting\textsuperscript{248}. Urine NGAL had the best diagnostic accuracy in the whole population, exhibiting the highest diagnostic odds ratios regardless of the absence or presence of urinary creatinine adjustment. Combined sensitivity and specificity of urine NGAL was 0.768 and 0.807, respectively, whereas sensitivity and specificity of urine NGAL/Cr was 0.716 and 0.865, respectively. Summary AUROC values for urine NGAL and urine NGAL/Cr were 0.852 and 0.914, respectively\textsuperscript{248}. In a meta-analysis of 53 studies conducted in hospitalized children, the combined specificity and sensitivity of urine NGAL for the prediction of AKI were 0.83 and 0.81, respectively, and summary AUROC was 0.89\textsuperscript{249}. Measurement time was an important factor in this study, as the performance of NGAL was observed to vary based on time of sample collection\textsuperscript{249}. Few studies have evaluated NGAL specifically in cisplatin-induced AKI. Urine NGAL shows variable performance in the discrimination of cisplatin-induced AKI, with reported AUROC values ranging from 0.61–0.87\textsuperscript{250–254}. Studies that measured NGAL within 24 hours of cisplatin infusion showed better performance compared to studies that measured NGAL at ≥ 3 days following cisplatin. In the same pan-Canadian cohort of 159 children mentioned above in
the KIM-1 section, the AUROC for discrimination of AKI using NGAL ranged from 0.56–0.72\textsuperscript{242}.

TIMP-2 and IGFBP7 are inducers of G1 cell cycle arrest produced in the early stages of cell injury\textsuperscript{255}. TIMP-2 and IGFBP7 were first identified as part of a discovery study to identify novel early protein biomarkers for AKI\textsuperscript{255}. The discovery study included 522 critically ill adults and characterized the performance of 340 protein biomarkers. TIMP-2 and IGFBP7 exhibited the best performance in the prediction of kidney injury within 12 to 36 hours of sample collection (AUROC of 0.75 and 0.77, respectively). The combination of TIMP-2 and IGFBP7 ([TIMP-2]•[IGFBP7]) was also found to add predictive value\textsuperscript{255}. Accordingly, the performance of TIMP-2 and IGFBP7 was further evaluated in a validation cohort of 744 adults\textsuperscript{255}. For the development of stage 2 or 3 KDIGO AKI within 12 hours of sample collection, TIMP-2 and IGFBP7 exhibited AUROC values of 0.79 and 0.76, respectively, while [TIMP-2]•[IGFBP7] yielded an AUROC of 0.80. When combined with other clinical variables, [TIMP-2]•[IGFBP7] improved risk prediction for stage 2 or 3 KDIGO AKI within 12 hours of sample collection\textsuperscript{255}. In a follow-up multicenter study with 420 critically ill adult patients, the AUROC for discriminating AKI and no AKI patients within 12 hours of sample collection was 0.82 when using [TIMP-2]•[IGFBP7]\textsuperscript{256}. The combination of [TIMP-2]•[IGFBP7] with clinical variables further improved risk prediction, yielding an AUROC of 0.86\textsuperscript{256}. These promising results led to FDA approval of a test of [TIMP-2]•[IGFBP7] to be marketed under the brand name Nephrocheck, to aid in risk assessment for moderate of severe AKI. Nephrocheck multiplies the concentration of the two biomarkers together and divides the result by 1000 to generate a single result in units of (ng/mL)\textsuperscript{2}/1000\textsuperscript{257}. A cutoff of > 0.3 and ≤ 2.0 (ng/mL)\textsuperscript{2}/1000 is used to indicate moderate risk for moderate/severe AKI, as [TIMP-2]•[IGFBP7] > 0.3 had a sensitivity of 0.92\textsuperscript{256}. It is worth noting that specificity using the >0.3 cutoff was 0.46, indicating the potential for frequent false positives\textsuperscript{256}. A result > 2.0 is associated with high risk for developing moderate/severe AKI. High urinary bilirubin and albumin interfere with Nephrocheck; as such, special care must be taken in interpreting results in patients with comorbidities leading to proteinuria\textsuperscript{257}. In pediatric patients, [TIMP-2]•[IGFBP7] was first used to predict AKI in children undergoing cardiac surgery, where urine levels of [TIMP-2]•[IGFBP7] measured 4 hours after surgery exhibited an AUROC of 0.85, with sensitivity
and specificity of 0.83 and 0.77, respectively\(^{258}\). In children undergoing liver transplantation, AUROC was 0.933 when \([\text{TIMP-2}] \cdot [\text{IGFBP7}]\) was measured within 6 hours of transplant\(^{259}\). In 237 neonates admitted to the neonatal ICU, an AUROC of 0.71 was observed when urine \([\text{TIMP-2}] \cdot [\text{IGFBP7}]\) was measured 0–5 days prior to AKI diagnosis\(^{260}\). Few studies of \([\text{TIMP-2}] \cdot [\text{IGFBP7}]\) have been conducted in the setting of cisplatin-induced AKI. In a study of 32 adults receiving cisplatin or carboplatin, \([\text{TIMP-2}] \cdot [\text{IGFBP7}]\) measured within 12 hours following platinum chemotherapy was predictive of AKI with an AUROC of 0.92\(^{261}\). However, in a study of lung cancer patients receiving cisplatin, urine \([\text{TIMP-2}] \cdot [\text{IGFBP7}]\) measured within 24 hours performed poorly in discriminating AKI\(^{262}\). In 156 pediatric patients, \([\text{TIMP-2}] \cdot [\text{IGFBP7}]\) showed poor to modest performance for detecting AKI following cisplatin chemotherapy\(^{263}\). This study measured TIMP-2 and IGFBP7 at two different cisplatin infusions (early vs late infusion), and measured the biomarkers prior to cisplatin infusion, morning after cisplatin infusion (~18–24 hours after infusion), and at hospital discharge. Urine \([\text{TIMP-2}] \cdot [\text{IGFBP7}]\) was a poor to modest discriminator of AKI in this pediatric cohort\(^{263}\). Based on these few studies, earlier measurement of \([\text{TIMP-2}] \cdot [\text{IGFBP7}]\) might be a better predictor of AKI in patients receiving cisplatin. More work is required to further characterize \([\text{TIMP-2}] \cdot [\text{IGFBP7}]\) in cisplatin-induced AKI.

Although these AKI biomarkers show promise, reported results have been subject to variability. This variability in biomarker performance may be in part due to inconsistencies in sample collection timepoint, assay used for biomarker quantification, usage of different biomarker thresholds in classification, differences in AKI definition, and differences in the clinical scenario and etiology of AKI. Additionally, there is a paucity of studies investigating biomarkers specifically in cisplatin-induced AKI; more investigations are required to characterize the utility of these biomarkers in patients receiving cisplatin chemotherapy. Each biomarker has its own limitations, and a single biomarker is unlikely to perform well in all settings, especially considering the complexity of AKI as a clinical syndrome. There is international consensus that a panel of biomarkers is likely necessary to improve AKI risk assessment, facilitate early AKI diagnosis or prediction, evaluate the progression of AKI, and predict long term outcomes\(^4\).
1.2.4.5 Animal Models of Cisplatin-induced AKI

Animal models of cisplatin-induced AKI are predominantly conducted in rodents. Most studies in rodent models utilize a single high dose intraperitoneal (i.p.) injection of cisplatin to induce nephrotoxicity (e.g., 20–30 mg/kg for mice). In a high dose i.p. model, functional impairment of the kidneys (typically measured by SCr and blood urea nitrogen), histological injury and morphological alterations usually starts to manifest 3–4 days after cisplatin injection. High dose cisplatin leads to death 7–10 days following cisplatin administration. In human patients, cisplatin is typically administered as multiple infusions that take place weeks apart over the course of many months. However, even in cisplatin-naïve patients, cisplatin can cause AKI after just one infusion, lending support to the use of a single dose to model cisplatin-induced AKI in rodents.

Concerns have been raised about the clinical applicability of the single dose model. The high dose single injection usually leads to severe toxicity and eventually death in the animals, which is not consistent with cisplatin chemotherapy in humans. Furthermore, as the high dose cisplatin often leads to death in the animals, the effects of repeated cisplatin dosing, as is the case in chemotherapy, cannot be tested in the high dose model. Additionally, the long-term effects of cisplatin-induced AKI cannot be studied in the single high dose model. Repeated low dose models of cisplatin may be more reflective of some chemotherapy regimens used in the clinic. Administration of multiple weekly low doses of cisplatin has allowed for the survival of mice over the course of cisplatin treatment and may allow for the evaluation of the long-term effects of cisplatin treatment.

Age and strain of the animals being used must also be considered, as both factors have been shown to play a role in susceptibility to cisplatin-induced nephrotoxicity. Most studies use young animals (typically 8–12 weeks old for mice), whereas the median age for cancer diagnosis is 66, according to the National Cancer Institute. Older age is a risk factor for cisplatin-induced AKI in human patients, and aged rats have exhibited increased susceptibility to cisplatin nephrotoxicity. Using aged animals may better reflect the nephrotoxic mechanisms responsible for AKI in older patients.
Finally, the vast majority of the investigations studying cisplatin-induced AKI have been in non-tumour bearing animals. Especially for studies evaluating therapeutic interventions against nephrotoxicity, it is important to ensure that the intervention does not affect the anti-tumour activity of cisplatin. Allograft and xenograft models of cancer or genetic induction of tumour formation are available options for tumour-bearing animal models.

1.3 Metabolomics

1.3.1 Overview of Metabolomics

Metabolomics is the comprehensive measurement of small molecules, otherwise known as metabolites, in the cells, tissues, organs, or biofluids of a living organism. Metabolomics studies typically define small molecules as molecules with molecular mass <1500 Da. The metabolome encompasses endogenous compounds encoded for by the host genome and produced by endogenous catabolic and anabolic metabolic processes, as well as exogenous metabolites that may be derived from the diet or environment. The human metabolome is large and highly complex and has been estimated to consist of over a million endogenous and exogenous compounds belonging to thousands of different chemical classes; the majority of these metabolites have yet to be characterized. As the metabolome is downstream of the genome, transcriptome, and proteome, metabolomics reflects alterations in all the preceding “-omes” and provides a snapshot of the metabolic processes occurring in a biological sample. This metabolic phenotype may be subject to a high degree of inter-individual variability, as the metabolome is sensitive to a wide array of internal variables such as age, gender, genetics, and disease, as well as external factors such as diet, medications, environmental exposure to pollutants/toxins, lifestyle, and gut microbiome composition. Metabolomics has been utilized to study the metabolic alterations that occur in the pathophysiology of human diseases such as metabolic diseases, cardiovascular disease, cancer, kidney diseases, and neurodegenerative diseases. One area of particular relevance to this dissertation is pharmacometabolomics, which aims to use metabolomics to predict or inform patient response to medications.
1.3.2 Analytical Techniques for Metabolomics

Due to the chemical complexity of the metabolome, various analytical techniques are utilized to conduct metabolomics studies. Nuclear magnetic resonance (NMR) or mass spectrometry (MS) are typically used for metabolomics analyses\textsuperscript{272,278,279}. MS methods are most frequently coupled with liquid chromatography (LC-MS)\textsuperscript{276,278}. The main advantage of using MS is its sensitivity. Due to the high sensitivity of MS, more metabolites are typically detected in MS studies compared to NMR, and small sample volumes are sufficient for MS-based experiments\textsuperscript{278}. A limitation of MS-based metabolomics is the requirement for metabolites to ionize in order for MS detection. There are many different ionization techniques, and no single ionization method can capture the entire metabolome\textsuperscript{278}. For compounds that do not readily ionize, chemical derivatization can be employed to enhance ionization\textsuperscript{280,281}. Furthermore, in LC-MS, the choice of LC column can greatly influence the separation of metabolites. Reverse-phase liquid chromatography (RPLC) is the most commonly used method for global profiling but is most suitable for non-polar or moderately polar compounds\textsuperscript{278,282}. In contrast, hydrophilic interaction liquid chromatography (HILIC) is better suited for the retention and separation of polar and charged compounds\textsuperscript{282}. Accordingly, a combination of multiple LC columns can be used for more comprehensive analysis of the metabolome. NMR is inherently quantitative, and although NMR based techniques require larger volumes of sample, it is non-destructive, and samples can be recovered for future analyses. Sample preparation also tends to be less intensive compared to LC-MS\textsuperscript{278,279}. NMR does not depend on ionization for sample detection and is highly reproducible\textsuperscript{278,279}. To facilitate comprehensive characterization of the metabolite, a combination of various MS techniques and NMR should be employed.

1.3.3 Data Processing and Analysis of LC-MS Metabolomics Datasets

LC-MS metabolomics experiments of complex biological matrices typically generate thousands of features within a single sample, many of which elute off the chromatography column at similar times. A total ion current chromatogram (TIC) sums up all of the intensities detected across the whole range of masses scanned by the MS. As compounds with similar chemical properties may co-elute, a single peak in the TIC typically consists
of multiple features. Peak picking is therefore conducted to separate the TIC chromatogram into distinct peak components. Open-source packages such as XCMS or MZmine are often used for processing of chromatographic data, including peak picking\textsuperscript{283,284}. In peak picking, extracted ion chromatograms (EICs) are generated for each mass to charge ratio ($m/z$), and then peak detection is conducted using the retention time\textsuperscript{284}. After peak detection, peaks are aligned across all samples, and gap filling is conducted for samples where the peak may not be present (integrate the retention time where the peak should be)\textsuperscript{285}. Putative annotation of isotopes or adducts can be conducted using an open-source package like CAMERA\textsuperscript{286}. A pooled sample is used as quality control (QC), where the pooled QC sample is injected into the LC-MS instrumentation at regular intervals throughout the run. Features that show low repeatability (i.e., relative standard deviation $>30\%$) in the pooled QC sample are filtered out. After generation of a feature list, the data can be normalized (e.g., normalize all features to a reference feature in each sample, such as an internal standard), transformed (e.g., log transformation), or scaled (e.g., pareto scaling) before further analysis\textsuperscript{287}.

Due to the complexity and high dimensionality of metabolomics datasets, multivariate analyses are necessary. Proprietary software tools such as SIMCA or EZInfo, or open-source R packages can be used to conduct multivariate statistical analyses. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) are commonly used for multivariate analysis of metabolomics data\textsuperscript{288}. PCA is an unsupervised (meaning the analysis is not aware of group labels) tool used to reduce the dimensionality of the complex metabolomics dataset, while preserving as much of the variance in the dataset as possible\textsuperscript{288,289}. The first principal component (PC) indicates a line in the multidimensional dataspace that captures the most variance in the data\textsuperscript{290}. The second PC is a line orthogonal to the first that explains the second most variance in the data\textsuperscript{290}. Subsequent PCs are generated in the same manner. PCA scores plot project each sample data point to a new coordinate space composed of two or three principal components, allowing for visualization of the largest variation in the dataset. OPLS-DA is a supervised analysis used for discrimination of known groups or classes\textsuperscript{288}. The goal of OPLS-DA is to maximize the covariance between the features and the class membership\textsuperscript{288}. From the PCA and OPLS-DA models, the features/metabolites most responsible for discrimination
between groups of interest can be identified. Metabolites of interest are putatively annotated by searching online metabolomics databases (Human Metabolome Database (HMDB), METLIN, or MassBank) using feature mass to charge ratio ($m/z$) values and comparing the fragmentation spectrum of each feature with experimental fragmentation spectral information found in the databases. If spectral information is not available, characteristic fragments generated through collision-induced dissociation can be used to narrow down potential metabolites. If characteristic fragments are not observed, predicted fragmentation spectra can be generated using CFM-ID or Waters MassFragment to aid with identification.

### 1.3.4 Pharmacometabolomics

Pharmacometabolomics is a field of metabolomics that seeks to understand how interindividual differences in the metabolome influence a patient’s response to drugs\cite{291}. Pharmacometabolomics studies have been conducted to study and predict drug efficacy and toxicity, and to identify biomarkers of drug transporters and drug metabolizing enzymes\cite{291-297}. One of the first studies to employ pharmacometabolomics evaluated lipid profiling of patients with schizophrenia before and after treatment with atypical antipsychotic drugs\cite{292}. The authors highlighted the lipidomic alterations induced by the antipsychotic agents olanzapine, risperidone, and aripiprazole, and proposed a set of baseline lipids that may discriminate between responders and non-responders to antipsychotic medication. Since then, many studies have utilized pharmacometabolomics, both independently and in conjunction with other -omics fields such as pharmacogenomics. One study that illustrates the utility of the combination of pharmacogenomics and pharmacometabolomics investigated responders and non-responders to citalopram/escitalopram treatment\cite{294}. Metabolomics analysis revealed glycine to be negatively associated with response to medication, and further genotyping for SNPs in genes involved in glycine synthesis identified the rs10975641 SNP in the glycine dehydrogenase gene as being associated with treatment outcome\cite{294}. Similarly, pharmacometabolomics and pharmacogenomics were combined in a study of aspirin resistance\cite{293}. Purine metabolites adenosine and inosine were found to be significantly higher in poor responders compared to good responders following aspirin therapy. Genes
involved with biosynthesis, degradation, or transport of purine metabolites were selected to identify SNPs associated with platelet aggregation. Of 51 SNPs found in the adenosine kinase gene region, rs16931294 showed the highest association with changes to platelet aggregation during aspirin therapy. The rs16931294 variant was associated with higher platelet aggregation following aspirin therapy (poor response). These studies highlight the use of pharmacometabolomics to inform pharmacogenomics. On the other hand, pharmacogenomics has also been used to guide pharmacometabolomics studies. CYP2D6 is an important drug metabolizing enzyme, responsible for the metabolism of approximately 25% of clinically used drugs. Individuals with the poor metabolizer CYP2D6 phenotype are at higher risk of adverse effects or non-response when receiving standard doses of CYP2D6-metabolized drugs. Solanidine and its metabolites have been proposed to be biomarkers of CYP2D6 poor metabolizer phenotype. A metabolomics study was conducted in patients genotyped for CYP2D6 to identify biomarkers for CYP2D6 activity. Solanidine and four metabolites of solanidine were proposed as potential biomarkers for CYP2D6 activity, as metabolites of solanidine were significantly higher in the CYP2D6 intermediate, normal, and ultra-rapid metabolizers compared to poor metabolizers, and solanidine levels were significantly higher in the poor metabolizers. Further studies of solanidine and its metabolites showed high predictive value in prediction of the poor metabolizer phenotype in both test and validation cohorts of CYP2D6 genotyped patients. Collectively, these studies highlight the utility of incorporating pharmacometabolomics into the precision medicine toolchest. Especially when combined with pharmacogenomics, pharmacometabolomics can provide insight into the mechanistic basis of variability in drug efficacy and toxicity and identify biomarkers indicative of patient response to medications.

1.4 Differences in Drug Response Between Adults and Children

As children develop and grow, they experience major physiological changes that may have profound effects on drug response. Although children commonly receive a wide variety of medications, many drugs have not been directly studied in children and are not labelled for use in children. For example, although cisplatin is commonly used in the treatment of
childhood cancer, its usage is off-label due to the absence of approved indications for children. When studying pharmacometabolomics, it is important to be cognizant of the physiological differences that exist between adults and children. The processes of absorption, distribution, metabolism, and excretion differ between children and adults and have consequences on the pharmacokinetic profiles of drugs in children.

Oral absorption can be affected by various physiological factors in the developing GI system. Gastric pH is practically neutral from birth and declines to reach adult values by the age of 3\textsuperscript{301,302}. Drugs degraded by acid have increased absorption in children compared to adults, while weak acids have shown decreased absorption. In the first few months of life, bile secretion is lower in very young children and may affect absorption of fat-soluble drugs\textsuperscript{301,302}. Traditionally, gastric emptying and intestinal transit time were believed to be prolonged in children, but recent evidence indicates that age has minimal effect on intestinal transit time\textsuperscript{303,304}. Data regarding ontogeny of drug metabolizing enzymes and drug transporters is scarce and often inconsistent. Studies on intestinal CYP3A expression have been conflicting, with one study reporting CYP3A4 expression increasing with age from neonates to adolescents, whereas expression decreased with age in a separate study\textsuperscript{305,306}.

The changes to body composition that occur in childhood development affect drug distribution. The proportion of extracellular water is highest in newborns, then gradually declines with age\textsuperscript{301,302,307}. Infants also have a higher proportion of body fat that eventually declines throughout development\textsuperscript{301,307}. Accordingly, neonates and infants may have larger volumes of distribution for both hydrophilic and lipophilic drugs. Additionally, plasma proteins are decreased in neonates and infants, and exhibit decreased binding capacity\textsuperscript{301,302}. Consequently, the unbound fraction of highly protein-bound drugs is higher, and distribution is increased.

Drug metabolizing enzymes generally increase from birth to adulthood. CYP enzymes are the most well characterized drug metabolizing enzymes with respect to drug metabolizing enzyme ontogeny. Total CYP content in the liver has been shown to progressively increase throughout development. The activity and expression of important drug metabolizing
CYPs (e.g., 1A2, 2A6, 2D6, 2E1, 3A4) typically reach adult levels around 1–5 years of age\textsuperscript{308}. Maturation of CYP2C9 appears to be delayed, as protein levels of CYP2C9 from 0–5 months of age were approximately 25% of adult levels, and approximately 50% from 5 months of age to 18 years of age\textsuperscript{309}. In the fetus, CYP3A7 is the dominant CYP3A enzyme, with CYP3A4 having little to no activity. Following birth, CYP3A7 activity progressively declines as CYP3A4 activity rises\textsuperscript{308}. Hepatic clearance of drugs can be higher in children that fall under the infant to pre-school age range compared to adults; liver blood flow is increased in this population of children, as liver to total body weight ratio is at its peak during this period of development\textsuperscript{301}. Differences in transporters may also play a role in hepatic metabolism, as age-dependent expression has been observed for hepatic drug transporters. For example, protein expression of organic anion transporting polypeptide (OATP) 1B3, an uptake transporter found on hepatocytes, was demonstrated to be high at birth to 3 months of age, decline from 3 months to 6 years age, and then increase from 6 to 12 years of age\textsuperscript{310}. The ontogeny of drug transporters is poorly characterized, and more research is needed to study age-related differences in expression and activity of transporters\textsuperscript{311}.

Excretion of drugs and drug metabolites are primarily facilitated by the kidneys. At birth, renal blood flow is approximately 6% of total cardiac output but increases to 15–25% by one year of age and reaches adult levels after two years\textsuperscript{312}. Glomerular filtration starts low after birth but reaches 50% of adult values 2 months after birth, and 90% by 1 year\textsuperscript{313}. It is important to consider that GFR lags behind in premature neonates, as initial GFR at birth is dependent on gestational age rather than postnatal age\textsuperscript{314}. Tubular secretion capacity increases over the first months of life and reaches adult levels at 7 months\textsuperscript{315}. Information regarding ontogeny of specific renal transporters is lacking and further studies are required to understand the pharmacokinetic consequences of age-related changes to transporter expression over the course of childhood development\textsuperscript{311}.
1.5 References


doi:10.1016/j.kjms.2012.10.004

doi:10.1177/1078155220901756


doi:10.1186/cc12503


287. van den Berg RA, Hoefsloot HC, Westerhuis JA, Smilde AK, van der Werf MJ. Centering, scaling, and transformations: improving the biological information


Chapter 2

2 Rationale, Aims, and Hypotheses

2.1 Overall Rationale

Cisplatin is an effective chemotherapeutic agent widely used for the treatment of a variety of solid tumours and hematologic cancers\textsuperscript{1-7}. Despite its effectiveness, cisplatin chemotherapy is complicated by a wide myriad of toxicity to non-cancerous tissues, including ototoxicity, neurotoxicity, gastrointestinal toxicity, and nephrotoxicity\textsuperscript{8}. Of these toxic effects, cisplatin-induced nephrotoxicity is often the dose limiting factor in cisplatin therapy\textsuperscript{1}. Acute kidney injury (AKI) is a common manifestation of cisplatin-induced nephrotoxicity, occurring in approximately 30\% of patients receiving cisplatin\textsuperscript{1}. AKI is an abrupt decline in kidney function that is associated with negative short-term outcomes such as increased in-hospital mortality, increased length of hospital stays, prolonged mechanical ventilation, and increased health care costs, as well as negative long-term outcomes such as recurrence of AKI, progression to CKD, cardiovascular events, and mortality\textsuperscript{9-14}. Cisplatin-induced AKI is currently diagnosed using the KDIGO guidelines, which define AKI as an increase in serum creatinine (SCr) ≥ 1.5 times baseline or an increase in SCr of ≥ 26.5 μmol/L\textsuperscript{15}. SCr has several limitations as a diagnostic marker of AKI, most notably, SCr can only diagnose AKI after significant kidney injury has already occurred\textsuperscript{16}. Furthermore, SCr is affected by several patient factors and is non-specific\textsuperscript{17-19}. Earlier diagnosis of AKI or prediction of high-risk patients is critical for successful prevention or management of AKI. Novel biomarkers are necessary to identify high-risk patients for cisplatin-induced AKI and to facilitate prediction or early diagnosis of cisplatin-induced AKI\textsuperscript{16}. To address these issues, we took an untargeted metabolomics approach to identify early diagnostic or predictive biomarkers for cisplatin-induced AKI. \textbf{The overall hypothesis of this dissertation was that evaluating metabolic differences prior to, and following cisplatin exposure will allow for the prediction or early diagnosis of cisplatin-induced AKI.}
2.2 Aim 1 (Chapter 3)

To investigate the metabolic alterations induced by cisplatin treatment in a mouse model of cisplatin-induced nephrotoxicity and identify early biomarkers of cisplatin-induced AKI.

Untargeted metabolomics was employed in a mouse model of cisplatin-induced nephrotoxicity to investigate the metabolic alterations induced by cisplatin in the blood, urine, and kidneys of cisplatin-treated mice. The objectives of this study were to: 1) characterize the metabolic alterations induced by cisplatin throughout the progression of kidney injury in C57BL/6 and FVB/N mice; 2) identify early biomarkers of cisplatin-induced nephrotoxicity (i.e., metabolites that are altered prior to elevation of SCr). We hypothesized that characterization of the mouse metabolome following cisplatin administration would reveal metabolites indicative of early kidney injury prior to elevations in SCr.

2.3 Aim 2 (Chapter 4)

To investigate the metabolic alterations induced by cisplatin chemotherapy in a cohort of adult head and neck cancer patients and assess the metabolic differences between AKI and non-AKI patients prior to and following cisplatin exposure.

Not all patients develop AKI after receiving cisplatin therapy. Biomarkers capable of predicting development of cisplatin-induced AKI prior to or shortly after cisplatin infusion will allow for careful monitoring and management of nephrotoxicity in patients at high risk of developing AKI. A cohort of 31 adult head and neck cancer patients receiving high dose cisplatin chemotherapy (100 mg/m²) were recruited at the London Regional Cancer Program between 2018-2020. Blood and urine samples were collected from patients prior to cisplatin infusion, 24–48 hours after cisplatin infusion, and 5–14 days after cisplatin infusion. Untargeted metabolomics was employed to: 1) characterize metabolic differences between patients who develop AKI after cisplatin infusion (AKI group) and those who do not (non-AKI group); 2) identify biomarkers that predict AKI prior to cisplatin infusion; 3) identify biomarkers that predict the development of AKI shortly after cisplatin infusion.
We hypothesized that differences in the metabolic profiles of AKI and non-AKI patients will predict the development of AKI prior to or shortly after cisplatin infusion.

2.4 Aim 3 (Chapter 5)

To investigate the metabolic alterations induced by cisplatin chemotherapy in a cohort of pediatric cancer patients and assess the metabolic differences between AKI and non-AKI patients prior to and following cisplatin exposure.

Children are not “small adults”. There are numerous physiological differences between children and adults that may affect the metabolome\(^{20-24}\). To account for age-associated differences in baseline biomarker concentrations, children and adults must be studied separately. A cohort of 159 pediatric cancer patients receiving cisplatin were recruited as part of a Canada-wide, multi-center prospective study to study the late effects of childhood cancer treatment. Blood and urine samples were collected from patients prior to cisplatin infusion, 24 hours after cisplatin infusion, and 3–5 days after cisplatin infusion. Similar to aim 2, we used untargeted metabolomics in a subset of this pediatric cohort to: 1) characterize metabolic differences between AKI and non-AKI patients; 2) identify biomarkers that predict AKI prior to cisplatin infusion; 3) identify biomarkers that predict the development of AKI shortly after cisplatin infusion. Sub-group analysis was also conducted to investigate sex differences and the effects of age stratification. As with aim 2, we hypothesized that differences in the metabolic profiles of AKI and non-AKI patients will predict the development of AKI prior to or shortly after cisplatin infusion.
2.5 References


Chapter 3

3 Metabolomics for the Identification of Early Biomarkers of Nephrotoxicity in a Mouse Model of Cisplatin-Induced Acute Kidney Injury

A version of this chapter has been published:


Reproduced with permission from Elsevier.
3.1 Introduction

Cisplatin is a platinum-based chemotherapeutic agent used in the treatment of a wide variety of malignancies. Although cisplatin is highly effective and widely used, clinical usage of cisplatin is often limited by toxicity to non-cancerous tissues. The kidneys are especially sensitive to cisplatin toxicity, and as such, cisplatin-induced nephrotoxicity is the dose-limiting factor in cisplatin therapy. Cisplatin is primarily excreted by the kidneys, through both glomerular filtration and tubular secretion. Transporter-mediated uptake of cisplatin by tubular epithelial cells results in the renal accumulation of cisplatin, subsequent biotransformation into highly reactive thiol metabolites, and ultimately cell injury.

Cisplatin-induced nephrotoxicity manifests as acute kidney injury (AKI) in approximately one-third of patients. AKI is characterised by a rapid decline in kidney function and is associated with increased risk for chronic kidney disease, major cardiovascular events, and mortality. AKI is defined by the Kidney Disease Improving Global Outcomes (KDIGO) as a ≥ 1.5-fold increase in serum creatinine (SCr) versus baseline, or as an increase in SCr ≥ 26.5 µmol/L. Though SCr is used for clinical diagnosis of AKI, detectable changes in SCr only occur after substantial kidney injury and functional impairment. Earlier identification of nephrotoxicity is necessary to initiate nephroprotective interventions for cisplatin-induced AKI. The search for diagnostic/prognostic biomarkers of AKI has yielded the discovery of several serum and urinary protein biomarkers, including neutrophil gelatinase-associated lipocalin, kidney injury molecule-1, cystatin C, tissue inhibitor of metalloproteinase 2, and insulin-like growth factor binding protein. However, these markers are not specific to AKI, nor are they specific for any one etiology of AKI. There is a consensus that the application of a panel of biomarkers is better suited for the early detection of AKI, evaluation of AKI severity/prognosis, and discrimination of AKI etiology, rather than the use of any one single biomarker.

In this study, we aimed to identify early biomarkers of cisplatin-induced AKI through metabolomics analysis of mouse plasma, kidney, and urine samples taken at multiple time points throughout AKI progression from two different strains of mice. We used C57BL/6 and FVB/N mice, as FVB/N mice were reported to be more sensitive to cisplatin-induced nephrotoxicity relative to C57BL/6 mice, as evidenced by greater percent increases in
both serum blood urea nitrogen and serum creatinine (SCr) 72 hours following cisplatin injection \(^{10}\). As mentioned previously, the disparity in sensitivity to cisplatin nephrotoxicity is observed in humans, where approximately one-third of patients receiving cisplatin chemotherapy develop clinical AKI. The two strains of mice were investigated to confirm the potential differences in the extent of kidney injury and to discover potential mechanisms responsible for the variability in sensitivity to cisplatin nephrotoxicity. To date, there have been few studies utilizing metabolomics in rodent models of cisplatin-induced AKI. Investigating metabolic alterations in all three of plasma, urine, and kidney tissue samples at multiple timepoints throughout AKI progression is needed to characterize biomarkers from initial cisplatin administration to the establishment of AKI.

### 3.2 Materials and Methods

#### 3.2.1 Chemicals and Reagents

Cisplatin (Accord Healthcare, 1 mg/mL) and 0.9.% saline were purchased from London Health Sciences Center - University Hospital Pharmacy (London, ON, Canada). Ketamine and xylazine were purchased from Western University Veterinary Services (London, ON, Canada). Chlorpropamide was purchased from Toronto Research Chemicals (Toronto, ON, Canada), atenolol-d7 from Sigma-Aldrich (Oakville, ON, Canada), flurazepam from Cerilliant (Round Rock, TX, USA), and DL-2-aminoheptanedioic acid from Bachem (Torrence, CA, USA). HPLC grade acetonitrile was purchased from Millipore Sigma (Oakville, ON, Canada). 10% formalin was purchased from VWR International (Mississauga, ON, Canada). Analytical standards for citric acid, creatine, equol, indole-3-carboxaldehyde, L-acetylcarnitine, phenylacetylglycine, phenylpropionylglycine, taurine, and tryptophan were purchased from Sigma-Aldrich (Oakville, ON, Canada). Analytical standards for phenylalanine, succinate, and trigonelline were purchased as part of the Mass Spectrometry Metabolite Library of Standards (MSMLS) kit (Sea Girt, NJ, USA). Analytical standards for equol 7-O-glucuronide, L-carnitine, and p-cresol glucuronide were purchased from Toronto Research Chemicals (Toronto, ON, Canada).
3.2.2 Animal Model

Wild-type male C57BL/6 and FVB/N mice (8-12 weeks old) were obtained from Charles River Laboratories. All experimental protocols and animal care procedures were conducted in accordance with the Canadian Council on Animal Care and were approved by the Animal Care Committee of Western University. All mice were housed in standard caging with a 12-hour light cycle and were given standard rodent chow and water *ad libitum*. Eight mice from both the FVB/N and C57BL/6 strains were used as baseline controls. Both C57BL/6 and FVB/N mice were randomly divided into “control” and “cisplatin” groups. Cisplatin groups were given a single intraperitoneal (i.p.) injection of cisplatin (15 mg/kg) and control mice were given an equal volume of 0.9% saline. A single i.p. injection of cisplatin at a dose of 15 mg/kg is well established in the literature to induce kidney injury 3-4 days following cisplatin injection in a mouse model, as measured by traditional indices of kidney damage such as elevated SCr, elevated blood urea nitrogen, and histological assessment\textsuperscript{10,11}. Mice were euthanized 1, 2, 3, and 4 days after treatment for sample collection. A ketamine/xylazine mixture (100 mg/kg and 12.5 mg/kg, respectively) was used for anesthesia, followed by intracardiac blood collection and bilateral thoracotomy for confirmation of euthanasia. Urine samples and both kidneys were also collected upon euthanasia. Samples were also isolated from untreated C57BL/6 and FVB/N mice to be used as baseline controls. Blood samples were collected in heparin-treated microcentrifuge tubes and centrifuged at 1000 g for isolation of plasma. The number of samples collected varied based on sample collection/availability and exact numbers of samples collected for each sample type in each experimental group can be found in Supplementary Table B1 (Appendix B).

3.2.3 Assessment of Renal Injury

Mouse plasma creatinine levels were measured with ultra performance liquid chromatography coupled with mass spectrometry (UPLC-MS). Ice-cold acetonitrile (ACN) containing 50 µM creatinine-d3 (internal standard) was added to plasma samples for protein precipitation (3:1 ratio). Following the addition of ACN, all samples were mixed with a vortex mixer, incubated at -20°C for 20 minutes, and centrifuged at 14000 g for 10 minutes. The resulting supernatant was isolated and used for creatinine quantification.
Chromatographic separation was achieved using a Waters ACQUITY UPLC BEH Amide column (1.7 µm particle size, 2.1 mm x 100 mm) maintained at 45°C in a Waters ACQUITY UPLC I-Class system. The mobile phase consisted of A) water + 0.1% formic acid and B) ACN + 0.1% formic acid. Mobile phase flow rate was set to 0.45 mL/min and the following mobile phase gradient was used: 0–0.5 mins, 90% B; 0.5–1 min, 90–60% B; 1–1.5 min, 60% B; 1.5–1.51 min, 60–90%B; 1.51–2.5 min, 90% B. Creatinine was measured in positive electrospray ionization mode using a Waters Xevo G2-S quadrupole time of flight (QToF) mass spectrometer. The creatinine signal was normalized to the creatinine-d3 internal standard and concentrations were quantified using a creatinine standard curve ranging from 0.78125 – 100 µM creatinine, using TargetLynx version 4.1 software.

Kidney samples were fixed in 10% formalin. All tissue processing, sectioning, and staining were performed by the Department of Pathology and Laboratory Medicine at Western University, Canada. Kidneys were dehydrated, embedded in paraffin, and cut into 5 µm sections using a microtome. Sections were then mounted on slides and subsequently stained with hematoxylin and eosin (H&E). The extent of renal injury was assessed using light microscopy by a trained pathologist who was blinded to the treatment conditions. Tubular injury was graded on an arbitrary scale of 0 – 5 (0, none; 1, <11%; 2, 11% to 25%; 3, 26% to 45%; 4, 46% to 75%; 5, >75%), based on the degree of observed proximal tubule dilation, brush-border damage, proteinaceous casts, interstitial widening, and necrosis.

### 3.2.4 Sample Preparation for Untargeted Metabolomics

Ice-cold acetonitrile containing chlorpropamide (4 µM), atenolol-d7 (1.8 µM), flurazepam (0.15 µM) and DL-2-aminoheptanedioic acid (100 µM) as internal standards, was added to plasma and urine samples for protein precipitation (3:1 ratio). Kidney samples (50 mg) were homogenized in 150 µL of ACN containing internal standards. Following the addition of ACN, all samples were mixed with a vortex mixer, incubated at -20°C for 20 minutes, and centrifuged at 14000 g for 10 minutes. Supernatant from the protein precipitation was diluted 1 in 5 with water. Diluted samples were transferred into glass vials for UPLC-MS analysis. A pooled sample was generated to serve as quality control (QC) throughout the metabolomics run.
3.2.5 Chromatography and Mass Spectrometry

Chromatography and mass spectrometry instrumentation were the same as described previously in “Assessment of Renal Injury”. 2 μL of sample was injected from each vial. Sample injection order was randomized, and the quality control pooled sample was injected after every 6 sample injections throughout the run. Chromatographic separation was achieved using a Waters ACQUITY UPLC HSS T3 column (1.8 μm particle size, 2.1 mm x 100 mm) maintained at 45°C. The mobile phase consisted of A) water + 0.1% formic acid and B) ACN + 0.1% formic acid. Mobile phase flow rate was set to 0.45 mL/min and the following mobile phase conditions were used: 0–2 mins, 1–60% B; 2–6 mins 60–85% B; 6–8 mins 85–99% B; 8–10 mins 99–1% B. Features were measured in both positive and negative electrospray ionization modes with the following mass spectrometer parameters: capillary voltage, 2kV; cone voltage, 40V; source temperature, 150°C; desolvation gas flow and temperature, 1200 L/h and 600°C; cone gas flow, 50 L/h. Data was acquired in centroid, using the MS² method in resolution mode. The MS² method generates both the precursor ion (function 1) and fragment ions (function 2) in one acquisition. The acquisition period was 11 minutes, with a 0.05 second scan time and a mass range of 50–1200 Da. Collision energy was set to 0 V for function 1 and was ramped from 15–50 V for function 2. Leucine-enkephalin (0.9 μM) was used as lockspray solution to ensure mass accuracy. The lockspray solution was infused at a flow rate of 10 μL/min. Lockmass was acquired at intervals of 10 seconds and averaged over 3 scans.

3.2.6 Metabolomics Data Processing

Data processing was performed using the R statistical programming language. Waters raw data files generated from the metabolomics analysis were converted to mzData files using the convert.waters.raw R package. The quality control pooled injections were used to find the optimal peak picking parameters, retention time corrections and grouping parameters with the isotopologue parameter optimization (IPO) package. The resulting parameters were input into the XCMS package for peak-picking, to integrate the area under the curve and to replace zero values. The CAMERA package was applied to XCMS processed features to annotate possible isotopes and adducts. XCMS and CAMERA packages were used to combine data from both positive and negative ionization modes. The resulting data
was subsequently normalized to internal standards, and features were filtered by applying a threshold of 30% relative standard deviation within quality control injections. Urinary metabolites were normalized to both urinary creatinine and internal standards, to account for differences in urine concentration. Features were grouped by retention time and correlation into “pcgroups” by the CAMERA package. Within each pgroup, only the feature with the highest mean raw intensity was kept for further data analysis.

### 3.2.7 Data and Statistical 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 analysis unless stated otherwise. The EZInfo 2.0 software (Umetrics, Umeå, Sweden) was used to perform multivariate analysis on the metabolomics data. Data was centered and pareto scaled upon import into EZInfo 2.0. Principal component analysis (PCA) was used to visualize unsupervised metabolic variation between saline and cisplatin treatment at each of the timepoints of the study. Orthogonal partial least squares discriminant analysis (OPLS-DA), a supervised discriminatory analysis, was used for the pairwise discrimination of treatment groups at each timepoint. For each OPLS-DA, metabolites were ranked by their correlation (p(corr)) values and variable importance in projection (VIP) values to select a subset of metabolites for identification. Features with 0.4 < p(corr) < -0.4 and VIP > 1 were considered as important discriminators of the groups being compared.

Features were analyzed by two-way ANOVA with Benjamini-Hochberg false discovery rate (FDR) correction to find features that were significantly different by saline vs. cisplatin treatment. The DEGreport (1.30.3) R package was used to generate hierarchical clusters of features that were determined by two-way ANOVA as being significantly altered by treatment. The Z-scores presented in time course analyses are calculated by centering each feature to its mean and dividing by the standard deviation of the feature. Clusters were selected for further analysis based on time course patterns of clinical interest, focusing on features that are differently expressed in the early timepoints (day 1 and 2) between the saline and cisplatin-treated mice. Individual features that were found to be significantly
different by two-way ANOVA and FDR correction were further analyzed by pairwise t-tests comparing saline vs. cisplatin treated mice at each timepoint, with p-values adjusted for multiple comparisons using Bonferroni correction. p<0.05 was considered as statistically significant for all univariate data analysis.

Univariate and multivariate receiver operating characteristic (ROC) curves were generated using MetaboAnalyst 5.0. Multivariate ROCs were generated using linear support vector machine classification, with features ranked by highest to lowest univariate area under the ROC (AUROC) values.

3.2.8 Putative Metabolite 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 and analyzed in parallel with biological samples to achieve a level 1 identification\textsuperscript{12}. 
3.3 Results

3.3.1 Assessment of Renal Injury

The extent of injury caused by cisplatin treatment was assessed in both mouse strains by plasma creatinine levels (Figure 3.1A, 3.1C) and histological evaluation by a clinical pathologist (Figure 3.1B, 3.1D). Plasma creatinine concentrations were 4.3-fold and 5.4-fold higher in day 4 cisplatin-treated C57BL/6 and FVB/N mice, respectively, compared to day 4 saline controls (p<0.05). In day 3 cisplatin-treated C57BL/6 and FVB/N mice, plasma creatinine concentrations were increased 2.3-fold and 2.0-fold compared to saline controls, although this difference was not statistically significant. In day 3 cisplatin-treated mice, 3/9 (33%) C57BL/6 mice and 5/8 (62.5%) FVB/N mice showed signs of histological renal injury (injury score ≥ 1), whereas on day 4, histological damage was observed in 6/8 (75%) and 5/7 (71.4%) cisplatin-treated C57BL/6 and FVB/N mice, respectively. Histological injury was not observed at any other timepoint, with the exception of one C57BL/6 mouse in the day 1 cisplatin group (1/9, 11.1%). Though FVB/N mice were expected to show higher sensitivity for cisplatin-induced nephrotoxicity, no notable differences were observed in extent of kidney injury between the two strains as evaluated by SCr and histological assessment.
Figure 3.1 Assessment of renal injury in untreated (black), saline-treated (blue), and cisplatin-treated (red) C57BL/6 mice (A, B), and FVB/N mice (C, D).

Plasma creatinine concentrations were normalized to creatinine-d3 standard before quantification (A, C). H&E-stained kidney sections were scored from 0-5 based on severity of renal injury by a pathologist blinded to treatment conditions (B, D). n=5-9 (exact sample sizes can be found in Supplementary Table B1, Appendix B) error bars represent mean ± SD. Statistical analysis was performed using two-way ANOVA, ***p < 0.001 compared to saline control.
3.3.2 Metabolomic Profiling of Saline-treated vs. Cisplatin-treated Mice Over Time

XCMS processing of chromatographic data and subsequent filtering by relative standard deviation of features in pooled quality control samples resulted in the selection of 2446 features for plasma, 2319 features for urine, and 3021 features for kidney. Following pcgroup filtering (as described in the methods section), 841 plasma features, 999 urine features, and 841 kidney features remained for analysis.

PCA score plots were generated to visualize the metabolic variation between saline-treated mice and cisplatin-treated mice at each of the four timepoints. Score plots of plasma, urine, and kidney samples from C57BL/6 mice all showed a clear separation between saline and cisplatin groups from days 2-4, with moderate separation observed at day 1 for all three sample types (Supplementary Figure B1, Appendix B). Similarly, PCA score plots of FVB/N mice exhibited clear separation between saline and cisplatin-treated mice from day 2-4 for all sample types and weak to moderate separation at day 1 (Supplementary Figure B2, Appendix B). Corresponding OPLS-DA models of pairwise comparisons between saline and cisplatin-treated mice at each timepoint confirmed the separation observed in PCA scores plots, with a high degree of fit (R²) and moderate predictive ability (Q²) between days 2-4 for both C57BL/6 (Figure 3.2) and FVB/N mice (Figure 3.3). OPLS-DA models of day 1 plasma samples for both strains of mice showed good fit and moderate predictive ability, but model statistics were poor for day 1 urine and kidney samples (Figure 3.2 and Figure 3.3).
Figure 3.2 Orthogonal partial least squares discriminant analysis scores plots comparing plasma (A-D), urine (E-H), and kidney (I-L) samples from saline-treated (black) and cisplatin-treated (red) C57BL/6 mice at each timepoint: day 1 (A, E, I), day 2 (B, F, J), day 3 (C, G, K), day 4 (D, H, L).

Model statistics for OPLS-DA were as follows: (A) $R^2 = 0.989$, $Q^2 = 0.369$ (B) $R^2 = 0.980$, $Q^2 = 0.542$ (C) $R^2 = 0.989$, $Q^2 = 0.467$ (D) $R^2 = 0.985$, $Q^2 = 0.580$ (E) $R^2 = 0.627$, $Q^2 = 0.033$ (F) $R^2 = 0.989$, $Q^2 = 0.649$ (G) $R^2 = 0.989$, $Q^2 = 0.295$ (H) $R^2 = 0.946$, $Q^2 = 0.581$ (I) $R^2 = 0.914$, $Q^2 = 0.101$ (J) $R^2 = 0.979$, $Q^2 = 0.611$ (K) $R^2 = 0.944$, $Q^2 = 0.426$ (L) $R^2 = 0.973$, $Q^2 = 0.569$. n=5-10, exact sample sizes can be found in Supplementary Table B1, Appendix B.
Figure 3.3 Orthogonal partial least squares discriminant analysis scores plots comparing plasma (A-D), urine (E-H), and kidney (I-L) samples from saline-treated (black) and cisplatin-treated (red) FVB/N mice at each timepoint: day 1 (A, E, I), day 2 (B, F, J), day 3 (C, G, K), day 4 (D, H, L).

Model statistics for OPLS-DA were as follows: (A) R² = 0.977, Q² = 0.386 (B) R² = 0.973, Q² = 0.579 (C) R² = 0.951, Q² = 0.702 (D) R² = 0.994, Q² = 0.815 (E) R² = 0.784, Q² = 0.303 (F) R² = 0.967, Q² = 0.597 (G) R² = 0.919, Q² = 0.604 (H) R² = 0.985, Q² = 0.534 (I) R² = 0.969, Q² = -0.320 (J) day 2: R² = 0.990, Q² = 0.805 (K) R² = 0.987, Q² = 0.348 (L) R² = 0.979, Q² = 0.706. n=6-8, exact sample sizes can be found in Supplementary Table B1, Appendix B.
In C57BL/6 mice, two-way ANOVA of individual features for each sample type revealed 181 plasma features, 683 urine features, and 66 kidney features that were significantly different by saline vs. cisplatin treatment after adjusting for multiple comparisons. Significant differences were found in 109 plasma features, 599 urine features, and 73 kidney features when comparing samples from saline and cisplatin-treated FVB/N mice. Within each sample type, significant features following similar time course patterns were grouped together by using hierarchical clustering, and clusters containing features showing early alterations were selected for further analysis (C57BL/6, Figure 3.4; FVB/N, Figure 3.5). To further narrow down important features, pairwise t-tests were performed between saline and cisplatin groups at each timepoint for each individual feature. Features that were significantly different in the early timepoints (day 1 or day 2) for either strain were selected for identification, summarized in Table 3.1 (C57BL/6) and Table 3.2 (FVB/N).
Figure 3.4 Time course cluster analysis of plasma (A), urine (B), and kidney (C) features that were significantly altered by cisplatin treatment in C57BL/6 mice.

The lighter, thinner lines represent change over time for each individual feature within each cluster, and the darker, thicker line represents the mean z-score for all features in each cluster. Boxes represent the median and interquartile range (IQR), and the whiskers represent 1.5x IQR.
Figure 3.5 Time course cluster analysis of plasma (A), urine (B), and kidney (C) features that were significantly altered by cisplatin treatment in FVB/N mice.

The lighter, thinner lines represent change over time for each individual feature within each cluster, and the darker, thicker line represents the mean z-score for all features in each cluster. Boxes represent the median and interquartile range (IQR), and the whiskers represent 1.5x IQR.
Table 3-1  Summary of metabolite alterations over time in plasma, urine, and kidney of C57BL/6 mice treated with cisplatin compared to C57BL/6 mice treated with saline. 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.
Cluster numbers are based on the time course cluster analysis, as shown in Figure 3.4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>p-Value</th>
<th>Cluster</th>
<th>Time</th>
<th>Relative Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cresol sulfate</td>
<td>C7H8O4S [M-H]</td>
<td>187.0063</td>
<td>1.91</td>
<td>Plasma</td>
<td>2 ↑ 12.55 1 ↑ 5.57 0.614 ↑ 21.18 0.054 ↑ 18.51 0.184</td>
</tr>
<tr>
<td>Phenylacetylglycine</td>
<td>C10H10NO3 [M-H]</td>
<td>192.0569</td>
<td>1.80</td>
<td>Urine</td>
<td>- ↓ 1.22 1 ↓ 1.27 0.0313 ↓ 1.17 1 ↑ 1.03 1</td>
</tr>
<tr>
<td>Phenylalanine (Fragment)</td>
<td>C8H10N+</td>
<td>120.0809</td>
<td>1.20</td>
<td>Urine</td>
<td>6 ↑ 1.55 0.258 ↑ 1.08 1 ↑ 2.37 0.026 ↑ 1.17 1</td>
</tr>
<tr>
<td>Phenylpropionylglycine</td>
<td>C11H12NO3 [M-H]</td>
<td>206.0818</td>
<td>1.97</td>
<td>Urine</td>
<td>- ↓ 1.81 0.196 ↓ 8.91 0.001 ↓ 3.28 0.012 ↓ 2.93 0.0001</td>
</tr>
<tr>
<td>Proline Betaine</td>
<td>C7H13NO2 [M-H]</td>
<td>144.1019</td>
<td>0.70</td>
<td>Plasma</td>
<td>- ↑ 1.15 1 ↑ 3.33 0.0001 ↓ 8.35 0.0001 ↓ 6.62 3.6E-06</td>
</tr>
<tr>
<td>Pyrocatechol Sulfate</td>
<td>C6H6O5S [M-H]</td>
<td>188.9860</td>
<td>1.64</td>
<td>Plasma</td>
<td>1 ↑ 20.67 1 ↑ 20.67 0.003 ↑ 1.33 1 ↓ 1.44 0.26</td>
</tr>
<tr>
<td>Succinate</td>
<td>C4H4O4 [M-H]</td>
<td>117.0187</td>
<td>1.13</td>
<td>Urine</td>
<td>- ↓ 1.35 1 ↓ 1.35 0.0001 ↓ 3.33 0.0001 ↓ 6.62 3.6E-06</td>
</tr>
<tr>
<td>Sulfoglycolitholic acid</td>
<td>C26H43NO3 [M-H]</td>
<td>512.2583</td>
<td>2.25</td>
<td>Plasma</td>
<td>1 ↑ 20.67 1 ↑ 20.67 0.003 ↓ 1.33 1 ↑ 1.74 1</td>
</tr>
<tr>
<td>Taurine</td>
<td>C2H6NO3S [M-H]</td>
<td>124.0065</td>
<td>0.51</td>
<td>Plasma</td>
<td>1 ↑ 20.67 1 ↑ 20.67 0.003 ↓ 1.33 1 ↑ 1.74 1</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>C2H6O4S [M-H]</td>
<td>514.2840</td>
<td>2.38</td>
<td>Plasma</td>
<td>1 ↑ 20.67 1 ↑ 20.67 0.003 ↓ 1.33 1 ↑ 1.74 1</td>
</tr>
<tr>
<td>Trigonelline</td>
<td>C7H8NO2 [M-H]</td>
<td>138.0553</td>
<td>0.62</td>
<td>Plasma</td>
<td>1 ↑ 14.8 0.016 ↑ 1.14 1 ↑ 1.43 0.048 ↑ 1.1 1</td>
</tr>
<tr>
<td>Tyrosol-4-sulfate</td>
<td>C8H10O5S [M-H]</td>
<td>217.0171</td>
<td>1.45</td>
<td>Plasma</td>
<td>- - - - - - - - - - - -</td>
</tr>
</tbody>
</table>

*Cluster numbers are based on the time course cluster analysis, as shown in Figure 3.4.*
<table>
<thead>
<tr>
<th>Metabolite Identity</th>
<th>ID Level</th>
<th>Chemical Formula</th>
<th>Observed m/z</th>
<th>t&lt;sub&gt;x&lt;/sub&gt; (min)</th>
<th>Sample Type</th>
<th>Cluster*</th>
<th>Change in Cisplatin-treated FVB/N Mice vs. Saline Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Direction</td>
</tr>
<tr>
<td>Cinnamoylglycerine 2</td>
<td>C1H15NO 3 [M+H]</td>
<td>204.0663</td>
<td>2.02</td>
<td>Plasma - - - - - - - - - -</td>
<td>2.09</td>
<td>0.189</td>
<td>↓</td>
</tr>
<tr>
<td>Citric Acid 1 C6H8O7 [M+H]</td>
<td>191.0392</td>
<td>1.10</td>
<td>Plasma - - - - - - - - - -</td>
<td>1.28</td>
<td>1</td>
<td>↓</td>
<td>3.57</td>
</tr>
<tr>
<td>Creatine 1 C4H10N3O2 [M+H]</td>
<td>132.0769</td>
<td>0.57</td>
<td>Plasma - - - - - - - - - -</td>
<td>1.28</td>
<td>0.022</td>
<td>↑</td>
<td>1.7</td>
</tr>
<tr>
<td>Equol 1 C15H15O3 [M+H]</td>
<td>243.1059</td>
<td>1.90</td>
<td>Plasma - - - - - - - - - -</td>
<td>1.72</td>
<td>0.288</td>
<td>↓</td>
<td>2.54</td>
</tr>
<tr>
<td>Equol 7-O- glucuronide 1 C21H21O9 [M+H]</td>
<td>417.1384</td>
<td>1.90</td>
<td>Plasma - - - - - - - - - -</td>
<td>1.31</td>
<td>0.167</td>
<td>↓</td>
<td>3.1</td>
</tr>
<tr>
<td>Equol 4'-sulfate 2 C15H13O6 [M+H]</td>
<td>321.0484</td>
<td>2.16</td>
<td>Plasma - - - - - - - - - -</td>
<td>1.39</td>
<td>0.292</td>
<td>↓</td>
<td>1.97</td>
</tr>
<tr>
<td>Homovanillic acid sulfate 2 C9H8O7S5 [M+H]</td>
<td>261.0072</td>
<td>1.43</td>
<td>Plasma - - - - - - - - - -</td>
<td>1.94</td>
<td>0.544</td>
<td>↑</td>
<td>1.8</td>
</tr>
<tr>
<td>Indole-3-carboxaldehyde 1 C9H8NO [M+H]</td>
<td>144.0448</td>
<td>2.10</td>
<td>Plasma - - - - - - - - - -</td>
<td>1.57</td>
<td>1</td>
<td>↓</td>
<td>2.38</td>
</tr>
<tr>
<td>Indole-3-carboxylic acid (Fragment) 1 C8H7N [M+H]</td>
<td>118.0655</td>
<td>1.69</td>
<td>Plasma - - - - - - - - - -</td>
<td>2.35</td>
<td>1</td>
<td>↓</td>
<td>2.51</td>
</tr>
<tr>
<td>L-acetylcarnitine 1 C9H8NO4 [M+H]</td>
<td>204.1232</td>
<td>0.78</td>
<td>Plasma - - - - - - - - - -</td>
<td>1.35</td>
<td>0.857</td>
<td>↑</td>
<td>1.12</td>
</tr>
<tr>
<td>L-carnitine 1 C7H15NO3 [M+H]</td>
<td>162.1126</td>
<td>0.53</td>
<td>Plasma - - - - - - - - - -</td>
<td>1.04</td>
<td>1</td>
<td>↓</td>
<td>1.23</td>
</tr>
<tr>
<td>lysoPC (20:3) 3 C28H52NO7P [M+H]</td>
<td>546.3556</td>
<td>4.10</td>
<td>Plasma - - - - - - - - - -</td>
<td>1.36</td>
<td>1</td>
<td>↓</td>
<td>1.63</td>
</tr>
<tr>
<td>lysoPC (20:3) 3 C30H50NO7P [M+H]</td>
<td>568.3377</td>
<td>4.23</td>
<td>Plasma - - - - - - - - - -</td>
<td>1.18</td>
<td>1</td>
<td>↑</td>
<td>1.19</td>
</tr>
<tr>
<td>P-cresol glucuronide 1 C13H15O7 [M+H]</td>
<td>283.0819</td>
<td>1.78</td>
<td>Plasma - - - - - - - - - -</td>
<td>2.30</td>
<td>1</td>
<td>↑</td>
<td>6.62</td>
</tr>
</tbody>
</table>

*Fold change was calculated using mean raw intensity values for each metabolite. All observed changes were calculated using mean raw intensity values for each metabolite. *p* values were < 5 ppm mass error relative to the theoretical m/z.

Table 3.2 Summary of metabolite alterations over time in plasma, urine, and kidney of FVB/N mice treated with cisplatin compared to FVB/N mice treated with saline.
Cluster numbers are based on the time course cluster analysis, as shown in Figure 3.5.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>p-cresol sulfate</td>
<td>C7H8O4S [M+H]</td>
<td>187.0063</td>
<td>191</td>
<td>1</td>
<td>5</td>
<td>151</td>
<td>1</td>
<td>6.76</td>
<td>0.004</td>
<td>↑</td>
<td>1942</td>
<td>6.50E-09</td>
<td>↑</td>
<td>117</td>
<td>4</td>
<td>1</td>
<td>1.44E-14</td>
<td>0.0324</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenolglycine</td>
<td>C10H12NO 3 [M-H]</td>
<td>192.0659</td>
<td>180</td>
<td>1</td>
<td>2</td>
<td>2.16</td>
<td>0.882</td>
<td>↑</td>
<td>8.17</td>
<td>0.008</td>
<td>↑</td>
<td>191</td>
<td>1</td>
<td>↑</td>
<td>16.24</td>
<td>9.80E-14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>C8H10N+</td>
<td>120.0809</td>
<td>120</td>
<td>1</td>
<td>2</td>
<td>1.19</td>
<td>1</td>
<td>4.45</td>
<td>1.87E-06</td>
<td>↑</td>
<td>4.24</td>
<td>4.00E-07</td>
<td>↑</td>
<td>24.88</td>
<td>1.26E-14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>C11H12NO 3 [M-H]</td>
<td>206.0818</td>
<td>197</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>1.44</td>
<td>0.002</td>
<td>↑</td>
<td>1.35</td>
<td>0.01</td>
<td>↑</td>
<td>1.24E-05</td>
<td>0.025</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proline Betaine</td>
<td>C7H13NO2 [M-H]</td>
<td>144.1019</td>
<td>070</td>
<td>3</td>
<td>2</td>
<td>1.22</td>
<td>0.298</td>
<td>↑</td>
<td>1.17</td>
<td>1</td>
<td>↑</td>
<td>3.65</td>
<td>0.026</td>
<td>↑</td>
<td>3.24</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyroacetohol Sulfate</td>
<td>C6H6O5S [M-H]</td>
<td>188.9860</td>
<td>164</td>
<td>2</td>
<td>5</td>
<td>1.31</td>
<td>0.196</td>
<td>↓</td>
<td>8.37</td>
<td>0.008</td>
<td>↓</td>
<td>9.79</td>
<td>0.012</td>
<td>↓</td>
<td>47.59</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>C4H6O4 [M-H]</td>
<td>117.0187</td>
<td>113</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>1.13</td>
<td>1</td>
<td>↓</td>
<td>2.49</td>
<td>0.001</td>
<td>↓</td>
<td>4.48</td>
<td>3.06E-05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfaglycolithoc acid</td>
<td>C26H43NO3 [M-H]</td>
<td>512.2683</td>
<td>225</td>
<td>3</td>
<td>1</td>
<td>1.04</td>
<td>1</td>
<td>5.88</td>
<td>0.009</td>
<td>↑</td>
<td>56.31</td>
<td>9.62E-05</td>
<td>↑</td>
<td>1.16</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>C2HNO3S [M-H]</td>
<td>124.0065</td>
<td>051</td>
<td>1</td>
<td>5</td>
<td>1.38</td>
<td>1</td>
<td>6.78</td>
<td>7.6E-02</td>
<td>↑</td>
<td>2.44</td>
<td>1</td>
<td>↑</td>
<td>14.77</td>
<td>0.043</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>C26H45NO3 [M-H]</td>
<td>514.2840</td>
<td>238</td>
<td>2</td>
<td>1</td>
<td>1.37</td>
<td>1</td>
<td>1.46</td>
<td>0.33</td>
<td>↑</td>
<td>3.94</td>
<td>0.0003</td>
<td>↑</td>
<td>3.94</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trigonelline</td>
<td>C7H8NO2 [M-H]</td>
<td>138.0553</td>
<td>062</td>
<td>1</td>
<td>5</td>
<td>1.38</td>
<td>1</td>
<td>3.02</td>
<td>0.009</td>
<td>↑</td>
<td>2.75</td>
<td>0.047</td>
<td>↑</td>
<td>1.85</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsophan</td>
<td>C11H10NO2 [M-H]</td>
<td>188.0709</td>
<td>135</td>
<td>1</td>
<td>5</td>
<td>1.01</td>
<td>1</td>
<td>1.95</td>
<td>0.003</td>
<td>↑</td>
<td>1.54</td>
<td>0.003</td>
<td>↑</td>
<td>1.07</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>C2H10O5S [M-H]</td>
<td>217.0171</td>
<td>145</td>
<td>2</td>
<td>9</td>
<td>1</td>
<td>1.79</td>
<td>0.023</td>
<td>↓</td>
<td>1.62</td>
<td>0.0189</td>
<td>↓</td>
<td>1.18</td>
<td>1.10E-02</td>
<td>0.0486</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.3 Early Plasma Biomarkers of Cisplatin-induced Acute Kidney Injury

Plasma creatine, L-acetylcarnitine, p-cresol sulfate, phenylalanine, taurine, sulfolithocholylglycine, taurocholate, and tryptophan were among identified plasma metabolites that were increased by cisplatin treatment (Figure 3.6 and Figure 3.7). In C57BL/6 mice, L-acetylcarnitine and taurine were significantly increased as early as day 1 and day 2, respectively, and remained elevated throughout day 3 and 4 (Figure 3.6C, 3.6H). Although L-acetylcarnitine and taurine were also elevated with cisplatin treatment in FVB/N mice, these changes were only significant on days 3 and 4 (Figure 3.7C, 3.7H). Amino acids phenylalanine and tryptophan exhibited early increases in both strains, though C57BL/6 mice showed significant changes earlier than FVB/N mice. Phenylalanine and tryptophan were significantly elevated on day 1 after cisplatin treatment in C57BL/6 mice (Figure 3.6F, 3.6J), whereas significant increases in both amino acids were observed two days following cisplatin treatment in FVB/N mice (Figure 3.7F, 3.7J). Bile acids sulfolithocholylglycine and taurocholic acid showed a similar pattern for both strains of mice, where a transient but significant elevation was observed on day 2 for C57BL/6 mice (Figure 3.6G, 3.6I) and days 2 and 3 for FVB/N mice (Figure 3.7G, 3.7I). Creatine and p-cresol sulfate were significantly elevated in cisplatin-treated FVB/N mice from days 2-4 (Figure 3.7A, 3.7E), and although they showed the same pattern of elevation in C57BL/6 mice, the changes were not statistically significant (Figure 3.6A, 3.6E).

2,3-dihydroxybenzoic acid, equol 4-sulfate, and LysoPC(20:3) were among plasma metabolites decreased by cisplatin treatment (Figure 3.6 and Figure 3.7). In C57BL/6 mice, 2,3-dihydroxybenzoic acid decreased in the early timepoints, but did not show significant early alterations in the plasma of FVB/N mice. Plasma LysoPC(20:3) was consistently decreased for all timepoints for C57BL/6 mice but was not significantly changed in FVB/N mice until day 3. Lastly, plasma levels of equol 4-sulfate were significantly lower in FVB/N mice from days 2-4, but no significant changes were observed in C57BL/6 plasma until day 4 (Figure 3.6 and Figure 3.7).
Figure 3.6 Log transformed relative intensity of features significantly affected by cisplatin treatment in plasma samples from C57BL/6 mice.

Data are presented as box and whisker plots, where boxes represent the median and interquartile range (IQR), and whisks represent 1.5x IQR. Statistical analysis was performed using two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, compared to saline control at each timepoint. n=5-9, exact sample sizes can be found in Supplementary Table B1, Appendix B.
Figure 3.7 Log transformed relative intensity of features significantly affected by cisplatin treatment in plasma samples from FVB/N mice.

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 two-way ANOVA, *p < 0.05 compared to saline control at each timepoint. n=7-8, exact sample sizes can be found in Supplementary Table B1, Appendix B.
3.3.4 Early Urine Biomarkers of Cisplatin-induced Acute Kidney Injury

The vast majority of urinary metabolites were decreased following cisplatin treatment. Of 683 metabolites significantly altered by cisplatin treatment in C57BL/6 mice, 665 were decreased (97.3%, Figure 3.4B), whereas 550/599 (91.8%) metabolites were decreased in FVB/N mice (Figure 3.5B). Out of the identified metabolites, L-carnitine was the only metabolite increased by cisplatin treatment (Figure 3.8G and Figure 3.9G) and was consistently elevated in the urine of C57BL/6 mice from day 2-4 (Figure 3.8G). In FVB/N mice, L-carnitine was also higher in the urine of cisplatin-treated mice throughout all timepoints, though only significant on day 1 (Figure 3.9G).

Citric acid, equol, equol 7-O-glucuronide, homovanillic acid sulfate, indole-3-carboxaldehyde, indole-3-carboxylic acid, phenylpropionylglycine, pyrocatechol sulfate, succinate, trigonelline, and tyrosol 4-sulfate were all significantly decreased by cisplatin treatment. In both strains of mice, equol, equol 7-O-glucuronide, indole-3-carboxaldehyde, succinate, and trigonelline were all significantly lower in the cisplatin groups from days 2-4 (Figure 3.8 and Figure 3.9). Citric acid, homovanillic acid sulfate, indole-3-carboxylic acid, phenylpropionylglycine, pyrocatechol sulfate, and tyrosol 4-sulfate showed a similar pattern, with decreased levels of these metabolites in cisplatin treated mice from days 2-4, albeit not achieving significance at all time points for both strains (Figure 3.8 and Figure 3.9).
Figure 3.8 Log transformed relative intensity of features significantly affected by cisplatin treatment in urine samples from C57BL/6 mice.

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 two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, compared to saline control at each timepoint. n=6-9, exact sample sizes can be found in Supplementary Table B1, Appendix B.
Figure 3.9 Log transformed relative intensity of features significantly affected by cisplatin treatment in urine samples from FVB/N mice.

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 two-way ANOVA, *p < 0.05 compared to saline control at each timepoint. n=6-8, exact sample sizes can be found in Supplementary Table B1, Appendix B.
3.3.5 Early Kidney Biomarkers of Cisplatin-induced Acute Kidney Injury

Hierarchical clustering revealed that the majority of significantly altered kidney metabolites were increased by cisplatin treatment, with 44/63 (69.8%) metabolites being increased in C57BL/6 mice (Figure 3.4C) and 50/73 (68.5%) metabolites increased in FVB/N mice (Figure 3.5C). Creatine, p-cresol glucuronide, p-cresol sulfate, phenylacetylglycine, phenylalanine, and tryptophan were found to be elevated by cisplatin treatment in kidney tissue. Creatine showed significant elevation throughout all four timepoints in FVB/N mice, and similarly was also increased in C57BL/6 mice, though only significantly increased at days 2 and 3 (Figure 3.10A, 3.11A). P-cresol glucuronide, p-cresol sulfate, and phenylacetylglycine were all significantly elevated from day 2-4 in cisplatin-treated FVB/N kidneys (Figure 3.11). These metabolites followed a similar pattern in C57BL/6 mice but were significantly altered only at day 3 and 4 for p-cresol glucuronide and sulfate, and days 1 and 4 for phenylacetylglycine (Figure 3.10). Kidney levels of phenylalanine (Figure 3.10G, 3.11G) and tryptophan (Figure 3.10J, 3.11J) were also generally increased by cisplatin treatment, but only tryptophan in C57BL/6 mice was shown to be significantly increased in the early timepoints.

Equol 4-sulfate, LysoPC(20:3), proline betaine, and trigonelline were kidney metabolites identified to be decreased by cisplatin treatment. In kidneys from FVB/N mice, levels of equol 4-sulfate were significantly decreased following cisplatin treatment for all timepoints (Figure 3.11B), and though equol 4-sulfate showed similar decreases in C57BL/6 kidneys, none of the changes were significant (Figure 3.10B). Proline betaine was consistently altered by cisplatin treatment in both strains, being significantly decreased from days 2-4 (Figure 3.10H, 3.11H). LysoPC(20:3) and trigonelline were also significantly decreased from days 2-4 in cisplatin-treated FVB/N mice (Figure 3.11). These metabolites were also decreased by cisplatin treatment in C57BL/6 kidneys, though the alterations were only significant on day 4 for both metabolites (Figure 3.10).
Figure 3.10 Log transformed relative intensity of features significantly affected by cisplatin treatment in kidney samples from C57BL/6 mice.

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 two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, compared to saline control at each timepoint. n=7-10, exact sample sizes can be found in Supplementary Table B1.
Figure 3.11 Log transformed relative intensity of features significantly affected by cisplatin treatment in kidney samples from FVB/N mice.

Data are presented as box and whisker plots, where boxes represent the median and interquartile range (IQR), and whisks represent 1.5x IQR. Statistical analysis was performed using two-way ANOVA, *p < 0.05 compared to saline control at each timepoint. n=7-8, exact sample sizes can be found in Supplementary Table B1.
3.3.6  Assessment of the Diagnostic Performance of Biomarkers

Univariate ROC curves were generated for each metabolite at each timepoint, and univariate AUROC was calculated to assess the diagnostic performance of each individual metabolite. All AUROC values for early plasma markers were summarized in Figure 3.12 and all AUROC values for early urine markers were summarized in Figure 3.13. Metabolites were ranked from highest to lowest univariate AUROC values. Multivariate ROCs were generated to assess the aggregate diagnostic performance of a combination of metabolites, ranging from a combination of the top two highest ranked metabolites to the top 10 and top 15 ranked metabolites for plasma (Figure 3.12) and urine (Figure 3.13), respectively.

An AUROC value ≥ 0.7 is generally considered to be acceptable. When discriminating saline vs. cisplatin treated C57BL/6 mice, plasma creatinine had an AUROC of 0.719 at day 1. Nine plasma metabolites had an AUROC ≥ 0.7 when discriminating saline vs. cisplatin-treated C57BL/6 mice on day 1 (creatine; 0.729, equol 4-sulfate; 0.736, L-acetylcarnitine; 0.944, L-carnitine; 0.944, LysoPC(20:3); 0.875, phenylalanine; 0.917, taurocholic acid; 0.708, trigonelline; 0.785, and tryptophan; 0.826). Eight of the nine metabolites with AUROC ≥ 0.7 on day 1 outperformed plasma creatinine, with the exception of taurocholic acid. In FVB/N mice, plasma creatinine had an AUROC of 0.562 on day 1. Five plasma metabolites in FVB/N mice had an AUROC ≥ 0.7 at day 1 (creatine; 0.773, pyrocatechol sulfate; 0.828, taurine; 0.719, taurocholic acid; 0.891, and trigonelline; 0.891). All 5 metabolites with AUROC ≥ 0.7 on day 1 outperformed plasma creatinine on day 1.

Multivariate ROCs generated for C57BL/6 plasma samples on day 1 revealed a maximum AUROC of 0.877, which was achieved in the ROC curve modeled using the top three ranked metabolites: L-acetylcarnitine, phenylalanine, and LysoPC(20:3) (Figure 3.12B). The maximum multivariate AUROC achieved in C57BL/6 urine was 0.545 on day 1 but reached AUROC values of 1 in all combinations of the top two to top 15 metabolites on day 2 (Figure 3.13B, 3.13C). In FVB/N plasma, a peak AUROC of 0.656 was obtained at day 1, whereas AUROC values of ≥ 0.991 were observed on day 2 for all combinations of metabolites (Figure 3.12E, 3.12F). In day 1 FVB/N urine samples, the highest multivariate
AUROC value of 0.940 was produced via the combination of all 15 early urine metabolites (Figure 3.13E). Diagnostic performance was further improved in day 2 FVB/N urine, with all metabolite combinations yielding an AUROC $\geq 0.953$ (Figure 3.13F).
Summary of area under the ROC curve values calculated for each plasma metabolite at all timepoints (A, D). Multivariate ROC curves were generated at day 1 (B, E) and day 2 (C, F) using a variable number of metabolites. ROC curves were generated using a sequential combination of the top two to top 10 ranked metabolites, where each metabolite was ranked using the calculated univariate AUROC at each respective timepoint.

**Figure 3.12** Receiver operating characteristic (ROC) analysis of early plasma biomarkers in C57BL/6 mice (A-C) and FVB/N mice (D-F).
Summary of area under the ROC curve values calculated for each urine metabolite at all timepoints (A, D). Multivariate ROC curves were generated at day 1 (B, E) and day 2 (C, F) using a variable number of metabolites. ROC curves were generated using a sequential combination of the top two to top 15 ranked metabolites, where each metabolite was ranked using the calculated univariate AUROC at each respective timepoint.

**Figure 3.13** Receiver operating characteristic (ROC) analysis of early urine biomarkers in C57BL/6 mice (A-C) and FVB/N mice (D-F).
3.4 Discussion

In this study, untargeted metabolomics was employed in a mouse model of cisplatin-induced AKI to investigate early metabolic changes following cisplatin administration. Previous studies have utilized untargeted metabolomics to study rodent models of cisplatin-induced AKI\textsuperscript{13–16}, but have typically focused on studying one or two biological matrices. Furthermore, though some of these studies have looked at the metabolic alterations induced by cisplatin-induced AKI over multiple timepoints, others have only studied a single timepoint after establishment of AKI. To our knowledge, our study is the first to comprehensively assess temporal metabolic alterations in all three of plasma, urine, and kidney samples throughout AKI progression. Additionally, our study used two separate strains of mice, allowing for the corroboration of metabolic changes observed in either strain. Metabolites that exhibited early alterations in plasma, urine, and kidney samples were identified as potential early biomarkers for cisplatin-induced nephrotoxicity; in total, 26 such metabolites were identified.

Our study used a single dose of cisplatin to induce nephrotoxicity in a mouse model. Although patients typically receive cisplatin infusions in multiple cycles over the course of multiple weeks, a single infusion is sufficient to induce acute kidney injury in approximately 1/3 of cisplatin-naïve patients\textsuperscript{17}. The clinical presentation of AKI after a single cisplatin infusion lends support for the suitability of a single dose model for an acute study of cisplatin toxicity. Previous studies have reported that FVB/N mice are more sensitive to cisplatin-induced AKI compared to C57BL/6 mice\textsuperscript{10}, but surprisingly, there were no differences between the two strains in this study with respect to either SCr or histological signs of injury. As we were unable to observe the differences in nephrotoxicity between the two strains in our study, the metabolic variation between the strains was not investigated. The two strains were instead used to confirm the consistency of change of early biomarkers and to exhibit the robustness of proposed biomarkers in two separate cohorts of mice.

The extent of renal injury throughout the day 1-4 timepoints was assessed by plasma creatinine and histological assessment. As expected based on previously published literature, kidney injury was established on day 4 after cisplatin injection, as evaluated by
SCr and histology. However, distinct metabolic alterations were observed between saline and cisplatin-treated mice as early as 1 – 2 days following injection, as evidenced by PCA/OPLS-DA and time course clustering analysis. These findings indicate the presence of metabolites that reflect cisplatin toxicity prior to the presentation of elevated SCr and histological injury. Metabolites exhibiting early alterations after cisplatin treatment were identified as potential early diagnostic biomarkers of cisplatin-induced nephrotoxicity. The metabolic alterations observed were due to cisplatin toxicity, as evidenced by accumulation of uremic toxins such as p-cresol sulfate and p-cresol glucuronide, especially in the late timepoints where kidney function was impaired. Uremic toxins accumulate in patients with impaired kidney function and have been particularly well documented in studies of chronic kidney disease\textsuperscript{18}. Furthermore, mitochondria are well established to be major targets of cisplatin toxicity\textsuperscript{19}. Many of the metabolites identified as potential early biomarkers were indicative of dysregulated mitochondrial function, providing further indication that cisplatin toxicity is driving the metabolic changes observed in our mouse model.

Mitochondrial dysfunction is a key component of the pathogenesis of AKI, with the kidneys being an organ with very high energy demand\textsuperscript{19}. Many metabolites identified as early biomarkers were related to mitochondrial energy metabolism, including metabolites related to fatty acid β-oxidation (FAO) and the tricarboxylic acid (TCA) cycle. Cisplatin has previously been shown to inhibit FAO by deactivating PPAR-α, a nuclear receptor that plays a crucial role in the regulation of FAO\textsuperscript{20,21}. L-carnitine plays a vital role in FAO, allowing for the transport of long chain fatty acids across the mitochondrial inner membrane\textsuperscript{22}. Plasma accumulation of acylcarnitines, formed by the conjugation of L-carnitine and fatty acids, are indicative of disorders FAO\textsuperscript{23}. Both L-carnitine and L-acetylcarnitine were altered by cisplatin treatment, with early elevations observed for urinary L-carnitine in both mouse strains and early increases in plasma L-acetylcarnitine levels in C57BL/6 mice. These cisplatin-induced alterations in urinary L-carnitine and plasma L-acetylcarnitine were sustained throughout the duration of the study. L-carnitine and acylcarnitines have previously been shown to be altered in other rodent models of cisplatin-induced nephrotoxicity\textsuperscript{13,14,16} and human patients undergoing cisplatin chemotherapy\textsuperscript{24,25}. In patients receiving cisplatin, urinary excretion of L-carnitine was significantly increased even on the first day of cisplatin treatment, with increased urine
levels being maintained for the next two days\textsuperscript{24,25}. Though studies have shown accumulation of acylcarnitines in the blood as early as 24 hours following cisplatin administration, our study is the first metabolomics study to show an early elevation of plasma L-acetylcarnitine, specifically, following cisplatin administration. In addition to markers of dysfunctional FAO, urine levels of TCA cycle intermediates citric acid and succinate showed significant decreases as early as two days after cisplatin treatment in C57BL/6 mice, in accordance with previous studies linking cisplatin and early alterations of TCA cycle intermediates in urine\textsuperscript{15,16,26}. Moreover, creatine, which plays a crucial role in the creatine kinase-phosphocreatine circuit, was consistently increased in the plasma, urine, and kidneys of both strains of mice throughout progression of kidney damage, though alterations were more pronounced in FVB/N mice. The creatine kinase system is responsible for coupling cellular sites of ATP production to sites of ATP consumption\textsuperscript{27} and is particularly sensitive to oxidative stress\textsuperscript{28,29}. An overall accumulation of creatine suggests an impairment in the generation of high energy phosphate molecules. These findings are in accordance with an untargeted metabolomics studied performed in adult head and neck cancer patients receiving cisplatin therapy, where early alterations were observed in numerous metabolites associated with FAO and the TCA cycle upon cisplatin administration. These similarities between the metabolic alterations observed in our mouse model compared to human patient samples corroborates the utility of the mouse model for studying the effects of cisplatin on mitochondrial energetics and exploring the mitochondria as a target for nephroprotective interventions\textsuperscript{17}.

Gut-derived metabolites of tryptophan indole-3-carboxaldehyde and indole-3-carboxylic acid\textsuperscript{30,31} were found to be significantly decreased as early as day 1 and day 2, respectively, in the urine of cisplatin-treated mice. Both indole-3-carboxaldehyde and indole-3-carboxylic acid have shown the ability to activate aryl hydrocarbon receptor (AhR)\textsuperscript{32,33}, which has been shown to induce renal fibrosis, podocyte injury, glomerular damage, inflammation, and is correlated with exacerbation of chronic kidney disease in both animal models and human patients\textsuperscript{34}. Phenylalanine and downstream gut-derived metabolites of phenylalanine also exhibited early alterations following cisplatin treatment in our study. Phenylalanine was elevated in plasma, urine, and kidney samples following cisplatin exposure, with plasma levels being significantly elevated as early as day 1 in C57BL/6
mice and day 2 in FVB/N mice. Phenylalanine is metabolized by intestinal bacteria to p-
cresol35, which is subsequently metabolized to p-cresol sulfate or p-cresol glucuronide in
the liver36,37. Both p-cresol conjugates were elevated in plasma, urine, and kidneys with
cisplatin treatment, where the most striking and consistent differences were observed in
FVB/N mice. P-cresol derivatives have predominantly been studied in the setting of
chronic kidney disease, where they have been associated with exacerbation of renal injury
and cardiovascular disease36,38. Additionally, urinary excretion of acylglycines
phenylpropionylglycine and phenylacetylglycine, gut-derived down-stream metabolites of
phenylalanine39–41, were found to be altered in cisplatin-treated mice. Urinary excretion of
phenylpropionylglycine was consistently decreased in both mouse strains throughout
cisplatin treatment, whereas phenylacetylglycine was shown to accumulate in the kidneys,
altogether suggesting tissue accumulation of acylglycine compounds. Acylglycines have
historically been used as markers of disorder of metabolism and FAO, where a defect in β-
odxidation is typically characterized by an increased urinary excretion of acylglycines42,43.
An accumulation of intracellular acyl-CoA due to disorders of FAO is associated with
toxicity and detrimental to mitochondrial function44,45, and conjugation of glycine with
these acyl-CoA compounds to form acylglycines has shown to have a detoxification
effect46.

Metabolomics analysis also revealed diet-derived secondary plant metabolites to be
affected by cisplatin treatment. In both strains of mice, urinary excretion of dietary phenolic
metabolites homovanillic acid sulfate, pyrocatechol sulfate, and tyrosol 4-sulfate47–49 were
significantly decreased two days after cisplatin treatment and remained decreased
throughout the study. Metabolomics studies in CKD rats and CKD patients have also
reported the accumulation of plasma pyrocatechol sulfate in the setting of renal decline50,51.
Polyphenolic compounds found in olive oil, including unconjugated precursors of
homovanillic acid sulfate and tyrosol 4-sulfate (homovanillic acid and tyrosol,
respectively) have routinely been reported to possess a multitude of health benefits in a
variety of pathophysiological conditions47,52,53. Trigonelline is a dietary plant alkaloid that
has been reported to possess hypoglycemic, anti-diabetic, antioxidant and overall
renoprotective properties. Trigonelline was consistently reduced in the urine and kidneys
of cisplatin treated mice. Studies have shown trigonelline to have beneficial effects against
diabetic nephropathy\textsuperscript{54,55}, and in attenuating epithelial-to-mesenchymal transition and ROS generation in an oxalate-induced \textit{in vitro} model of renal fibrosis\textsuperscript{56}. Our study adds to previous findings from a metabolomics study in a mouse model of cisplatin-induced AKI which reported an early decrease in urinary trigonelline\textsuperscript{15}, by showing decreased levels of trigonelline in the plasma and kidneys of cisplatin-treated mice in addition to urine.

Taurine is a $\beta$-amino acid that has a protective role against oxidative stress, reducing mitochondrial production of oxidants\textsuperscript{57}. Additionally, taurine plays a crucial role in post-translation modification of mitochondrial tRNAs, which are important in leucine and lysine synthesis, and by extension mitochondrial protein translation of electron transport chain complexes\textsuperscript{58,59}. In our study, taurine was significantly higher in the plasma of day 2 cisplatin treated C57BL/6 mice, though a significant elevation was not observed until day 3 in the plasma of FVB/N mice. Taurine enters tubular cells via uptake by taurine transporter (TauT). TauT is found on both apical and basolateral membranes of renal tubular cells, with expression varying based on tubular cell type\textsuperscript{60}. Cisplatin exposure to proximal tubule cells for 24 hours \textit{in vitro} has been shown to downregulate gene and protein expression of TauT and reduce the function of TauT, potentially through stimulation of p53\textsuperscript{61}. Alterations in taurine transport may help to explain the plasma accumulation of taurine observed in our study.

As previously discussed, the main strength of our study was the comprehensive metabolomic analysis of three biological matrices sampled at multiple timepoints throughout the progression of kidney injury. Moreover, two separate strains of mice were investigated, demonstrating that cisplatin-induced metabolic alterations could be reproduced in two different strains of mice. There were also some limitations to this study. Firstly, only male mice were studied, and sex differences could not be assessed. We used male mice in our study as the initial objective was to focus on the strain differences in susceptibility to cisplatin-induced nephrotoxicity, which were reported in a study of male mice. Additionally, the most common indication for high dose cisplatin is head and neck cancer which predominantly affects males. As we only used male mice in this investigation, we recognize that this limits the generalizability of our findings, and sex differences must be taken into consideration in future validation studies for our proposed candidate
biomarkers. Additionally, diet is known to affect metabolomics; mice had free access to chow ad libitum, and cisplatin-treated mice were observed to eat less compared to saline controls.

3.5 Conclusion

Current clinical markers of AKI are incapable of detecting cisplatin-induced nephrotoxicity prior to the establishment of significant renal injury and functional impairment, and there is a need for biomarkers that are capable of earlier detection. In this study, a total of 26 plasma, urine, and kidney metabolites were identified as potential early biomarkers of cisplatin-induced acute kidney injury. Alterations in these metabolites over time following cisplatin administration were consistent in two separate strains of mice. These markers may help to better understand the pathophysiological mechanisms behind cisplatin nephrotoxicity and AKI in general. Many of these metabolites are indicative of dysregulated mitochondrial dysfunction, highlighting the detrimental effects of cisplatin on mitochondria. Many metabolites were dietary and gut-derived, indicating the need to investigate the crosstalk between the gut and the kidney in the setting of acute kidney injury. Our study provides a large panel of metabolites that can be targeted for future clinical studies of early detection of AKI and may provide guidance in the selection of therapeutic targets against cisplatin-induced nephrotoxicity. Further studies are necessary to validate the applicability, utility, and translatability of these metabolites in a clinical setting.
3.6 References


42. Ruiz-sala P, Peña-quintana L. Biochemical Markers for the Diagnosis of Mitochondrial Fatty Acid Oxidation Diseases. Published online 2021.


45. Ellis JM, Bowman CE, Wolfgang MJ. Metabolic and Tissue-Specific Regulation of Acyl-CoA Metabolism. Published online 2015. doi:10.1371/journal.pone.0116587


Chapter 4

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

A version of this chapter has been published:


Reproduced with permission from Wiley.
4.1 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\(^1\). Cisplatin is primarily eliminated by the kidneys through tubular secretion and glomerular filtration, and consequently accumulates in the kidneys to cause kidney injury\(^1,2\). Cisplatin-induced nephrotoxicity presents as acute kidney injury (AKI) in approximately one-third of patients receiving cisplatin\(^1,3\). 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\(^4\). Clinical diagnosis of AKI is based on increases in serum creatinine (SCr) concentrations or a decrease in urine output\(^5\). However, serum creatinine and decreased urine output are markers of functional impairment, only manifesting after significant kidney injury and impairment of glomerular filtration\(^6\). 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\(^7,8\). 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\(^7,9\).

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\(^10–13\), to our knowledge, our study is the first to use untargeted metabolomics in a cohort of patients receiving cisplatin therapy.
4.2 Materials and Methods

4.2.1 Chemicals and Reagents

The following reagents, solvents and standards were purchased from Millipore Sigma (Oakville, ON, Canada): HPLC grade acetonitrile, atenolol-d7, betaine, butylated hydroxytoluene (BHT), creatinine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 3-nitrophenylhydrazine (3-NPH), octanoylcarnitine, phenylisothiocyanate (PITC), pyridine, and taurine. Chlorpropamide and 3-hydroxydecanedioic acid were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Ammonium acetate and histidine were purchased from Bio Basic Inc. (Markham, ON, Canada). Indoxyl sulfate was purchased from Gold Biotechnology (St. Louis, MO, USA).

4.2.2 Study Participants and Sample Collection

Participants were recruited as part of a pilot for the ACCENT (A Canadian study of Cisplatin mEtabolomics and NephroToxicity) 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/m²) for head and neck cancer. Exclusion criteria were chronic kidney disease (GFR < 60 ml/min) 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. All patients recruited in this pilot study were cisplatin naïve and received a cisplatin dose of 100 mg/m². Patient demographic information (Table 4.1) 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 published14.

4.2.3 Classification of AKI

Patients were classified as “no AKI” or “AKI” based on the Kidney Disease Improving Global Outcomes (KDIGO) guidelines5, 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. Serum creatinine concentrations measured at the post timepoint were compared to those measured at the pre timepoint to classify AKI.

4.2.4 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 in section 3.2.4. A pooled sample was generated to serve as quality control (QC) throughout the metabolomics run.

4.2.5 Chemical Derivatization of Serum and Urine Samples

Chemical 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. al15. ACN was added to serum and urine samples for protein precipitation and supernatant was isolated. Isolated supernatant was used for both PITC and NPH derivatization reactions.

For PITC derivatization, 50 µL of LC-MS grade water containing internal standard (10 µM chlorpropamide and 18 µm atenolol-d7) was added to 10 µL of supernatant, followed by evaporation in a 37 °C degree water bath under a constant stream of air. A 5% PITC (%v/v) solution was prepared in a 1:1:1 ethanol:water:pyridine mixture, and 50 µL of the PITC reaction mix was added to each evaporated sample and incubated at room temperature for
20 minutes. Excess PITC solution was removed by evaporation and reconstituted in 300 µL of methanol solution containing 5 mM ammonium acetate by shaking at 450 rpm for 30 minutes. Each sample was subsequently diluted with the addition of 300 µL of LC-MS grade water and transferred to glass chromatography vials for LC-MS injection.

For 3-NPH derivatization, 50 µL of LC-MS grade water containing internal standard (10 µM chlorpropamide and 18 µm atenolol-d7) and 30 µL of methanol were added to 10 µL of supernatant, followed by the addition of 25 µL of each of the following: 250 mM solution of 3-NPH in 50:50 methanol:water, 150 mM solution of EDC in methanol, and 7.5% pyridine (%v/v) solution in 75:25 methanol:water. The reactions were shaken at 450 rpm for 2 hours at room temperature. Each reaction was diluted and stabilized by adding 350 µL of water and 25 µL of a 2 mg/mL solution of BHT in methanol, then transferred to glass chromatography vials for LC-MS injection.

For both PITC and 3-NPH reactions, pooled samples were generated to serve as QC.

4.2.6 Chromatography and Mass Spectrometry

Chromatographic separation was achieved using a Waters ACQUITY UPLC HSS T3 column (1.8 µm particle size, 2.1 mm x 100 mm) maintained at 45°C in a Waters ACQUITY UPLC I-Class system. Mobile phase A consisted of water + 0.1% formic acid and mobile phase B consisted of ACN + 0.1% formic acid. 5 µL of sample was injected for un-derivatized samples, whereas 10 µL of sample was injected for derivatized samples. Mobile phase gradient used for chromatographic separation is summarized in Supplementary Table C1 (Appendix C). For untargeted metabolomics, samples were analyzed in both positive and negative electrospray ionization (ESI) modes with the mass spectrometer parameters described in Supplementary Table C2 (Appendix C). Data was acquired in centroid mode, using the MS\(^E\) method in resolution mode. The MS\(^E\) method generates both the precursor ion (function 1) and fragment ions (function 2) in one acquisition. Data acquisition period was 11 minutes, with scan time of 0.05 seconds and a mass range of 50 – 1200 Da. Collision energy was set to 0 V for function 1 and was ramped from 15 – 50 V for function 2. Leucine-enkephalin (500 ng mL\(^{-1}\)) was used as lockspay solution to ensure mass accuracy. The lockspay solution was infused at a flow rate of 10
μL/min. Lockmass was acquired at intervals of 10 seconds and averaged over 3 scans. PITC derivatized samples were analyzed in positive ESI mode, and 3-NPH derivatized samples were analyzed in negative ESI mode, using the same mass spectrometer parameters used for untargeted metabolomics.

### 4.2.7 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.

### 4.2.8 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 ($R^2$ and $Q^2$ 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\textsuperscript{16}. Multivariate ROCs were generated using linear support vector machine classification, with features ranked by highest to lowest univariate AUROC values.

4.2.9 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\textsuperscript{17}.
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).

4.3 Results

4.3.1 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 4.1). Baseline demographics including age, BMI, baseline SCr/eGFR, and cigarette packs/day were nearly identical between no AKI and AKI patients (Table 4.1). 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).

4.3.2 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 by m/z and retention time with the library of standards.
Table 4-1 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. Groups were not statistically different from each other for any of the baseline characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Sex (M/F)</th>
<th>Age (years)</th>
<th>Body mass index (kg/m²)</th>
<th>Ethnicity (C/B/A/I/O)</th>
<th>Baseline SCr (µmol/L)</th>
<th>Baseline eGFR (mL/min/1.73m²)</th>
<th>Smoking (packs/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No AKI</strong> (n=20)</td>
<td>18/2</td>
<td>59.5 (7.1)</td>
<td>26.7 (5.2)</td>
<td>20/0/0/0/0</td>
<td>69.2 (13.4)</td>
<td>101.9 (9.0)</td>
<td>0.8 (0.6)</td>
</tr>
<tr>
<td><strong>AKI</strong> (n=11)</td>
<td>11/0</td>
<td>61.7 (3.8)</td>
<td>26.7 (6.0)</td>
<td>11/0/0/0/0</td>
<td>63.2 (10.4)</td>
<td>104.3 (6.8)</td>
<td>0.7 (0.6)</td>
</tr>
</tbody>
</table>
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 4.1A) and 24-48h timepoints (Figure 4.1B), and clear separation at the post timepoint (Figure 4.1C). In serum, strong separation was observed in both the pre (Figure 4.2A) and post (Figure 4.2C) timepoints, with moderate separation observed at the 24-48h timepoint (Figure 4.2B). 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 (R²) and predictive ability (Q²) at the post timepoint for both urine and serum (Figure 4.1F, 4.2F), as well as the pre timepoint for serum (Figure 4.2D). Moderate model statistics were observed for the 24-48h timepoint for both urine and serum (Figure 4.1E, 4.2E), as well as the pre timepoint for urine (Figure 4.1D). Features with 0.4 < 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 3.2.
Figure 4.1 Principle component analysis (A-C) and orthogonal partial least squares discriminant analysis (D-F) scores plots comparing urine samples from no AKI (black) and AKI (red) patients at each timepoint: pre (A, D), 24-48h (B, E), and post (C, F).

Model statistics for OPLS-DA were as follows: (D) pre: $R^2 = 0.784$, $Q^2 = 0.389$ (E) 24-48h: $R^2 = 0.752$, $Q^2 = 0.384$ (F) post: $R^2 = 0.969$, $Q^2 = 0.700$. n=18-20 for no AKI patients and n=10-11 for AKI patients.
Figure 4.2 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: pre (A, D), 24-48h (B, E), and post (C, F).

Model statistics for OPLS-DA were as follows: (D) pre: $R^2 = 0.991$, $Q^2 = 0.668$ (E) 24-48h: $R^2 = 0.776$, $Q^2 = 0.317$ (F) post: $R^2 = 0.995$, $Q^2 = 0.813$. $n=18-20$ for no AKI patients and $n=10-11$ for AKI patients.
<table>
<thead>
<tr>
<th>Metabolite Identity</th>
<th>ID Level</th>
<th>Chemical Formula</th>
<th>Observed m/z</th>
<th>tR (min)</th>
<th>Sample Type</th>
<th>Change in AKI Patients vs. No AKI Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pre</td>
</tr>
<tr>
<td>Aminoisobutanoate (NPH)</td>
<td>1</td>
<td>C4H9NO2 + C6H5N3O [M-H]</td>
<td>237.0987</td>
<td>1.44</td>
<td>Serum</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Betaine</td>
<td>1</td>
<td>C5H11NO2 [M-H]</td>
<td>118.0863</td>
<td>0.62</td>
<td>Serum</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td>1</td>
<td>C4H7N3O [M-H]</td>
<td>114.0664</td>
<td>0.61</td>
<td>Serum</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine (fragment)</td>
<td>1</td>
<td>C5H7N3O3 [M-H]</td>
<td>130.0489</td>
<td>0.56</td>
<td>Serum</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine (PITC)</td>
<td>1</td>
<td>C2H5NO2 + C7H5NS [M-H]</td>
<td>211.0542</td>
<td>1.94</td>
<td>Serum</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glyocolate (NPH)</td>
<td>1</td>
<td>C2H4O3 + C6H5N3O [M-H]</td>
<td>210.0514</td>
<td>1.74</td>
<td>Serum</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippuric acid sulfate</td>
<td>2</td>
<td>C9H9NO6S [M-H]</td>
<td>258.0072</td>
<td>1.36</td>
<td>Serum</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>1</td>
<td>C6H9N3O2 [M-H]</td>
<td>154.0616</td>
<td>0.53</td>
<td>Serum</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Hydroxydecanoic acid</td>
<td>1</td>
<td>C10H18O5 + C6H5N3O [M-H]</td>
<td>217.1075</td>
<td>1.76</td>
<td>Serum</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indole-3-acetate (NPH)</td>
<td>1</td>
<td>C10H9NO2 + C6H5N3O [M-H]</td>
<td>309.0987</td>
<td>2.55</td>
<td>Serum</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indoxyl sulfate</td>
<td>1</td>
<td>C8H7NO45 [M-H]</td>
<td>212.0017</td>
<td>1.87</td>
<td>Serum</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>3</td>
<td>C18H30O2 [M-H]</td>
<td>279.2319</td>
<td>4.36</td>
<td>Serum</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Octanoyl carnitine</td>
<td>1</td>
<td>C15H29NO4 [M+Na]</td>
<td>310.2015</td>
<td>2.00</td>
<td>Serum</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Octenoyl carnitine</td>
<td>2</td>
<td>C15H27NO4 [M-H]</td>
<td>286.2016</td>
<td>1.88</td>
<td>Serum</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Charge</th>
<th>Molecule Formula</th>
<th>[M+H] Mass (Da)</th>
<th>p&lt;sub&gt;M&lt;/sub&gt;</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
<th>Serum</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine (PITC)</td>
<td>1</td>
<td>C3H7NO3 + C7H5NS [M+H]</td>
<td>241.0647</td>
<td>1.86</td>
<td>-</td>
<td>1.39</td>
<td>-</td>
<td>1.37</td>
<td>-</td>
<td>0.6135</td>
<td>-</td>
<td>2.80</td>
<td>-</td>
<td>0.0176</td>
<td>-</td>
<td>0.0176</td>
<td>-</td>
<td>0.0176</td>
<td>-</td>
<td>0.0176</td>
<td>-</td>
<td>0.0176</td>
<td>-</td>
<td>0.0176</td>
<td>-</td>
<td>0.0176</td>
<td>-</td>
<td>0.0176</td>
<td>-</td>
<td>0.0176</td>
<td>-</td>
<td>0.0176</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suberate (NPH)</td>
<td>1</td>
<td>C8H14O4 + C6H5N3O [M-H]</td>
<td>443.1676</td>
<td>2.59</td>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>1</td>
<td>C2H7NO3S [M-H]</td>
<td>124.006</td>
<td>0.57</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine (PITC)</td>
<td>1</td>
<td>C4H9NO3 + C7H5NS [M+H]</td>
<td>255.0803</td>
<td>1.86</td>
<td>Serum</td>
<td>-</td>
<td>1.03</td>
<td>-</td>
<td>1.35</td>
<td>-</td>
<td>0.7733</td>
<td>-</td>
<td>2.90</td>
<td>-</td>
<td>0.0201</td>
<td>-</td>
<td>0.0201</td>
<td>-</td>
<td>0.0201</td>
<td>-</td>
<td>0.0201</td>
<td>-</td>
<td>0.0201</td>
<td>-</td>
<td>0.0201</td>
<td>-</td>
<td>0.0201</td>
<td>-</td>
<td>0.0201</td>
<td>-</td>
<td>0.0201</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMAP</td>
<td>1</td>
<td>C11H20N2O3 [M+H]</td>
<td>229.1554</td>
<td>1.15</td>
<td>Serum</td>
<td>-</td>
<td>1.58</td>
<td>1</td>
<td>1.30</td>
<td>0.1353</td>
<td>-</td>
<td>1.87</td>
<td>0.0195</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tyramine (PITC)</td>
<td>1</td>
<td>C8H11NO + C7H5NS [M+H]</td>
<td>273.1061</td>
<td>2.52</td>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

This table provides a summary of the masses and proportions for various compounds, along with their relative changes in serum and urine samples.
4.3.3 Predictive and Early Urinary Markers of Cisplatin-induced Acute Kidney Injury

In urine, glycine, 3-hydroxydecanedioic acid, hippuric acid sulfate, and suberate were found to be significantly different between no AKI and AKI patients prior to cisplatin infusion (Figure 4.3). 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 4.2, Figure 4.3C). 3-hydroxydecanedioic 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 4.2, Figure 4.3B, 4.3D). 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 4.2, Figure 4.3A).

Diagnostic performance of individual markers was assessed by calculation of univariate AUROC. AUROC values $\geq 0.7$ 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 4.3E). Glycine, 3-hydroxydecanedioic 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 $> 0.8$. Multivariate ROCs were generated to assess the aggregate diagnostic performance of a combination of metabolites at the pre timepoint. Metabolites were ranked based on their respective univariate AUROC values at the pre timepoint from highest AUROC to lowest. A maximum AUROC of 0.879 was achieved in the multivariate ROC curve modeled using all four metabolites: glycine, 3-hydroxydecanedioic acid, hippuric acid sulfate, and suberate (Figure 4.3F).
Figure 4.3 Log transformed relative intensity of urine features significantly different between no AKI and AKI patients at the pre timepoint (A-D).

Data are presented as box and whisker plots, where boxes represent the median and interquartile range (IQR), and whiskers represent 1.5x IQR. (E) Diagnostic performance of urine metabolites as assessed by the area under the receiver operating curve (AUROC). (F) Multivariate receiver operating characteristic (ROC) curves generated using a variable number of metabolites. ROC curves were generated using a sequential combination of the top two to top four ranked metabolites, where each metabolite was ranked using its respective calculated univariate AUROC at the pre timepoint. 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.
4.3.4 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-fold), and tyramine (↓2.87-fold) were all significantly lower at the post timepoint in the AKI group relative to the no AKI group (Table 4.2, Figure 4.4). 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 4.4K). 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 4.4B, 4.4F, 4.4I). 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 4.4A, 4.4F, 4.4J). Urine metabolites were ranked based on their respective univariate AUROC values at the post timepoint from highest to lowest AUROC and multivariate ROCs were generated using sequential combinations of ranked metabolites. A combination of the top five ranked metabolites – glycolate, tyramine, betaine, serine, and taurine – yielded the maximum AUROC of 0.978 (Figure 4.4L).
Figure 4.4 Log transformed relative intensity of urine features significantly different between no AKI and AKI patients at the post timepoint (A-J).

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 (AUROC). (L) Multivariate receiver operating characteristic (ROC) curves generated using a variable number of metabolites. ROC curves were generated using a sequential combination of the
top two to top 10 ranked metabolites, where each metabolite was ranked using its respective calculated univariate AUROC at the post timepoint. 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.
4.3.5 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 4.5), with the exception of glutamine, which was 1.55-fold lower in AKI patients at the post timepoint (Table 4.2, Figure 4.5B). 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 4.2, Figure 4.5). 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 4.5H). Additionally, the AUROC values of octanoylcarnitine 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 4.5H). Serum metabolites were ranked based on their respective univariate AUROC values at the post timepoint from highest to lowest AUROC and multivariate ROCs were generated using sequential combinations of ranked metabolites. A maximum AUROC value of 0.989 was observed using a combination of the top five ranked metabolites – octanoylcarnitine, indoxyl sulfate, octenoylcarnitine, TMAP, linolenic acid (Figure 4.5I).
Figure 4.5 Log transformed relative intensity of serum features significantly different between no AKI and AKI patients at the post timepoint (A-G).

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 serum metabolites as assessed by the area under the receiver operating curve (AUROC). (I) Multivariate receiver operating characteristic (ROC) curves generated using a variable number of metabolites. ROC curves were generated using a sequential combination of the top two to top six ranked metabolites, where each metabolite was ranked using its respective calculated univariate AUROC at the post timepoint. 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.
4.3.6 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-hydroxydecanedioic 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 4.6). 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 4.6). 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 4.6). Urinary succinate and serum 4-hydroxycinamnic 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-hydroxycinamnic acid and phenylalanine, respectively), with the alterations being sustained at the post timepoint (Figure 4.6). Similar metabolite alterations were observed over time for patients in the AKI group (Supplementary Figure C1 and C2, Appendix C).
**Figure 4.6** 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.
4.4 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\textsuperscript{14} 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\textsuperscript{10–13,18}. 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-hydroxydecanedioic 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 4.2).

Glycine is an amino acid component of the potent antioxidant molecule glutathione and has been associated with beneficial effects in reducing oxidative stress\textsuperscript{19,20}. 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\textsuperscript{21}. 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 lower in patients with moderate to severe AKI compared to mild AKI\textsuperscript{22}. 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 \textit{in vivo} when administered to rats before cisplatin treatment or ischemic insult\textsuperscript{23,24}. 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-hydroxydecanedioic acid and suberate are dicarboxylic acids that have been associated with fatty acid β-oxidation disorders. Increased urinary excretion of 3-hydroxydecanedioic acid and suberate have been used to diagnose medium-chain acyl-CoA dehydrogenase deficiency (MCAD) and indicates a block in fatty acid oxidation\textsuperscript{25-27}. Dysfunctional mitochondrial fatty acid oxidation is believed to be a crucial mechanism in cisplatin-induced AKI\textsuperscript{28}. 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\textsuperscript{29,30}. An accumulation of intracellular acyl-CoAs due to disorders of fatty acid β-oxidation is associated with lipotoxicity and detrimental to mitochondrial function\textsuperscript{31-33}. 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 4.5D, 4.5E). Elevation of serum acylcarnitines is also a marker of dysfunction in fatty acid β-oxidation\textsuperscript{34}. Taken together, the elevation of urinary 3-hydroxydecanedioic 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 to 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\textsuperscript{35,36} and AKI\textsuperscript{37,38}, hippuric acid sulfate has yet to be implicated with kidney disease.
Tubular transport is known to be affected in various etiologies of AKI, including cisplatin-induced AKI\textsuperscript{39,40}. Alterations of renal uptake and efflux transporters have been observed in rodent models of cisplatin-induced AKI, including uptake transporters such as organic anion transporters 1 and 3 (OAT1/OAT3), OCT2, multidrug and toxin extrusion 1 (MATE1), and multidrug resistance-associated protein 2 (MRP2)\textsuperscript{39,40}. Alterations in expression of renal transporters may contribute to changes in the observed levels of endogenous substrates of renal transporters. For example, uremic toxins indoxyl sulfate and indole-3-acetate are known substrates of OAT1/3\textsuperscript{41,42}, and cisplatin-mediated inhibition of renal uptake through OAT1/3 may contribute to the serum accumulation of indoxyl sulfate and decreased urinary excretion of indole-3-acetate observed in our study. Organic cation/carnitine transporter 2 (OCTN2), found on the apical membrane of the proximal tubule, plays a key role in the reabsorption of L-carnitine\textsuperscript{43}. L-carnitine plays an essential role in the delivery of long chain fatty acids into the mitochondrial matrix for β oxidation\textsuperscript{44}. Cisplatin was found to reduce the expression of Octn2 \textit{in vivo}, possibly through cisplatin-mediated modulation of peroxisome proliferator-activated receptor alpha (PPARα), a transcription factor heavily involved in regulating fatty acid oxidation\textsuperscript{43}. Downregulation of Octn2 and a consequent reduction in L-carnitine reabsorption may help to explain the transient increase in urinary L-carnitine excretion observed at the 24-48h timepoint.

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 do not progress to AKI (Figure 4.6). 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\textsuperscript{45}, 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 4.5G). 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\textsuperscript{46}. 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\textsuperscript{6}. 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 (\textbf{Table 4.1}). 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.

\section*{4.5 Conclusion}

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-hydroxydecanedioic 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.
4.6 References


Chapter 5

5 Metabolomic Investigation of Cisplatin-induced Acute Kidney Injury in Pediatric Cancer Patients
5.1 Introduction

Cisplatin is a chemotherapeutic agent widely used for the treatment of solid tumours, such as testicular, ovarian, bladder, cervical, lung, and head/neck cancer\textsuperscript{1}. Specifically in children, cisplatin is used for the treatment of neuroblastomas, medulloblastomas, osteosarcomas, germ cell tumors, and hepatoblastomas, amongst other cancer types\textsuperscript{2}. Despite its effectiveness against a multitude of malignancies, cisplatin treatment is often limited due to toxicity to non-cancerous tissues. Adverse side effects of cisplatin include ototoxicity, neurotoxicity, nausea and vomiting, and nephrotoxicity; of these, cisplatin-induced nephrotoxicity is the main dose-limiting factor in cisplatin therapy\textsuperscript{3}. Cisplatin is predominantly cleared by the kidneys through glomerular filtration and secretion\textsuperscript{1}. This high degree of renal clearance, coupled with the innate tendency of the kidneys to concentrate its filtrates, leads to the accumulation of cisplatin in renal tubule cells and subsequent renal injury\textsuperscript{4}. Following transporter-mediated uptake into renal tubular cells, cisplatin undergoes biotransformation and subsequently mediates toxicity in the renal tubule cells through several mechanisms, including generation of reactive oxygen species (ROS), mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and inflammation\textsuperscript{1,5}.

Cisplatin-induced nephrotoxicity manifests as acute kidney injury (AKI) in approximately 1/3 of patients receiving cisplatin therapy\textsuperscript{1-3}. The incidence of AKI may be higher in pediatric patients receiving cisplatin; a recent prospective study of 159 pediatric receiving cisplatin chemotherapy reported that 46% participants developed AKI during cisplatin chemotherapy\textsuperscript{6}. AKI is an abrupt or rapid decline in renal function occurring over the course of hours to days, and is associated with future chronic kidney disease, cardiovascular events, and mortality\textsuperscript{6-8}. The current standardized definition of AKI is outlined in the 2012 Kidney Disease: Improving Global Outcomes (KDIGO) guidelines\textsuperscript{9}. The KDIGO guidelines define AKI based on serum creatinine (SCr) and urinary output (UO), where AKI is diagnosed by an increase in SCr ≥ 1.5 times baseline within 7 days, an increase in SCr ≥ 26.5 μmol/L within 48 hours, or a reduction in UO < 0.5 ml/kg/h for over 6 hours.
Though current definitions of AKI are based on SCr and UO, these markers have several limitations in AKI diagnosis. Increased SCr and decreased UO are delayed responses to functional impairment of the kidney, and only manifest when significant renal injury is already present. Furthermore, SCr and UO are non-specific markers of AKI and are influenced by various non-renal confounding factors. Cisplatin-induced nephrotoxicity is also typically non-oliguric, and thus, UO is not a reliable marker of cisplatin-associate AKI. Biomarkers capable of earlier detection of AKI, prediction of AKI onset, or even prediction of patient risk for AKI prior to insult are necessary for guiding cisplatin chemotherapy, improving prognosis of kidney injury, and testing appropriate nephroprotective interventions against AKI. Several AKI biomarkers are currently being investigated to facilitate early diagnosis of AKI but have yet to be incorporated into routine clinical practice. It is likely that a combination of biomarkers is necessary to improve AKI diagnosis, delineate AKI etiology, assess AKI severity, and evaluate the prognosis of AKI.

We employed untargeted metabolomics to analyze urine and serum samples from a cohort of pediatric cancer patients receiving cisplatin chemotherapy. The purpose of this study was to investigate the metabolic differences between pediatric patients who developed clinical AKI (based on KDIGO SCr definition) following cisplatin infusion and patients who did not develop clinical AKI. We aimed to identify serum or urine markers of cisplatin-induced AKI that are capable of early AKI detection, and biomarkers that predict patient risk of AKI development even prior to nephrotoxic insult.

5.2 Materials and Methods

5.2.1 Chemicals and Reagents

The following reagents, solvents and standards were purchased from Millipore Sigma (Oakville, ON, Canada): HPLC grade acetonitrile, atenolol-d7, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 3-nitrophenylhydrazine (3-NPH), phenylisothiocyanate (PITC), and pyridine. Chlorpropamide was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Ammonium acetate was purchased from Bio Basic Inc. (Markham, ON, Canada).
5.2.2 Study Participants and Sample Collection

Participants were recruited as part of the Applying Biomarkers to Long-term Effects in Child and Adolescent Cancer Treatment (ABLE) study, a nationwide, multi-center prospective study evaluating late effects of child cancer treatment. ABLE was comprised of four arms: ototoxicity, nephrotoxicity, thrombosis, and Graft-versus-host disease. This biomarker discovery study was conducted using patient samples collected as part of the nephrotoxicity arm, with the purpose of identifying metabolites associated with cisplatin-induced AKI.

The ABLE study was conducted in accordance with the World Medical Association Declaration of Helsinki. Patient recruitment was conducted at 12 different sites across Canada. After obtaining informed written consent/assent, a cohort of 159 pediatric cancer patients receiving cisplatin were recruited to the study. Inclusion criteria were designed to maximize sample size. Patients were included in the study if they were less than 18 years of age when initiating cisplatin treatment and had at most 1 prior cycle of cisplatin (recruitment prior to the second cycle was allowed to maximize patient recruitment). Patients with pre-existing renal transplantation or estimated glomerular filtration rate (GFR) <30 mL/min/1.73m² were excluded from the study. Cisplatin therapies typically involve 3 to 8 infusion cycles given every 3 to 6 weeks. Patients underwent two separate “acute visits”, where acute visit 1 (AV1) occurred during the first (or second) cisplatin cycle, and acute visit 2 (AV2) occurred during the last (or before) cycle. At each acute visit, urine and blood samples were collected from patients prior to cisplatin infusion (“pre”), morning after infusion (“post”), and 3–5 days following (“discharge”) cisplatin infusion. Blood samples were centrifuged at 1000 g for 10 minutes at room temperature, and the supernatant was isolated to obtain serum. Serum was subsequently aliquoted and stored at -80 °C. Urine samples were stored directly at -80 °C. Samples from AV1 were used for this discovery study, since patients were more likely to be cisplatin naïve at AV1.

5.2.3 Classification of AKI

Patients were classified as “no AKI” or “AKI” based on the Kidney Disease Improving Global Outcomes (KDIGO) guidelines, which define AKI as a ≥ 1.5 times increase in
serum creatinine (SCr) versus baseline or as an increase in SCr ≥ 26.5 µmol/L. SCr levels were monitored for 10 days following cisplatin infusion, and patients were classified as having AKI if they met the KDIGO criteria for AKI diagnosis at any point during the monitoring period. Urine output was not used, as cisplatin-induced AKI is typically non-oliguric.

### 5.2.4 Sample Preparation for Untargeted Metabolomics

Serum and urine samples were thawed on ice prior to sample preparation. Ice-cold acetonitrile (ACN) containing chlorpropamide (5 µM) and atenolol-d7 (1.8 µM) was added to serum and urine samples for protein precipitation, as described in section 3.2.4. Pooled samples were generated for each matrix type to serve as quality control (QC) throughout LC-MS analysis.

### 5.2.5 Chemical Derivatization of Patient Serum and Urine Samples

Chemical derivatization was utilized to increase the coverage of the metabolome. As described in section 4.2.5, 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. Pooled samples were generated for samples derivatized with PITC and 3-NPH, respectively, to serve as QC throughout LC-MS analysis.

### 5.2.6 Chromatography and Mass Spectrometry

Prepared samples were injected at a volume of 5 µL for untargeted metabolomics and a volume of 10 µL for derivatized samples. Chromatographic separation was achieved using a Waters ACQUITY UPLC HSS T3 reverse-phase column (1.8 µm particle size, 2.1 mm x 100 mm) and MS data acquisition was conducted using a Waters Xevo G2S QToF mass spectrometer. LC-MS parameters were identical to those described in section 4.2.6. The mobile phase gradient used for chromatographic separation and MS parameters used for data acquisition are summarized in **Supplementary Table C1** and **Supplementary Table C2** (Appendix C), respectively.
5.2.7 Processing of LC-MS Chromatographic Data

Data files generated from untargeted metabolomics were converted to mzData files using the convert.waters.raw package in R. The XCMS R package was used for peak picking, retention time correction, grouping, and integration of metabolomics data. The isotopologue parameter optimization (IPO) R package was used in conjunction with QC injections to optimize parameters used for XCMS processing. All negative features were normalized to the internal standard chlorpropamide, and all positive features were normalized to atenolol-d7. Urine samples were further normalized to their own urine creatinine signals, to account for variability in patient urine concentration. Following normalization, features found to have ≥30% relative standard deviation within QC injections were excluded from analysis. The CAMERA R package was used to annotate possible isotopes and adducts, and to group related features 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. For the derivatized metabolomics dataset, duplicate features present in both the untargeted and derivatized datasets were removed from the derivatized dataset. Intensity values of all features were log 10 transformed to reduce heteroscedasticity and correct for skewed data distribution. Any zero values encountered during log transformation were treated as 1/5 of the minimum intensity values of each feature. Log transformed intensity values were used for all analyses unless stated otherwise (e.g., fold change calculations).

5.2.8 Statistical Analysis

Principal component analysis (PCA) was used for dimensionality reduction of the metabolomics dataset, and PCA score plots were generated to visualize unsupervised separation of no AKI and AKI patients at each of the sample collection timepoints (pre, post, discharge). Individual orthogonal partial least squares discriminant analysis (OPLS-DA) models were generated at each timepoint to compare no AKI and AKI patients using a supervised approach. For each OPLS-DA, metabolites were ranked by their variable importance in projection (VIP) values, which assesses the importance of each feature in the discrimination of the classes being compared in an OPLS-DA model. Features with VIP values ≥ 1 were considered to be important contributors in the model. This exclusion
of features based on VIP values was repeated, generating a new OPLS-DA model each time, until OPLS-DA model statistics ($R^2$ and $Q^2$ values) were maximized. The final optimized OPLS-DA model was used to select the most important features for annotation at each timepoint, using a VIP value threshold of ≥ 1 and correlation ($p_{corr}$) values less than -0.4 and greater than 0.4.

Features that were important in discriminating no AKI vs. AKI patients at each timepoint were further analyzed by two-way ANOVA with Benjamini-Hochberg false discovery rate (FDR) correction. Features showing statistical significance based on AKI classification following two-way ANOVA and FDR correction were further analyzed using pairwise t-tests to compare no AKI and AKI patients at each sample collection timepoint. p-values obtained in the pairwise t-test were adjusted for multiple comparisons using Bonferroni correction. Univariate and multivariate receiver operating characteristic (ROC) curves were generated and area under the ROC (AUROC) values for each ROC curve were calculated using the pROC package in R. Multivariate ROC models were generated using a logistic regression model with repeated k-folds cross validation (10 repeats of 5-folds).

5.2.9 Putative Annotation and Identity Confirmation of Metabolites

Discriminatory features were putatively annotated by searching online metabolomics databases (Human Metabolome Database (HMDB), METLIN, or MassBank) using feature $m/z$ values and comparing the fragmentation spectrum of each feature with experimental fragmentation spectral information found in the databases. If spectral information was not available, characteristic fragments generated through collision-induced dissociation were used to narrow down potential metabolites. If characteristic fragments were not observed, predicted fragmentation spectra were consulted using CFM-ID or Waters MassFragment, which generate proposed theoretical fragment information based on the observed fragmentation spectrum of each feature and the likelihood of bond breakages based on the molecular structure of the candidate metabolite. Analytical standards were purchased for putatively annotated metabolites and analyzed in parallel with pooled patient samples to compare $m/z$, retention time, and fragmentation spectra between the analytical standard and feature observed in patient samples. A level 1 identification was achieved if all three parameters were matched between analytical standard and the feature in patient samples.
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).

5.3 Results

5.3.1 Patient Demographics

Though patients were also diagnosed with AKI using electrolyte abnormality criteria in the ABLE study, our study focused on patients with either no clinical presentation of AKI or patients who were classified as having AKI based on the KDIGO SCr definition of AKI; thus, 86 patients were included in our metabolomics investigation. Baseline patient demographics are summarized in Table 5.1. Of the 86 patients, 34 patients presented with clinical AKI (39.5%) based on SCr concentrations measured over the course of 10 days following cisplatin infusion. The study cohort consisted of 48 males and 38 females (55.8% male). The majority of patients recruited to this study were Caucasian (64/86, 74.4%). Baseline demographic characteristics age, body mass index (BMI), and baseline SCr were not significantly different between no AKI and AKI groups (Table 5.1). Baseline estimated glomerular filtration rate (eGFR) was significantly higher in the AKI group compared to the no AKI group (Table 5.1). Out of the 34 patients in the AKI group, 25 patients (73.5%) had stage 1 AKI, 6 patients (17.6%) had stage 2 AKI and 3 patients (8.8%) had stage 3 AKI.

5.3.2 Metabolic Profiling of No AKI vs. AKI Patients

Following feature exclusion by VIP values as described in section 5.2.7, PCA score plots were generated to visualize the metabolic differences between no AKI and AKI patients at each timepoint. Overall, PCA scores plots of both serum and urine samples showed poor separation between no AKI and AKI patients at all timepoints (Supplementary Figure D1). Corresponding supervised OPLS-DA models comparing serum samples from no AKI and AKI patients showed good model performance (goodness of fit R² and predictive ability Q²) at the pre and discharge timepoints, but weak performance at the post timepoint.
Poor OPLS-DA model performance was observed for urine samples at all timepoints (Figure 5.1).
Table 5-1 Baseline demographics of study population. Patient sex is presented as a ratio of males to females (M/F). Demographic characteristics of age, body mass index, cisplatin dose, baseline serum creatinine (SCr), and baseline estimated glomerular filtration rate (eGFR) are presented as median ± standard deviation. Baseline eGFR was significantly higher in the AKI group vs. the no AKI group, as determined by an unpaired t-test (*p<0.05). No other characteristic was significantly different between the two groups.

<table>
<thead>
<tr>
<th></th>
<th>No AKI (n=52)</th>
<th>AKI (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex (M/F)</strong></td>
<td>30/22 (57.7% M)</td>
<td>18/16 (52.9% M)</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>2.67 ±4.28</td>
<td>4.96 ±4.77</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian (n=64)</td>
<td>35 (67.3%)</td>
<td>29 (85.3%)</td>
</tr>
<tr>
<td>Asian (n=7)</td>
<td>5 (9.6%)</td>
<td>2 (5.9%)</td>
</tr>
<tr>
<td>Mixed Race (n=7)</td>
<td>6 (11.5%)</td>
<td>1 (2.9%)</td>
</tr>
<tr>
<td>First Nations Canadian (n=4)</td>
<td>3 (5.8%)</td>
<td>1 (2.9%)</td>
</tr>
<tr>
<td>Black or African American (n=3)</td>
<td>3 (5.8%)</td>
<td>0</td>
</tr>
<tr>
<td>Other (n=1)</td>
<td>0</td>
<td>1 (2.9%)</td>
</tr>
<tr>
<td><strong>Cancer Diagnosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuroblastoma (n=35)</td>
<td>22 (42.3%)</td>
<td>13 (38.2%)</td>
</tr>
<tr>
<td>Medulloblastoma (n=18)</td>
<td>12 (23.1%)</td>
<td>6 (17.6%)</td>
</tr>
<tr>
<td>Osteosarcoma (n=10)</td>
<td>9 (17.3%)</td>
<td>1 (2.9%)</td>
</tr>
<tr>
<td>Germ cell tumor (n=7)</td>
<td>4 (7.7%)</td>
<td>3 (8.8%)</td>
</tr>
<tr>
<td>Hepatoblastoma (n=5)</td>
<td>2 (3.8%)</td>
<td>3 (8.8%)</td>
</tr>
<tr>
<td>Other (n=11)</td>
<td>3 (5.8%)</td>
<td>8 (23.5%)</td>
</tr>
<tr>
<td><strong>Body mass index (kg/m²)</strong></td>
<td>15.77 ±3.21</td>
<td>15.88 ±2.43</td>
</tr>
<tr>
<td><strong>Cisplatin Dose (mg/m²)</strong></td>
<td>51.84 ±17.54</td>
<td>64.79 ±20.09</td>
</tr>
<tr>
<td><strong>Baseline SCr (µmol/L)</strong></td>
<td>29 ±11.87</td>
<td>19.5 ±16.09</td>
</tr>
<tr>
<td><strong>Baseline eGFR (mL/min/1.73m²)</strong></td>
<td>142.47 ±29.84</td>
<td>†166.93* ±54.53</td>
</tr>
</tbody>
</table>

†Three outlier eGFR values were removed.
Figure 5.1 Orthogonal partial least squares discriminant analysis scores plots comparing serum (A, B, C) and urine (D, E, F) samples from no AKI (black) and AKI (red) patients at the pre (A, D), post (B, E), and discharge (C, F) timepoints.

Model statistics for OPLS-DA were as follows: (A) R^2 = 0.976, Q^2 = 0.607 (B) R^2 = 0.572, Q^2 = 0.227 (C) R^2 = 0.984, Q^2 = 0.542 (D) R^2 = 0.339, Q^2 = -0.075 (E) R^2 = 0.416, Q^2 = 0.010 (F) R^2 = 0.556, Q^2 = 0.006. n=20-46, exact sample sizes can be found in Supplementary Table D1, Appendix D.
5.3.3 Discriminatory Metabolites for AKI in Serum

Discriminatory metabolites between the no AKI and AKI group at each timepoint were selected using the final optimized OPLS-DA model and applying a VIP value threshold of ≥ 1 and correlation (p(corr)) threshold values of less p(corr) ≤ -0.4 or p(corr) ≥ 0.4. Metabolites that met the VIP and p(corr) threshold requirements were putatively identified and further subjected to a two-way ANOVA with FDR correction to select for metabolites that are significantly different based on AKI classification. Significant metabolites are summarized in Table 5.2. Cortisol, creatine, dodecanamide, lauryl diethanolamide, phenylalanyl-phenylalanine, piperine, proline, suberoyl-L-carnitine, taurine, tauroursdeoxycholic acid, and trans-4-hydroxy-L-proline were significantly different based on AKI classification (Figure 5.2, Table 5.2). Pairwise t-tests comparing no AKI and AKI patients at each timepoint, revealed phenylalanyl-phenylalanine (Figure 5.2E) and suberoyl-L-carnitine (Figure 5.2H) levels to be significantly lower in the serum of AKI patients at the pre timepoint compared to no AKI patients (↓1.62-fold and ↓1.61-fold in AKI patients, respectively). Lauryl diethanolamide (Figure 5.2D) was significantly higher in the serum samples of AKI patients at the pre timepoint (↑2.66-fold). At the post timepoint, phenylalanyl-phenylalanine (Figure 5.2E) and piperine (Figure 5.2F) were significantly lower in AKI patients (↓1.69-fold and ↓4.06-fold, respectively), whereas dodecanamide (Figure 5.2C) was significantly higher in AKI patients (↑1.22-fold).
Table 5-2

<table>
<thead>
<tr>
<th>Metabolite Identity</th>
<th>ID Level</th>
<th>Chemical Formula</th>
<th>m/z (min)</th>
<th>Sample Type*</th>
<th>Change in AKI Patients vs. No AKI Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pre</td>
<td>24-48 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Direction</td>
<td>Fold</td>
</tr>
<tr>
<td>3-amino-4-hydroxybenzoate</td>
<td>2</td>
<td>C7H7NO3 [M+H]</td>
<td>154.0506</td>
<td>1.15</td>
<td>Serum</td>
</tr>
<tr>
<td>Arginine [PITC]</td>
<td>1</td>
<td>C6H14N4O2 + C7H5NS [M+H]</td>
<td>310.1339</td>
<td>1.51</td>
<td>Serum</td>
</tr>
<tr>
<td>Citramalate [NPH]</td>
<td>1</td>
<td>C9H8O5 + C6H5N3O [M+H]</td>
<td>417.1152</td>
<td>2.29</td>
<td>Serum</td>
</tr>
<tr>
<td>Cortisol</td>
<td>2</td>
<td>C21H30O5 [M+H]</td>
<td>363.2172</td>
<td>2.28</td>
<td>Serum</td>
</tr>
<tr>
<td>Creatine [NPH]</td>
<td>1</td>
<td>C4H9N3O2 + C6H5N3O [M+H]</td>
<td>265.1051</td>
<td>1.44</td>
<td>Serum</td>
</tr>
<tr>
<td>Cystathionine [PITC]</td>
<td>1</td>
<td>C7H14N2O4S</td>
<td>358.0902</td>
<td>1.58</td>
<td>Serum</td>
</tr>
<tr>
<td>Dodecanamide</td>
<td>3</td>
<td>C12H25NO [M+H]</td>
<td>200.2011</td>
<td>3.93</td>
<td>Serum</td>
</tr>
<tr>
<td>Ethanolamine phosphate [NPH]</td>
<td>1</td>
<td>C2H8NO4P + C6H5N3O [M+H]</td>
<td>275.0545</td>
<td>1.39</td>
<td>Serum</td>
</tr>
<tr>
<td>Glucuronate [NPH]</td>
<td>1</td>
<td>C6H10O7 + 2x C6H5N3O [M+H]</td>
<td>463.1207</td>
<td>2.28</td>
<td>Serum</td>
</tr>
<tr>
<td>Kynurenate [NPH]</td>
<td>1</td>
<td>C10H7NO3 + C6H5N3O [M+H]</td>
<td>323.0766</td>
<td>2.26</td>
<td>Serum</td>
</tr>
<tr>
<td>Lauryl diethanolamide</td>
<td>2</td>
<td>C16H33NO3 [M+H]</td>
<td>288.2539</td>
<td>3.42</td>
<td>Serum</td>
</tr>
<tr>
<td>1-methylhypoxanthine</td>
<td>3</td>
<td>C6H8N4O [M+H]</td>
<td>151.0618</td>
<td>1.19</td>
<td>Serum</td>
</tr>
<tr>
<td>1-methylnicotinamide</td>
<td>2</td>
<td>C7H8N2O2+</td>
<td>137.0713</td>
<td>0.56</td>
<td>Serum</td>
</tr>
<tr>
<td>N-(3-acetamidopropyl) pyrrolidin-2-one</td>
<td>3</td>
<td>C9H16N2O2 [M+H]</td>
<td>185.1287</td>
<td>1.36</td>
<td>Serum</td>
</tr>
</tbody>
</table>

Fold change was calculated using mean raw intensity values for each metabolite. Bolding denotes significant p-values (p<0.05). All observed m/z values were <5 ppm mass error relative to the theoretical m/z.
<table>
<thead>
<tr>
<th>N-acetylaspartate [NPH]</th>
<th>1</th>
<th>C6H9N6O5 + C6H5N3O5 [M-H]</th>
<th>441.1260</th>
<th>2.23</th>
<th>Serum</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>↑</td>
<td>2.56</td>
<td>0.7345</td>
<td>↑</td>
<td>2.40</td>
<td>0.1207</td>
<td>↑</td>
<td>2.28</td>
<td>0.1448</td>
</tr>
<tr>
<td>N-acetyleneuraminat e [NPH]</td>
<td>1</td>
<td>C11H19N9O9 + C6H5N3O5 [M-H]</td>
<td>443.1407</td>
<td>1.48</td>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>↑</td>
<td>2.41</td>
<td>0.5068</td>
<td>↑</td>
<td>2.61</td>
<td>0.0881</td>
<td>↑</td>
<td>2.23</td>
<td>0.1777</td>
</tr>
<tr>
<td>Phenylalan-phenylaniline</td>
<td>2</td>
<td>C18H20N2O3 [M+H]</td>
<td>313.1551</td>
<td>1.68</td>
<td>Serum</td>
<td>↓</td>
<td>1.62</td>
<td>0.0495</td>
<td>↓</td>
<td>1.69</td>
<td>0.0081</td>
<td>↓</td>
<td>1.25</td>
<td>0.3639</td>
</tr>
<tr>
<td>Phosphoenolpyruvate [NPH]</td>
<td>1</td>
<td>C3H5O6P + C6H5N3O5 [M-H]</td>
<td>302.0174</td>
<td>1.67</td>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>↑</td>
<td>1.70</td>
<td>0.9251</td>
<td>↑</td>
<td>5.46</td>
<td>0.0575</td>
<td>↑</td>
<td>2.22</td>
<td>0.4046</td>
</tr>
<tr>
<td>Piperine</td>
<td>2</td>
<td>C17H19N3O3 [M+H]</td>
<td>286.1442</td>
<td>3.17</td>
<td>Serum</td>
<td>↓</td>
<td>1.70</td>
<td>0.1155</td>
<td>↓</td>
<td>4.06</td>
<td>0.0222</td>
<td>↑</td>
<td>1.75</td>
<td>0.5996</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proline [PITC]</td>
<td>1</td>
<td>C5H9NO2 + C7H5NS [M+H]</td>
<td>251.0862</td>
<td>2.11</td>
<td>Serum</td>
<td>↑</td>
<td>1.52</td>
<td>1.0</td>
<td>↑</td>
<td>1.62</td>
<td>1.0</td>
<td>↑</td>
<td>2.20</td>
<td>0.1631</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>↑</td>
<td>1.95</td>
<td>0.1912</td>
<td>↑</td>
<td>1.19</td>
<td>1.0</td>
<td>↑</td>
<td>2.14</td>
<td>0.0224</td>
</tr>
<tr>
<td>2-PY</td>
<td>2</td>
<td>C7H8N2O2 [M+H]</td>
<td>153.0661</td>
<td>1.16</td>
<td>Serum</td>
<td>↑</td>
<td>1.02</td>
<td>1.0</td>
<td>↑</td>
<td>1.20</td>
<td>1.0</td>
<td>↑</td>
<td>1.67</td>
<td>0.0726</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>↑</td>
<td>1.21</td>
<td>1.0</td>
<td>↑</td>
<td>1.80</td>
<td>0.0341</td>
<td>↑</td>
<td>1.64</td>
<td>0.0193</td>
</tr>
<tr>
<td>Quinolinolate [NPH]</td>
<td>1</td>
<td>C7H5N4O4 + 2x C6H5N3O5 [M-H]</td>
<td>436.0999</td>
<td>2.53</td>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>↑</td>
<td>2.72</td>
<td>0.8694</td>
<td>↑</td>
<td>2.92</td>
<td>0.0746</td>
<td>↑</td>
<td>2.81</td>
<td>0.1317</td>
</tr>
<tr>
<td>Suberoyl-L-carnitine</td>
<td>3</td>
<td>C15H27N6O6 [M+K]</td>
<td>356.1478</td>
<td>1.11</td>
<td>Serum</td>
<td>↓</td>
<td>1.61</td>
<td>0.0133</td>
<td>↓</td>
<td>1.30</td>
<td>1.0</td>
<td>↓</td>
<td>1.34</td>
<td>0.1550</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Taurine [PITC]</td>
<td>1</td>
<td>C2H7NO3S + C7H5NS [M-H]</td>
<td>261.0371</td>
<td>1.71</td>
<td>Serum</td>
<td>↑</td>
<td>2.05</td>
<td>0.4756</td>
<td>↑</td>
<td>1.67</td>
<td>1.0</td>
<td>↑</td>
<td>2.48</td>
<td>0.0728</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>↑</td>
<td>1.16</td>
<td>1.0</td>
<td>↑</td>
<td>1.30</td>
<td>1.0</td>
<td>↑</td>
<td>1.69</td>
<td>0.6688</td>
</tr>
<tr>
<td>Tauroursodeoxycholic acid</td>
<td>2</td>
<td>C6H4S5O6S [M+H]</td>
<td>498.2882</td>
<td>3.25</td>
<td>Serum</td>
<td>↑</td>
<td>1.11</td>
<td>0.3664</td>
<td>↑</td>
<td>1.89</td>
<td>0.0930</td>
<td>↑</td>
<td>2.04</td>
<td>0.2085</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trans-4-hydroxy-L-proline [PITC]</td>
<td>1</td>
<td>C5H9N3O3 + C7H5NS [M-H]</td>
<td>267.0806</td>
<td>1.72</td>
<td>Serum</td>
<td>↑</td>
<td>1.66</td>
<td>1.0</td>
<td>↑</td>
<td>1.35</td>
<td>1.0</td>
<td>↑</td>
<td>2.01</td>
<td>0.1101</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 5.2 Log transformed relative intensity of serum features significantly different between no AKI and 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 two-way ANOVA, followed by pairwise t-tests at each individual timepoint; *p<0.05, **p<0.01, ***p<0.001, compared to no AKI patients at each timepoint. n=22-37, exact sample sizes can be found in Supplementary Table D1, Appendix D.
5.3.4 Discriminatory Metabolites for AKI in Urine

Following two-way ANOVA for urine metabolites, citramalate, ethanolamine phosphate, glucuronate, kynurenate, 1-methylhypoxanthine, 1-methylnicotinamide, N-(3-acetamidopropyl) pyrroldin-2-one, N-acetylaspartate, N-acetylneuraminate, phosphoenolpyruvate, proline, 2-PY, and quinolinate were found to be significantly different based on AKI classification (Figure 5.3, Table 5.2). In urine, none of the identified metabolites were significantly different at the pre timepoint between no AKI and AKI patients. At the post timepoint, the relative abundance of kynurenate (Figure 5.3F) and 2-PY (Figure 5.3N) were significantly higher in AKI patients compared to no AKI patients (↑2.72-fold and ↑1.80-fold, respectively). Kynurenate and 2-PY also remained significantly elevated in the urine of AKI patients at the discharge timepoint (↑2.15-fold and ↑1.64-fold, respectively). Additionally, proline (Figure 5.3M) was also significantly higher in the urine of AKI patients compared to no AKI patients at the discharge timepoint (↑2.14-fold).

5.3.5 Diagnostic Performance of Biomarkers in Classification of No AKI and AKI Patients

To assess the performance of each metabolite as a classifier between no AKI and AKI patients, univariate receiver operating characteristic (ROC) curves were generated for each individual metabolite at each timepoint. The area under the ROC curve (AUROC) values were calculated for each ROC to serve as a measure of the classification performance of each ROC model. AUROC values and corresponding confidence intervals are summarized in Table 5.3 for serum metabolites and Table 5.4 for urine metabolites. An AUROC value of 0.5 indicates lack of discrimination, and as a general guideline, an AUROC value ≥ 0.7 is generally considered as acceptable classification performance. In serum, lauryl diethanolamide and suberoyl-L-carnitine exhibited AUROC > 0.7 at the pre timepoint, dodecanamide and phenylalanylphenylalanine exhibited AUROC > 0.7 at the post timepoint, and creatine, and lauryl diethanolamide exhibited AUROC > 0.7 at the discharge timepoint (Table 5.3). In urine, only kynurenate exhibited an AUROC > 0.7 at the post timepoint (Table 5.4).
**Figure 5.3** Log transformed relative intensity of urine features significantly different between no AKI and 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 two-way ANOVA, followed by pairwise t-tests at each individual timepoint; *p<0.05, **p<0.01, compared to no AKI patients at each timepoint. n=20-46, exact sample sizes can be found in *Supplementary Table D1, Appendix D.*
Table 5-3 Diagnostic performance of individual serum metabolites for classification between no AKI and AKI patients at each timepoint, as assessed by the area under the receiver operating curve (AUC) and corresponding confidence intervals (CI).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pre</th>
<th></th>
<th></th>
<th>Post</th>
<th></th>
<th></th>
<th>Disc</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC</td>
<td>CI</td>
<td>AUC</td>
<td>CI</td>
<td>AUC</td>
<td>CI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>0.598</td>
<td>0.457-0.75</td>
<td>0.610</td>
<td>0.468-0.73</td>
<td>0.672</td>
<td>0.517-0.823</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.591</td>
<td>0.435-0.741</td>
<td>0.631</td>
<td>0.504-0.767</td>
<td>0.675</td>
<td>0.532-0.803</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatine</td>
<td>0.561</td>
<td>0.412-0.699</td>
<td>0.594</td>
<td>0.459-0.731</td>
<td>0.702</td>
<td>0.57-0.832</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dodecanamide</td>
<td>0.549</td>
<td>0.391-0.699</td>
<td>0.728</td>
<td>0.599-0.846</td>
<td>0.566</td>
<td>0.417-0.716</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lauryl Diethanolamide</td>
<td>0.701</td>
<td>0.571-0.837</td>
<td>0.559</td>
<td>0.422-0.706</td>
<td>0.721</td>
<td>0.58-0.849</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanylphenylalanine</td>
<td>0.663</td>
<td>0.519-0.8</td>
<td>0.707</td>
<td>0.574-0.822</td>
<td>0.615</td>
<td>0.47-0.766</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piperine</td>
<td>0.648</td>
<td>0.509-0.799</td>
<td>0.689</td>
<td>0.561-0.81</td>
<td>0.609</td>
<td>0.466-0.759</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suberoyl-L-carnitine</td>
<td>0.745</td>
<td>0.6-0.875</td>
<td>0.535</td>
<td>0.396-0.669</td>
<td>0.650</td>
<td>0.504-0.788</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>0.600</td>
<td>0.458-0.749</td>
<td>0.527</td>
<td>0.388-0.663</td>
<td>0.684</td>
<td>0.582-0.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tauroursodeoxycholic acid</td>
<td>0.621</td>
<td>0.472-0.76</td>
<td>0.573</td>
<td>0.437-0.705</td>
<td>0.626</td>
<td>0.493-0.777</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans-4-hydroxy-L-proline</td>
<td>0.529</td>
<td>0.398-0.682</td>
<td>0.519</td>
<td>0.364-0.661</td>
<td>0.692</td>
<td>0.551-0.824</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5-4 Diagnostic performance of individual urine metabolites for classification between no AKI and AKI patients at each timepoint, as assessed by the area under the receiver operating curve (AUC) and corresponding confidence intervals (CI).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pre</th>
<th></th>
<th>Post</th>
<th></th>
<th>Disc</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC</td>
<td>CI</td>
<td>AUC</td>
<td>CI</td>
<td>AUC</td>
<td>CI</td>
</tr>
<tr>
<td>3-amino-4-hydroxybenzoate</td>
<td>0.570</td>
<td>0.394-0.718</td>
<td>0.638</td>
<td>0.491-0.778</td>
<td>0.661</td>
<td>0.505-0.809</td>
</tr>
<tr>
<td>Citramalate</td>
<td>0.554</td>
<td>0.402-0.706</td>
<td>0.651</td>
<td>0.516-0.779</td>
<td>0.670</td>
<td>0.524-0.802</td>
</tr>
<tr>
<td>Cystathionine</td>
<td>0.634</td>
<td>0.493-0.776</td>
<td>0.584</td>
<td>0.421-0.723</td>
<td>0.667</td>
<td>0.549-0.801</td>
</tr>
<tr>
<td>Ethanolamine phosphate</td>
<td>0.600</td>
<td>0.448-0.75</td>
<td>0.622</td>
<td>0.466-0.77</td>
<td>0.659</td>
<td>0.53-0.782</td>
</tr>
<tr>
<td>Glucuronate</td>
<td>0.590</td>
<td>0.454-0.736</td>
<td>0.636</td>
<td>0.484-0.78</td>
<td>0.670</td>
<td>0.525-0.805</td>
</tr>
<tr>
<td>Kynurenate</td>
<td>0.589</td>
<td>0.446-0.739</td>
<td><strong>0.749</strong></td>
<td>0.601-0.874</td>
<td>0.695</td>
<td>0.564-0.83</td>
</tr>
<tr>
<td>1-methylhypoxanthine</td>
<td>0.645</td>
<td>0.512-0.8</td>
<td>0.660</td>
<td>0.521-0.797</td>
<td>0.563</td>
<td>0.411-0.725</td>
</tr>
<tr>
<td>1-methylnicotinamide</td>
<td>0.671</td>
<td>0.538-0.808</td>
<td>0.636</td>
<td>0.492-0.771</td>
<td>0.634</td>
<td>0.475-0.765</td>
</tr>
<tr>
<td>N-(3-acetamidopropyl)pyrrolidin-2-one</td>
<td>0.552</td>
<td>0.402-0.693</td>
<td>0.656</td>
<td>0.509-0.811</td>
<td>0.620</td>
<td>0.465-0.756</td>
</tr>
<tr>
<td>N-acetylaspartate</td>
<td>0.568</td>
<td>0.433-0.714</td>
<td>0.639</td>
<td>0.502-0.787</td>
<td>0.666</td>
<td>0.527-0.809</td>
</tr>
<tr>
<td>N-acetylneuraminate</td>
<td>0.594</td>
<td>0.433-0.76</td>
<td>0.657</td>
<td>0.523-0.804</td>
<td>0.657</td>
<td>0.509-0.785</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>0.546</td>
<td>0.392-0.69</td>
<td>0.679</td>
<td>0.533-0.813</td>
<td>0.655</td>
<td>0.503-0.794</td>
</tr>
<tr>
<td>Proline</td>
<td>0.668</td>
<td>0.505-0.797</td>
<td>0.518</td>
<td>0.324-0.635</td>
<td>0.665</td>
<td>0.52-0.837</td>
</tr>
<tr>
<td>2PY or 4PY</td>
<td>0.548</td>
<td>0.403-0.694</td>
<td>0.677</td>
<td>0.542-0.79</td>
<td>0.679</td>
<td>0.537-0.818</td>
</tr>
<tr>
<td>Quinolinate</td>
<td>0.554</td>
<td>0.391-0.702</td>
<td>0.645</td>
<td>0.51-0.784</td>
<td>0.653</td>
<td>0.51-0.779</td>
</tr>
</tbody>
</table>
Multivariate ROC models were generated to assess the aggregate diagnostic performance of a combination of metabolites. For each sample type at each timepoint, metabolites were ranked from highest to lowest univariate AUROC values. Multivariate ROC models were generated by sequentially combining the top 2 highest ranked metabolites to the top 11 metabolites for serum and top 2 to top 15 ranked metabolites for urine (rankings were assigned individually at each timepoint). At the pre timepoint, a combination of the top 6 ranked serum metabolites (suberoyl-L-carnitine, lauryl diethanolamide, phenylalanylphenylalanine, piperine, taouroursodeoxycholic acid, and taurine) yielded the best ROC model with an AUROC of 0.829 (Figure 5.4A, 5.4D), whereas the top 2 ranked urine metabolites (1-methylnicotinamide and proline) yielded the highest AUROC at 0.660 (Figure 5.5A, 5.5D). At the post timepoint, combining the top 2 ranked metabolites achieved the highest AUROC for both serum (dodecanamide and phenylalanylphenylalanine) and urine metabolites (kynurenate and 2-PY), with an AUROC of 0.724 (Figure 5.4B, 5.4E) and 0.709 (Figure 5.5B, 5.5E) respectively. As with the post timepoint, combining the top 2 ranked metabolites achieved the best ROC models for both serum (lauryl diethanolamide and creatine) and urine (kynurenate and 2-PY) at the discharge timepoint, with AUROC values of 0.717 (Figure 5.4C, 5.4F) and 0.650 (Figure 5.5C, 5.5F), respectively.
Multivariate ROC curves were generated using a variable number of metabolites, using a sequential combination of the top 2 to top 11 ranked metabolites, where each metabolite was ranked at each timepoint using the calculated univariate AUROC at each respective timepoint (Table 5.3).

**Figure 5.4** Multivariate receiver operating characteristic (ROC) curves of serum metabolites (A-C) and corresponding area under the curve and confidence intervals (D-F) at the pre (A, D), post (B, E), and discharge (C, F) timepoints.

Multivariate ROC curves were generated using a variable number of metabolites, using a sequential combination of the top 2 to top 11 ranked metabolites, where each metabolite was ranked at each timepoint using the calculated univariate AUROC at each respective timepoint (Table 5.3).
Multivariate ROC curves were generated using a variable number of metabolites, using a sequential combination of the top 2 to top 15 ranked metabolites, where each metabolite was ranked at each timepoint using the calculated univariate AUROC at each respective timepoint (Table 5.4).

**Figure 5.5** Multivariate receiver operating characteristic (ROC) curves of urinary metabolites (A-C) and corresponding area under the curve and confidence intervals (D-F) at the pre (A, D), post (B, E), and discharge (C, F) timepoints.
5.3.6 Investigation of Sex Differences in Metabolic Alterations

Metabolites found to be significantly different between no AKI and AKI groups at each timepoint were separated by sex to assess sex differences in metabolite levels in serum (Supplementary Figure D2, Appendix D) and urine (Supplementary Figure D3, Appendix D). Two-way ANOVA analyses were conducted for all metabolites after separation of patients by sex. The relative abundance of serum arginine, creatine, cortisol, lauryl diethanolamide, phenylalanine, piperine, suberoyl-L-carnitine, taurine, and trans-4-hydroxy-L-proline were found to be significantly different between no AKI and AKI patients in females, but not in male patients (Supplementary Figure D2, Appendix D). When comparing relative abundance of urinary metabolites in no AKI and AKI patients for each sex separately, similar overall trends (with respect to higher or lower levels of metabolites in AKI vs. no AKI patients) were observed between the male and female patients, though the extent of the differences in urine metabolite relative abundance between no AKI and AKI patients varied between the sexes (Supplementary Figure D3, Appendix D). After separating by sex, the relative abundance of urine metabolites 3-amino-4-hydroxybenzoate, ethanolamine phosphate, kynurenate, 1-methylnicotinamide, N-(3-acetamidopropyl)pyrroloidin-2-one, proline, and 2-PY were significantly different between no AKI and AKI patients in male patients only. In contrast, citramalate, cystathionine, N-acetylaspartate, N-acetylneuraminate, and quinolinate were significantly different between no AKI and AKI patients in female patients, but not male (Supplementary Figure D3, Appendix D). Glucuronate was the only metabolite significantly different between no AKI and AKI patients in both male and female patients after separation by sex (Supplementary Figure D3, Appendix D).
5.3.7 Stratification by Patient Age

As a sub-analysis, patients were stratified by age into groups of patient age ≤ 3 (“under 3”) and patient age > 3 (“over 3”). PCA score plots were generated to visualize the metabolic differences between no AKI and AKI patients at each timepoint, for both the under 3 and over 3 groups (Supplementary Figure D4 and Supplementary Figure D5, respectively). Overall, PCA score plots of both serum and urine samples showed strong visual clustering based on AKI status in the under 3 patients (Supplementary Figure D4, Appendix D), and moderate visual clustering in over 3 patients (Supplementary Figure D5, Appendix D). OPLS-DA modelling of the under 3 patients displayed strong discrimination between no AKI and AKI patients for all timepoints in both urine and serum samples, exhibiting robust model performance with respect to $R^2$ and $Q^2$ (Figure 5.6). In the over 3 patients, OPLS-DA models of serum samples showed excellent discrimination between no AKI and AKI patients at all timepoints, with strong model performance (Figure 5.7). OPLS-DA models of urine samples from the over 3 patients performed strongly for the post timepoint but showed moderate performance for the pre and discharge timepoints (Figure 5.7).

Figure 5.8 shows the relative abundance of urine metabolites from the under 3 patients. Though only hydroxytyrosol glucuronide, hydroxytyrosol sulfate, and vanilloylglycine were significantly different by two-way ANOVA between no AKI and AKI patients in the under 3 group, there was a consistent trend of increased metabolite abundance in AKI patients compared to no AKI patients (Figure 5.8).
Figure 5.6 Orthogonal partial least squares discriminant analysis scores plots comparing serum (A, B, C) and urine (D, E, F) samples from no AKI (black) and AKI (red) patients under the age of 3 at the pre (A, D), post (B, E), and discharge (C, F) timepoints.

Model statistics for OPLS-DA were as follows: (A) $R^2 = 0.988$, $Q^2 = 0.709$ (B) $R^2 = 0.799$, $Q^2 = 0.483$ (C) $R^2 = 0.993$, $Q^2 = 0.604$ (D) $R^2 = 0.993$, $Q^2 = 0.597$ (E) $R^2 = 0.971$, $Q^2 = 0.491$ (F) $R^2 = 0.999$, $Q^2 = 0.666$. $n=5-18$, exact sample sizes can be found in Supplementary Table D1.
Figure 5.7 Orthogonal partial least squares discriminant analysis scores plots comparing serum (A, B, C) and urine (D, E, F) samples from no AKI (black) and AKI (red) patients over the age of 3 at the pre (A, D), post (B, E), and discharge (C, F) timepoints.

Model statistics for OPLS-DA were as follows: (A) $R^2 = 0.988$, $Q^2 = 0.503$ (B) $R^2 = 0.941$, $Q^2 = 0.555$ (C) $R^2 = 0.999$, $Q^2 = 0.534$ (D) $R^2 = 0.897$, $Q^2 = 0.223$ (E) $R^2 = 0.901$, $Q^2 = 0.589$ (F) $R^2 = 0.867$, $Q^2 = 0.320$. n=8-25, exact sample sizes can be found in Supplementary Table D1.
Figure 5.8 Log transformed relative intensity of urine features in no AKI and AKI patients under the age of 3.

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 two-way ANOVA. n=5-11, exact sample sizes can be found in Supplementary Table D1.
Univariate ROCs were generated to assess performance for each urine metabolite in the discrimination of no AKI vs. AKI patients under the age of 3. At the pre timepoint, alanine, citrate, ethylmalonate, hydroxytyrosol glucuronide, hydroxytyrosol sulfate, malate, N-acetyllalanine, and pantothenate achieved AUROC > 0.7, and lactate, N-acetylserine, and vanilloylglycine achieved AUROC > 0.8 (Supplementary Table D2, Appendix D). At the post timepoint, ethylmalonate and glucuronate achieved an AUROC > 0.7 (Supplementary Table D2, Appendix D). Finally, at the discharge timepoint, univariate ROC models for citrate, ethylmalonate, glucuronate, hydroxytyrosol glucuronide, malate, N-acetyllalanine, N-acetylserine, pantothenate, proline, and vanilloylglycine yielded AUROC values > 0.7, with alanine, glycerate, lactate, and N-alpha-acetyllysine achieving AUROC > 0.8 (Supplementary Table D2, Appendix D). It is worth noting that sample sizes for the under 3 patients were small (n=5-11, Supplementary Table D1, Appendix D) and the corresponding confidence intervals for the univariate ROC models were large (Supplementary Table D2, Appendix D).

As before, each metabolite was assigned a rank at each timepoint based on the calculated univariate AUROC values. Multivariate ROC models were generated by iteratively combining the top 2 highest ranked metabolites to the top 15 metabolites. Starting from the top 2 ranked metabolites, multivariate ROC models were sequentially generated by incorporating the next highest-ranking metabolite at each iteration. At the pre timepoint, the maximum AUROC of 0.821 was achieved by combining the top 5 ranked metabolites: vanilloylglycine, lactate, N-acetylserine, hydroxytyrosol sulfate, and malate (Figure 5.9A, 5.9D). The maximum AUROC at the post timepoint was 0.644, which was obtained from the combination of the top 5 ranked metabolites: ethylmalonate, N-acetyllalanine, lactate, glucuronate, and hydroxytyrosol glucuronide malate (Figure 5.9B, 5.9E). At the discharge timepoint, a combination of glycerate and lactate yielded the highest AUROC, at 0.792 malate (Figure 5.9C, 5.9F).
Multivariate ROC curves were generated using a variable number of metabolites, using a sequential combination of the top 2 to top 16 ranked metabolites, where each metabolite was ranked at each timepoint using the calculated univariate AUROC at each respective timepoint (Supplementary Table D2, Appendix D).

**Figure 5.9** Multivariate receiver operating characteristic (ROC) curves of urinary metabolites (A-C) and corresponding area under the curve and confidence intervals (D-F) at the pre (A, D), post (B, E), and discharge (C, F) timepoints.

Multivariate ROC curves were generated using a variable number of metabolites, using a sequential combination of the top 2 to top 16 ranked metabolites, where each metabolite was ranked at each timepoint using the calculated univariate AUROC at each respective timepoint (Supplementary Table D2, Appendix D).
5.4 Discussion

In this discovery study, we used untargeted metabolomics to analyze serum and urine samples from pediatric cancer patients receiving cisplatin chemotherapy to identify potential predictive or early diagnostic biomarkers of cisplatin-induced AKI. Untargeted metabolomics studies have previously been conducted to investigate metabolic alterations in the context of cisplatin-induced AKI but have primarily utilized in vitro or pre-clinical in vivo models; although we have studied cisplatin-induced AKI in a cohort of adult head and neck cancer patients, to our knowledge, our study is the first to assess metabolomic alterations in a cohort of pediatric patients\(^{19-24}\). We demonstrate that metabolic differences are present between pediatric patients who develop AKI following cisplatin infusion and patients who do not develop AKI and highlight metabolites that may have predictive value in discriminating those two groups of patients.

Multivariate metabolic profiling of serum samples exhibited poor (post timepoint) to good (pre and discharge timepoint) overall discrimination between no AKI and AKI pediatric patients. In urine, overall metabolic separation between no AKI and AKI patients was poor at all study timepoints. One of the factors that may contribute to poor discrimination is the heterogeneity of the patient cohort. Though heterogeneity enhances the generalizability of results, it poses a challenge in proper interpretation of metabolomics data. The metabolome is affected by numerous endogenous and exogenous factors, including genetics, age, gender, diet, lifestyle, drugs, environmental xenobiotics, gut microbiome composition, and even the type of cancer itself\(^{25}\). The pediatric cohort in this investigation was heterogeneous with respect to age, cancer type, cisplatin dose, number of cisplatin infusions prior to AV1, and number of medications received (nephrotoxic or otherwise), all of which may have influenced the metabolome during chemotherapy.

Kynurenate, 1-methylnicotinamide, 2-PY, and quinolinate, metabolites that are constituents of nicotinamide adenine dinucleotide (NAD\(^+\)) synthesis pathways\(^{26,27}\), were consistently elevated in the urine of AKI patients compared to no AKI patients. Univariate AUROC values for the aforementioned metabolites ranged from 0.548-0.671 at the pre timepoint, 0.636-0.749 at the post timepoint, and 0.634-0.695 at the discharge timepoint. Urinary kynurenate at the post timepoint showed the strongest discrimination between no
AKI and AKI patients out of any metabolite identified in this study, with an AUROC of 0.749. In urine, a combination of kynurenate and 2-PY yielded the strongest multivariate ROC model at the post timepoint with an AUROC of 0.709. NAD+ is an essential coenzyme for redox reactions, acting as an electron acceptor in various metabolic processes including glycolysis, oxidative phosphorylation, and fatty acid β-oxidation. In addition to energy metabolism, NAD+ has been implicated in DNA repair, modulation of epigenetic modifications, circadian rhythm, and inflammation. NAD+ biosynthesis occurs through two pathways: de novo synthesis and the salvage pathway. De novo synthesis involves the catabolic metabolism of tryptophan through the kynurenine pathway. In the kynurenine pathway, tryptophan is converted to kynurenine, which is subsequently converted to quinolinate through a series of enzymatic reactions. Quinolinate is then converted to NAD+ following two enzymatic reactions. An alternate branch of the kynurenine pathway results in the conversion of kynurenine to kynurenate through the actions of kynurenine amino transferase enzymes. Nicotinamide is generated as a by-product of NAD+ consumption in metabolic reactions, and the salvage pathway uses the nicotinamide by-product to regenerate NAD+ through two sequential enzymatic reactions. Excess nicotinamide that is not recycled to form NAD+ is metabolized into 1-methylnicotinamide, which can subsequently be metabolized into 2-PY. Previous studies have shown disruptions in NAD+ homeostasis in the setting of AKI, through both accelerated consumption of NAD+ as well as defective biosynthesis of NAD+. Results from our study suggest disruptions in NAD+ homeostasis begin to occur prior to functional impairment of kidney function and elevation of traditional biomarkers.

Similar to our study, urine levels of quinolinate were observed to be elevated in ischemic AKI, both in mice and in human patients receiving cardiac surgery. Higher urinary levels of quinolinate were found in patients receiving cardiac surgery who developed post-operative AKI compared to patients who did not develop AKI; the authors proposed that urinary quinolinate/tryptophan ratio could be used as an indicator of NAD+ metabolism in the context of AKI. Numerous pre-clinical and clinical studies have also observed alterations in the levels of kynurenate in the serum and plasma of AKI patients. A study of ICU patients with AKI reported that increased urinary excretion of kynurenate was associated with increased AKI severity, longer AKI duration, increased need for renal
replacement therapy (RRT), and non-recovery from AKI. In a metabolomics study of urine samples collected from combat injury patients, 1-methylnicotinamide was associated with mortality, need for RRT, and AKI severity. 2-PY is currently most known for being classified as a uremic toxin; however, a recent study has reported 2-PY to be protective against renal fibrosis in kidney fibroblasts and renal tubular epithelial cells and refuted the status of 2-PY as a uremic toxin. In addition to these studies showing disruption of NAD+ homeostasis in various etiologies of AKI, we show that the NAD+ pathway is similarly altered in the setting of nephrotoxic AKI. Indeed, studies in cisplatin-treated mice have demonstrated the association between cisplatin and reduced NAD+ and boosting NAD+ levels has shown to be protective against cisplatin nephrotoxicity.

Age was a factor of particular interest, as younger age has previously been associated with increased risk of clinical AKI. On top of the physiological and metabolic changes that occur throughout childhood development, age has implications in exogenous variables such as diet and lifestyle. Age has been shown to affect baseline levels of metabolites and is an important variable to account for in metabolomic studies. Patients were separated into ≤ 3 years of age and > 3 years of age, as patient age ≤ 3 years was previously associated with the highest risk of developing clinical AKI following cisplatin infusion in the ABLE pediatric cohort. Furthermore, urine concentrations of kidney injury molecule 1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL), two biomarkers of AKI, were found to be consistently higher in patients aged < 3 years. Metabolic profiling following separation by age resulted in greatly improved discrimination between no AKI and AKI patients, especially in patients under the age of 3. Upon examination of the relative abundance of urine metabolites in under 3 patients, we found that although only a few metabolites were statistically different between no AKI and AKI patients, there was a consistent trend of increased metabolite abundance in the urine of AKI patients compared to no AKI patients. The lack of statistical significance may be partly explained by the resultant small sample sizes following stratification by age. Univariate ROC values for urine metabolites were also higher after separation by age, but the corresponding confidence intervals were large, presumably due to the small sample size. Collectively, our results corroborate the importance of accounting for age in metabolomics and biomarker discovery. Though we used an age cut-off of 3 years of age in this study, future studies
should have larger sample sizes to allow for further stratification of patients into additional strata of narrower age ranges to comprehensively evaluate the effects of age.

We also observed the presence of sex differences when comparing metabolite abundance across timepoints for male and female patients separately. In the ABLE cohort, NGAL was previously observed to be 2-3-fold higher in female pediatric patients compared to male\textsuperscript{45}. These findings reiterate the importance of accounting for sex differences in biomarker studies to evaluate if the observed metabolic alterations are applicable for both sexes, and to ensure appropriate baselines are established for each sex. As with age, future validation studies require larger sample sizes to appropriately study the effects of sex on metabolite levels.

The main limitation of the study was the relatively small sample size. The sample size was not large enough to perform comprehensive subgroup analyses to assess factors such as age, sex, and cisplatin dose. Another limitation of the study was the frequency of sample collection; following cisplatin infusion, samples were only collected at two separate timepoints: the morning after cisplatin infusion and 2-5 days after cisplatin infusion. Previous studies have reported that AKI biomarkers such as NGAL are elevated in a matter of hours following an insult to the kidneys, peaking within 12 hours\textsuperscript{49,50}. Potential metabolic alterations that occurred prior to the post timepoint (the morning after cisplatin infusion) could have been missed, in addition to metabolic changes that may have occurred between the post and discharge timepoints. It is worth considering that out of the hundreds of thousands of metabolites in the metabolome, we were only able to detect a small fraction of the metabolome. Of the features that are detected, many are left unannotated due to the complexity of the metabolome and gaps in metabolomics databases. Incorporating other analytical techniques such as nuclear magnetic resonance or gas chromatography for future studies will permit a more comprehensive analysis of the metabolome.

5.5 Conclusion

Though SCr is currently used to define AKI, it is a poor diagnostic marker of AKI due to the delayed increase in SCr following kidney injury. In this study, we highlight metabolites of the NAD\textsuperscript{+} biosynthesis pathway as being different between pediatric cancer patients
who develop AKI following cisplatin infusion vs. patients who do not. Kynurenate was able to modestly discriminate between AKI and no AKI patients at the post timepoint and may help predict the development of AKI shortly after cisplatin infusion. We also outline the challenges of conducting metabolomics in a pediatric cohort and important covariates to account for. Future research should aim to study a larger cohort to facilitate subgroup analyses. Metabolic alterations observed in this study should be validated in a separate cohort, and the clinical utility of biomarkers of interest should be assessed to determine whether or not incorporation of the biomarkers improves AKI diagnosis, prediction, or prognosis.
5.6 References


Chapter 6

6 Discussion and Conclusions

6.1 Discussion and Significance of Research

Though cisplatin continues to see widespread clinical use due to its effectiveness in the treatment of solid tumours and hematologic cancers, its use is complicated by off-target toxicity to non-cancerous tissues\textsuperscript{1–7}. Of these, cisplatin-induced nephrotoxicity is typically the major dose-limiting toxicity, leading to the clinical presentation of acute kidney injury (AKI) in approximately one-third of patients receiving cisplatin as part of their chemotherapy regimen\textsuperscript{1,8}. While the short- and long-term adverse outcomes of AKI are well documented, management and treatment of AKI are not optimal\textsuperscript{9–15}. One of the major limitations in the management of AKI is the delayed diagnosis of kidney injury. The current markers used for AKI diagnosis, serum creatinine (SCr) and urine output (UO), are markers of impaired kidney function and are only detectable after significant injury to the kidneys\textsuperscript{16}. With respect to drug-induced AKI mediated by cisplatin, impaired kidney function assessed by creatinine isn’t detectable until 5-8 days following the dose. Furthermore, neither SCr nor UO are specific to kidney injury and are confounded by numerous patient-specific factors\textsuperscript{17–20}. Early renoprotective intervention against cisplatin-induced AKI is necessary to improve AKI management and patient outcomes\textsuperscript{15}. Novel biomarkers are necessary to facilitate early detection of AKI and allow for implementation of preventative measures in a timely manner. Additionally, biomarkers that can predict high risk patients prior to cisplatin therapy will allow for the stratification of patients into different risk levels and implementation of appropriate supportive measures or adjustments to chemotherapy regimens as necessary\textsuperscript{15}. Untargeted metabolomics was employed to identify early and predictive biomarkers of cisplatin-induced AKI. The overall hypothesis of this dissertation was that evaluating metabolic differences prior to, and following cisplatin exposure will allow for the prediction or early diagnosis of cisplatin-induced AKI. In this dissertation, we explore the metabolic pathways altered by cisplatin administration and highlight potential predictive or early diagnostic biomarkers of cisplatin-induced AKI that can be further validated in future pre-clinical and clinical investigations.
6.2 Chapter 3

6.2.1 Summary of Chapter 3

In chapter 3, we used a mouse model of cisplatin-induced nephrotoxicity to characterize the metabolic alterations induced by cisplatin throughout the progression of kidney injury and to identify early biomarkers of cisplatin-induced nephrotoxicity. C57BL/6 and FVB/N mice were injected (i.p.) with a 15 mg/kg dose of cisplatin to induce kidney injury. Mice were sacrificed 1, 2, 3, and 4 days after cisplatin or saline injection, and blood, urine, and kidney samples were collected. In this model, functional impairment of kidney function was observed 4 days after cisplatin in most mice, as evidenced by significantly increased plasma creatinine concentrations and histological signs of injury. Untargeted metabolomics was used to investigate the metabolic alterations induced by cisplatin treatment and to identify early biomarkers of nephrotoxicity that are altered prior to impairment of kidney function (as indicated by elevated plasma creatinine). Metabolic profiling of plasma, urine, and kidney samples using multivariate analyses revealed strong discrimination between saline and cisplatin-treated mice before loss of kidney function was observed. In total, we identified 26 metabolites that exhibit early alterations prior to traditional measures of kidney injury. There was a high degree of concordance between C57BL/6 and FVB/N mice with respect to the metabolic alterations induced by cisplatin treatment over the course of the study. Of the 26 metabolites discussed, several metabolites were associated with mitochondrial energy metabolism, including metabolites related to fatty acid β-oxidation (carnitine/L-acetylcarnitine and acylglycine metabolites) and intermediates of the TCA cycle (citric acid and succinate). Mitochondria are one of the primary targets of toxicity inside proximal tubule cells, and cisplatin is known to induce mitochondrial dysfunction\textsuperscript{21}. Previous studies have also shown alterations in metabolites associated with fatty acid β-oxidation and the TCA cycle, including our metabolomics study in adult head and neck cancer patients (Chapter 4)\textsuperscript{22–29}. We also observed a number of alterations in gut-derived metabolites of tryptophan and phenylalanine. Though the interplay between the gut microbiome and kidney disease has been investigated in recent years, it has primarily been studied in the context of CKD\textsuperscript{30}. Recent evidence indicates an association between AKI and gut dysbiosis. Cisplatin has been shown to induce gut dysbiosis in rodent models.
Supplementation with probiotics containing *Lactobacillus reuteri* and *Clostridium butyricum* was demonstrated to improve cisplatin-induced gut dysbiosis by enriching the population of beneficial bacteria, alleviate the cisplatin-induced impairment of the intestinal barrier and protect against the nephrotoxic effects of cisplatin\(^{31}\). A study of cisplatin-induced hepatotoxicity showed that ablation of the gut microbiome with antibiotics protected against cisplatin hepatotoxicity\(^{32}\). Moreover, fecal microbiota transplant from cisplatin-treated mice was shown to exacerbate cisplatin hepatotoxicity\(^{32}\). From these studies, the gut microbiome appears to play an important role in cisplatin toxicity, where the composition of the gut microbiome can modulate the toxic effects of cisplatin. Elevations of gut-derived metabolites may be indicative of gut dysbiosis. Further studies are necessary to investigate if the elevations in indole-3-carboxaldehyde, indole-3-carboxylic acid, p-cresol sulfate, and p-cresol glucuronide are indeed mediated by cisplatin-induced gut dysbiosis. This study identified potential early diagnostic biomarkers of cisplatin-induced AKI, reinforces the central role of mitochondrial dysfunction in the nephrotoxic mechanisms of cisplatin, and demonstrates potential cross-talk between the injured kidney and gut dysbiosis.

### 6.2.2 Chapter 3 Limitations and Future Directions

A primary limitation of this study was that only male mice were studied, and sex differences in the metabolic alterations induced by cisplatin were not evaluated. The influence of sex on sensitivity of cisplatin nephrotoxicity is unclear, with contrasting reports of each sex being more susceptible than the other\(^{33}\). Female sex has been reported to be a risk factor in human patients. As such, future metabolomics investigations should include both sexes to evaluate sex differences in the metabolome in response to cisplatin and enhance the generalizability of results. Another limitation was that the effect of diet was also not considered in this mouse study. Diet is known to affect the metabolome. Mice used in this study were also not tumour bearing; as the cancer itself
can affect the metabolome, tumour bearing animals should be used to more closely reflect a cancer patient being treated with cisplatin. Coverage of the metabolome can also be increased in future studies by employing HILIC chromatography, chemical derivatization, or NMR-based metabolomics analysis. This mouse study provides a large panel of potential biomarkers for the early detection of AKI. These metabolites also provide mechanistic insight into important pathways in the nephrotoxic mechanisms of cisplatin. Biomarkers must be validated in the clinical setting to evaluate the applicability/translatability of these findings. A targeted metabolomics approach should be applied to future clinical studies to validate our findings and better elucidate specific metabolite groups or metabolic pathways that can be targeted for analysis. For instance, in addition to untargeted metabolomics, future studies may conduct targeted quantification of acylcarnitines, intermediates of the TCA cycle, and gut-derived indole metabolites.

6.3 Chapter 4

6.3.1 Summary of Chapter 4

In chapter 4, an untargeted metabolomics investigation was conducted in a cohort of 31 adult head and neck cancer patients receiving cisplatin chemotherapy. Participants were recruited as part of a pilot study for the Canada-wide, multi-center, ACCENT study. Adult patients (>18 years of age) initiating high dose cisplatin treatment (dose ≥ 70 mg/m²) for head and neck cancer were recruited for this pilot study. Urine and blood samples were collected from patients at three separate timepoints: prior to (“pre”), 24–48 hours after (“24-48h”), and 5-14 days following (“post”) cisplatin infusion. Patients were classified as no AKI or AKI based on the KDIGO SCr definition of AKI by comparing SCr concentrations quantified at the post timepoint to concentrations measured at the pre timepoint. Untargeted metabolomics was employed to characterize metabolic differences between patients who develop AKI after cisplatin infusion (AKI group) and those who do not (no AKI group). Additionally, we identified biomarkers that predict AKI prior to cisplatin infusion and biomarkers that predict the development of AKI shortly after cisplatin infusion. Of 31 patients, 11 patients developed clinical AKI and 20 patients did not. Multivariate analyses revealed moderate to strong separation between no AKI and AKI patients at all three timepoints in both serum and urine metabolomics. Glycine, 3-
hydroxydecanedioc acid, hippuric acid sulfate, and suberate were significantly different between no AKI and AKI patients prior to cisplatin infusion and were identified as predictive markers of cisplatin-induced AKI. All four metabolites exhibited univariate AUROC > 0.7 at the pre timepoint; the strongest discriminator between no AKI and AKI patients was suberate with an AUROC of 0.839. A maximum AUROC of 0.879 was achieved for discrimination of no AKI and AKI patients by using a multivariate ROC model that included all four metabolites at the pre timepoint. Similar to our mouse study (Chapter 3), we observed alterations in metabolites associated with dysregulated fatty acid β-oxidation. Previous studies have demonstrated that cisplatin inhibits mitochondrial fatty acid β-oxidation by deactivating PPAR-α34,35. Increased urinary excretion of 3-hydroxydecanedioc acid and suberate are used to diagnose medium-chain acyl-CoA dehydrogenase deficiency (MCAD) and indicates a block in fatty acid β-oxidation36–38. Moreover, alterations were also observed in the serum levels of acylcarnitines (octanoylcarnitine and octenoylcarnitine); elevation in serum acylcarnitine levels also indicates dysfunctional fatty acid β-oxidation39. The elevation of urinary 3-hydroxydecanedioc acid, urinary suberate, and serum acylcarnitines in AKI patients suggest a lower capacity for fatty acid β-oxidation in patients who develop clinical AKI following cisplatin therapy. Lower glycine levels in the urine were previously associated with the need for renal replacement therapy, and moderate to severe AKI (vs. mild) in combat injury patients40. Glycine pre-treatment has been shown to be protective against cisplatin nephrotoxicity and ischemic AKI in mice, potentially due to its antioxidant role as a constituent of glutathione41–44. We also observed that cisplatin induces early metabolic changes in both the urine and serum even in patients who do not develop clinical AKI. Many of these metabolites were TCA cycle intermediates or associated with fatty acid β-oxidation. These metabolites may indicate the presence of subclinical AKI (kidney damage without substantial loss of function) in all patients receiving cisplatin45. In this study, glycine, 3-hydroxydecanedioc acid, hippuric acid sulfate, and suberate were identified as potential predictive markers of cisplatin-induced AKI.
6.3.2 Chapter 4 Limitations and Future Directions

The major limitation of this study was the small sample size. Future metabolomics studies will be conducted in much larger cohorts as part of the ACCENT study. Patient recruitment for the ACCENT study is ongoing at 6 different oncology centers across Canada and aims to recruit 300 adult and 300 children\textsuperscript{46}. Adult and pediatric patients will be divided into a discovery (200 adults and 200 children) and validation cohort (100 adults and 100 children). Due to a combination of small sample size and the much higher prevalence of head and neck cancer in men compared to women, our study cohort was predominantly male (93.5%); consequently, we could not assess sex differences in our cohort\textsuperscript{47,48}. Future metabolomics studies conducted in the ACCENT cohorts should study sex differences in biomarkers in response to cisplatin chemotherapy if sample sizes allow. Ideally, samples should be collected more frequently throughout the cisplatin cycle to evaluate the temporal changes in biomarker expression following cisplatin therapy. As with our animal study, various analytical techniques should be used to characterize the metabolome more comprehensively (e.g., HILIC chromatography and NMR-based metabolomics). Though the homogeneity of our pilot cohort is ideal for minimizing confounding variables in the metabolome, it is not reflective of the general population of head and neck cancer patients. Thus, our proposed predictive biomarkers of cisplatin-induced AKI must be validated using larger cohorts of more diverse patients and employing fully quantitative methods. In subsequent metabolomics investigations in the large ACCENT cohort, the predictive metabolites and related pathways found in this study should be used to guide targeted quantification of metabolites, in combination with metabolites and pathways we identified as potential early diagnostic markers of AKI in Chapter 3.

6.4 Chapter 5

6.4.1 Summary of Chapter 5

Chapter 5 describes a metabolomic investigation conducted in a cohort of pediatric cancer patients receiving cisplatin therapy. Patients were recruited as part of the nephrotoxicity arm of the ABLE study, a Canada-wide, multi-center investigation of the long-term effects of childhood and adolescent cancer treatment. Children (<18 years of age) initiating
cisplatin treatment with at most 1 prior cycle of cisplatin were recruited to this study. In our metabolomic investigation, we analyzed samples collected at the first acute visit (AV1), which took place on the first or second cisplatin infusion. At AV1, patient blood and urine samples were collected prior to cisplatin infusion (“pre”), the morning after cisplatin infusion (“post”), and 3-5 days following cisplatin infusion (“discharge”). Patients were classified as “no AKI”, or “AKI” based on SCr-based KDIGO criteria; SCr levels were monitored daily for 10 days following cisplatin infusion, and AKI was diagnosed if patients met the KDIGO SCr criteria at any point within this timeframe. As with our adult cohort, we used untargeted metabolomics to characterize the metabolic differences between no AKI and AKI patients, to identify biomarkers predictive of AKI prior to cisplatin infusion or early biomarkers capable of predicting AKI development shortly after cisplatin infusion. It is important to study children separate from adults for biomarker studies to account for age-associated differences in the baseline concentrations of biomarkers. Of 86 patients studied, 34 patients presented with clinical AKI (39.5%). Multivariate metabolic profiling exhibited poor overall discrimination between no AKI and AKI patients. We observed that four metabolites (kynurenate, 2-PY, 1-methylnicotinamide, and quinolinate) associated with NAD+ synthesis were consistently elevated in the urine of AKI patients compared to no AKI patients. The relative abundance of kynurenate at the post timepoint was 2.72-fold higher in AKI patients compared to no AKI and showed the greatest discriminatory value with a post timepoint univariate ROC of 0.749. Previous studies have reported disturbances in NAD+ homeostasis in various settings of AKI as well as cisplatin-treated mice; augmenting NAD+ synthesis by supplementation of the salvage precursor nicotinamide has been shown to protect against AKI49–53. Here, we show that the NAD+ pathway may be similarly altered in pediatric patients receiving cisplatin chemotherapy. The heterogeneity of the cohort may partially explain the poor discrimination between no AKI and AKI patients observed in our pediatric cohort compared to our adult cohort. Age was a factor that was particularly variable, with participants ranging from a few months old to 17 years old. Stratification of patients into ≤ 3 years of age and > 3 years of age greatly improved discrimination between no AKI and AKI patients, especially for patients under the age of 3. In this study, we highlight constituents of the NAD+ biosynthesis pathway as being different between pediatric
patients no AKI and AKI patients following cisplatin treatment. The changes in these metabolites were observed prior to elevation of SCr. We also demonstrate the challenges of metabolomics studies in a pediatric cohort and show that age is an important variable to account for.

6.4.2 Chapter 5 Limitations and Future Directions

The main limitations of the study were the relatively small sample size and heterogeneity of the study population. Future studies with larger cohorts will allow for comprehensive subgroup analyses to be conducted to assess factors such as age, sex, and cisplatin dose. To increase patient recruitment, acute visit 1 included both cisplatin naïve patients and patients with a prior cycle of cisplatin. Prior exposure to cisplatin may change metabolite levels, and future studies should aim to recruit cisplatin naïve patients. The ACCENT study aims to address both previously mentioned limitations by recruiting larger cohorts of exclusively cisplatin naïve patients. As discussed for the adult cohort, increasing the frequency of sample collection would allow for a better understanding of the changes in metabolite levels over time throughout the acute visit. Increasing metabolome coverage with additional analytical techniques may also reveal more discriminatory metabolites. It is worth noting that the multivariate ROC models generated using logistic regression did not account for confounding variables such as age, sex, and cisplatin dose. Incorporation of these factors into the multivariate ROC models may improve performance of biomarkers in the discrimination of no AKI and AKI patients. As mentioned in the previous sections, increasing the coverage of the metabolome with various analytical techniques may reveal additional metabolites capable of discriminating between no AKI and AKI patients. A panel of metabolites involved in NAD+ biosynthesis should be targeted in future metabolomics studies in larger cohorts to validate their utility for predicting the development of AKI shortly after cisplatin infusion. Interestingly, we did not observe impairment of fatty acid β-oxidation in our pediatric cohort. Mitochondrial dysfunction is a natural hallmark of aging, and aged mitochondria may be more prone to injury after cisplatin insult due to heightened baseline dysfunction. When evaluating the relative abundance of metabolites after age stratification (Figure 5.8), we observed increased urine levels of citrate, malate, and lactate in patients under the age of 3, which may indicate
disturbances in mitochondrial energy metabolism; these alterations were previously not detected when evaluating the full cohort and may have been masked by age-associated differences in the metabolome.

### 6.5 Conclusions

Serum creatinine is a poor biomarker of cisplatin-induced AKI. Biomarkers capable of early AKI detection or prediction of high-risk patients prior to cisplatin infusion are necessary for prevention and management of AKI. This dissertation used an untargeted metabolomics approach to characterize the metabolic alterations induced by cisplatin in a mouse model of cisplatin-induced nephrotoxicity, a cohort of adult head and neck cancer patients, and a cohort of pediatric patients. Our mouse model of cisplatin-nephrotoxicity identified markers of mitochondrial dysfunction (TCA cycle intermediates and markers of impaired fatty acid β-oxidation) and gut-derived metabolites of tryptophan and phenylalanine to exhibit early alterations following cisplatin treatment, prior to kidney function impairment and histological injury. The central role of the mitochondria was further reinforced in the adult cohort, where we observed that 3-hydroxydecanedioic acid and suberate, markers of impaired mitochondrial fatty acid β-oxidation were elevated in the urine of AKI patients prior to cisplatin infusion. Furthermore, glycine was lower in the urine of AKI patients prior to cisplatin infusion, which may indicate susceptibility to oxidative stress. We proposed 3-hydroxydecanedioic acid, suberate, glycine, and hippuric acid sulfate as predictive markers of cisplatin-induced AKI. Finally in our pediatric cohort, we observed that urine levels of kynurenate, 2-PY, 1-methylnicotinamide, and quinolinate, metabolites associated with NAD+ biosynthesis, were consistently higher in urine of AKI patients compared to no AKI patients. A summary of proposed early and predictive metabolite markers is provided in Table 6.1. Future metabolomics studies in larger pediatric and adult cohorts are necessary to validate these observations; these studies should combine untargeted metabolomics and targeted metabolomics. The findings reported in this thesis should be used to guide targeted quantification of the metabolites previously discussed and metabolites from related metabolic pathways. For example, a panel of metabolites consisting of markers of impaired fatty acid β-oxidation, TCA cycle intermediates, gut-derived metabolites of tryptophan and phenylalanine metabolism, and
metabolites involved in NAD+ biosynthesis can be targeted in subsequent metabolomics studies. Additionally, these findings can be used to guide pre-clinical experiments evaluating nephroprotective interventions. When testing protective interventions against cisplatin nephrotoxicity, it is important to consider that nephrotoxic mechanisms of cisplatin overlap with many of the anti-cancer mechanisms of cisplatin. Nanoparticle-based drug delivery systems targeted for the kidneys may facilitate kidney protection without compromising tumour killing activity of cisplatin\textsuperscript{55,56}. For example, a kidney-targeted delivery of mitochondrial antioxidants could protect against mitochondrial dysfunction and alleviate cisplatin nephrotoxicity. A recent study used ceria nanoparticles with tunable catalytic activity to confer protection to kidneys by inducing the Nrf2 antioxidant pathway\textsuperscript{55}. These nanoparticles are inactive in acidic tumour environments but active in the neutral environment in the kidney cortex. Enhancing \textit{de novo} NAD+ synthesis or salvage pathways of NAD+ could also confer protection against cisplatin nephrotoxicity. Inhibition of aminocarboxymuconate semialdehyde decarboxylase (ACMSD) has been demonstrated to boost NAD+ \textit{de novo} synthesis, enhance mitochondrial function, and protect against cisplatin-induced kidney injury\textsuperscript{53}. The \textit{de novo} synthesis involves the formation of an unstable metabolite amino-β-carboxymuconate-ε-semialdehyde that can either cyclize spontaneously to form quinolinate and feed into NAD+ synthesis or be metabolized by ACMSD. ACMSD expression is reported to be primarily localized to the kidney and liver, and inhibition of ACMSD may confer kidney-specific protection against nephrotoxicity while maintaining the anti-cancer effects of cisplatin\textsuperscript{53}. These therapeutic interventions should be studied in tumour-bearing animals to confirm that the anti-cancer effects of cisplatin are not compromised.
Table 6-1 Summary of proposed early and predictive metabolite markers of AKI.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Study</th>
<th>Sample Type</th>
<th>Compound Class</th>
<th>Biomarker Type</th>
<th>Related Metabolic Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid</td>
<td>Mouse</td>
<td>Urine</td>
<td>Weak organic acid</td>
<td>Early</td>
<td>TCA cycle intermediate</td>
</tr>
<tr>
<td>Creatine</td>
<td>Mouse</td>
<td>Plasma</td>
<td>Amino acid derivative</td>
<td>Early</td>
<td>Creatine kinase system for cellular energy shuttling</td>
</tr>
<tr>
<td>Indole-3-carboxaldehyde</td>
<td>Mouse</td>
<td>Urine</td>
<td>Indole</td>
<td>Early</td>
<td>Gut-derived metabolite of tryptophan</td>
</tr>
<tr>
<td>Indole-3-carboxylic acid</td>
<td>Mouse</td>
<td>Urine</td>
<td>Indole</td>
<td>Early</td>
<td>Gut-derived metabolite of tryptophan</td>
</tr>
<tr>
<td>L-acetylcarnitine</td>
<td>Mouse</td>
<td>Plasma</td>
<td>Acylcarnitine</td>
<td>Early</td>
<td>Dysregulation of fatty acid β-oxidation</td>
</tr>
<tr>
<td>L-carnitine</td>
<td>Mouse</td>
<td>Urine</td>
<td>Amino acid derivative</td>
<td>Early</td>
<td>Dysregulation of fatty acid β-oxidation</td>
</tr>
<tr>
<td>P-cresol sulfate</td>
<td>Mouse</td>
<td>Plasma</td>
<td>Phenylsulfate</td>
<td>Early</td>
<td>Gut-derived metabolite of phenylalanine</td>
</tr>
<tr>
<td>Phenylpropionyl-glycine</td>
<td>Mouse</td>
<td>Urine</td>
<td>Acylglycine</td>
<td>Early</td>
<td>Dysregulation of fatty acid β-oxidation</td>
</tr>
<tr>
<td>Succinate</td>
<td>Mouse</td>
<td>Urine</td>
<td>Dicarboxylic acid</td>
<td>Early</td>
<td>TCA cycle intermediate</td>
</tr>
<tr>
<td>Taurine</td>
<td>Mouse</td>
<td>Plasma</td>
<td>Amino sulfonic acid</td>
<td>Early</td>
<td>Mitochondrial antioxidant, mitochondrial translation</td>
</tr>
<tr>
<td>Glycine</td>
<td>ACCENT adults</td>
<td>Urine</td>
<td>Amino acid</td>
<td>Predictive</td>
<td>Constituent of glutathione</td>
</tr>
<tr>
<td>Hippuric acid sulfate</td>
<td>ACCENT adults</td>
<td>Urine</td>
<td>Benzoic acid derivative</td>
<td>Predictive</td>
<td>Gut-derived metabolite of dietary polyphenols</td>
</tr>
<tr>
<td>3-Hydroxy-decanedioic acid</td>
<td>ACCENT adults</td>
<td>Urine</td>
<td>Medium chain fatty acid</td>
<td>Predictive</td>
<td>Dysregulation of fatty acid β-oxidation</td>
</tr>
<tr>
<td>Suberate</td>
<td>ACCENT adults</td>
<td>Urine</td>
<td>Dicarboxylic acid</td>
<td>Predictive</td>
<td>Dysregulation of fatty acid β-oxidation</td>
</tr>
<tr>
<td>Kynurenate</td>
<td>ABLE children</td>
<td>Urine</td>
<td>Quinoline carboxylic acid</td>
<td>Early*</td>
<td>Metabolic byproduct of the kynurenine pathway of tryptophan metabolism</td>
</tr>
<tr>
<td>1-methyl-nicotinamide</td>
<td>ABLE children</td>
<td>Urine</td>
<td>Nicotinamide</td>
<td>Early*</td>
<td>Metabolite of nicotinamide</td>
</tr>
<tr>
<td>2-PY</td>
<td>ABLE children</td>
<td>Urine</td>
<td>Nicotinamide</td>
<td>Early*</td>
<td>Metabolite of 1-methylnicotinamide</td>
</tr>
<tr>
<td>Quinolinate</td>
<td>ABLE children</td>
<td>Urine</td>
<td>Pyridine dicarboxylic acid</td>
<td>Early*</td>
<td>Metabolic byproduct of the kynurenine pathway used for de novo NAD+ biosynthesis</td>
</tr>
</tbody>
</table>

*Early denotes metabolites that were different between AKI and no AKI patients at the 24h timepoint (post).
6.6 References


Appendices

Appendix A: Ethics Approvals

From: eSiriusWebServer
Date: Thursday, November 24, 2016 at 3:55 PM
To: Bradley Lyle Urquhart
Cc: Animal Care Committee
Subject: eSirius Notification - New Animal Use Protocol is APPROVED2016-071::1

Western

AUP Number: 2016-071
PI Name: Urquhart, Brad
AUP Title: Metabolomic Investigation Of Drug Induced Acute Kidney Injury
Approval Date: 11/24/2016

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Metabolomic Investigation Of Drug Induced Acute Kidney Injury" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal 2016-071::1.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

The University of Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Western University Health Science Research Ethics Board
HSREB Full Board Initial Approval Notice

Principal Investigator: Dr. Brad Urquhart
Department & Institution: Schulich School of Medicine and Dentistry
Physiology & Pharmacology, Western University

Review Type: Full Board
HSREB File Number: 107220
Study Title: Pharmacometabolic identification of early predictors of chemotherapy-induced kidney injury in oncology patients

HSREB Initial Approval Date: December 10, 2015
HSREB Expiry Date: December 10, 2016

Documents Approved and/or Received for Information:

<table>
<thead>
<tr>
<th>Document Name</th>
<th>Comments</th>
<th>Version Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other</td>
<td>Appendix. CTCAM 4.0</td>
<td>2015/09/23</td>
</tr>
<tr>
<td>Western University Protocol</td>
<td></td>
<td>2015/11/20</td>
</tr>
<tr>
<td>Assent</td>
<td></td>
<td>2015/11/20</td>
</tr>
<tr>
<td>Data Collection Form/Case Report Form</td>
<td></td>
<td>2015/11/20</td>
</tr>
</tbody>
</table>

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above named study, as of the HSREB Initial Approval Date noted above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice Practices (ICH E6 R1), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Ethics Officer to Contact for Further Information: Edita Besle | Nicole Kario | Grace Kelly | Mona Mehdi | Vikki Tran

This is an official document. Please retain the original in your files.
**Appendix B: Supplementary Information for Chapter 3**

**Supplementary Table B1.** Summary of group sizes for each sample type at all timepoints.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Sample Type</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>Saline</td>
<td>Plasma</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>8</td>
<td>9</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Cisplatin</td>
<td>Plasma</td>
<td>9</td>
<td>9</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>9</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>10</td>
<td>7</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>FVB/N</td>
<td>Saline</td>
<td>Plasma</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Cisplatin</td>
<td>Plasma</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>
Supplementary Figure B1. Principal component analysis scores plots comparing plasma (A-D), urine (E-H), and kidney (I-L) samples from saline-treated (black) and cisplatin-treated (red) C57BL/6 mice at each timepoint: day 1 (A, E, I), day 2 (B, F, J), day 3 (C, G, K), day 4 (D, H, L). n=5-10, exact sample sizes can be found in Supplementary Table B1.
Supplementary Figure B2. Principal component analysis scores plots comparing plasma (A-D), urine (E-H), and kidney (I-L) samples from saline-treated (black) and cisplatin-treated (red) FVB/N mice at each timepoint: day 1 (A, E, I), day 2 (B, F, J), day 3 (C, G, K), day 4 (D, H, L). n=6-8, exact sample sizes can be found in Supplementary Table B1.
**Supplementary Table B2.** Area under the receiver operating characteristic curve (AUROC) at each timepoint.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Observed m/z</th>
<th>Sample Type</th>
<th>Area Under the Receiver Operating Characteristic Curve (AUROC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C57BL/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Plasma Creatinine</td>
<td>114.067</td>
<td>Plasma</td>
<td>0.719</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plasma</td>
<td>-</td>
</tr>
<tr>
<td>Cinnamoylglycine</td>
<td>204.0663</td>
<td>Plasma</td>
<td>0.792</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>-</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>191.0192</td>
<td>Plasma</td>
<td>0.556</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>-</td>
</tr>
<tr>
<td>Creatine</td>
<td>132.0769</td>
<td>Plasma</td>
<td>0.729</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>0.778</td>
</tr>
<tr>
<td>Equol</td>
<td>243.1019</td>
<td>Plasma</td>
<td>0.736</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>-</td>
</tr>
<tr>
<td>Equol 7-O-gluconuride</td>
<td>417.1184</td>
<td>Plasma</td>
<td>0.708</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>-</td>
</tr>
<tr>
<td>Equol 4'-sulfate</td>
<td>321.0434</td>
<td>Plasma</td>
<td>0.528</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>0.625</td>
</tr>
<tr>
<td>Homovanillic acid</td>
<td>261.0072</td>
<td>Plasma</td>
<td>0.625</td>
</tr>
<tr>
<td>Sulfate</td>
<td></td>
<td>Kidney</td>
<td>0.625</td>
</tr>
<tr>
<td>Indole-3-carboxaldehyde</td>
<td>144.0448</td>
<td>Plasma</td>
<td>0.625</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>-</td>
</tr>
<tr>
<td>Indole-3-carboxylic acid (fragment)</td>
<td>118.0655</td>
<td>Plasma</td>
<td>0.667</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>-</td>
</tr>
<tr>
<td>L-acetylcarnitine</td>
<td>204.1232</td>
<td>Plasma</td>
<td>0.944</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>0.619</td>
</tr>
<tr>
<td>L-carnitine</td>
<td>162.1126</td>
<td>Plasma</td>
<td>0.944</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>0.778</td>
</tr>
<tr>
<td>LysoPC (20:3)</td>
<td>546.3556</td>
<td>Plasma</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>-</td>
</tr>
<tr>
<td>LysoPC (20:3) [M+Na]</td>
<td>568.3377</td>
<td>Plasma</td>
<td>0.875</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>-</td>
</tr>
<tr>
<td>P-cresol glucuronide</td>
<td>283.0819</td>
<td>Plasma</td>
<td>0.514</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>0.550</td>
</tr>
<tr>
<td>P-cresol sulfate</td>
<td>187.0063</td>
<td>Plasma</td>
<td>0.528</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>0.667</td>
</tr>
<tr>
<td>Phenylacetylglycine</td>
<td>192.0659</td>
<td>Plasma</td>
<td>0.775</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>0.542</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>120.0809</td>
<td>Plasma</td>
<td>0.819</td>
</tr>
<tr>
<td>(fragment)</td>
<td></td>
<td>Kidney</td>
<td>-</td>
</tr>
<tr>
<td>Compound</td>
<td>Plasma</td>
<td>Unmeasured</td>
<td>Unmeasured</td>
</tr>
<tr>
<td>---------------------------</td>
<td>----------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>Phenylpropionylglycine</td>
<td>206.0818</td>
<td>0.800</td>
<td>0.698</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>0.722</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proline Betaine</td>
<td>144.1019</td>
<td>0.681</td>
<td>0.889</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>0.556</td>
<td>0.797</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>0.588</td>
<td>0.952</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pyrocatechol Sulfate</td>
<td>188.9860</td>
<td>0.639</td>
<td>0.875</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>0.611</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Succinate</td>
<td>117.0187</td>
<td>0.569</td>
<td>0.938</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sulfoglycolithocholic acid</td>
<td>512.2683</td>
<td>0.542</td>
<td>0.889</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>0.528</td>
<td>0.889</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>0.667</td>
<td>0.750</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.575</td>
<td>0.651</td>
</tr>
<tr>
<td>Taurine</td>
<td>124.0065</td>
<td>0.708</td>
<td>0.931</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>0.613</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>514.2840</td>
<td>0.785</td>
<td>0.736</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>0.569</td>
<td>0.906</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>0.750</td>
<td>0.825</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.826</td>
<td>0.667</td>
</tr>
<tr>
<td>Trigonelline</td>
<td>138.0553</td>
<td>0.667</td>
<td>0.547</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>0.800</td>
<td>0.873</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>0.556</td>
<td>0.922</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Appendix C: Supplementary Information for Chapter 4

Supplementary Table C1. Mobile phase gradient for RPLC separation of metabolites.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>99.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2.0</td>
<td>40.0</td>
<td>60.0</td>
</tr>
<tr>
<td>6.0</td>
<td>15.0</td>
<td>85.0</td>
</tr>
<tr>
<td>8.0</td>
<td>1.0</td>
<td>99.0</td>
</tr>
<tr>
<td>10.0</td>
<td>99.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Supplementary Table C2. Mass spectrometer parameters used in metabolomics analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary voltage</td>
<td>2 kV</td>
</tr>
<tr>
<td>Cone voltage</td>
<td>40 V</td>
</tr>
<tr>
<td>Source temperature</td>
<td>150°C</td>
</tr>
<tr>
<td>Desolvation gas flow</td>
<td>1000 L h⁻¹</td>
</tr>
<tr>
<td>Desolvation gas temp.</td>
<td>500°C</td>
</tr>
<tr>
<td>Cone gas flow</td>
<td>50 L h⁻¹</td>
</tr>
</tbody>
</table>
Supplementary Figure C1. Log transformed relative intensity of urine and serum features significantly altered over time in 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=10-11.
Supplementary Figure C2. Log transformed relative intensity of additional urine 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.
Supplementary Figure C3. Log transformed relative intensity of additional urine features significantly altered over time in 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=10-11.
Appendix D: Supplementary Information for Chapter 5

**Supplementary Table D1.** Summary of sample sizes for each sample type at all timepoints, separated by sex, and separated by under 3 and over 3 years of age (U3/O3).

<table>
<thead>
<tr>
<th>AKI Diagnosis</th>
<th>Sample Type</th>
<th>Pre</th>
<th></th>
<th>Post</th>
<th></th>
<th>Discharge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>M/F</td>
<td>U3/O3</td>
<td>Total</td>
<td>M/F</td>
</tr>
<tr>
<td>No AKI</td>
<td>Serum</td>
<td>34</td>
<td>20 M 10</td>
<td>14 F 24</td>
<td>37</td>
<td>22 M 13</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>40</td>
<td>24 M 5</td>
<td>16 F 35</td>
<td>41</td>
<td>26 M 5</td>
</tr>
<tr>
<td>AKI</td>
<td>Serum</td>
<td>25</td>
<td>12 M 15</td>
<td>13 F 10</td>
<td>30</td>
<td>14 M 18</td>
</tr>
<tr>
<td></td>
<td>Urine</td>
<td>23</td>
<td>13 M 10</td>
<td>10 F 13</td>
<td>22</td>
<td>14 M 9</td>
</tr>
</tbody>
</table>

**Supplementary Figure D1.** Principal component analysis (PCA) scores plots comparing serum (A, B, C) and urine (D, E, F) samples from no AKI (black) and AKI (red) patients at the pre (A, D), post (B, E), and discharge (C, F) timepoints. n=20-46, exact sample sizes can be found in Supplementary Table D1.
Supplementary Figure D2. Log transformed relative intensity of serum features in no AKI and AKI patients separated by sex. For each metabolite, the figure on the left
represents relative abundance of the metabolite in serum samples from male patients (denoted by M) and the figure on the right represents relative abundance in serum samples of female patients (denoted by F). 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 two-way ANOVA, followed by pairwise t-tests at each individual timepoint; *p<0.05, **p<0.01, compared to no AKI patients at each timepoint. n=11-22 for males and n=11-16 for females; exact sample sizes can be found in Supplementary Table D1.
**Supplementary Figure D3.** Log transformed relative intensity of urine features in no AKI and AKI patients separated by sex. For each metabolite, the figure on the left represents relative abundance of the metabolite in urine samples from male patients (denoted by M) and the figure on the right represents relative abundance in urine samples of female patients (denoted by F). 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 two-way ANOVA, followed by pairwise t-tests at each individual timepoint; *p<0.05, **p<0.01, compared to no AKI patients at each timepoint. n=11-26 for males, n=8-20 for females; exact sample sizes can be found in **Supplementary Table D1.**
Supplementary Figure D4. Principal component analysis (PCA) scores plots comparing serum (A, B, C) and urine (D, E, F) samples from no AKI (black) and AKI (red) patients under the age of 3 at the pre (A, D), post (B, E), and discharge (C, F) timepoints. n=5-18, exact sample sizes can be found in Supplementary Table D1.
Supplementary Figure D5. Principal component analysis (PCA) scores plots comparing serum (A, B, C) and urine (D, E, F) samples from no AKI (black) and AKI (red) patients over the age of 3 at the pre (A, D), post (B, E), and discharge (C, F) timepoints. n=8-25, exact sample sizes can be found in Supplementary Table D1.
**Supplementary Table D2.** Diagnostic performance of individual urine metabolites for classification between no AKI and AKI patients under the age of 3 at each timepoint, as assessed by the area under the receiver operating curve (AUC) and corresponding confidence intervals (CI).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pre</th>
<th></th>
<th>Post</th>
<th></th>
<th>Disc</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC</td>
<td>CI</td>
<td>AUC</td>
<td>CI</td>
<td>AUC</td>
<td>CI</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.74</td>
<td>0.43-1</td>
<td>0.578</td>
<td>0.261-0.895</td>
<td>0.803</td>
<td>0.576-1</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.74</td>
<td>0.385-1</td>
<td>0.689</td>
<td>0.34-1</td>
<td>0.758</td>
<td>0.515-1</td>
</tr>
<tr>
<td>Ethylmalonate</td>
<td>0.7</td>
<td>0.379-1</td>
<td>0.756</td>
<td>0.404-1</td>
<td>0.727</td>
<td>0.472-0.982</td>
</tr>
<tr>
<td>Glucuronate</td>
<td>0.62</td>
<td>0.254-0.986</td>
<td>0.711</td>
<td>0.357-1</td>
<td>0.788</td>
<td>0.561-1</td>
</tr>
<tr>
<td>Glycerate</td>
<td>0.62</td>
<td>0.216-1</td>
<td>0.689</td>
<td>0.337-1</td>
<td>0.848</td>
<td>0.655-1</td>
</tr>
<tr>
<td>Hydroxytyrosol glucuronide</td>
<td>0.72</td>
<td>0.443-0.997</td>
<td>0.711</td>
<td>0.344-1</td>
<td>0.788</td>
<td>0.467-1</td>
</tr>
<tr>
<td>Hydroxytyrosol sulfate</td>
<td>0.78</td>
<td>0.431-1</td>
<td>0.667</td>
<td>0.312-1</td>
<td>0.697</td>
<td>0.379-1</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.8</td>
<td>0.486-1</td>
<td>0.733</td>
<td>0.417-1</td>
<td>0.818</td>
<td>0.61-1</td>
</tr>
<tr>
<td>Malate</td>
<td>0.76</td>
<td>0.408-1</td>
<td>0.689</td>
<td>0.338-1</td>
<td>0.758</td>
<td>0.516-0.999</td>
</tr>
<tr>
<td>N-acetylalanine</td>
<td>0.72</td>
<td>0.331-1</td>
<td>0.756</td>
<td>0.404-1</td>
<td>0.72</td>
<td>0.443-0.997</td>
</tr>
<tr>
<td>N-acetylserine</td>
<td>0.8</td>
<td>0.408-1</td>
<td>0.678</td>
<td>0.312-1</td>
<td>0.788</td>
<td>0.561-1</td>
</tr>
<tr>
<td>N-alpha-acetyllysine</td>
<td>0.54</td>
<td>0.236-0.844</td>
<td>0.689</td>
<td>0.397-0.981</td>
<td>0.803</td>
<td>0.552-1</td>
</tr>
<tr>
<td>Pantothenate</td>
<td>0.7</td>
<td>0.351-1</td>
<td>0.667</td>
<td>0.32-1</td>
<td>0.758</td>
<td>0.458-1</td>
</tr>
<tr>
<td>Proline</td>
<td>0.64</td>
<td>0.33-0.95</td>
<td>0.667</td>
<td>0.326-1</td>
<td>0.727</td>
<td>0.38-1</td>
</tr>
<tr>
<td>Vanilloylglycine</td>
<td>0.84</td>
<td>0.591-1</td>
<td>0.689</td>
<td>0.323-1</td>
<td>0.795</td>
<td>0.512-1</td>
</tr>
</tbody>
</table>
Curriculum Vitae

Name: Yong Jin (James) Lim

Post-secondary Education and Degrees:
The University of Western Ontario
London, Ontario, Canada
2012-2016 BMSc

The University of Western Ontario
London, Ontario, Canada
2016-2023 Ph.D.

Honours and Awards:
Canadian Society of Pharmacology and Therapeutics (CSPT)
Peter Dresel Trainee Presentation Award
2021

CSPT-American Society of Pharmacology and Therapeutics (ASPET) Conference Travel Award – Poster presentation
2021

CSPT Travel Bursary
2017-2019

Related Work Experience:
Teaching Assistant
The University of Western Ontario
2016-2021
- 2016-2017 – Pharmacology 4660a
- 2017-2021 – Physiology 1021

Work Study Research Assistant
The University of Western Ontario
2013-2015

Publications:


