December 2015

Regulation of Hepatic Drug Metabolizing Enzymes in Chronic Kidney Disease

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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REGULATION OF HEPATIC DRUG METABOLIZING ENZYMES IN CHRONIC KIDNEY DISEASE

(Thesis format: Integrated Article)

by

Thomas J. Velenosi

Graduate Program in Pharmacology and Toxicology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
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Abstract

Chronic kidney disease (CKD) occurs as a result of declining renal function for 3 or more months. CKD effects 1 in 10 Canadians and is associated with a number of co-morbidities including diabetes and cardiovascular disease. To manage CKD and associated co-morbidities, patients take an average of 12 medications with a median pill burden of 19. Indeed, renal drug elimination is compromised in CKD, as declining glomerular filtration reduces drug excretion into urine. More recently, studies have provided evidence of altered non-renal drug clearance in CKD. The majority of drug clearance occurs in the liver by CYP2C and CYP3A drug metabolizing enzymes. Hepatic CYP2C and CYP3A drug metabolizing enzymes are tightly regulated by nuclear receptors. The majority of CKD patients have mild to moderate degrees of CKD and the potential for altered hepatic drug metabolism at these earlier stages is unknown. As renal function declines, patients begin to experience the uremic condition, which consists of metabolic waste product accumulation in the blood. A number of studies suggest that these uremic toxins may mediate the downregulation of hepatic CYP2C and CYP3A; however, the mechanism by which this occurs remains to be determined. My overall hypothesis is that hepatic drug metabolism is altered in CKD.

Herein, we evaluate the effects of moderate CKD on hepatic drug metabolism and determine a possible mechanism of CYP2C and CYP3A downregulation in rats with CKD. In a rat model of moderate CKD, hepatic CYP2C and CYP3A function and expression were significantly decreased demonstrating a negative exponential correlation with kidney function. Transcriptional activation in both the CYP2C and CYP3A promoters was reduced as a result of decreased nuclear receptor binding and histone acetylation. Untargeted metabolomics was utilized to identify potential uremic mediators of hepatic CYP2C and CYP3A altered expression and function. Gut-derived uremic toxins accounted for the most significant metabolic signatures defining plasma and liver tissue in CKD, leading to the evaluation of these toxin effects on hepatic CYP2C and CYP3A. Reduction of gut-derived uremic toxins did not recover CYP2C and CYP3A function and expression in CKD. In conclusion, these studies further our understanding of hepatic drug metabolizing enzyme downregulation in the setting of CKD.
Keywords

Drug metabolism, Pharmacokinetics, Cytochrome P450, Chronic Kidney Disease, End-stage renal disease, Uremia, AST-120, Indoxyl Sulfate, Chromatin Immunoprecipitation, Metabolomics.
Co-Authorship Statement

Chapter 1:
A portion of the introduction was reproduced from a review article:


TJV and BLU wrote and revised the manuscript and approved of the final version of this review article.

Chapter 3:


TJV and BLU designed the experiments. TJV, AF and SL conducted experiments. TJV and BLU analyzed and interpreted data. TJV and BLU wrote the manuscript and all authors approved of the final version.

Chapter 4:


TJV and BLU designed the experiments. TJV, DAF and GS conducted experiments. TJV, DBH and BLU analyzed and interpreted data. TJV and BLU wrote the manuscript and all authors approved of the final version.

Chapter 5:


TJV and BLU contributed to the experimental design. Animal experiments and procedures were conducted by TJV, AH, DAF and AT. UPLC-QToFMS experiments were conducted by TJV, AH, DAF, AT, ASK and BLU. Compound standards were synthesized by PK and MAK. Data and statistical analysis were performed by TJV, AH, DAF, AT, ASK, LEM, SEN and BLU. Reagents and analytical tools were contributed by BLU. The manuscript was drafted by TJV, with additions and revisions by all authors.
Chapter 6:


TJV and BLU designed the experiments. Animal experiments and procedures were conducted by TJV, AT and AK. All other experiments were conducted by TJV. TJV and BLU analyzed and interpreted data. TJV and BLU wrote the manuscript.
Dedication

To Nonno and Nonna
Acknowledgments

I would first like to express my deepest thanks to Dr. Brad Urquhart, who guided me into research as an undergraduate student and has been an amazing mentor throughout graduate school. From the day I began in his lab, Brad has created a positive environment that is conducive to learning and has always been available to give advice. I am incredibly privileged to be his first graduate student and be involved in establishing his research program. Throughout the 5 years I have spent in his lab, doing research has been more fun than work and has motivated me to pursue a career in science. His great vision and approach to research has left a tremendous impression on me and, for that, I am most grateful.

Dr. David Freeman, imparting your expertise in liquid chromatography and analytical pharmacology has provided me with a strong basis for my graduate studies. Dr. Murray Cutler, teaching me to design and develop my own experiments has been a great asset in grad school. Thank you for your mentorship as an undergraduate supervisor, and as a friend.

Thank you to the past and present members of the Urquhart Lab. Dave Feere, your assistance in the lab and your invaluable friendship has made my time as a graduate student an enjoyable experience. May your handicap never go above 10 and your “hot hands” keep your dentistry skills up to par. I am also grateful for the teamwork and contributions provided by Alvin Tieu and Andrew Kucey. Alvin, I hope that you continue to put the same effort into research as you do into fashion and always be the best dressed. Andrew Kucey, may you continue to work as hard at achieving your career goals as you do at scoring in the top corner in hockey, and settle for nothing but the best. I have no doubt that you will both succeed in your future endeavors. This work could not have been made possible without other previous members of the Urquhart Lab, including Angel Fu and Anzel Hennop.

I would also like to thank Dr. Nica Borradaile for her mentorship as well as the past and current members of her lab. To Alex Stoianov, I appreciate our life discussions both during and after your time at Western. Your ability to give good advice will be an asset to your career as a physician. Alex Hetherington, your generous baking and positive attitude have always been uplifting in the lab. You’re always the most organized and helpful to everyone in the lab. I wish you both the best of luck.
To the members of my advisory committee, Drs. Dan Hardy, Wei-Yang Lu and Ruud Veldhuizen for your discussion about my project and guidance throughout graduate school.

I am also grateful for the advice I have received from the many faculty members, staff and students in the department of Physiology and Pharmacology including Dr. Peter Chidiac, Dr. Rommel Tirona, Dr. Angela Nissen, Dr. Christopher Pin, Dr. John DiGuglielmo, Buon Thai, Dr. Moshi Bhattacharya, Dr. Timothy Regnault, Dr. Dean Betts, Dr. Gurjeev Sohi and many others.

I would like to thank the Schulich School of Medicine and Dentistry and the Natural Sciences and Engineering Research Council of Canada for the financial support of this thesis.

Last but not least, I would like to thank my family including my parents and brother who have encouraged and supported me throughout the hardships of graduate school. To my grandparents, who have inspired me to pursue higher education and my aunts, uncles and cousins who have always been highly supportive of my career goals.
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## Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAG</td>
<td>Alpha 1 acidic glycoprotein</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-Binding Cassette</td>
</tr>
<tr>
<td>ADR</td>
<td>Adverse drug reaction</td>
</tr>
<tr>
<td>AcH3</td>
<td>Acetylated histone 3</td>
</tr>
<tr>
<td>AcH4</td>
<td>Acetylated histone 4</td>
</tr>
<tr>
<td>ACR</td>
<td>Albumin to creatinine ratio</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive androstane receptor</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum serum concentration</td>
</tr>
<tr>
<td>CMPF</td>
<td>3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
</tr>
<tr>
<td>EPPIC</td>
<td>Evaluating Prevention of Progression in CKD</td>
</tr>
<tr>
<td>ESRD</td>
<td>End stage renal disease</td>
</tr>
<tr>
<td>EuTox</td>
<td>European Uremic Toxin Work Group</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>Histone 3 Lysine 27 tri-methylation</td>
</tr>
<tr>
<td>H3K4me2</td>
<td>Histone 3 Lysine 4 dimethylation</td>
</tr>
<tr>
<td>H3K9me3</td>
<td>Histone 3 Lysine 9 tri-methylation</td>
</tr>
<tr>
<td>H4R3me2a</td>
<td>Histone 4 arginine 3 asymmetric dimethylation</td>
</tr>
<tr>
<td>HD</td>
<td>Hemodialysis</td>
</tr>
<tr>
<td>HMDB</td>
<td>Human Metabolome Database</td>
</tr>
<tr>
<td>HNF</td>
<td>Hepatocyte nuclear factor</td>
</tr>
<tr>
<td>hPCFT</td>
<td>Human proton coupled folate transporter</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>Huh7</td>
<td>Human hepatoma 7 cells</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
</tbody>
</table>
IgG  Immunoglobulin G
IL  Interleukin
Km  Michaelis constant
MAPK  Mitogen-activated protein kinases
miR  Micro RNAs
MS  Mass spectrometer
NADPH  Nicotinamide adenine dinucleotide phosphate (reduced)
NAT2  N-acetyltransferase 2
NF-κB  Nuclear factor kappa B
NHANES  National Health and Nutrition Examination Survey
NMR  Nuclear magnetic resonance
OAT  Organic anion transporter
OPLS-DA  Orthogonal partial least squares discriminate analysis
P-gp  P-glycoprotein
P450  Cytochrome P450
PCA  Principal component analysis
PD  Peritoneal dialysis
PDA  Photodiode array detection
PRMT1  Protein arginine methyltransferase 1
PXR  Pregnane X receptor
QToF  Quantitative time of flight
ROS  Reactive oxygen species
$r_s$  Spearman's rank correlation coefficient
RNA Pol II  RNA polymerase II
RXR  Retinoid X receptor
SEM  Standard error of the mean
SLC  Solute carrier
STAT5b  Signal transducer and activator of transcription-5b
T1/2  Half-life
TGF  Transforming growth factor
TMAO  Trimethylamine N-oxide
TNF-α  Tumour necrosis factor α
UPLC  Ultra performance liquid chromatography
VIP  Variable importance in projection
$V_{max}$  Maximum enzyme velocity
'1 INTRODUCTION

1Portions of this chapter are reproduced with permission from: Velenosi TJ and Urquhart BL (2014) Pharmacokinetic considerations in chronic kidney disease and patients requiring dialysis. Expert Opin Drug Metab Toxicol 10:1131-1143.
1.1 Overview of Chronic Kidney Disease

1.1.1 Prevalence and Stages

Chronic kidney disease (CKD) is defined as abnormalities of kidney structure or function present for over three months that have implications for health (Stevens et al., 2013). Approximately 1 in 10 Canadians have CKD, which is similar to other countries including the USA (10-14%) and China (11%) (Coresh et al., 2007; Zhang et al., 2012a; Arora et al., 2013; Saran et al., 2015). The majority of patients with CKD are over 60, where prevalence of the disease is approximately 25% (Saran et al., 2015).

CKD is divided into 5 stages and classified based on estimated glomerular filtration rate (eGFR). There are several equations used to estimate GFR; however, the current method recommended by the National Kidney Foundation is the CKD Epidemiology Collaboration (CKD-EPI) (Stevens et al., 2011). This equation uses serum creatinine as a biomarker as well as age, gender and race to estimate GFR. In early stages of CKD, patients may not present with altered GFR and therefore, an albumin to creatinine ratio (ACR) in urine is used to assess the initial stages of kidney damage. Glomerular filtration of albumin is restricted due to its large size (68 kDa) so its presence in the urine is suggestive of kidney damage. In CKD, albumin is the most abundant protein found in urine (National Kidney Foundation, 2002). The amount of albumin in urine is measured as a ratio to creatinine with micro- (3-30 mg/g creatinine) and macroalbuminuria (≥ 30 mg/g creatinine) levels defining individual risk for CKD progression along with eGFR (Table 1.1). Stages 1 and 2 of CKD are defined by kidney damage (including elevated ACR) with normal eGFR (stage 1, ≥ 90 ml/min/1.73 m²) or slightly decreased eGFR (stage 2, 60 – 89 ml/min/1.73 m²). Complications generally begin when eGFR is 30 – 59 ml/min/1.73 m² in stage 3, which contains more than half of all CKD patients (Arora et al., 2013). CKD progresses when eGFR continues to decline until stage 5 (< 15 ml/min/1.73 m²) or end-stage renal disease (ESRD). Patients with ESRD require renal replacement therapy such as hemodialysis (HD) or transplant to support life. In Canada, the risk of death during the first year of ESRD is 16.4% (Quinn et al., 2011). Other markers of kidney damage, if present for > 3 months, can also be considered CKD.
### Table 1.1 Risk of progression as determined by eGFR and albuminuria at various stages of CKD.

<table>
<thead>
<tr>
<th>Stages</th>
<th>eGFR (ml/min/1.73 m²)</th>
<th>Description</th>
<th>Albuminuria Categories</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal to mildly increased (30-300 mg/g)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Moderately Increased (&lt; 30 mg/g)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Severely Increased (≥300 mg/g)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>≥ 90</td>
<td>Normal</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>2</td>
<td>60–89</td>
<td>Mild ↓GFR</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>3</td>
<td>30–59</td>
<td>Moderate ↓GFR</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>4</td>
<td>15–29</td>
<td>Severe decreased</td>
<td></td>
<td>Very high</td>
</tr>
<tr>
<td>5</td>
<td>&lt;15 or anuric</td>
<td>Kidney failure</td>
<td></td>
<td>Very high</td>
</tr>
</tbody>
</table>

Adapted from: (National Kidney Foundation, 2002; KDIGO, 2013)
including structural abnormalities detected by histology or imaging, tubular disorders, and urinary sediment aberrations (KDIGO, 2013).

1.1.2 Causes, Comorbidities, Signs and Symptoms

1.1.2.1 Diabetes in CKD

Diabetes is the primary cause of CKD and ESRD diagnosis, accounting for the largest proportion of ESRD patients at 36% (CIHI, 2015). When patients are diagnosed with type II diabetes, 7.3% have microalbuminuria, which progresses at a rate of 2% per year (Adler et al., 2003). Microalbuminuria in diabetic patients also progresses to macroalbuminuria at a rate of 2.8% per year. Hyperglycemia is thought to be a major cause of diabetic nephropathy and a number of clinical trials have suggested glycemic control as an important factor in delaying the progression of CKD in diabetic patients (KDIGO, 2013). Glycated hemoglobin (HbA1c) is the standard measure for hyperglycemia over long periods. The National Kidney Foundation guidelines recommend HbA1c < 7.0% for CKD patients, which is consistent with recommendations for the general population (KDOQI, 2007). In the US, approximately 35% of ESRD patients are above this value (Williams et al., 2006). It is difficult to maintain glycemic control in CKD patients with diabetes. These patients have insulin resistance, impaired insulin secretion as well as decreased insulin degradation (Williams and Garg, 2014). When eGFR decreases below 20 mL/min/1.73 m², renal insulin clearance also decreases, prolonging its activity. The liver and kidney are major sites of insulin degradation, which also declines in CKD due to kidney damage (Williams and Garg, 2014).

1.1.2.2 Cardiovascular Disease in CKD

Renal vascular disease, including high blood pressure, causes ESRD in 23% of new cases and is the second leading cause of CKD (CIHI, 2015). Progression of CKD can occur as a result of poorly controlled hypertension. Over 70% of patients with stage 3-5 CKD have hypertension and patients who have a baseline blood pressure of 180/100 mmHg or higher are 15 times more likely to develop ESRD than those with a baseline blood pressure of 110/50 mmHg (Tozawa et al., 2003; Inker et al., 2011). The interaction
between CKD and cardiovascular disease is complex. These patients experience the traditional cardiovascular risk factors including hypertension, advanced age and dyslipidemia as well as CKD-specific cardiovascular risk factors such as volume overload, malnutrition, anemia, oxidative stress and inflammation (Segall et al., 2014). As a result, the standard treatment of cardiovascular disease in the general population can be ineffective in patients with CKD (Segall et al., 2014). Vascular calcification and thickening are also CKD-specific risk factors that contribute to cardiovascular disease. Left ventricular hypertrophy is the predominant clinical indicator of cardiovascular disease when ESRD patients begin dialysis (Foley et al., 1995). Cardiac fibrosis is also a common cardiovascular complication that occurs in patients with CKD. Inter-myocardiocyte fibrosis occurs in hypertensive CKD patients, which is not seen in hypertensive patients that have normal renal function (Aoki et al., 2005). The combination of left ventricular hypertrophy and cardiac fibrosis can be detrimental to both CKD and ESRD patients. These clinical manifestations are considered the highest priority for treatment in ESRD patients to prevent sudden cardiac death and congestive heart failure (Glassock et al., 2009).

Other major causes of ESRD include polycystic kidney disease (5%), pyelonephritis (2%), and drug induced CKD (2%) (CIHI, 2015).

### 1.1.2.3 Other Co-morbidities and Treating CKD

The majority of complications in CKD begin to arise in stage 3. Screening for anemia, bone abnormalities and metabolic acidosis is recommended at this stage of the disease (National Kidney Foundation, 2002). Bone abnormalities can occur as a result of secondary hyperparathyroidism. As renal function declines, vitamin D levels decrease leading to less calcium absorption (de Francisco, 2004). Phosphate clearance is also compromised, resulting in hyperphosphatemia. High serum phosphate causes depletion of serum calcium stores through the formation of insoluble calcium phosphate. When this occurs, serum calcium stores deplete, causing increased production of parathyroid hormone and the development of secondary hyperparathyroidism (de Francisco, 2004). Patients generally begin to develop secondary hyperparathyroidism when parathyroid hormone blood levels increase during stage 3 of CKD (National Kidney Foundation,
Increases in parathyroid hormone cause increased bone resorption and can lead to various types of bone disease. Anemia occurs in CKD as a result of decreased erythropoietin production in the kidney and the pro-inflammatory condition associated with CKD. Anemia can develop in the early stages of CKD, but is most common in ESRD (Iseki and Kohagura, 2007). Inflammation is also highly prevalent in CKD and is measured using serum c-reactive protein (CRP). CRP levels are increased in a large percentage of CKD patients and, once increased, remain consistently high (Ortega et al., 2002). A number of non-specific signs and symptoms can also develop as early as stage 3 of CKD including nausea and vomiting, loss of appetite, weight loss, confusion, lethargy, gastrointestinal ulcers and impaired homeostasis (Depner, 2001). Although present in many patients, it is difficult to attribute these non-specific signs and symptoms as CKD without prior knowledge of serum and/or urine biochemistry changes. In the 1999 to 2006 National Health and Nutrition Examination Survey (NHANES), approximately 40% of subjects with undiagnosed diabetes or prediabetes had CKD (Plantinga et al., 2010). It is estimated that the majority of patients with CKD are undiagnosed, which may be a result of under-diagnosed co-morbidities that contribute to CKD as well as the many nonspecific signs and symptoms of the disease (Coresh et al., 2005).

The National Kidney Foundation recommends CKD testing for all patients with diabetes, hypertension, family history of CKD and age > 60. This includes assessing proteinuria as well as eGFR. In the Modified Diet in Renal Disease Study, CKD progressed in 85% of patients at a rate of 4 mL/min annually for all stages (Hunsicker et al., 1997; Murphree and Thelen, 2010). There are modifiable and non-modifiable risk factors that contribute to the decline in renal function. Treatment is aimed to reduce modifiable risk factors such as proteinuria, low levels of serum albumin, high glycemic index and blood pressure (Murphree and Thelen, 2010). Non-modifiable risk factors include male gender, increasing age and African-American race (Murphree and Thelen, 2010).
1.2 Renal Replacement Therapy for ESRD Treatment

If a patient progresses to ESRD, renal replacement therapy is required in order to sustain life and can include HD, peritoneal dialysis (PD) or kidney transplantation. The time to initiate dialysis is controversial and dependent on many factors. Currently, there are no laboratory values that dictate the requirement to begin dialysis, although two approaches are typically used which include “early-start” or “late-start”. In early start, patients begin on renal replacement therapy once their eGFR is reduced to less than 15 mL/min/1.73 m². In contrast, late start dialysis is initiated once the patient begins to experience signs of uremia or their eGFR drops to less than 7 mL/min/1.73 m². A recent randomized controlled trial found no difference in survival or clinical outcomes when comparing early start and late start dialysis (Cooper et al., 2010). Accordingly, nephrologists in some countries are now recommending an intent-to-defer approach to starting dialysis (Nesrallah et al., 2014). Over one million patients worldwide now receive renal replacement therapy to treat kidney failure (Coresh et al., 2007). Institutional HD is the most commonly used method of renal replacement therapy and has consistently accounted for 77% of all new patients initiating dialysis in Canada over the last 10 years (CIHI, 2015). This method involves the removal of solutes and excess water from the blood using a semi-permeable dialysis membrane and dialysate solution. Blood travels out of the body, generally through a catheter or fistula, and into a dialysis machine where liquid, solutes and ions are exchanged across a synthetic membrane using a counter-current flow of dialysate. Dialysate is a physiological salt solution containing a number of components such as potassium, sodium and calcium as well as bicarbonate and glucose (Locatelli et al., 2015). The composition of dialysate is prescribed to an individual patient to optimize the amount of fluid removal during a dialysis session. The filtered blood then travels back into the patient. The extracorporeal filtration of solutes is dependent on pore size, surface area and material of the dialysis membrane as well as blood and dialysate flow rate. Low-flux dialysis membranes with small pore sizes were used for conventional dialysis; however, they have recently been replaced by larger pore size high-flux dialysis membranes. The advantages of high-flux dialysis include clearance of larger solutes (called middle molecules) including β-2 microglobulin (11, 800 Da) and shorter duration of dialysis session (Schneider and Streicher, 1985). Continuous ambulatory and
automated PD account for approximately 20% of the initial renal replacement treatment for patients diagnosed with ESRD (CIHI, 2015). PD involves the surgical insertion of a catheter into the peritoneal cavity. For continuous ambulatory PD, peritoneal cavity is filled via catheter allowing solutes to diffuse through the catheter membrane into a hypertonic dialysis solution prior to emptying, which must occur multiple times per day. Automated PD uses a machine to exchange fluid at night while the patient sleeps. Dialysis is the most frequently used renal replacement modality; however, in Canada, 42.5% of ESRD patients have a functioning kidney transplant (CIHI, 2015). Transplant patients no longer require dialysis once the kidney allograft begins to function and their risk of cardiovascular events are significantly decreased (Zolty et al., 2008). Erythropoietin levels increase in the first few days after kidney transplant and hemoglobin can reach target levels (11 to 12 g/dL) around 3 months after transplant (Joist et al., 2006). Quality of life is also greatly improved in transplant patients; however these patients must take immunosuppressants to inhibit rejection of the transplanted kidney (Laupacis et al., 1996).

1.3 Uremia

The definition of uremia has evolved in the literature over many years. A condition originally defined by its literal meaning of “urine in the blood”, has been used as an umbrella term to encompass all signs and symptoms of advanced CKD. More recently, anemia, hypocalcemic tetany and hypertension due to volume overload have been removed from the definition of uremia, which is now described as the illness associated with the accumulation of organic waste products that are normally cleared by the kidney (Meyer and Hostetter, 2007). If untreated, uremia can lead to death; however, the advent of renal replacement therapy has prolonged life expectancy in ESRD patients. As a result, a number of the small molecular weight compounds cleared in dialysis have been implicated in uremic toxicity (Meyer and Hostetter, 2007). Despite the intermittent removal of organic waste products by dialysis, patients continue to suffer from a number of uremic symptoms known as the “residual syndrome” (Depner, 2001). Possible causes of the residual syndrome include hormone imbalances, incomplete removal of dialyzable
solute as well as the accumulation of protein-bound metabolic waste products that are poorly cleared in dialysis (Depner, 2001). In this section, uremic toxins will be described and characterized according to the European Uremic Toxin Work Group (EUTox). This collaborative research effort focuses on the deleterious effects of solute retention on biological systems in CKD as well as effective removal of these toxins (Vanholder et al., 2009). The EUTox has identified over 90 uremic toxins, which are classified based on size and protein binding properties (Duranton et al., 2012).

1.3.1 Free water-soluble uremic toxins

Free water-soluble low molecular weight compounds (< 500 Da) account for 46% of identified uremic toxins in patients with CKD (Duranton et al., 2012). These compounds include reactive carbonyl compounds such as alkanals, alkenals, and 4-OH-alkenals, which are produced as a result of the increased oxidative state in uremia (Alhamdani et al., 2006). Guanidine based molecules such as guanidinosuccinic acid and methylguanidine are generated from arginine metabolism and are among the uremic toxins with the highest fold change in CKD in relation to patients with normal kidney function (Duranton et al., 2012). Asymmetric dimethyl arginine also accumulates in CKD and these toxins have been implicated in cardiovascular morbidity and mortality (Taes et al., 2008; Sibal et al., 2010). Trimethylamine N-oxide (TMAO) is another free water-soluble uremic toxin derived from choline and trimethylamine. A recent study discovered a novel pathway linking TMAO to cardiovascular disease and atherosclerosis (Wang et al., 2011).

1.3.2 Middle Molecules

Uremic toxins > 500 Da are classified as middle molecules. These account for 28% of identified uremic toxins and mostly include proteins and cytokines (Duranton et al., 2012). As described above, parathyroid hormone accumulates in CKD and is considered a middle molecule. Implementation of high-flux dialyzers from conventional have allowed for the removal of other large proteins including β-2 microglobulin, which can cause amyloidosis and has been associated with increased mortality in long-term dialysis patients (Cheung et al., 2006). Cytokines are also a fairly large class of middle molecules
that accumulate in plasma of patients with CKD. These include interleukin (IL)-6, IL-8, and IL-10 as well as tumour necrosis factor α (TNF-α)(Duranton et al., 2012). High cytokine levels increase the risk of cardiovascular complications and mortality in CKD patients and can be elevated in the absence of clinically apparent inflammation (Zoccali et al., 2006).

1.3.3 Protein-bound and gut-derived uremic toxins

Solutes classified as protein-bound uremic toxins represent 25% of all identified uremic toxins (Duranton et al., 2012). These solutes are highly bound to plasma protein and therefore, poorly cleared in dialysis (Lesaffer et al., 2000). Unlike many free water-soluble toxins, the plasma concentration of protein bound uremic toxins increases as CKD progresses and are highest in ESRD (Vanholder et al., 2003a). The majority of protein-bound uremic toxins are also derived from aromatic amino acid putrefaction, involving bacterial decomposition in the colon. These protein-bound gut-derived metabolic products are further classified into indole, phenol and hippurate groups.

1.3.3.1 Indoles

Indole is produced from tryptophan metabolism by bacterial tryptophanase. The majority of bacteria-produced indole is absorbed and hydroxylated in the liver by cytochrome P450 2E1 to form 3-hydroxy indole (Banoglu et al., 2001). 3-Hydroxy indole undergoes sulfation by sulfotransferase 1A1 to form indoxyl sulfate (Banoglu and King, 2002) (Fig. 1.1). Plasma levels of indoxyl sulfate are reduced in ESRD patients, similar to levels in normal renal function, when these patients undergo partial colectomies (Aronov et al., 2011). This indicates that the colon is the major site of indole generation and is required for the production of indoxyl sulfate (Aronov et al., 2011). A number of intestinal bacteria contain tryptophanase, which is inducible in the presence of tryptophan. In ESRD, Clostridiaceae, Enterobacteriaceae, and Verrucomicrobiaceae tryptophanase containing bacterial families are found in higher abundance than in patients with normal kidney function, which can contribute to elevated levels of indoxyl sulfate in these patients (Wong et al., 2014).
Figure 1.1 Generation of indoxyl sulfate, p-cresyl sulfate and hippuric acid from gut-derived aromatic amino acid putrefaction.

Fig. 1.1 images were modified from Servier Medical Art (http://www.servier.co.uk/medical-art-gallery).
Once in circulation, approximately 93% of indoxyl sulfate is protein-bound to albumin (Devine et al., 2014). Indoxyl sulfate has been shown to have a number of deleterious effects in CKD, contributing to nephrotoxicity and cardiovascular disease. In proximal tubule cells, indoxyl sulfate has been shown to induce reactive oxygen species (ROS) and cause renal fibrosis in CKD (Saito et al., 2014). Indoxyl sulfate increases the p53-transforming growth factor (TGF) β1-Smad3 pro-fibrotic pathway through p53 activation in the proximal tubule (Shimizu et al., 2013). ROS production by indoxyl sulfate in both renal tubular cells and glomerular mesangial cells activates nuclear factor (NF)-κB, enhancing infiltration of monocytes into uremic kidneys (Miyazaki et al., 1997).

In the clinic, indoxyl sulfate has been correlated with cardiovascular events in ESRD patients (Cao et al., 2015). Aortic calcification and left ventricle systolic dysfunction are also associated with high levels of indoxyl sulfate in patients with CKD (Barreto et al., 2009; Sato et al., 2013). Indoxyl sulfate plays a role in cardiovascular remodeling of cardiac myocytes and cardiac fibroblasts. This occurs through activation of pro-fibrotic, pro-hypertrophic and pro-inflammatory pathways including the NF-κB pathway (Lekawanvijit et al., 2010; Niwa, 2013). Indoxyl sulfate has a strong association of cardiovascular mortality in ESRD and therefore, plays a significant role in cardiovascular disease (Barreto et al., 2009).

Due to its strong protein binding, indoxyl sulfate does not undergo significant glomerular filtration. Instead, organic anion transporters (OAT) 1 and 3 on the basolateral membrane of the proximal tubule mediate indoxyl sulfate transcellular transport. On the apical membrane, OAT4 is responsible for secretion into urine (Enomoto and Niwa, 2007). It is estimated that 3% of tryptophan is excreted by the kidney as indoxyl sulfate (Bender, 1983; Keszthelyi et al., 2009). Therefore, although production of indoxyl sulfate is increased in ESRD, as mentioned above, elevated plasma levels are mainly a result of decreased clearance.

Indole that is not absorbed can also be converted into indole acid derivatives, which are generated by gut bacteria and found in smaller quantities in plasma than indoxyl sulfate.
These include indole-3-acetic acid, indolyl-acetyl-glutamine and indolyl-propionic acid (Keszthelyi et al., 2009).

1.3.3.2 Phenols

Gut bacterial metabolism of tyrosine produces a number of phenol derivatives that accumulate in patients with CKD. Similar to indoxyl sulfate, Clostridiaceae and Enterobacteriaceae bacteria are more abundant in the colon of patients with ESRD and produce p-cresol (Wong et al., 2014). In these bacteria, p-hydroxyphenylacetate is generated from hydroxyphenylacetate decarboxylase mediated metabolism of tyrosine, which can be further metabolized to p-cresol (Smith and Macfarlane, 1997). The majority of p-cresol is subsequently absorbed and sulfated in the liver (Fig. 1.1). ESRD patients with partial colectomies have reduced plasma levels of p-cresyl sulfate suggesting that the colon is the major site of p-cresol formation (Aronov et al., 2011). P-cresyl sulfate is also 90% protein bound to albumin in circulation and therefore, poorly removed by dialysis (De Smet et al., 1998; Martinez et al., 2005). Clearance of p-cresyl sulfate mainly occurs in the kidneys via OATs (Miyamoto et al., 2011).

Indeed, elevated plasma levels of p-cresyl sulfate in ESRD have been shown to cause many of the same toxic effects as indoxyl sulfate. Interestingly, in patients with ESRD, high levels of unbound plasma p-cresyl sulfate increase the risk of all cause mortality and cardiovascular mortality beyond the traditional risk in uremia (Wu et al., 2012). Unbound p-cresyl sulfate is also a predictor of mortality in earlier stages of CKD (Liabeuf et al., 2010). P-cresyl sulfate can induce pro-fibrotic TGF-β1 in human proximal tubule cells and ROS production via NADPH oxidase 4 activation. Kidney damage is also exacerbated in animals with CKD supplemented with p-cresyl sulfate (Watanabe et al., 2013).

P-cresol can also be glucuronidated to form p-cresyl glucuronide; however, research on p-cresyl glucuronide is limited mostly to its identification in urine or plasma (Lesaffer et al., 2003; Aronov et al., 2011). Therefore, there is insufficient evidence for pathophysiologic effects of p-cresyl glucuronide, although one study suggested that it
might be an indicator of altered gut microbiota during inflammation (Zhang et al., 2012b).

The majority of studies assessing the accumulation of tyrosine derivatives in CKD have focused on p-cresyl sulfate. However, phenol is another gut-derived tyrosine metabolite that accumulates in CKD, detected in plasma and urine as phenyl sulfate (Kikuchi et al., 2010; Akiyama et al., 2012). Like the other sulfated uremic toxins, phenyl sulfate is highly protein bound (90.7%), and transported by OATs (Wikoff et al., 2011; Itoh et al., 2012). Conversely, phenyl sulfate is more efficiently removed by HD, possibly due to a weak interaction with albumin (Itoh et al., 2012).

1.3.3.3 Hippurate

Phenylalanine is converted to benzoic acid by intestinal bacteria and subsequently combined with glycine to form hippuric acid in the intestine or the liver (Fig. 1.1) (Chiba et al., 1994; Mulder et al., 2005). Hippuric acid is also strongly bound to albumin and plasma levels of this uremic toxin are highly elevated in CKD. Elevated levels of hippuric acid have been shown to cause insulin resistance and glucose intolerance, possibly contributing to diabetic complications (Spustova et al., 1989). Endothelial cell dysfunction has also been attributed to increased plasma levels of hippuric acid, which may promote cardiovascular disease prior to the development of uremia (Addabbo et al., 2013).

1.4 Removal of gut-derived uremic toxins in uremia

1.4.1 Probiotics, Prebiotics, and dietary fibre

As described above, not only is the clearance of gut-derived uremic toxins decreased in CKD, but indole and p-cresol producing bacteria are also more abundant in the colon microbiota for these patients. Therefore, a therapeutic strategy to reduce plasma levels of these uremic toxins is to manipulate the colon microbiota in patients with CKD. This can be accomplished by improving gut dysbiosis in CKD using probiotics, prebiotics or the combination of both, known as symbiotics. Probiotics are defined as microorganisms that
express exogenous enzymes and increase the colon concentrations of symbiontons to provide a health benefit (Food and Agriculture Organization, 2002). Administration of non-indole producing bacteria including *Lactobacillus* and *Bifidobacillus* have demonstrated decreased level of plasma indoxyl sulfate and p-cresyl sulfate in CKD and ESRD (Hida et al., 1996; Guida et al., 2014). However, there are concerns of increased inflammation due to urease hydrolysis of urea in these probiotics (Koppe et al., 2015). The use of probiotics can also be supplemented with prebiotics. Prebiotics are carbohydrates that are not digested or absorbed in the gut but can be used by intestinal bacteria. Lactulose is a common example of a prebiotic and has been shown to lower indoxyl sulfate and p-cresyl sulfate levels in healthy patients (Terada et al., 1992). Finally, increasing dietary fibre can also reduce plasma indoxyl sulfate and p-cresyl sulfate levels in ESRD patients (Sirich et al., 2014). In CKD, lower dietary protein to fibre ratios have been shown to associate with decreased plasma levels of both indoxyl sulfate and p-cresyl sulfate (Rossi et al., 2015).

### 1.4.2 AST-120

Another method used to remove gut-derived uremic toxins is to prescribe AST-120 to patients with CKD. AST-120 is a compound that began development for the treatment of uremia in 1975 and was approved in Japan in 1991 (Niwa et al., 1991; Schulman et al., 2015). It is also approved in both Korea (2005) and the Philippines (2010). AST-120 is a spherical carbon adsorbent, 0.2-0.4 mm in diameter, composed of carbon with a surface oxide. Unlike activated charcoal, AST-120 is capable of binding small molecules (100-10,000 Da) with negligible effects on amylase, pepsin, lipase, and chymotrypsin enzymes (Niwa et al., 1991). It has also been reported to have limited adsorption to fat-soluble vitamins (Schulman et al., 2006). AST-120 is indicated in Japan for delaying the onset of dialysis and mitigating symptoms of progressive CKD. It has minimal adverse effects, which can include constipation, nausea and diarrhea; however, the frequency of these adverse events is similar in placebo treated patients (Schulman et al., 2015). AST-120 acts by adsorbing gut-derived uremic toxin precursors in the colon, thereby inhibiting their absorption. A number of studies have demonstrated reduced plasma concentration of indoxyl sulfate, p-cresyl sulfate, indole-3-acetic acid, phenyl sulfate and hippuric acid
following administration of AST-120 in CKD (Kikuchi et al., 2010; Akiyama et al., 2012; Wu et al., 2014). In the colon, AST-120 administration to rats mitigated the depletion of epithelial tight-junction proteins (Vaziri et al., 2013). AST-120 has also been shown to ameliorate a number of effects caused by gut-derived uremic toxins described above including endothelial dysfunction, CKD progression, serum cytokine accumulation, and inflammation (Iida et al., 2006; Ito et al., 2013; Inami et al., 2014).

There is conflicting evidence for the beneficial use of AST-120 in clinical trials for patients with CKD. AST-120 has been shown to slow the decline in renal function for patients with CKD over a 12 month and 56 week period in Japanese patients (Konishi et al., 2008; Nakamura et al., 2011). However, these data are not consistent with other clinical trials. In a Phase II clinical trial, AST-120 was given at doses of 2.7, 6.3 and 9 g/d resulting in a dose-dependent decrease in serum indoxyl sulfate with no effect on serum creatinine levels for patients with CKD (Schulman et al., 2006). The largest clinical trials for AST-120 were the Evaluating Prevention of Progression in CKD (EPPIC) trials -1 and -2, which were separate trials conducted simultaneously with a total 5840 patients (Schulman et al., 2006). The primary endpoint for these trials was a triple composite and included dialysis initiation, serum creatinine doubling and kidney transplantation. EPPIC-1, EPPIC-2 and a pooled analysis of both studies showed that AST-120 did not delay the primary endpoints and therefore, did not support its use as therapy for moderate and severe CKD (Schulman et al., 2015).

1.5 Metabolomics

There are a number of methods to measure small molecules in biological matrices using known standards; however, recent advances in technology have allowed for the identification of unknown metabolites. Metabolomics is the study of all metabolites generated in a biological system and currently the newest of the “-omic” technologies. Metabolites are the final product in response to genetic and environmental changes and can provide signatures for genotype-phenotype and genotype-environmental relationships (Corona et al., 2012). Metabolomics is also a powerful technique for identifying altered
metabolic pathways that are affected by chronic disease. Prior to the advent of metabolomics, hypothesis driven studies were required to measure known biomarkers and assess their potential to cause complications in disease. However, untargeted metabolomics allows for the measurement of unknown small molecules. In the context of uremia, metabolomics is ideal for the measurement and identification of small molecules that accumulate as a result of declining renal function (Rhee, 2015).

### 1.5.1 Metabolomics Methods

Strategies to assess metabolomics are divided into targeted and untargeted approaches. Targeted analysis characterizes known groups of metabolites and can be quantitative with the use of internal standards. Metabolites measured using this approach are predefined, which allows for a refined sample preparation to enrich a targeted group of metabolites and reduce analytical artifacts (Roberts et al., 2012). In contrast, untargeted metabolomics allows for the discovery of novel metabolites. The comprehensive identification of metabolites by this method is determined by sample preparation and analytical instrumentation, which can limit the overall coverage of metabolites measured in a sample. Untargeted metabolomics requires either nuclear magnetic resonance (NMR) or mass spectrometry (MS) to identify compounds. NMR is highly quantitative and reproducible but has low-sensitivity. MS is highly sensitive and reliable for metabolite identification; however, metabolites must be separated using either liquid or gas chromatography prior to detection and matrix effects can influence signal intensities (Pan and Raftery, 2007). The majority of recent studies are employing the use of quantitative time-of-flight (QToF) mass spectrometry coupled to ultra performance liquid chromatography (UPLC) or gas chromatography. Electrospray ionization (ESI) probes are commonly used to ionize metabolites by adding or removing a proton using a positive or negative voltage, respectively. Ions are then accelerated and their accurate mass is detected based on the time between acceleration and detection. During a metabolomics method, new QToF mass spectrometers, such as Waters Xevo™ G2S-QToFMS, can simultaneously acquire both MS and MS\(^E\) data. In the MS function, data is acquired by scanning for a range of masses to detect small molecules. In the MS\(^E\) function, the same range is scanned; however, argon and collision energy are used to fragment ions and
detect the resulting daughter ions. Acquiring simultaneous MS and MS\textsuperscript{E} data for a metabolite allows for the determination of elemental composition and structural information, respectively.

### 1.5.2 Data Processing and Analysis

Metabolomics using UPLC-QToFMS generates an enormous amount of data that must be processed prior to analysis. The first step is peak-picking, which integrates the area of chromatographic peaks based on a minimum threshold. This results in a list of ion masses, retention times and peak areas for each sample. Peaks with similar retention time and ion mass are then aligned for each sample. For a given ion mass and retention time, some samples will have a peak area below the minimum threshold set in the peak-picking step and contain a value of zero. To avoid problems during statistical analysis, the area below the peak-picking threshold is calculated. Each ion with the same mass and retention time is called a feature. The data are then normalized across samples prior to statistical analysis.

A number of proprietary and open software packages are available for processing and data analysis including Markerlynx (with EZinfo) (Umetrics, Umea, Sweden) and XCMS Online (Tautenhahn et al., 2012). Markerlynx with EZinfo is Waters and Umetrics proprietary software package that can be used for multivariate statistical analysis. It allows for data scaling using unit variance or pareto scaling to reduce noise and analysis by principal component analysis (PCA) as well as orthogonal partial least squares discriminant analysis (OPLS-DA). PCA allows for visualization of the largest variation in the data set and OPLS-DA demonstrates the variation between experimental groups separate from variation within experimental groups. Finally, EZinfo can be used to construct S-plot projections, which show features with the largest difference in magnitude between two experimental groups combined with the least variation within groups (Wiklund et al., 2008). The XCMS Online open software package allows for univariate statistical analysis (Tautenhahn et al., 2012). After uploading and processing data using the XCMS Online site, statistical analysis by ANOVA can be performed to determine significantly different peaks across multiple experimental groups. This analysis not only reports p-values for each feature, but also reports q-values as a measure of false discovery.
rate (Benjamini and Hochberg, 1995). Features can be filtered for isotopes and p-value and q-value thresholds are set to determine highly significant features. Further analysis of significant features is necessary to identify them as metabolites.

1.5.3 Metabolite Identification

Metabolites can be identified using the accurate ion mass to determine elemental composition as well as fragmentation pattern to confirm molecular structure. A number of databases exist with metabolite spectral libraries including, METLIN (Smith et al., 2005), the Human Metabolome Database (HMDB) (Wishart et al., 2013) and MassBank (Horai et al., 2010); however, the spectral information is fairly limited and relative fragment intensity can vary between mass spectrometers. Therefore, for unknown compounds with missing published spectral information, putative fragmentation \textit{in silico} is used to aid in identification. The potential elemental composition is first determined from accurate mass, which can be used to query biological compounds. The fragmentation pattern of the unknown metabolite is then compared to the putative fragmentation of biological compounds based on structural information. Putative fragmentation can be done using Waters MassFragment® or online using MetFrag and MetFusion (Wolf et al., 2010; Gerlich and Neumann, 2013). Finally, fragmentation pattern and retention time of a purchased compound standard is compared to the unknown sample to confirm its identity.

There are four levels of metabolite identification found in published metabolomics literature (Sumner et al., 2007). Level 1 completes all of the above steps to the gold standard of comparing unknown metabolites to authentic standards using a minimum of two independent methods including retention time, fragmentation spectrum and accurate mass. Level 2 putatively annotates metabolites by comparing the unknown metabolite fragmentation pattern to \textit{in silico} fragmentation of a biological compound or using spectral libraries. Level 3 also putatively annotates a compound but can only identify the class of compound and not the specific identity. This commonly occurs when identifying unsaturated fatty acids, as fragmentation pattern rarely provides information for the location of alkene or alkyne functional groups. Metabolites that remain unknown despite the use of \textit{in silico} fragmentation and database queries are classified as level 4.
1.6 Drug Therapy for CKD Management

Patients with CKD and ESRD have several co-morbidities, as discussed above, that require pharmacological management including diabetes, hypertension, bone disease and anemia. In early stages of CKD, the strongest evidence exists for treatment benefits using angiotensin converting enzyme inhibitors and angiotensin II receptor blockers, which are supported by multiple randomized controlled trials (Ruggenenti et al., 1998; Rodby et al., 2000; Brenner et al., 2001). Lipid lowering drugs may also reduce the risk of cardiovascular events in CKD; however, the association between LDL cholesterol and myocardial infarction in stages 3-5 of CKD is weaker than the general population (Tonelli et al., 2013). As patients progress to later stages of CKD, they require medications to treat bone disease and anemia (Wouters et al., 2015). Drug therapy intensifies for ESRD patients that require renal replacement therapy, as more complications arise in this final stage of kidney disease. It is estimated that ESRD patients take an average of 12 different medications (Manley et al., 2004) with a median daily pill burden of 19 (Chiu et al., 2009). Many prescribed medications are required for these patients to manage an average of 6 comorbidities along with ESRD (Manley et al., 2005). Common medications to treat CKD can be classified into a number of categories (Manley et al., 2003). Bone disease medications include vitamin D analogues, phosphate binders, calcium salts. Iron and erythropoietin are used to treat anemia, and cardiac medications include drugs to treat hypertension, arrhythmia, coronary artery disease and congestive heart failure. Endocrine medications comprise agents for diabetes and thyroid disorders, and anti-infective drugs are used for bacteria and viral infections (Manley et al., 2003).

A consequence of the polypharmacy experienced by ESRD patients is heightened risk for medication related problems. It has been reported that one medication related problem occurs for every 2.7 medication exposures in dialysis patients (Manley et al., 2005). Despite widespread evidence in the literature, dosing errors in patients with CKD still occur at an alarming rate (Farag et al., 2014).
1.7 Drug Metabolism and Disposition

In order for a drug to be effective, it must reach the site of action at an appropriate concentration. This can depend on a number of factors associated with drug administration including the route, dose, and formulation. Once inside the body, drug concentration at the site of action depends on absorption, distribution, metabolism and excretion. The majority of drug elimination occurs through hepatic drug metabolism but can also occur in the intestines and kidney (Wienkers and Heath, 2005). Drugs can enter cells by traversing the plasma membrane either directly through the lipid bilayer or via uptake transporters. This depends on the physicochemical characteristics of the drug, as large hydrophilic drugs are more likely to require transport. Once inside the cell, drugs can undergo biotransformation by phase I and/or phase II drug metabolizing enzymes. Phase I drug metabolism generally results in oxidation through cytochrome P450 enzymes (P450s). Phase II drug metabolism involves the conjugation of drugs by adding large hydrophilic functional groups including glutathione, glucuronic acid and sulfate. Conjugation can occur directly with the parent drug or may require initial phase I metabolism. Efflux transporters also exist to excrete parent drugs or drug metabolites, which requires active transport. Therefore, drug concentrations at the site of action depend on the interplay between drug metabolizing enzymes and transporters.

1.7.1 P450 Enzyme Function and Regulation

1.7.1.1 P450 Enzyme Function

P450 enzymes are a large group of major drug metabolizing enzymes. In humans, 18 families and 44 subfamilies exist; however only P450 families 1, 2 and 3 are involved in the biotransformation of drugs. These enzymes contain a heme functional group, which is an iron-containing protoporphyrin IX molecule. Monooxygenation reactions account for the majority of P450 function, which occurs in a number of steps using the heme functional group and oxygen as well as an electron and proton source (Zhu and Silverman, 2008). P450 enzymes are embedded in the smooth endoplasmic reticulum membrane along with NAPDH-cytochrome P450 oxidoreductase, which is required for enzymatic function. NADPH-cytochrome P450 oxidoreductase transfers electrons from
NADPH to the P450 heme group, reducing the iron to its ferrous state. The reduced iron can bind O\textsubscript{2} and transfer one oxygen atom to a substrate, releasing the other oxygen after protonation as water (Denisov et al., 2005). Oxidation of drugs by P450 enzymes generally reduces activity. Substrates for P450 enzymes are mostly hydrophobic and poorly soluble; therefore, oxidation of these substrates increases hydrophilicity to promote excretion from the body (Denisov et al., 2005). \textit{In vitro} P450-mediated metabolism can be assessed using Michaelis-Menten kinetics. This requires an experimental set-up in which the substrate concentration is much greater than the enzyme concentration allowing for a steady state of enzyme-substrate complex formation to enzyme-product decay. If this occurs, then the enzyme kinetics can be modeled to this simplified equation:

\[
\nu = \frac{V_{\text{max}}}{1 + \frac{K_m}{[S]}}
\]

Where \( V_{\text{max}} \) is the maximum enzyme velocity and \( K_m \) is defined as the substrate concentration at half of the \( V_{\text{max}} \), obtained under saturating conditions. The \( K_m \) is also known as the Michaelis constant and varies between enzymes and between substrates. The above equation creates a hyperbolic curve, which approaches an asymptote defining the \( V_{\text{max}} \) (Shou et al., 2001).

\subsection*{1.7.2 Nuclear Receptor Regulation of CYP2C and CYP3A}

Transcription of hepatic drug metabolizing enzymes and transporters is highly regulated by nuclear receptors. These transcription factors contain three protein domains. Interaction with co-activators and other nuclear receptors occurs at the n-terminus, which is the ligand independent activating domain (Urquhart et al., 2007). A central DNA binding domain can interact directly with a recognition sequence in the DNA to regulate transcription. A c-terminus ligand-binding domain associates with a number of ligands including drugs, bile acids, hormones, and lipids (Urquhart et al., 2007). There are six evolutionary groups of nuclear receptors, the largest of which are type I nuclear receptors (Germain et al., 2006). Localization of type I nuclear receptors begins in the cytosol, where these proteins are bound to chaperones. Upon ligand activation, these nuclear
receptors translocate to the nucleus. This group includes the pregnane X receptor (PXR) and constitutive androstane receptor (CAR). Conversely, type II nuclear receptors are bound to co-repressors in the nucleus when inactive and include the retinoid X receptor (RXR) and hepatocyte nuclear factor (HNF)-4α. RXR forms heterodimers with PXR and CAR in the nucleus prior to DNA binding. HNF-4α forms a homodimer when activated (Urquhart et al., 2007). Once active, these nuclear receptors bind to specific motifs in the DNA.

In humans, hepatic CYP3A4 is regulated by a number of nuclear receptors including PXR, CAR, RXR, glucocorticoid receptor and HNF-4α (Urquhart et al., 2007). PXR is a major regulator of CYP3A4 and can bind to two separate sites in the CYP3A4 promoter. A proximal PXR response element exists at -160 bp and a distal binding site is located between -7800 and -7200 bp. The most frequent PXR binding motif is AG(G/T)TCA, which is connected by a spacer to a similar sequence. The CYP3A4 proximal PXR binding site is an everted repeat with a 6 bp spacer (ER6) of AGGTCA. The distal promoter contains an ER6 site flanked by two direct repeats linked with a 3 base pair spacer (DR3) (Goodwin et al., 1999). Binding of PXR to these regions of the CYP3A4 promoter induces transcription. CAR can also bind to the proximal ER6 motif in the CYP3A4 promoter to activate transcription (Sueyoshi et al., 1999). The glucocorticoid receptor can increase PXR mRNA, which has been linked to increases in CYP3A4 expression but can also activate CYP3A4 expression through a PXR-independent pathway (Pascussi et al., 2000; El-Sankary et al., 2002). HNF-4α is critically involved in PXR and CAR activation of CYP3A4. HNF-4α binds upstream of the distal PXR/CAR binding site to a response element containing a DR1 repeat and regulates both induction and basal transcription of CYP3A4 (Tirona, 2010).

Regulation of rat CYP3A2 is slightly different than human CYP3A4. CYP3A2 contains only a proximal PXR binding site, which exists as a DR3 of AGTTCA instead of an ER6 motif. In rats, HNF-4α binds Site A in the proximal promoter of CYP3A2, which regulates both basal and induced transcriptional activity (Huss and Kasper, 2000). Similar to CYP3A4 regulation, the glucocorticoid receptor activation also increases PXR
and RXR expression and can activate CYP3A2 expression through a PXR-dependent pathway (Miyata et al., 1995).

Human CYP2C9 is regulated by PXR, CAR and HNF-4α. The CYP2C9 promoter contains a proximal HNF-4α binding site and is thought to bind to an HPF-1 motif (Ibeanu and Goldstein, 1995). A PXR response element is located between -1839 and -1824 bp in the CYP2C9 promoter. Induction of CYP2C9 has been shown to occur through this increased binding of PXR to this response element (Chen et al., 2004). Phenobarbital, a stereotypical CAR inducer, has also been shown to increase CYP2C9 mRNA expression. However, phenobarbital is not a potent inducer of CYP2C9 as large concentrations are required for induction. At high concentrations, phenobarbital has been shown to activate PXR and therefore, it is unknown whether PXR or CAR is responsible for phenobarbital-mediated induction of CYP2C9 (Gerbal-Chaloin et al., 2001; Chen et al., 2004). CYP2C9 is also regulated by the glucocorticoid receptor and the CYP2C9 promoter contains two glucocorticoid receptor elements between -1684 and -1654 bp.

Conversely, a major physiological regulator of the sexually dimorphic pattern of hepatic CYP2C11 expression in rats is growth hormone (Wauthier and Waxman, 2008). This occurs as a result of the differences between male and female pituitary release of growth hormone. A continuous release of growth hormone in female rats suppresses CYP2C11 expression and activates the female specific CYP2C12. The male rat pituitary has a pulsatile release of growth hormone, which stimulates CYP2C11 expression (Wauthier and Waxman, 2008). CYP2C11 is also regulated by a number of transcription factors including: HNF-1α, HNF-3β, HNF-4α, and HNF-6 (Park and Waxman, 2001).

1.7.3 Epigenetic Regulation of CYP2C and CYP3A

Epigenetic regulation of hepatic drug metabolizing enzymes has been shown to occur through histone post-translational modifications, DNA methylation and micro RNAs (miR). CpG islands exist in the HNF-4α and distal PXR binding site regions of the CYP3A4 promoter (Kacevska et al., 2012). PXR and HNF-4α can also be inhibited by micro RNAs. Overexpression of miR-148a decreases protein levels of PXR (Takagi et
al., 2008). Similarly, overexpression of miR-24 and miR-34a downregulate HNF-4α and target genes including CYP3A4.

Post-translational modifications of histones also regulate CYP3A4. Protein arginine methyltransferase 1 (PRMT1) is involved in PXR-mediated transcriptional activation of CYP3A4 (Tian, 2013). PRMT1 functions by asymmetrically dimethylating histone 4 arginine 3 (H4R3) using s-adenosyl methionine as a methyl donor. H4R3 asymmetric dimethylation (H4R3me2) facilitates H4 acetylation by p300 and transcriptional activation of CYP3A4 (Xie et al., 2009). The process of H4R3me2 and subsequent H4 acetylation is suggested to be unidirectional (Tian, 2009). PRMT1 also facilitates HNF-4α dependent transcriptional activation (Barrero and Malik, 2006).

Epigenetic modifications associated with PXR have not been studied in rats; however, in mice, PXR binding overlaps with histone 3 lysine 4 dimethylation (H3K4me2) (Cui et al., 2010). This study also found that PXR binding did not associate with the histone 3 lysine 27 tri-methylation (H3K27me3) epigenetic signature. H3K4me2 is a signature of transcriptional activation and H3K27me3 is a marker of gene silencing (Lorincz et al., 2004; Shilatifard, 2006).

There is minimal information in the literature specifically identifying epigenetic modifications in the human CYP2C9, rat CYP2C11 and CYP3A2 promoters.

1.8 Pharmacokinetic Changes in CKD

1.8.1 Drug Absorption and Bioavailability

Drug absorption following oral dosing is primarily mediated by the physicochemical characteristics of the drug. Bioavailability can be further impacted by the balance between uptake and efflux drug transporter activity as well as pre-systemic metabolism mediated by intestinal and hepatic P450s or gut bacteria. Paracellular intestinal drug absorption is usually minimal due to tight junctions in the intestinal epithelium (Groschwitz and Hogan, 2009) and the fact that the surface area for paracellular transport
represents only approximately 0.01% of the intestinal surface area (Pappenheimer and Reiss, 1987).

The absorption and bioavailability of drugs is highly variable in patients with ESRD. The mechanism for variable absorption and bioavailability is multifactorial and begins with the intestinal pH. Urea retention in ESRD results in a high influx of urea into the gut, which can be hydrolyzed by bacterial urease into ammonia (Ramezani and Raj, 2014). According to the FDA, the target pH range for a drug to be considered highly soluble drug is 1 to 7.5 (CDER/FDA, 2000); therefore, an increase in pH due to high concentrations of ammonia may alter the absorption of weakly basic drugs. Studies by Magnusson et al. assessed the integrity of the intestinal barrier in uremic rats (Magnusson et al., 1990) and human patients with CKD (Magnusson et al., 1991) by using polyethylene glycols that ranged in size from 326 – 1162 Da. They found increased polyethylene glycol leakage in both uremic rats and human patients with CKD and conclude that these relatively large molecules are able to undergo paracellular transport across the intestinal epithelium in kidney disease. To evaluate the impact of CKD on intestinal barrier function mechanistically, Vaziri et al. evaluated the expression of proteins that make up the intestinal tight junction complex in a rodent model of CKD. They found a greater than 50% decrease in the expression of the tight junction complex proteins claudin-1, occludin and zonula occludens-1 (Vaziri et al., 2012). This was further confirmed using T84 tight junction forming enterocytes. Similar to the studies in uremic rats, treatment of T84 cells with uremic serum impaired intestinal barrier function secondary to decreases in claudin-1, occludin and zonula occludens-1 expression. It has been proposed that uremic toxins and inflammation mediate the decreased intestinal barrier function in renal failure. This was evaluated by treating CKD rats with AST-120 and assessing intestinal barrier function (Vaziri et al., 2013). Treatment of CKD rats with AST-120 partially restored the protein expression of claudin-1, zonula occludens-1 and occludin demonstrating that uremic toxins are at least partially responsible for the disruption in intestinal barrier function.

Data from studies in rodents and human patients with CKD suggest that intestinal barrier function is reduced in CKD. Given that the absorption of polyethylene glycols are
increased in CKD, it is likely that increased paracellular absorption of drugs partially accounts for the increased and variable drug absorption observed in dialysis. Experimental confirmation of increased paracellular drug absorption and the impact on drug pharmacokinetics in patients on HD still requires validation.

Aside from the intestinal tight junction barrier complex, transport proteins also play an essential role in mediating the barrier function of the intestine. The intestinal epithelium is lined with a multitude of uptake and efflux transporters. These transporter proteins, along with the physicochemical characteristics of the drug, are primary determinants of intestinal drug absorption. Although there are numerous transporters expressed in the intestine, members of the solute carrier (SLC) and ATP-binding cassette (ABC) families are the most important in mediating the uptake and efflux of drugs, respectively. The SLC transporters SLCO2B1 (OATP2B1), SLCO1A2 (OATP1A2) and SLC15A1 (PEPT1) mediate the uptake of substrate drugs across intestinal epithelia into the enterocyte (Ho and Kim, 2005; Glaeser et al., 2007). In contrast, ATP binding cassette (ABC) efflux transporters such as ABCB1 (P-glycoprotein, P-gp), ABCG2 (Breast Cancer Resistance Protein, BCRP) and ABCC2 (Multidrug Resistance-Associated Protein 2, MRP2) restrict drug absorption by actively pumping drugs from the enterocyte to the intestinal lumen. Efflux transporters such as ABCC1 (MRP1) and ABCC3 (MRP3) mediate the efflux of drugs from the intestine into the portal circulation. In addition, enterocytes express relatively large amounts of CYP3A4, which acts to metabolize substrate drugs thereby decreasing oral bioavailability.

The expression and activity of intestinal drug transporters was evaluated in a rodent model of CKD (Naud et al., 2007). A decrease in the protein expression of P-gp, MRP2 and MRP3 and a consistent reduction in transport activity using everted gut sacs were observed. In addition, intestinal CYP3A2 (rat ortholog of human CYP3A4) expression and activity is decreased in rats with CKD (Leblond et al., 2002). Collectively these data suggest that drug absorption and bioavailability are increased in CKD. It has also been demonstrated that the intestinal folate uptake transporter SLC46A1 (human proton coupled folate transporter, hPCFT) is responsible for the uptake transport of some drugs (Urquhart et al., 2010). Studies using the 5/6 nephrectomy model of CKD demonstrate
that hPCFT expression is decreased (Bukhari et al., 2011). Although no functional assays were conducted in this study, decreased expression of hPCFT in rats with CKD suggests that the absorption of drugs that are substrates for hPCFT such as methotrexate and pemetrexed will be decreased.

1.8.2 Distribution

Once absorbed, drugs are carried to target tissues and sites of elimination (e.g. liver, kidney) in the systemic circulation. Many drugs are extensively bound to plasma proteins such as albumin and alpha 1 acidic glycoprotein (AAG), which limits their extravascular distribution. HD is commonly associated with hypoalbuminemia. Multiple factors affect plasma protein levels including malnutrition and low-protein diet regimes. Albumin is the most abundant plasma protein and albumin levels are an indication of plasma protein pool size (Steinman, 2000). Albumin, which predominantly binds weakly acidic drugs, exhibits fluctuations in concentration due to changes in plasma volume that occur during the intra-dialytic period. A previous study demonstrated a 0.6 g/dL increase in plasma albumin concentration during 2 to 4 hour HD. The other major plasma protein AAG binds weakly basic drugs. In contrast to albumin, the concentration of AAG is typically increased in ESRD as a result of chronic inflammation experienced in this patient population (Vasson et al., 1991). AAG is thought to bind inflammatory mediators and modulate the immune response (Moore et al., 1997). Further to changes in the concentration of plasma proteins, accumulation of inflammatory factors and protein-bound uremic toxins such as indoxyl sulfate, p-cresyl sulfate and hippuric acid can decrease plasma protein binding of drugs (Sakai et al., 2001). A combination of low serum albumin along with competition for plasma protein binding sites by uremic toxins increases the free fraction of drugs.

One of the most highly studied drugs with regard to alteration in protein binding in dialysis is the anti-epileptic drug phenytoin. Phenytoin is important to study in dialysis as seizure disorders are relatively common in this patient population, phenytoin has a narrow therapeutic window, and exhibits non-linear kinetics whereby a small increase in dose can result in a large increase in plasma concentration. Free phenytoin concentration is an important determinant of both efficacy and toxicity. Multiple studies have reported
that the free fraction of phenytoin is greater in dialysis patients than healthy controls (Steele et al., 1979; Liponi et al., 1984; Dasgupta and Abu-Alfa, 1992). Phenytoin is weakly acidic so hypoalbuminemia may contribute to this phenomenon. In addition, the uremic toxins hippuric acid and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) inhibit the binding of phenytoin to plasma protein (Mabuchi and Nakahashi, 1988; Vanholder et al., 1988). Changes in the protein binding of drugs like phenytoin can have a profound influence on dosing regimens and dialyzability.

Another important consequence of dialysis is the fluid removal and hemoconcentration that can occur. In terms of pharmacokinetics of drugs, the concentration of albumin actually increases post-dialysis as a result of hemoconcentration. Aside from changes in plasma protein concentration, which may impact free drug concentration, hemoconcentration has also been shown to increase plasma drug concentrations in post-dialysis plasma samples. For example, plasma concentration of the non-dialyzed monoclonal antibody rituximab has been shown to increase 1.4 to 4.8-fold following a HD session (Jillella et al., 2002).

### 1.8.3 Drug Metabolism

While it is well appreciated that renal excretion of drugs is decreased in dialysis patients, 73% of drugs undergo metabolism prior to excretion (Wienkers and Heath, 2005). The majority of drug metabolism occurs in the liver. Emerging evidence has focused on alterations in non-renal clearance pathways in kidney disease and their impacts on drug disposition. Several reviews have addressed this important issue (Nolin et al., 2003; Dreisbach and Lertora, 2008; Nolin et al., 2008; Naud et al., 2012; Velenosi and Urquhart, 2014; Yeung et al., 2014). P450 enzymes in the small intestine and liver mediate the majority of drug metabolism. CYP3A4 and CYP2C9 metabolize approximately 43% of clinically used drugs (Zanger and Schwab, 2013). Altered function of these drug metabolizing enzymes has been assessed in animal studies and in patients with CKD. Pichette and colleagues have demonstrated that rats with severe CKD have decreased hepatic expression of phase I drug metabolizing enzymes CYP2C11 and CYP3A2 and the phase II drug metabolizing enzyme N-acetyltransferase 2 (NAT2) (Leblond et al., 2000; Guevin et al., 2002; Simard et al., 2008). These studies suggest
uremic toxins that accumulate in kidney disease downregulate the expression of cytochrome P450 enzymes. Studies have also demonstrated that aside from decreases in P450 expression, uremic solutes that accumulate in CKD may also directly inhibit P450 mediated metabolism. For example, the uremic toxins CMPF and indoxyl sulfate inhibit the CYP3A mediated metabolism of erythromycin (Sun et al., 2004).

The impact of CKD on drug metabolism in humans is not as clear as the studies in animal models of CKD. Early studies using non-selective P450 probe drugs lidocaine (CYP1A2 and CYP3A4) and nifedipine (CYP3A4 and CYP2D6) revealed a decrease in hepatic drug metabolism for CKD patients. Interestingly, metabolism appears to be recovered in patients on HD (Ahmed et al., 1991; De et al., 2006). The CYP2C9 mediated metabolism of S-warfarin is also decreased in CKD patients. Consistent with these findings, patients with CKD have decreased S-warfarin clearance and require a lower warfarin dose than patients with normal kidney function (Dreisbach et al., 2003; Limdi et al., 2010; Gong et al., 2011). Selegiline is a monoamine oxidase inhibitor that is dependent on P450 mediated metabolism prior to excretion. Selegiline pharmacokinetics are substantially altered in patients with kidney disease with significant increases in $C_{\text{max}}$ and AUC (Anttila et al., 2005). As selegeline is metabolized by CYP2B6, CYP2C19 and CYP1A2, these data suggest that one or more of these enzymes is impacted by kidney disease.

More recent clinical studies have provided conflicting results with regard to the impact of kidney disease on drug metabolism. The generally accepted gold standard in vivo probe to assess CYP3A4 activity in humans is evaluation of midazolam clearance. Nolin et al. investigated the non-renal clearance of oral midazolam in HD patients and healthy controls (Nolin et al., 2009). In contrast to studies in animal models of CKD, midazolam clearance was unaltered in this study, suggesting CYP3A4 activity is unchanged in HD patients. A follow-up study assessing midazolam metabolism in non-dialysis dependent CKD, HD and PD patients, revealed significantly higher midazolam exposure for the HD group only, which did not support the previous study (Thomson et al., 2015). The impact of kidney disease on the pharmacokinetics of new drugs approved by the FDA between 2003 and 2007 has been evaluated demonstrating similar inconsistent results (Zhang et al., 2009). The study identified several drugs whose clearance was predominantly
mediated by non-renal mechanisms such as metabolism. When assessing the specific contribution of individual P450s, no clear pattern could be delineated. Of the 13 drugs that exhibit significant non-renal clearance mediated by CYP3A4 in the study, 6 had altered pharmacokinetics in kidney disease and 7 had no alterations in kidney disease. This was evident, even in drugs within the same class. For example, tadalafil has a fractional excretion of less than 0.3% and is metabolized by CYP3A4. Patients on HD have a 2-fold increase in $C_{\text{max}}$ and 2.7 to 4.1-fold increase in AUC compared to healthy controls. Accordingly, the dosage recommendation for tadalafil in HD patients is 5 mg no more than once every 72 hours. In contrast, vardenafil has a fractional excretion of 2-6% and is also metabolized by CYP3A4 and to a minor extent by CYP2C9. Vardenafil exhibits only a 30% increase in AUC and therefore, no dosage adjustment is required in HD. It is clear that the impact of kidney disease on hepatic drug metabolism is complicated and addition experimental evidence is required to delineate pathways that are altered in patients with kidney disease.

1.8.4 Other Routes of Elimination

1.8.4.1 Urinary Excretion

The most obvious consequence of ESRD is the absence of renal drug excretion. Indeed, the dosages of drugs that are predominantly excreted in the urine invariably require reduction to avoid accumulation in dialysis patients. There are many publications with recommendations on dosage adjustment for patients with kidney disease (Lam et al., 1997; Gabardi and Abramson, 2005; Munar and Singh, 2007; Brater, 2009; Verbeeck and Musuamba, 2009; Matzke et al., 2011). Most often dosage recommendations are made based on the patient’s eGFR and should be based on clinical pharmacokinetic studies in patients with kidney disease. A notable example is the recent characterization of the pharmacokinetics of baclofen (Vlavonou et al., 2014). The authors evaluated baclofen’s pharmacokinetics in patients across the stages of CKD. As a result, they were able to recommend dosage reductions of 1/3, 1/2, and 2/3 for patients with mild, moderate or severe kidney dysfunction, respectively.
It is also important to consider the pharmacokinetics of metabolites that may have pharmacological activity. The majority of drugs undergo some degree of metabolism prior to renal excretion. Phase II drug metabolism typically adds a polar functional group to drug molecules, which promotes excretion by the kidney. Although glucuronidated metabolites typically have reduced pharmacological activity, there are important exceptions. For example, morphine-6-glucuronide is more potent than the parent drug morphine (Shimomura et al., 1971). Morphine pharmacokinetics are altered in patients with renal failure. Dialysis patients have a 2-fold increase in morphine AUC and are susceptible to morphine intoxication such as respiratory depression (Osborne et al., 1986; Osborne et al., 1993). Possibly of greater importance is the accumulation of the active metabolite morphine-6-glucuronide. Morphine-6-glucuronide AUC is over 4-fold greater in dialysis patients than patients with normal kidney function and high blood levels persist well beyond 24 hours (Osborne et al., 1993).

An important consideration when contemplating dosage alteration in CKD is the method used to estimate GFR. It is important to note that the dosage recommendations for many drugs were made prior to standardization of laboratory techniques to measure serum creatinine. Therefore, it is possible that some recommendations may not be suitable for use with serum creatinine that has calibration traceable to an isotope dilution mass spectrometry method.

Aside from decreases in GFR, kidney disease also impacts the expression of drug transporters that play a crucial role in renal drug elimination. Naud et al. determined the expression and activity of renal drug transporters in rats that underwent a 5/6 nephrectomy (Naud et al., 2011). They found pronounced decreases in the expression of uptake transporters OAT1, OAT2, OAT3 and OATP4C1 along with a decreased expression of P-gp. They also found significantly increased expression of the efflux transporters MRP2, MRP3 and MRP4. These data demonstrate that in addition to decreases in the filtration of drugs, kidney disease also changes the tubular secretion of drugs. The uremic toxin indoxyl sulfate has been shown to play a key role in the downregulation of renal OATP4C1 expression (Akiyama et al., 2013). Indoxyl sulfate causes increased expression of the transcription factor GATA3, which in turn
downregulates OATP4C1 expression. Therefore as renal disease progresses, indoxyl sulfate accumulates and in effect, shuts down OATP4C1 mediated renal transport. Human patients also appear to have decreased renal drug transporter expression. Patients with mild to moderate decreases in creatinine clearance have decreased OAT1 expression (Sakurai et al., 2004). It is currently unknown to what extent renal transporter expression changes in more severe kidney disease in human patients.

### 1.8.4.2 Biliary Excretion

The other major route of drug elimination is biliary excretion. Hepatic drug transporters predominantly mediate biliary drug excretion. Transporters such as OATP1B1, OATP1B3 and OATP2B1 are expressed on the sinusoidal membrane of hepatocytes and mediate the uptake of drugs into the hepatocyte. Multiple efflux transporters including P-gp, BCRP and MRP2 are expressed on the canalicular membrane of hepatocytes and mediate the active efflux of drugs from the hepatocyte into the bile. Rats with kidney disease have decreased protein expression of the uptake transporter OATP2 (Naud et al., 2008). Similarly, there is evidence to suggest that OATP1B1 and OATP1B3 transporters are downregulated in humans. When normal human hepatocytes are incubated with uremic serum from patients, expression of OATP1B1 and OATP1B3 were decreased by approximately 60% (Fujita et al., 2014). These data suggest that hepatic uptake of OAT substrates is decreased in patients on dialysis. There is also evidence supporting the direct inhibition of drug transporters by uremic toxins. The protein bound uremic toxin, CMPF, has been shown to directly inhibit uptake of erythromycin in rat hepatocytes and SN-38, the active metabolite of irinotecan, in human hepatocytes (Sun et al., 2004; Fujita et al., 2014). Other protein bound uremic toxins including indoxyl sulfate, indole acetate and hippuric acid have demonstrated inhibition of OATP1B1 mediated uptake transport (Fujita et al., 2014).

### 1.8.5 Altered Non-Renal Drug Clearance in Dialysis Patients

Aside from dialytic clearance, prescribers must also consider that the non-renal clearance of several drugs is impacted by kidney disease. This is most likely a result of changes in the expression and activity of drug transporters and drug metabolizing enzymes and may
result in drug accumulation and unpredictable pharmacokinetics in HD patients. There are several clinical pharmacokinetic studies that have been done in HD patients. While a few examples are covered here, many others have been reviewed elsewhere (Nolin et al., 2003; Dreisbach and Lertora, 2008; Nolin et al., 2008; Naud et al., 2012; Yeung et al., 2014).

Erythromycin is a macrolide antibiotic with minimal renal excretion. Erythromycin undergoes hepatic clearance and was once used as an indicator of CYP3A4 mediated metabolism (Watkins et al., 1989). More recent studies have identified an important role of the drug transporters OATP1B1 and P-gp in erythromycin clearance (Kurnik et al., 2006; Lancaster et al., 2012). A study investigating the pharmacokinetics of erythromycin in anuric HD patients revealed a 31% decrease in hepatic clearance along with a 68% increase in the dose and weight adjusted AUC (Sun et al., 2010). Earlier studies also showed that HD patients had a 28% reduction in the erythromycin breath test, which was shown to partially recover following an HD session (Dowling et al., 2003; Nolin et al., 2006). The data from these studies suggest that erythromycin hepatic clearance is decreased in HD as a result of decreased CYP3A4 mediated metabolism, OATP/P-gp mediated transport or a combination of both. The fact that dialysis restores the erythromycin breath test suggests that dialyzable uremic toxins are at least partially responsible for the altered hepatic clearance. Although the uremic toxin CMPF has been shown to decrease hepatic uptake of erythromycin (Sun et al., 2004), it is generally considered to be minimally dialyzed suggesting that other toxins are likely involved (Vanholder et al., 2003b; Neirynck et al., 2013).

Other recent studies investigated the pharmacokinetics of fexofenadine in HD patients (Nolin et al., 2009; Thomson et al., 2015). Fexofenadine is widely used as a non-specific probe of drug transporters and exhibits negligible renal excretion. Fexofenadine pharmacokinetic parameters were substantially altered in HD patients compared to healthy controls (Nolin et al., 2009; Thomson et al., 2015). HD patients had a 63% reduction in fexofenadine clearance, accompanied by significant increases in Cmax, T1/2, and AUC. These changes are likely mediated by a combination of increased bioavailability and decreased hepatic clearance. Multiple transporters are involved in the
disposition of fexofenadine including OATP1B1 (Niemi et al., 2005), OATP1B3 (Shimizu et al., 2005), P-gp (Cvetkovic et al., 1999), BSEP and MRP transporters (Matsushima et al., 2008). It is therefore difficult to delineate the specific transport pathways that are impacted in HD patients.

Rosuvastatin is a hydroxy-methylglutaryl coenzyme A reductase inhibitor used in the treatment of hypercholesterolemia. Rosuvastatin is another drug that exhibits minimal renal excretion, is minimally metabolized, and largely undergoes transporter-mediated hepatic elimination into the bile. Despite not being renally eliminated, CKD patients with creatinine clearance less than 30 mL/min have been reported to have plasma concentrations 3-fold higher than healthy controls and accordingly, dose adjustments are recommended (Zhang et al., 2009). In contrast, studies in HD and PD patients demonstrate no clinically relevant changes in rosuvastatin pharmacokinetics compared to patients with normal kidney function (Bologa et al., 2009; Birmingham et al., 2013). These data highlight that the dialysis process itself impacts the interdialytic pharmacokinetics of drugs and the timing of the dose in relation to dialysis is likely important in determining the pharmacokinetics.

It is important to note, that comorbidities associated with dialysis and non-dialysis dependent patients may also impact non-renal drug clearance. In addition to data implicating altered drug metabolism in CKD, patients with diabetes have been demonstrated to have decreased CYP3A4 mediated drug metabolism and increased CYP2E1 mediated metabolism (Dostalek et al., 2011). Accordingly, when considering pharmacokinetics in patients with kidney disease, it is important to consider the comorbidities of these patients and how they may impact drug disposition. In addition to pre-existing conditions, it is also possible that unintended aspects of the dialysis process itself may impact non-renal drug clearance. For example, there is evidence from studies in rodents that phthalate esters (e.g. di(2-ethylhexyl)phthalate) that leach from plastic tubing such as those used in dialysis can alter the expression of cytochrome P450 enzymes (Parmar et al., 1994). This requires confirmation in human studies with concentrations of phthalates found in dialysis patients.
1.9 Animal Models of CKD

To understand the effects of CKD, animal models are commonly used with rapid onset pathogenesis. This allows for delineation of mechanisms in CKD that may take years to develop in patients. Ureteric obstruction of kidneys is a commonly used surgical model of CKD. Obstructing the ureter for greater than 72 hours results in permanent kidney damage and the development of CKD (Cochrane et al., 2005). A major disadvantage of this model is rapid and severe loss of kidney function that limits the study length. To extend the study length, partial ureteric obstruction has been used; however, this is generally performed in neonatal animals to limit complications and leads to variable and often mild CKD (Chevalier et al., 2009). The sub-total 5/6 nephrectomy is a well established model of progressive CKD (Hewitson and Becker, 2009). The 5/6 nephrectomy is most commonly performed in rats and can be achieved using a ligation model or renal mass resection. The ligation model involves complete removal of one kidney and ligation of 2/3 of renal artery branches. The same unilateral nephrectomy is performed in the ablation model, along with a 2/3 nephrectomy of the other kidney for a 5/6 total nephrectomy (Hewitson and Becker, 2009). The partially nephrectomized kidney keeps the animal alive while mimicking CKD. Drug induced models of kidney disease have also been characterized. The use of doxorubicin, a nephrotoxic chemotherapeutic agent, can result in interstitial fibrosis after 6 weeks of treatment in mice (Wang et al., 2000).

The use of adenine-induced tubular obstruction is also a common model for the induction of progressive CKD. In this model, animals are orally administered up to 0.75% adenine supplemented into the diet. The adenine metabolite 2,8-dihydroxyadenine precipitates in the proximal tubule epithelia, causing tubule damage (Terai et al., 2008). Adenine induced CKD is reversible after 2 weeks of treatment; however 4-6 weeks of adenine supplemented diet results in permanent kidney damage. Rats are commonly used for this model and after 4-6 weeks of treatment they experience dilation of tubules, interstitial fibrosis and calcification of tubule basement membranes (Terai et al., 2008; Hewitson and Becker, 2009). The adenine-induced kidney disease model results in a more consistent progression of CKD with less variability than the 5/6 nephrectomy and does
not require the technical expertise of performing surgery (Terai et al., 2008). However, there are disadvantages to using the adenine model, most notably the limited food consumption in the first week. This results in minimal weight gain during this period and therefore, control animals must be pair-fed to CKD animals (Terai et al., 2008).
1.10 References


Mulder TP, Rietveld AG, and van Amelsvoort JM (2005) Consumption of both black tea and green tea results in an increase in the excretion of hippuric acid into urine. *Am J Clin Nutr* 81:256S-260S.


2 HYPOTHESES AND SPECIFIC AIMS
2.1 Focus of the thesis

In Canada, 1 in 10 people have some form of CKD and these patients take many medications to manage their CKD and associated illnesses. Therefore, understanding altered pharmacokinetics for patients with CKD is an important health concern.

The pharmacokinetics of many drugs are altered in CKD (Bianchetti et al., 1976; Osborne et al., 1993; Uehlinger et al., 1996; Marbury et al., 2000; Dowling et al., 2003; Dreisbach et al., 2003; De Martin et al., 2006; Periclou et al., 2006; Nolin et al., 2009; Zhang et al., 2009; Sun et al., 2010). The most common alteration is a decrease in drug elimination resulting in increased plasma concentrations and heightened risk for drug toxicity. For many years, it has been appreciated that drugs primarily excreted by the kidney require dosage adjustment in patients with ESRD. More recently, there are several examples of altered non-renal drug clearance in ESRD patients complicating effective pharmacotherapy in this highly medicated patient population (Nolin et al., 2009; Zhang et al., 2009; Sun et al., 2010). Highlighting the importance of altered pharmacokinetics in CKD, the Food and Drug Administration (FDA) published a guidance document in 1998 in order to help direct industry to conduct appropriate pharmacokinetic studies in the setting of kidney disease (Food and Drug Administration, 1998). This initial document was updated in 2010 but currently remains as a draft and has not been formally implemented. The updated draft includes the recommendation to assess drugs that are primarily secreted or metabolized in the liver. Therefore, research is required to provide information for regulatory bodies, such as the FDA and Health Canada, to implement appropriate drug development guidelines and ensure drug safety in patients with CKD.

My overall hypothesis is that hepatic drug metabolism is altered in CKD
2.2 Specific Aim 1

To determine if hepatic drug metabolism is altered in early stages of CKD

Many of the studies assessing hepatic drug metabolism in CKD have evaluated patients or animals with ESRD or severe renal disease, respectively. Although this research has been essential in establishing a premise for the effect of CKD on hepatic drug metabolism, the majority of patients with CKD are in stages 1 to 3. Therefore, in Chapter 3, we evaluate hepatic drug metabolizing enzyme function and expression using a model of surgically induced moderate CKD.

I hypothesize that induction of moderate and severe CKD will produce a corresponding decrease in liver CYP2C11 and CYP3A2 function and expression using rat partial nephrectomy models. Sprague-Dawley rats were subjected to surgical procedures that allowed the generation of three distinct models of kidney function: normal kidney function, moderate and severe kidney disease. Forty-two days following surgery, rats were sacrificed and hepatic CYP2C11 and CYP3A expression was evaluated by western blot and qPCR. In addition, enzymatic activity was determined in liver microsomes by evaluating midazolam (CYP3A), testosterone (CYP3A and CYP2C) and tolbutamide (CYP2C) Michaelis-Menten kinetics in CKD. When the degree of kidney disease was correlated with metabolic activity, an exponential decline in CYP2C and CYP3A mediated metabolism was observed. Our data suggest that drug metabolism is significantly decreased in earlier stages of CKD and imply that patients with moderate CKD may be subject to unpredictable pharmacokinetics.

2.3 Specific Aim 2

To identify possible mechanisms by which hepatic drug-metabolizing enzyme function and expression are altered in CKD

CYP2C and CYP3A drug-metabolizing enzymes play a critical role in determining the pharmacokinetics of many prescribed medications. These enzymes are transcriptionally
regulated by the nuclear receptors PXR and HNF4-α. Expression of CYP2C and CYP3A is decreased in CKD; however, the mechanism by which this occurs is unknown. Therefore, in Chapter 4, we investigate possible mechanisms of CYP2C and CYP3A downregulation in CKD. I hypothesize that altered drug metabolism in CKD is secondary to decreased nuclear receptor binding to the promoters of CYP2C and CYP3A as a result of histone modulation. We induced CKD in rats by 5/6 nephrectomy and used chromatin immunoprecipitation (ChIP) to determine nuclear receptor and epigenetic alteration mediated differences in the promoter region of the CYP2C and CYP3A genes. This study suggests that decreased nuclear receptor binding and histone acetylation may contribute to the mechanism of drug-metabolizing enzyme down-regulation and altered pharmacokinetics in CKD.

2.4 Specific Aim 3

To determine the metabolic changes in plasma, liver, heart and kidney tissue as well as the metabolic axis between these biological matrices in rats with CKD.

CKD results in the accumulation of metabolic waste products that are normally cleared by the kidney, known as uremia. Many of these waste products are derived from bacteria metabolites in the gut. Accumulation of uremic toxins in plasma and tissue, as well as the gut-plasma-tissue metabolic axis is important for understanding pathophysiological mechanisms of comorbidities and altered hepatic drug metabolism in CKD.

I hypothesize that gut-derived uremic toxins accumulate in both plasma and tissue in CKD. In Chapter 5, an untargeted metabolomics approach was used to determine uremic toxin accumulation in plasma, liver, heart and kidney tissue in rats with adenine-induced CKD. Rats with CKD were also given AST-120, a spherical carbon adsorbent, to assess metabolic changes in plasma and tissues with the removal of gut-derived uremic toxins. A combination of multivariate and univariate statistics was used to identify significant metabolic signatures in uremia. CKD was primarily defined by 8 gut-derived uremic toxins, which were significantly increased in plasma and all studied tissues. Our
results highlight the importance of diet and gut-derived metabolites in the accumulation of uremic toxins and define the gut-plasma-tissue metabolic axis in CKD.

### 2.5 Specific Aim 4

To determine which uremic toxins cause down-regulation of hepatic drug metabolizing enzymes in CKD.

In Aim 3 (Chapter 5), we identify a number of gut-derived uremic toxins that accumulate in both plasma and liver as a result of CKD. Therefore, it is possible that these uremic toxins are involved in the downregulation of hepatic drug metabolizing enzymes. To investigate this further, the first aim of this study was to identify which uremic toxin(s) cause the down-regulation of hepatic CYP3A \textit{in vitro}. The second aim of this study was to determine if gut-derived uremic toxins down regulate hepatic drug metabolizing enzymes in rats with CKD.

I hypothesize that the removal of gut-derived uremic toxins, using AST-120, would recover expression and function of hepatic CYP2C and CYP3A in rats with CKD. Prior to testing this hypothesis, various uremic toxins were screened for their effects on CYP3A4 expression using human hepatoma (Huh7) cells. This screen provided evidence of indoxyl sulfate causing a concentration-dependent decrease of CYP3A4 expression. Subsequently, hepatic drug metabolizing enzymes were evaluated \textit{in vivo} using an adenine-induced model of CKD followed by administration of AST-120 to remove gut-derived uremic toxins. AST-120 did not significantly recover CYP2C and CYP3A function and expression in rats with CKD. Our \textit{in vitro} results suggest that indoxyl sulfate may contribute to the downregulation of CYP3A4 in CKD; however, hepatic CYP2C and CYP3A function and expression were not significantly recovered in CKD by removal of gut-derived uremic toxins \textit{in vivo}.
2.6 References


Down-Regulation of Hepatic CYP3A and CYP2C Mediated Metabolism in Rats with Moderate Chronic Kidney Disease

3.1 Introduction

CKD is a progressive condition characterized by the decrease in GFR over time. The incidence of CKD is increasing at an alarming rate and these patients require more than seven medications to manage both their CKD and associated co-morbidities (Talbert, 1994). Drug therapy in patients with CKD is complicated as these patients frequently experience unpredictable pharmacokinetics resulting in an increased incidence of medication-related adverse events (Manley et al., 2005). Although it is appreciated that renal clearance of drugs is compromised in CKD, recent studies have demonstrated that kidney failure also affects non-renal drug clearance (Leblond et al., 2001; Naud et al., 2008; Nolin et al., 2009). The non-renal clearance of drugs is principally mediated by hepatic drug metabolism. CYP3A4, a member of the cytochrome P450 family of drug metabolizing enzymes, is involved in the oxidative metabolism of up to 50% of all drugs on the market (Wrighton et al., 1996). Elegant studies in experimental animal models of severe CKD have demonstrated a reduction in the function and expression of CYP3A and other members of the CYP family (Leblond et al., 2000; Leblond et al., 2002).

The Kidney Early Evaluation Program (KEEP, 2000 to 2010) and the National Health and Nutritional Examination Survey (NHANES, 1996 to 2006) have estimated the prevalence of kidney disease to be greater than 14% in the United States (KEEP, 2011; McCullough et al., 2011; Stevens et al., 2011). The majority of animal and clinical studies investigating the effect of CKD on non-renal drug clearance have focused solely on severe CKD. Patients studied in the majority of these publications are on dialysis despite the fact that severe CKD patients comprise approximately 3% of the total number of patients with CKD (KEEP 2000 to 2009, NHANES 1999 to 2006). According to KEEP, 11%, 19% and 67% of patients with CKD are in stages 1, 2 and 3, respectively (KEEP, 2011). Therefore, patients with mild and moderate kidney disease comprise the majority (97%) of the population of patients with CKD. Few studies have evaluated pharmacokinetics and drug metabolism in varying degrees of kidney disease despite the fact that CKD is known to be a progressive disorder whereby kidney function declines over time. Clinical studies in patients with varying degrees of CKD have demonstrated altered lidocaine and nicardipine pharmacokinetics; however, these drugs are metabolized
by multiple P450 enzymes and are influenced by other pharmacokinetic factors in kidney disease, such as changes in plasma protein binding (Ahmed et al., 1991; De et al., 2006). Consequently, there is a lack of data investigating the effects of CKD on specific drug metabolizing enzymes in the majority of the patient population. This void in the literature has been highlighted in a recent review (Naud et al., 2012).

The objective of this study was to determine if decreases in hepatic drug metabolizing enzymes observed in severe CKD are also seen at earlier stages of disease progression. To address this, we employed a combination of surgical kidney resection and partial renal artery ligation in rats to establish a novel model of CKD with two different degrees of kidney disease. Upon establishing this model, we assessed hepatic expression and function of the predominant P450 isoforms, CYP3A and CYP2C. In order to fully elucidate the effect of CKD progression on enzyme function, full enzyme kinetics of selected probe drugs was used to characterize the metabolic activity of these enzymes across the continuum of disease progression.
3.2 Methods

3.2.1 Chemical Reagents and Drugs

Midazolam, 1’OH-midazolam, 4’OH-midazolam, OH-tolbutamide and ketoconazole were purchased from Toronto Research Chemicals (Toronto, ON). Testosterone, 6βOH-testosterone, 16αOH-testosterone were purchased from Steraloids Inc. (Newport, RI). Flurazepam and carbamazepine were purchased from Cerilliant (Round Rock, TX) and tolbutamide was purchased from Sigma (St. Louis, MO).

3.2.2 Experimental Model

Seventeen male Sprague-Dawley rats, weighing 150 g, were obtained from Charles River Laboratories, Inc. (Wilmington, MA). The animals were maintained on standard rat chow and water ad libitum on a 12 hour light cycle. Rats were allowed to acclimatize for at least 3 days before any experimental procedure was undertaken. The animal protocols were approved by the University of Western Ontario Animal Care Committee (Appendix A).

3.2.3 Surgical Induction of Kidney Disease

Studies were performed in three experimental groups, which simulated normal kidney function (n = 6) as well as moderate (n = 7) and severe (n = 4) kidney disease. Moderate and severe kidney disease were surgically induced by modification of a standard two-stage partial nephrectomy. Atropine (0.04 mg/kg SC) was given prior to anaesthesia in addition to intramuscular xylazine (5 mg/kg) and ketamine (100 mg/kg). Anaesthesia was maintained with 2% isoflurane in oxygen via facemask throughout all surgical procedures. On day zero, rats in the moderate kidney disease group underwent a one-third partial nephrectomy of the left kidney. Rats in the severe kidney disease group were subjected to the same procedure plus ligation of one branch of the left renal artery to mitigate the perfusion of blood to the remaining kidney. Seven days later, a complete right nephrectomy was performed on both moderate and severe kidney disease groups as previously reported (Leblond et al., 2000). Rats in the control group underwent sham laparotomies. All rats were given ketoprofen (5 mg/kg, IM) pre-operatively and every 24
hours post-operation for 3 days. All rats were weighed daily to monitor health. Control and moderate kidney disease rats were pair-fed the same amount of standard rat chow that was ingested by severe kidney disease rats on the previous day. Forty two days after the initial surgery rats were sacrificed and liver tissue was harvested, flash frozen in liquid nitrogen and stored at -80°C. Serum creatinine and urea concentrations were determined by the London Laboratory Services Group by standard methods (London, ON).

3.2.4 Real-time PCR Analysis

Total RNA was extracted using Trizol Reagent (Life Technologies Inc. Burlington, ON) according to the manufacturer’s instructions. RNA concentration and purity were measured by spectrophotometry. Total RNA was reverse transcribed using iScript RT-qPCR Supermix (Bio-rad Hercules, CA) in a 20 µL reaction volume. Primer pairs used were as follows: CYP3A2 (forward) 5’-GCTCTTGATGCATGGTTAAGATTTG-3’ and (reverse) 5’-ATCACAGACCTTGCCAACCTCCTT-3’ (Baldwin et al., 2006), CYP2C11 (forward) 5’-CCCTGAGGACTTTTGGGATGGGC-3’ and (reverse) 5’-AGGGGCACCTTTGCTCTCTC-3’ and β-actin (forward) 5’-ACGAGGCCCAGAGCAAG-3’ and (reverse) 5’-TTGGTTACAATGCGCTGGTTC-3’ (Sohi et al., 2011). Real-time PCR was performed using SsoFast Evagreen® (Bio-rad Hercules, CA) and expression was analyzed using the ΔΔCt normalized to β-actin.

3.2.5 Hepatic Microsome Isolation

Liver microsomes were isolated by differential centrifugation according to a slightly modified method by Kurosawa et al. (Kurosawa et al., 2009). Briefly, liver tissue was rinsed in 0.9% NaCl solution and homogenized in 1.15% KCl containing 1 mM ethylenediaminetetraacetic acid (EDTA) using a Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 9000 g for 20 min at 4°C and the subsequent supernatant was centrifuged at 105 000 g for 60 min at 4°C. The microsomal pellet was resuspended in 100 mM potassium phosphate buffer containing 20% glycerol at pH 7.4 and protein concentration was determined by Pierce BCA protein assay (Fisher Scientific, Whitby, ON, Canada). Microsomal protein was stored at -80°C until analysis.
3.2.6 Western Blot Analysis

Protein expression of CYP3A2 and CYP2C11 was assessed in hepatic microsomal fractions using Western blot analysis. Twenty five µg of microsomal protein was electrophoresed on a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. Proteins were transferred to nitrocellulose and immunoblots were performed according to antibody manufacturer’s recommendation. Immune complexes were revealed by horseradish peroxidase (Millipore, Billerica, MA) and band intensity was determined by densitometry (Quantity One 1-D Analysis Software on a VersaDoc Imaging System, Bio-Rad, Hercules, CA). Primary antibodies for CYP3A2 (monoclonal rabbit anti-rat) were obtained from Millipore (Billerica, MA), CYP2C11 (monoclonal mouse anti-rat) from Detroit R&D Inc. (Detroit, MI) and actin (polyclonal rabbit anti-rat, chicken, mouse human) from Sigma (St. Louis, MO). Horseradish peroxidase (HRP)-linked secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Luminata™ Forte Western HRP substrate was obtained from Millipore (Billerica, MA). CYP450 antibody specificities were determined using Supersomes™ obtained from BD Biosciences (Mississauga, ON) (Supplementary Fig. C1, Appendix C).

3.2.7 Hepatic Metabolism of CYP3A and CYP2C substrates

Metabolic activity of CYP3A and CYP2C in hepatic microsomes was determined using specific probe substrates. Midazolam and testosterone were selected as probes for CYP3A, and testosterone and tolbutamide were selected as a probe for CYP2C enzyme activity. These probe substrates were selected based on their previously documented selective metabolism by specific rat P450 isozymes (Chovan et al., 2007). Reactions (final volume 250 µL) were conducted in 50 mM potassium phosphate buffer, 2 mM MgCl₂ (pH 7.4) with 1 mg/mL of hepatic microsomal protein. Prior to experimentation, linear rate of production of metabolites was determined by varying time, protein and relevant substrate concentrations. Metabolism was determined to be linear at 10 minutes for formation of testosterone metabolites (6β-OH testosterone and 16α-OH testosterone) and 30 minutes for formation of midazolam (1'-OH midazolam, 4'-OH midazolam) and
tolbutamide (OH-tolbutamide) metabolites. All reactions were conducted at 37°C and were started by the addition of a relevant concentration of substrate drug (midazolam, testosterone or tolbutamide) followed by 1 mM NADPH. At the end of the incubation, reactions were terminated with 50 µL of ice-cold acetonitrile followed by a 15 minute incubation on ice and centrifugation to pellet precipitated protein (Chovan et al., 2007).

3.2.8 Metabolite Analysis by UPLC-PDA

Metabolite analysis was conducted by solid phase extraction followed by ultra-performance liquid chromatography with photodiode array detection (UPLC-PDA). Solid phase extraction cartridges (C18, Strata-X Polymeric Reverse Phase 33 µm, Phenomenex, Torrance, CA) were conditioned according to manufacturer’s specifications. Flurazepam was used as an internal standard for midazolam and tolbutamide quantification and carbamazepine was used as an internal standard for testosterone quantification. Analytes and internal standard were passed across the packing by gravity after which the cartridges were washed with 1 mL of nano-pure water followed by 1 mL of 50/50 methanol/water (for midazolam and testosterone extraction) or 1 mL of 5% methanol/water (for tolbutamide extraction). Analytes were eluted into clean glass test tubes with 1 mL of methanol containing 0.1% triethylamine and 0.1% trifluoroacetic acid. The eluent was dried, reconstituted in mobile phase and injected on either a Waters Acquity UPLC™ BEH C18 column (1.7 µm particle size, 50 X 2.1 mm) for testosterone analyte separation or a Waters Acquity UPLC™ BEH Shield RP C18 column (1.7 µm particle size, 50 X 2.1 mm) for midazolam and tolbutamide analyte separation. The columns were maintained at 40°C in a Waters Acquity UPLC™ H-Class System. The mobile phase flow was maintained at 0.8 mL/min and consisted of 10 mM KH2PO4 (pH = 3.0), acetonitrile and methanol. Mobile phase ratios and gradients for each assay are summarized in Supplementary Table C1 (Appendix C). An ACQUITY UPLC® PDA detector was used to detect midazolam (254 nm), tolbutamide (230 nm) and testosterone (245 nm for testosterone and 290 nm for carbamazepine) for quantification. The inter-assay coefficients of variation for 1’OH-midazolam, 4’OH-midazolam, 6β-OH testosterone, 16α-OH testosterone, and OH-tolbutamide were 10.7%, 23.1%, 3.1%, 3.2%, and 5.6%, respectively. The intra-assay coefficients of variation for
1’OH-midazolam, 4’OH-midazolam, 6β-OH testosterone, 16α-OH testosterone and OH-tolbutamide were 2.7%, 5.0%, 3.2%, 3.2% and 8.6%, respectively.

3.2.9 Data Analysis and Statistical Procedures

A Michaelis-Menten model was used to fit the formation of midazolam, testosterone and tolbutamide metabolites. Maximum reaction rate of enzyme ($V_{\text{max}}$) and Michaelis constant ($K_m$) were calculated using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, CA). Correlations were performed using Spearman’s rank test. Statistical analysis between control, moderate and severe kidney disease groups was performed using one-way ANOVA followed by Tukey’s multiple comparison tests. Data are presented as mean ± SEM and a $p$-value less than 0.05 was considered significant.
3.3 Results

3.3.1 Serum Biochemistry

A rat 5/6 nephrectomy model has been used extensively as an animal model for severe CKD (Leblond et al., 2000; Michaud et al., 2006; Michaud et al., 2010). We employed variations of this surgical procedure to generate rats with moderate or severe CKD. Rats that underwent sham laparotomy (control), 2/3 nephrectomy and 2/3 nephrectomy plus vessel ligation surgeries resulted in three distinct degrees of kidney function (control, moderate CKD and severe CKD; respectively). This is represented by 1.65-fold and 4.78-fold higher serum creatinine levels in moderate and severe CKD, respectively, compared to control ($p < 0.05$, Table 3.1). Rats in the severe CKD group also had significantly higher serum creatinine levels than those with moderate kidney disease ($p < 0.05$). Serum urea concentrations were higher in severe kidney disease compared to control and moderate CKD rats ($p < 0.05$). There was no difference in body weight between the groups (Table 3.1). One rat with severe CKD was euthanized prior to completion of the study.

3.3.2 CYP3A2 and CYP2C11 mRNA and Protein Expression

An 88% and 99.6% decrease in hepatic CYP3A2 mRNA expression was demonstrated in moderate and severe kidney disease rats, respectively, compared to controls (Fig. 3.1A, $p < 0.05$). Similarly, CYP2C11 mRNA expression in moderate and severe CKD was decreased by 77% and 95%, respectively, compared to controls (Fig. 3.1B). When mRNA expression was correlated with serum creatinine, there was a significant exponential decrease in both CYP3A and CYP2C mRNA expression as serum creatinine levels increased (Fig. 3.1C $r_s = -0.785$ and Fig. 3.1D $r_s = -0.809$, respectively; $p < 0.05$).

Similar to mRNA expression, CYP3A2 and CYP2C11 protein expression were significantly decreased in both moderate and severe CKD. CYP3A2 protein expression was decreased by 75% and 91% in moderate and severe kidney disease rats, respectively, compared to controls (Fig. 3.2A, $p < 0.05$). Likewise, a reduction in CYP2C11 expression was also demonstrated in moderate (41%) and severe (68%) kidney disease
**Table 3.1** Physical and biochemical characteristics of control, moderate and severe kidney disease rats.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Moderate CKD</th>
<th>Severe CKD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Rats</td>
<td>6</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>478 ± 20</td>
<td>448 ± 17</td>
<td>419 ± 10</td>
</tr>
<tr>
<td>Serum Creatinine (µM)</td>
<td>23 ± 1</td>
<td>38 ± 1*</td>
<td>110 ± 10***†††</td>
</tr>
<tr>
<td>Serum Urea (mM)</td>
<td>6.1 ± 0.5</td>
<td>9.1 ± 0.6</td>
<td>23.35 ± 3.1***†††</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM.

* $p < 0.05$ compared to control.

*** $p < 0.001$ compared to control.

††† $p < 0.001$ compared to moderate CKD.
**Figure 3.1** mRNA expression of hepatic P450s in control, moderate and severe kidney disease rats.

mRNA expression of hepatic CYP3A2 (A) and CYP2C11 (B) in control, moderate and severe kidney disease rats. Correlation between hepatic CYP3A2 ($p < 0.001$) (C) and CYP2C11 ($p < 0.001$) (D) mRNA expression with serum creatinine levels of control (○), moderate (□) and severe (Δ) kidney disease rats. Expression of CYP3A2 and CYP2C11 was normalized to expression of the housekeeping gene β-actin. Analysis was performed in triplicate and results are presented as mean ± SEM, $n \geq 4$ (A, B) or means of triplicates (C, D), **$p < 0.01$** compared to control.
Figure 3.2 Protein expression of hepatic P450s in control, moderate and severe kidney disease rats.

Protein expression of hepatic CYP3A2 (A) and CYP2C11 (B) in control, moderate and severe kidney disease rats. Correlation between hepatic CYP3A2 \( (p < 0.001) \) (C) and CYP2C11 \( (p < 0.01) \) (D) protein expression with serum creatinine of control (○), moderate (□) and severe (Δ) kidney disease rats. Protein bands are expressed in densitometry units (%) and are standardized to actin. Control bands were arbitrarily defined as 100%. Experiments were performed in duplicate and results are presented as mean ± SEM, \( n \geq 4 \) (A, B) or means of duplicates (C, D), \* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) compared to control. Representative blots are shown.
compared to controls (Fig. 3.2B, \( p < 0.05 \)). Both CYP3A2 and CYP2C11 protein expression showed a significant inverse exponential relationship with serum creatinine levels. (Fig. 3.2C \( r_s = -0.820 \) and Fig. 3.2D \( r_s = -0.665 \), respectively; \( p < 0.05 \)).

### 3.3.3 Hepatic CYP3A and CYP2C Mediated Drug Metabolism

To determine the effect of varying degrees of kidney disease on hepatic CYP3A function, we evaluated the metabolism of CYP3A probe drugs, midazolam and testosterone, using rat liver microsomes. Both 1’-OH midazolam and 4’-OH midazolam are produced by CYP3A (Chovan et al., 2007) and full enzyme kinetics of these metabolites were determined in this study. \( V_{\text{max}} \) values for 1’-OH midazolam production was significantly reduced by 62% and 72%, respectively, in moderate and severe kidney disease groups compared to control (Fig. 3.3A; \( p < 0.05 \)). 4’OH-midazolam production was reduced by 63% and 75%, respectively, in moderate and severe CKD compared to controls (Fig. 3.3B; \( p < 0.05 \)). The formation of 6βOH-testosterone is also specifically catalyzed by CYP3A dependent mechanisms (Chovan et al., 2007). A 66% and 68% decrease in 6βOH-testosterone \( V_{\text{max}} \) was demonstrated in moderate and severe kidney disease groups, respectively, compared to controls (Fig. 3.3C). Michaelis-Menten kinetic parameters for 1’-OH midazolam, 4’-OH midazolam and 6βOH-testosterone are summarized in Table 3.2. A significant inverse correlation was found between \( V_{\text{max}} \) values for 1’-OH midazolam, 4’-OH midazolam and 6βOH-testosterone formation and serum creatinine levels (Fig. 3.3D \( r_s = -0.818 \), Fig. 3.3E \( r_s = -0.750 \) and Fig 3.3F \( r_s = -0.742 \), respectively; \( p < 0.05 \)). Hepatic CYP2C enzyme activity was evaluated using the probe drugs testosterone and tolbutamide. Testosterone is specifically metabolized to 16αOH-testosterone by CYP2C11. CYP2C11 \( V_{\text{max}} \) was significantly decreased by 67% and 82% in moderate and severe kidney disease rats, respectively (Fig. 3.4A). An inverse correlation was also demonstrated between \( V_{\text{max}} \) values for 16αOH-testosterone production and serum creatinine (Fig. 3.4C \( r_s = -0.742 \), \( p < 0.05 \)). Hepatic CYP2C function was also characterized by evaluating tolbutamide metabolism. Formation of OH-tolbutamide was unchanged between control, moderate and severe kidney disease groups (Fig. 3.4B) and there was no correlation between OH-tolbutamide \( V_{\text{max}} \) and serum
creatinine (Fig. 3.4D). Michaelis-Menten kinetic parameters for $16\alpha$-OH-testosterone and OH-tolbutamide are presented in Table 3.3.
Figure 3.3 Michaelis-Menten kinetics of CYP3A enzyme function in rat liver microsomes from control, moderate and severe kidney disease groups.

Michaelis-Menten plots of 1’OH-midazolam (A), 4’OH-midazolam (B) and 6β-OH testosterone (C) after incubation of rat liver microsomes with 1 mM NADPH and various concentrations of midazolam (A and B) or testosterone (C). Correlation between 1’OH-midazolam Vmax (D) ($p < 0.001$), 4’OH-midazolam Vmax (E) ($p < 0.01$) and 6β-OH testosterone Vmax (F) ($p < 0.001$) with serum creatinine of control (○), moderate (□) and severe (Δ) kidney disease rats. Experiments were performed in duplicate and results are presented as mean ± SEM, $n \geq 4$ (A, B, C) or means of duplicates (D, E, F).
Table 3.2 Michaelis-Menten kinetic values for CYP3A probe substrates in control, moderate and severe kidney disease rat liver microsomes.

<table>
<thead>
<tr>
<th></th>
<th>1’OH-midazolam</th>
<th>4’OH-midazolam</th>
<th>6β-OH Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$ (pmol min$^{-1}$ mg protein$^{-1}$)</td>
<td>$K_m$ (µM)</td>
</tr>
<tr>
<td>Control</td>
<td>39.98 ± 7.33</td>
<td>19.64 ± 2.38</td>
<td>29.11 ± 2.78</td>
</tr>
<tr>
<td></td>
<td>178.7 ± 26.9</td>
<td>186.10 ± 17.76</td>
<td></td>
</tr>
<tr>
<td>Moderate CKD</td>
<td>16.63 ± 2.18*</td>
<td>7.41 ± 1.04***</td>
<td>16.18 ± 4.00</td>
</tr>
<tr>
<td></td>
<td>329.8 ± 60.4</td>
<td>62.38 ± 13.30***</td>
<td></td>
</tr>
<tr>
<td>Severe CKD</td>
<td>28.66 ± 9.94</td>
<td>5.57 ± 0.32***</td>
<td>20.41 ± 9.06</td>
</tr>
<tr>
<td></td>
<td>411.4 ± 125.9</td>
<td>58.80 ± 11.90***</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.94</td>
<td>5.12 ± 1.14</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM.

* $p < 0.05$ compared to control.
** $p < 0.01$ compared to control.
*** $p < 0.001$ compared to control.
Figure 3.4 Michaelis-Menten kinetics of CYP2C enzyme function in rat liver microsomes from control, moderate and severe kidney disease groups.

Michaelis-Menten plots of $16\alpha$OH-testosterone (A) and OH-tolbutamide (B) after incubation of rat liver microsomes with 1 mM NADPH and various concentrations of testosterone and tolbutamide, respectively. Relationship between $16\alpha$OH-testosterone $V_{\text{max}}$ (C) ($p < 0.001$) and OH-tolbutamide $V_{\text{max}}$ (D) with serum creatinine of control (○), moderate (□) and severe (Δ) kidney disease rats. Experiments were performed in duplicate and results are presented as mean ± SEM, n ≥ 4 (A, B) or means of duplicates (C, D).
Table 3.3 Michaelis-Menten kinetic values for CYP2C probe substrates in control, moderate and severe kidney disease rat liver microsomes.

<table>
<thead>
<tr>
<th></th>
<th>16α-OH Testosterone</th>
<th>OH-Tolbutamide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$ (pmol min$^{-1}$mg protein$^{-1}$)</td>
</tr>
<tr>
<td>Control</td>
<td>215.1 ± 64.6</td>
<td>432.6 ± 75.7</td>
</tr>
<tr>
<td>Moderate CKD</td>
<td>257.8 ± 37.3</td>
<td>144.4 ± 38.3$^{**}$</td>
</tr>
<tr>
<td>Severe CKD</td>
<td>233.3 ± 81.8</td>
<td>76.83 ± 20.9$^{**}$</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM.

$^{**}p < 0.01$ compared to control.
3.4 Discussion

A number of studies have shown altered hepatic CYP expression in severe CKD (Uchida et al., 1995; Leblond et al., 2000; Leblond et al., 2001). Major rat drug metabolizing isoforms investigated have included CYP1A2, CYP2C6, CYP2C11, CYP2D, CYP2E1 and CYP3A1/2. Of these isoforms, CYP2C11 and CYP3A1/2 continually appear downregulated in severe CKD (Uchida et al., 1995; Leblond et al., 2001; Guevin et al., 2002; Rege et al., 2003). Our results indicate that CYP3A2 and CYP2C11 protein expression are decreased by 75% and 40% in moderate kidney disease, respectively. This profound decrease in protein expression is similar to the decrease seen in severe kidney disease. Clinically, this suggests that patients with earlier stages of CKD are likely to experience variable and unpredictable pharmacokinetics and drug response when taking standard doses of drugs that are substrates for CYP3A and CYP2C. These results may help explain the increased incidence of medication related adverse events in patients with CKD (Manley et al., 2005).

Previous studies have suggested that protein downregulation is the result of a reduction in mRNA for hepatic CYP3A2 and CYP2C11 (Leblond et al., 2001). Our study provides further evidence of this notion as both moderate and severe kidney disease resulted in reduced CYP3A2 and CYP2C11 protein expression that is secondary to decreased mRNA. A possible cause for this decrease may be due to uremic toxin accumulation in moderate and severe CKD. Recent studies have shown that uremic toxins can cause direct inhibition or down-regulation of drug metabolizing enzymes and drug transporters (Tsujimoto et al., 2010; Reyes and Benet, 2011). In this study, it appears that uremic toxins are interfering with the regulation of drug metabolizing enzyme transcription. Indeed, rat hepatocytes treated with pre-dialysis serum from human ESRD patients exhibit down-regulation of CYP3A and CYP2C. This affect was alleviated by the addition of an NF-κB inhibitor suggesting that uremic toxins may be activating the NF-κB pathway (Michaud et al., 2005). The uremic toxin, indoxyl sulfate, has been shown to activate NF-κB in proximal tubule cells and enhance the infiltration of monocytes to uremic kidneys (Miyazaki et al., 1997). Furthermore, down-regulation of hepatic CYP
expression has been noted in inflammation-associated pathological states occurring in other organs (Renton and Nicholson, 2000).

The majority of studies demonstrating decreased hepatic CYP3A function in severe CKD have used erythromycin as a CYP3A probe (Leblond et al., 2000; Leblond et al., 2001; Sun et al., 2004). Although erythromycin is metabolized by CYP3A, its disposition is determined by the interplay between transport and metabolism (Sun et al., 2010). This confounds its use as a specific CYP3A probe for whole cell and in vivo studies (Sun et al., 2004). Pharmacokinetic studies in patients with varying degrees of kidney function have also used a variety of non-selective probe substrates for P450 enzymes (Ahmed et al., 1991; De et al., 2006). Although these studies clearly highlight that drug metabolism is differentially affected as kidney function declines, it is impossible to draw mechanistic conclusions about which specific P450 isozymes are affected. An alternate approach is to use midazolam, a selective phenotypic probe for CYP3A, which is not a substrate for uptake or efflux drug transporters (Nolin et al., 2009). In the present study, we evaluated the function of CYP3A using the well-established probe substrates, midazolam and testosterone (Guengerich, 1999; Dostalek et al., 2011). Our results demonstrate that as kidney function declines, there is a rapid decrease in CYP3A enzymatic activity.

CYP2C function has been assessed in severe CKD using the aminopyrine breath test (Leblond et al., 2000). Although aminopyrine is metabolized by CYP2C enzymes, it is also metabolized by many other P450 isoforms, such as CYP1A2 and CYP3A (Tanaka and Breimer, 1997). In our study, CYP2C mediated metabolism of tolbutamide to OH-tolbutamide was unchanged in CKD. Tolbutamide is a CYP2C family drug probe but is not specific for CYP2C11 (Cribb et al., 1995; Brown et al., 2007; Dostalek et al., 2007). Therefore, other CYP2C isoforms that are unaffected by CKD may have accounted for the lack of effect observed with regard to tolbutamide metabolism. This theory is supported by studies which show no effect of severe CKD on CYP2C6 protein expression (Leblond et al., 2001). To characterize the effect of progressive decline in kidney function on CYP2C11 activity, we analyzed the formation of 16αOH-testosterone, a specific metabolite of CYP2C11. The formation of 16αOH-testosterone was decreased in hepatic microsomes of both moderate and severe CKD. These data support other
studies, which demonstrate that CYP2C11 function is diminished in severe kidney disease (Uchida et al., 1995; Leblond et al., 2000). This also suggests that earlier stages of kidney disease result in a significant decrease in CYP2C11 function, similar to the outcome seen in severe kidney disease.

Few studies have evaluated the correlation between the progression of kidney disease and extent of drug metabolism. After demonstrating that CYP3A and CYP2C11 enzyme function and expression were decreased in moderate CKD, we examined the correlation of this affect with the degree of kidney function. Our study shows a significant inverse exponential correlation between kidney function and CYP3A2 and CYP2C11 protein and mRNA expression. A similar inverse correlation was also demonstrated between kidney function and CYP3A and CYP2C11 enzyme function. Surprisingly, these correlations are not linear as our data clearly demonstrates that a small decrease in kidney function during earlier stages of CKD produces a pronounced decrease in enzyme function and expression. The mechanism by which this occurs is unknown but may be due to the exponential increase in serum uremic mediators as CKD progresses. For example, the uremic toxin indoxyl sulfate increases exponentially as kidney disease progresses (Niwa and Ise, 1994; Barreto et al., 2009). A similar trend has also been demonstrated for the uremic toxin $p$-cresyl sulfate (Liabeuf et al., 2010). Our results suggest that the rapid decrease in kidney function may coincide with the exponential increase in uremic toxins that occurs in earlier stages of CKD.

Altered non-renal drug clearance has been studied in patients with varying levels of kidney disease including ESRD (Nolin et al., 2006; Nolin et al., 2009; Sun et al., 2010). A recent study by Nolin et al. (2009) administered oral midazolam to ESRD patients on hemodialysis. Surprisingly, the metabolism of midazolam was unchanged in ESRD patients compared to healthy controls. The authors suggest that as ESRD patients undergo routine hemodialysis, it is possible that uremic toxins modulating the expression and/or direct inhibition of CYP3A4 are removed and restore drug metabolism to levels observed in healthy controls (Nolin et al., 2009). This is supported by other clinical studies that show no change in nicardipine metabolism in ESRD patients on dialysis, yet significant reductions in patients with CKD not requiring dialysis (Ahmed et al., 1991).
In conclusion, this study demonstrates that changes in drug metabolism are not restricted to severe CKD and that, in fact, milder forms of CKD significantly decrease the expression and activity of CYP3A and CYP2C enzymes. To our knowledge this is the first study to systematically evaluate the effect of varying degrees of CKD on hepatic drug metabolism using variations of the commonly used remnant kidney model. In addition, this is the first study to evaluate CYP3A and CYP2C activity in CKD by performing full enzyme kinetics of substrate drugs. The results of our study suggest that metabolism and disposition of drugs that are substrates of CYP3A or CYP2C may be altered in earlier stages of kidney disease. This may have profound implications in the variable pharmacokinetics of medications in kidney disease. A recent assessment by the US Food and Drug Administration highlights the importance of evaluating the impact of kidney impairment on the pharmacokinetics of drugs (Zhang et al., 2009). Our data are in agreement with this assessment and suggest that the pharmacokinetics of drugs in all stages of kidney impairment should be investigated, not just ESRD.
3.5 References


DECREASED NUCLEAR RECEPTOR ACTIVITY AND EPIGENETIC MODULATION ASSOCIATES WITH DOWNREGULATION OF HEPATIC DRUG METABOLIZING ENZYMES IN CHRONIC KIDNEY DISEASE.³

4.1 Introduction

Patients with CKD take many medications to manage their CKD and co-morbidities (Talbert, 1994). Sub-therapeutic dosing and overdosing result in adverse drug events commonly associated with CKD, which translates into therapeutic ineffectiveness or drug-induced toxicity. It is estimated that 1 medication related problem occurs with every 2.7 medication exposures in dialysis patients (Manley et al., 2005). Several reports suggest that altered pharmacokinetics in CKD at least partially mediates this increase in adverse drug events (Leblond et al., 2000; Nolin et al., 2009; Sun et al., 2010b). Although it is well known that the renal clearance of drugs is altered in CKD, the effect on non-renal drug clearance is not well understood.

Hepatic drug metabolism is the major route of drug elimination and is mediated predominantly by the P450 superfamily of oxidizing enzymes. The P450 isoforms, CYP2C and CYP3A, metabolize the majority (43%) of clinically used drugs (Zanger and Schwab, 2013). Several studies have reported downregulation of CYP2C and CYP3A enzymes in CKD (Leblond et al., 2000; Leblond et al., 2001; Leblond et al., 2002; Michaud et al., 2005). In addition, in vitro studies incubating rat primary hepatocytes with uremic serum demonstrate a decrease in CYP2C11 and CYP3A2 expression, which are rat orthologs for human CYP2C9 and CYP3A4, respectively (Michaud et al., 2008). Consistent with these studies, surgical induction of CKD in rats results in a pronounced decrease of hepatic CYP2C and CYP3A function and expression (Leblond et al., 2000; Leblond et al., 2001; Velenosi et al., 2012). Although decreased CYP2C and CYP3A function and protein expression appear to be secondary to decreased mRNA expression, the molecular mechanism(s) by which this occurs in CKD is unclear.

Hepatic P450 enzymes are transcriptionally regulated by nuclear receptors. These xenosensing and hormonal regulators of gene expression include PXR and HNF-4α (Tirona et al., 2003; Urquhart et al., 2007). PXR contains a promiscuous ligand-binding domain that can be activated by chemically diverse compounds to regulate CYP3A expression. When activated, PXR translocates into the nucleus and heterodimerizes with RXR. This heterodimer binds to regulatory regions on DNA to modulate transcription.
HNF-4α is an essential transcriptional regulator of CYP3A and CYP2C expression (Ibeanu and Goldstein, 1995; Tirona et al., 2003). In contrast to PXR, HNF-4α binds to the promoter of these enzymes as a homodimer to activate transcription (Ibeanu and Goldstein, 1995; Tirona et al., 2003). In addition to altered CYP expression, numerous clinical and animal studies suggest that adverse drug events occur due to altered pharmacokinetics in patients with CKD (Ahmed et al., 1991; Leblond et al., 2000; Leblond et al., 2001; Leblond et al., 2002; Nolin et al., 2009; Sun et al., 2010b). Although the mechanism(s) remain controversial, several reports indicate that altered drug pharmacokinetics in CKD is mediated by accumulation of uremic waste products (Nolin et al., 2008; Naud et al., 2012; Velenosi and Urquhart, 2014). These uremic waste products become toxins at high levels and include amino acid metabolites, hormones, cytokines and other metabolic waste products. Many of these toxins are cleared in dialysis; however, some circulate bound to plasma proteins and are poorly cleared (Duranton et al., 2012).

Altered transcriptional regulation of several genes has also been associated with epigenetic modifications in the setting of CKD (Noh et al., 2009; Sun et al., 2010a). Previous studies have suggested that the uremic environment of CKD produces epimutations including DNA methylation and histone posttranslational modifications, which result in altered gene expression (Dwivedi et al., 2011). In the context of regulating CYP enzymes, transcriptional activation occurs in tandem with the binding of PXR and HNF-4α as well as post-translational histone modifications leading to a permissive chromatin environment (Barrero and Malik, 2006; Xie et al., 2009; Cui et al., 2010). Therefore, it is possible that alterations in histone modifications play an important role in the mechanism of CKD-induced changes in hepatic CYP2C and CYP3A expression.

The objective of this study was to identify possible mechanisms by which hepatic drug metabolizing enzyme function and expression are altered in CKD. We hypothesize that altered drug metabolism in CKD is secondary to decreased nuclear receptor binding to the promoters of CYP2C and CYP3A as a result of histone modulation. In this study, we
investigate the nuclear receptor and epigenetic regulation of hepatic drug metabolizing enzymes in a well-characterized rat model of CKD.
4.2 Methods

4.2.1 Experimental Rat Model

Male Sprague-Dawley rats, weighing 150 g, were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA). Twelve rats were randomly separated into two groups, which simulated normal kidney function and CKD. CKD was surgically induced by a two-stage 5/6 subtotal nephrectomy (Leblond et al., 2000). Control rats were pair-fed the same amount of standard rat chow that was ingested by CKD rats on the previous day. Six weeks after initial surgery, rats were sacrificed by isoflurane anesthesia followed by decapitation. Liver tissue was harvested, snap frozen in liquid nitrogen and stored at -80°C prior to analysis. Serum creatinine and urea were determined by the London Laboratory Services Group by standard methods (London, Ontario, Canada). The animal protocols were approved by the University of Western Ontario Animal Care Committee (Appendix A).

4.2.2 Testosterone Metabolism and Analyte Quantification

Liver microsomal fractions were isolated by differential centrifugation as previously published (Velenosi et al., 2012). Testosterone was selected as a probe substrate to determine hepatic CYP2C11 and CYP3A enzyme function as described previously (Velenosi et al., 2012).

4.2.3 RNA Extraction and Real-time PCR Analysis

RNA was extracted from rat livers using TRIzol according to the manufacturers protocol. cDNA was synthesized from 1 µg of total RNA according to manufacturers protocol with qScript cDNA Supermix (Quanta BioSciences, Inc., Gaithersburg, MD, USA) and relative mRNA expression quantified by real-time PCR with PerfeCta SYBR Green Fastmix (Quanta BioSciences, Inc., Gaithersburg, MD, USA). Primer sets for CYP1A1, CYP1A2, CYP2C11, CYP3A2, PXR, HNF-4α, CAR, and RXR were generated using NCBI Primer-Blast (Table 4.1). Gene expression was normalized to β-actin using the ΔΔCT method.
<table>
<thead>
<tr>
<th>Gene/Promoter</th>
<th>Primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drug Metabolizing Enzymes</strong></td>
<td></td>
</tr>
<tr>
<td>Rat CYP1A1</td>
<td>Forward ATGTCCAGCTCTCAGATGATAAAGGTC Reverse ATCCCTGCCAATCAGCTGTCTAACC</td>
</tr>
<tr>
<td>Rat CYP1A2</td>
<td>Forward CAACCCAGCCCTCAAGAGGTAAAAG Reverse GCGCCTGTAGTCTCTGGATAC</td>
</tr>
<tr>
<td>Rat CYP2C11</td>
<td>Forward CCGTGGAGACCTTTTGGGATGGGC Reverse AGGGGACCTTTGCTCTTTCCTC</td>
</tr>
<tr>
<td>Rat CYP3A2</td>
<td>Forward GCTCTTGATGCATGGTAAAAGATTTG Reverse ATCACAGACCTTGCCAACCTCCTT</td>
</tr>
<tr>
<td><strong>Nuclear Receptors</strong></td>
<td></td>
</tr>
<tr>
<td>Rat PXR</td>
<td>Forward TGCACACAGGTTCCTGTTCTTGA Reverse GGGGTGCGTGTCTGGATGC</td>
</tr>
<tr>
<td>Rat HNF-4α</td>
<td>Forward GGGATCGGATCAGCAGCAGG Reverse GGGGGAGGTGATCTGAGAGACA</td>
</tr>
<tr>
<td>Rat CAR</td>
<td>Forward CTTTTCCGTTCCCTGACCA Reverse AGGCAGAAGCTGTATGAGT</td>
</tr>
<tr>
<td>Rat RXR</td>
<td>Forward AACCCCTCTAGGCCCTCAAT Reverse TAGTGGTTTGAGGGAGGAGG</td>
</tr>
<tr>
<td><strong>Nuclear Receptor and RNA Pol II Binding Sites</strong></td>
<td></td>
</tr>
<tr>
<td>-292 to -172 Rat CYP3A2 Promoter</td>
<td>Forward GGCTCACCTGGGACTTTTGGTACTCT Reverse CCCATGTAGCAGCTGTGTTTACTGA</td>
</tr>
<tr>
<td>-793 to -692 Rat CYP2C11 Promoter</td>
<td>Forward CTTCACTGCTAAAACACTCAGTC Reverse CCCAGAGGTTTCAACATACA</td>
</tr>
<tr>
<td>-145 to -22 Rat CYP2C11 Promoter</td>
<td>Forward ACAGGTCAGGGTCCAAAGAGAAA Reverse CTGGAGCTGTGCTGAGAGACTT</td>
</tr>
<tr>
<td>Rat RNA Pol II GAPDH Binding Site</td>
<td>Forward CGTAGCTCAGGGCTCTGCGCCTTTT Reverse CTGGCAGCAGCAGAGATAGGCTG</td>
</tr>
</tbody>
</table>

*Table 4.1 Real-time PCR Primers*
4.2.4 Western Blot Analysis

Protein expression of CYP2C11 and CYP3A2 was assessed in hepatic microsomal fractions using western blot analysis as previously described (Chapter 3). HNF-4α and PXR western blots were performed in hepatic lysates. Immunoblots were performed according to antibody manufacturer’s recommendation for CYP2C11 (Detroit R&D Inc., Detroit, MI, USA), CYP3A2 (Millipore, Billerica, MA, USA), HNF-4α (GeneTex, Irvine, CA, USA) and PXR (Abcam, Cambridge, MA, USA). Secondary HRP-linked antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Dallas, TX, USA). Immune complexes were revealed by horseradish peroxidase (Millipore, Billerica, MA, USA) and band intensity determined by densitometry (Quantity One 1-D Analysis Software on a VersaDoc Imaging System, Bio-Rad, Hercules, CA, USA).

4.2.5 Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed on rat liver tissue using a previously published method with slight modification (Sohi et al., 2011). Briefly, DNA was cross-linked to protein with 1% formaldehyde and subsequently sheared by sonication. Chromatin samples were pre-cleared with protein A/G agarose then incubated with antibodies to RNA polymerase II (RNA Pol II, 1 µg per aliquot, 17-620, Millipore, Billerica, MA, USA), PXR (5 µg per aliquot, sc-25381, Santa Cruz, Dallas, TX, USA), HNF-4α (5 µg per aliquot, sc-8987, Santa Cruz, Dallas, TX, USA), acetyl histone H3 (5 µg per aliquot, K9, K14, #06-559, Millipore, Billerica, MA, USA), acetyl histone H4 (5 µg per aliquot, K5, K8, K12, K16, #06-886, Millipore, Billerica, MA, USA), H3K27me3 (5 µg per aliquot, #07-449, Millipore, Billerica, MA, USA), H3K9me3 (Billerica, MA, USA), H4R3me2 (5 µg per aliquot, #39705, ActivMotif, Carlsbad, CA, USA) or H3K4me2 (5 µg per aliquot, #07-030, Millipore, Billerica, MA, USA) overnight at 4°C. Separate aliquots were treated with the same amount of non-immune IgG from the same host species to determine non-specific binding (Santa Cruz Biotechnology or Millipore). The binding of RNA Pol II, PXR (-217 to -208) and HNF-4α (-190 to -180) to the rat CYP3A2 promoter (Huss et al., 1999) was quantified by real-time PCR using primers to amplify -292 to -172 (Table 4.1). This primer set was also used to determine histone
modifications in the *CYP3A2* promoter. GeneInspector™ was used to identify putative rat HNF-4α binding sites in the *CYP2C11* promoter (-1 to -10,000 bp). A proximal putative HNF-4α binding site was identified from -755 to -751 bp and was evaluated using forward and reverse primers amplifying -793 to -692 bp (Table 4.1). A site upstream of the CYP2C11 HNF-4α binding site binding site (-4438 to -4296 bp) was also amplified as a negative control. -793 to -692bp was also amplified to determine histone H4 acetylation and H4R3me2 in the CYP2C11 promoter. PCR primers were also used to amplify -145 to -22 to assess *CYP2C11* promoter binding of RNA Pol II as well as H3K4me2, H3K9me3 and H3K27me3 epigenetic markers (Table 4.1).

### 4.2.6 Statistical Analysis

Statistical differences between control and CKD rats were assessed using the unpaired Student’s t-test. Results are expressed as mean ± SEM and *p* < 0.05 was considered statistically significant.
4.3 Results

4.3.1 Serum Biochemistry and Body Weight

Serum levels of creatinine and urea in rats with surgically induced CKD were 2.74-fold and 1.97-fold higher, respectively, compared to control ($p < 0.05$, Table 4.2). There was no difference in body weight between CKD and control rats.

4.3.2 Hepatic CYP3A and CYP2C Mediated Drug Metabolism

To confirm decreased CYP2C and CYP3A mediated metabolism in CKD, we evaluated metabolism of testosterone, using rat liver microsomes. A 68% decrease in the CYP2C11 mediated production of $16\alpha$-OH testosterone was demonstrated in rats with CKD compared to controls (Fig. 4.1A, $p < 0.05$). CYP3A activity assessed by measuring $6\beta$-OH testosterone formation was also significantly decreased by 78% in CKD rats, compared to controls (Fig. 4.1B, $p < 0.05$).

4.3.3 Protein and mRNA expression of Drug Metabolizing Enzymes

Microsomal protein expression of CYP2C11 and CYP3A2 was decreased by 65.6% and 88.8%, respectively, in CKD rats compared to controls (Fig. 4.1C, $p < 0.05$). Real-time PCR indicated a 70.5% and 97.8% decrease in hepatic CYP2C11 and CYP3A2 mRNA expression in CKD rats, respectively, compared to controls (Fig. 4.1D, $p < 0.05$).

4.3.4 RNA Pol II Recruitment to the CYP3A2 and CYP2C11 Promoters

A potential transcriptional mechanism of CYP2C11 and CYP3A2 downregulation was investigated by measuring the binding of RNA Pol II to the promoters of CYP2C11 and CYP3A2 using ChIP. Rats with CKD had greater than a 76% and 71% decrease in recruitment of RNA Pol II to the CYP2C11 and CYP3A2 promoters, respectively ($p < 0.05$, Fig. 4.2). Non-immune IgG ChIP resulted in negligible binding to the CYP2C11 and CYP3A2 promoters.
Table 4.2 Characteristics of control and CKD rats.

Data are represented as mean ± SEM, n = 6.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Chronic Kidney Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>478 ± 20</td>
<td>451 ± 12</td>
</tr>
<tr>
<td><strong>Serum Creatinine (µM)</strong></td>
<td>23 ± 1</td>
<td>63 ± 12*</td>
</tr>
<tr>
<td><strong>Serum Urea (µM)</strong></td>
<td>6.1 ± 0.5</td>
<td>12.0 ± 0.3*</td>
</tr>
</tbody>
</table>

* *p* < 0.05 compared to control.
Figure 4.1 Hepatic P450 enzyme function and expression in control and chronic kidney disease (CKD) rats.

Hepatic CYP2C (A) and CYP3A (B) enzyme activity in control and CKD rats. Production of 16α-OH testosterone (CYP2C11) and 6β-OH testosterone (CYP3A2) was measured after incubation of rat liver microsomes with 1 mM NADPH and 200 µM testosterone. Hepatic protein (C) and mRNA (D) expression of drug metabolizing enzymes in control and CKD rats. Protein and mRNA are standardized to β-actin. Controls were arbitrarily defined as 100%. Results are presented as mean ± SEM, n = 6, *p < 0.05 compared to control. Representative western blots are shown.
**Figure 4.2** RNA Pol II chromatin immunoprecipitation

RNA Polymerase II (RNA Pol II) binding to the initiation site of hepatic CYP2C11 (A) and CYP3A2 (B) in control and chronic kidney disease (CKD) rats. Chromatin Immunoprecipitation (ChIP) was carried out on rat liver tissue using an RNA Pol II specific antibody. All target genes were normalized to RNA Pol II binding to GAPDH. Relative binding of RNA Pol II is expressed as a percentage of control binding. Controls were arbitrarily defined as 100%. Results are presented as mean ± SEM, n = 6, *p < 0.05 compared to control.
4.3.5 Hepatic Nuclear Receptor Expression and Recruitment to the CYP2C11 and CYP3A2 Promoters in CKD

To determine the effect of nuclear receptors on transcriptional regulation of CYP2C11 and CYP3A2 in CKD, we assessed the steady-state levels of nuclear receptor mRNA and protein along with promoter recruitment. CKD did not significantly alter the hepatic mRNA expression of PXR, HNF-4α, CAR or RXRα (Fig. 4.3A). Protein expression of PXR and HNF-4α was also unchanged (Fig. 4.3B and Fig. 4.3C). While a previously described binding site for HNF-4α in the CYP3A2 promoter has been established (Huss et al., 1999), we used GeneInspector™ to identify a novel putative binding site at -757 of the CYP2C11 promoter. ChIP was used to assess the binding of HNF-4α to the promoter of both CYP2C11 and CYP3A2 in control and CKD rats. Binding of HNF-4α to its binding sites in the CYP2C11 and CYP3A2 promoters was significantly decreased by 57% and 77%, respectively in CKD rats compared to controls (p < 0.05, Fig. 4.4A, B). Recruitment of PXR to the CYP3A2 promoter was also significantly decreased greater than 57% in CKD rats compared to controls (p < 0.05, Fig. 4.4C).

4.3.6 Decreased Histone Acetylation in the CYP2C11 and CYP3A2 Promoters of Rats with CKD.

Epigenetic modulation can cause gene silencing through decreased histone acetylation (Jenuwein and Allis, 2001). To assess the possibility that altered histone acetylation in the promoters of CYP2C11 and CYP3A2 occurs in tandem with decreased nuclear receptor binding in CKD, ChIP was employed using antibodies against acetyl histone H3 and acetyl histone H4 in control and CKD liver tissue. Interestingly, histone H3 acetylation was decreased in both the CYP2C11 (-145 to -22) and CYP3A2 (-292 to -172) promoter regions by 77% (p < 0.05, Fig. 4.5A, B). Histone H4 acetylation was also decreased in the CYP3A2 promoter (p < 0.05, Fig. 4.5D); however, there was no change in H4 acetylation of the putative HNF-4α binding site in the CYP2C11 promoter (-793 to -751, Fig. 4.5C). Given alterations in histone methylation can also influence gene expression (Shilatifard, 2006), we next assessed if activating and/or inhibiting histone methylation modifications occurred in hepatic CYP2C11 and CYP3A2 promoters. H3K4me2 and H4R3me2 activating modifications were examined by ChIP in the
Figure 4.3 Nuclear receptor expression in control and chronic kidney disease (CKD) rats.

(A) mRNA expression of nuclear receptors regulating drug metabolizing enzymes in control and CKD rats. Pregnane X Receptor (PXR) (B) and Hepatocyte Nuclear Factor 4 α (HNF-4α) (C) protein expression in control and CKD rats. Protein is standardized to β-actin and controls were arbitrarily defined as 100%. Results are presented as mean ± SEM, n = 6. Representative western blots are shown.
Figure 4.4 Nuclear receptor binding to the CYP2C11 and CYP3A2 promoter in control and chronic kidney disease (CKD) rats.

Hepatocyte Nuclear Factor 4 α binding to the promoter regions of CYP2C11 (A) and CYP3A2 (B) drug metabolizing enzymes in control and CKD rats. Pregnane X Receptor (PXR) binding to the promoter of hepatic CYP3A2 in control and CKD rats (C). Chromatin Immunoprecipitation (ChIP) was carried out on rat liver tissue using a PXR or HNF-4α specific antibody. The relative level of immunoprecipitated genomic DNA was normalized to total genomic DNA. Relative binding of PXR and HNF-4α are expressed as a percentage of control binding. Results are presented as mean ± SEM, n = 6, *p < 0.05 compared to control.
Figure 4.5 Histone acetylation at the initiation site of hepatic CYP2C11 and CYP3A2.

Histone 3 acetylation at the initiation sites of hepatic CYP2C11 (A) and CYP3A2 (B) in control and chronic kidney disease rats (CKD). Histone 4 acetylation at the nuclear receptor binding sites of CYP2C11 (C) and CYP3A2 (D) in control vs. CKD rats. Chromatin Immunoprecipitation (ChIP) was carried out on rat liver tissue using an Acetyl-H3 (AcH3) or Acetyl-H4 (AcH4) specific antibody. The relative level of immunoprecipitated genomic DNA was normalized to total genomic DNA. Relative levels of AcH3 and AcH4 are expressed as a percentage of control. Results are presented as mean ± SEM, n = 6, *p < 0.05 compared to control.
CYP2C11 and CYP3A2 promoters of control and CKD rat livers. ChIP revealed no significant change in H3K4me2 and H4R3me2 in promoters of these enzymes for rats with CKD (Fig. 4.6A, C). Histone methylation silencing modifications assessed included H3K9me3 and H3K27me3. No significant differences in silencing methylation modifications were found in the promoters of hepatic CYP2C11 and CYP3A2 for rats with CKD compared to controls (Fig. 4.6B, D).
Histone 3 lysine 4 dimethylation (H3K4me2, A), histone 3 lysine 9 trimethylation (H3K9me3, B) and histone 3 lysine 27 trimethylation (H3K27me3, D) at the initiation site of hepatic CYP2C11 and CYP3A2 in control rats and rats with chronic kidney disease (CKD). Asymmetric histone 4 arginine 3 dimethylation (H4R3me2, C) at the nuclear receptor binding sites of CYP2C11 and CYP3A2 in control and CKD rats. Chromatin Immunoprecipitation (ChIP) was carried out on rat liver tissue using an H3K4me2, H3K9me3, H3K27me3 or H4R3me2 specific antibody. The relative level of immunoprecipitated genomic DNA was normalized to total genomic DNA. Relative
levels of H3K4me2, H3K9me3, H3K27me3 and H4R3me2 are expressed as a percentage of control. Results are presented as mean ± SEM, n = 6, *p < 0.05 compared to control.
4.4 Discussion

In this study, we explored the possible underlying transcriptional factor (e.g. nuclear receptor activity) and epigenetic (e.g. posttranslational histone modifications) alterations that accompany downregulation of the important drug metabolizing enzymes CYP2C and CYP3A in CKD. Hepatic CYP2C11 and CYP3A2 function and expression are decreased in CKD similar to levels observed in previous studies. Decreased protein expression and function of these drug metabolizing enzymes have been consistently supported as secondary to the decrease in mRNA expression (Leblond et al., 2000; Okabe et al., 2003; Velenosi et al., 2012).

Activity of PXR and HNF-4α nuclear receptors commonly occurs through post-translational modifications and protein interactions; however, some studies have indicated that changes in the expression of these nuclear receptors can alter CYP3A mRNA expression (Souidi et al., 2005; Ni et al., 2009). For example, rats exposed to depleted uranium exhibited increased hepatic PXR expression, which occurred concomitantly with increased CYP3A2 expression (Souidi et al., 2005). Moreover, inflammatory animal models, such as intraperitoneal lipopolysaccharide injection in mice, have demonstrated decreased PXR and RXRα expression, which leads to impaired CYP3A11 expression (Xu et al., 2006). CKD can be considered an inflammation-associated pathological state and although there was a slight trend towards decreased nuclear receptor expression, there was no significant change in the expression of nuclear receptors that regulate CYP2C11 and CYP3A2 in CKD compared to controls.

While the expression of these nuclear receptors was not altered in our study, we demonstrated that the recruitment of hepatic PXR and HNF-4α to the CYP3A promoter was substantially decreased in CKD. To our knowledge, this is the first study in an in vivo disease model to implicate alterations in PXR and HNF-4α binding along with changes in the chromatin environment associated with recruitment of these nuclear receptors. Previous in vitro studies suggest that decreased PXR binding may be a result of protein-protein interactions, epigenetic changes, reduced recruitment of co-activators or a combination of these mechanisms (Tian, 2013). Protein-protein interactions with NF-κB
and PXR have been demonstrated through direct binding with the RXRα DNA binding domain to inhibit transcriptional activity (Gu et al., 2006). Moreover, NF-κB has been shown to play a role in mediating uremic toxin induced downregulation of CYP2C and CYP3A expression. In a previous study, NF-κB inhibitors mitigated the downregulation of these enzymes in rat hepatocytes treated with pre-dialysis serum. However, post-dialysis serum had no effect on drug metabolizing enzyme expression suggesting the toxins involved may be cleared in dialysis (Michaud et al., 2008).

Basal expression of rat CYP2C11 is regulated by growth hormone in a complex interaction between signal transducer and activator of transcription-5b (STAT5b), HNF-1α, HNF-3β, HNF-4α and HNF-6 (Park and Waxman, 2001; Wauthier and Waxman, 2008). In humans, the major regulators of CYP2C9 are PXR, CAR and HNF-4α (Zanger and Schwab, 2013). Therefore, although this study demonstrates a decrease in binding of a common nuclear receptor regulating both CYP2C11 and CYP2C9, these enzymes display distinct species differences in regulation.

The focus of our study was to also examine the associated epigenetic changes in the promoter region of these enzymes as a result of CKD. *In vitro* studies have shown that both PXR and HNF-4α activation occurs through the activity of PRMT1, which asymmetrically dimethylates histone H4R3 causing subsequent H4 acetylation and transcriptional activation (Barrero and Malik, 2006; Xie et al., 2009). This pathway is thought to be unidirectional as acetylation of histone H4 has been shown to inhibit H4R3me2 (Tian, 2009; Tian, 2013). Our study demonstrated that histone H4R3me2 was not significantly altered in the promoters of hepatic *CYP2C11* (-793 to -751) and *CYP3A2* (-292 to -172) in CKD (Fig. 4.6C). Further, PRMT1 mRNA was unchanged in CKD rats compared to control (Supplementary Fig. C2, Appendix C). Our results indicate that histone H4 acetylation is significantly decreased alongside RNA Pol II recruitment in the promoter region of hepatic *CYP3A2* in rats with CKD suggesting decreased transcriptional activation (Fig. 4.5D). Tian categorized chromatin remodeling by PRMT1 in a three state cycle: “transcriptionally silent” with no chromatin markers, “ready state” which occurs after asymmetric dimethylation of H4R3 by PRMT1 and “active state” which occurs during H4 acetylation and the removal of H4R3me2 markers...
Therefore, it is possible that the percentage of CYP3A2 promoter in H4R3me2 “ready state” (Fig. 4.6C) is unaffected by CKD while the percentage of histone H4 acetylation is significantly decreased (Fig 4.5D) resulting in a higher percentage in the transcriptionally silent state. The mechanism by which H4 acetylation is decreased may be a result of NF-κB activation and histone deacetylases (HDACs) causing deacetylation to the transcriptionally silent state (Tian, 2009; Tian, 2013). A previous study demonstrated reduced HDAC1 binding to the CYP3A4 promoter in the presence of the CYP3A4 inducer, carbamazepine (Wu et al., 2012). However, HDAC1 was not enriched in the rat CYP3A2 promoter for control or CKD rats (data not shown).

Decreased recruitment of RNA Pol II is associated with increased H3K9 methylation silencing modifications and decreased H3K9 acetylation activating modifications (Sohi et al., 2011). A maternal protein restriction model of intrauterine growth restriction has shown long term gene silencing of hepatic CYP7A1. CYP7A1 gene silencing occurred as a result of decreased RNA Pol II binding in rats (Sohi et al., 2011). This study also showed decreased H3 acetylation and increased H3K9me3 in the CYP7A1 promoter. Other studies have associated these histone modifications with decreased RNA Pol II recruitment (Lorincz et al., 2004; Shilatifard, 2006). In our study, H3K9me3 was unaltered in rat hepatic CYP2C11 and CYP3A2 promoters; however, H3 acetylation was decreased further suggesting the possibility of increased HDAC activity in the promoter of these enzymes. Studies examining the epigenetic changes associated with phenobarbital-induced CAR induction of CYP2B1 have also noted increases in H3 acetylation as well as methylation modifications including increased H3K4me2 and decreased H3K27me3 (Lempainen et al., 2011). H3K4me2 is associated with activation of transcription while H3K27me3 is a marker of silencing heterochromatin (Lorincz et al., 2004; Shilatifard, 2006). In our study, no significant changes in H3K4me2 and H3K27me3 were found in the promoters of CYP2C11 or CYP3A2 in CKD. Another potential silencing mechanism that could explain our results is silencing via direct DNA methylation. Although direct DNA methylation silencing modifications have been associated with PXR binding sites in mice (Cui et al., 2010), our in silico screen did not identify any CpG islands in the rat CYP2C11 or CYP3A2 promoters as predicted by CpG island searcher software (Takai and Jones, 2002).
Emerging evidence strongly suggests that alterations in non-renal clearance pathways in CKD impact the pharmacokinetics of drugs. Our study describes a possible mechanism by which these enzymes are downregulated. We therefore propose a mechanistic hypothesis to explain how uremia induces altered nuclear receptor binding and epigenetic modifications to downregulate drug disposition genes (Fig. 4.7). We show for the first time that hepatic CYP2C11 and CYP3A2 downregulation in CKD are associated with both decreased nuclear receptor binding and decreased acetylation in the promoter region of these enzymes. Future studies should address the temporal and severity relationships between changes in nuclear receptor binding and histone acetylation in CKD. Given the complex nature of uremia and the several putative uremic toxins that likely contribute to this effect, future studies are also needed to elucidate the role of specific uremic toxins in regulation of drug metabolism.

In summary, CYP2C and CYP3A enzymes are involved in the oxidative metabolism of almost half of all prescribed medications (Zanger and Schwab, 2013). They also play essential roles in cholesterol, steroid, and lipid homeostasis (Luoma, 2008). Our study provides evidence for an association between decreased transcription factor binding and histone acetylation and downregulation of these important genes in the setting of CKD.
Figure 4.7 Schematic of mechanistic hypothesis describing the downregulation of drug metabolizing enzymes in chronic kidney disease (CKD).

In the absence of CKD (left), PXR, HNF-4α and RNA polymerase II bind to the promoter region of CYP2C11 and CYP3A2 to drive transcription. In CKD (right), we propose that uremic toxins accumulate in the blood and impact transcriptional processes in the hepatocyte. This results in decreased PXR, HNF-4α and RNA Pol II binding to the promoter of CYP2C11 and CYP3A2. In addition, histone acetylation in the promoters of CYP2C11 and CYP3A2 is decreased promoting a transcriptionally silent state. Collectively, changes in nuclear receptor binding and epigenetic histone acetylation mediate decreased transcription. Images used to generate Fig. 4.7 were modified from Servier Medical Art (http://www.servier.co.uk/medical-art-gallery).
4.5 References


Lempainen H, Muller A, Brasa S, Teo SS, Roloff TC, Morawiec L, Zamurovic N, Vicart A, Funhoff E, Couttet P, Schubeler D, Grenet O, Marlowe J, Moggs J, and


5 UNTARGETED PLASMA AND TISSUE METABOLOMICS IN RATS WITH CHRONIC KIDNEY DISEASE GIVEN AST-120.³

5.1 Introduction

The National Kidney Foundation defines CKD as the presence of kidney damage or a progressive decline in renal function lasting for three or more months (National Kidney Foundation, 2002). The prevalence of CKD is estimated to be upwards of 14% in the United States (KEEP, 2011; McCullough et al., 2011; Stevens et al., 2011). CKD is divided into 5 stages, which are classified based on glomerular filtration rate. In end stage renal disease, patients experience fatigue, impaired vascular reactivity, hypothermia, malnutrition and psychological disturbances associated with being on dialysis, known as the “residual syndrome” (Depner, 2001). Many of these signs and symptoms can develop in early stages of CKD but are not specific to the disease and therefore, are difficult to identify (Meyer and Hostetter, 2007). Although biochemical mechanisms of the residual syndrome are not completely understood, uremia is considered a major cause of this condition (Depner, 2001).

Uremia is the accumulation of solutes that are normally cleared by the kidneys in CKD (Meyer and Hostetter, 2007). At high concentrations, the accumulating solutes are considered uremic toxins as they begin to induce the residual syndrome. Many of these highly concentrated toxins are gut-derived metabolites generated via protein fermentation by bacteria in the colon (Meyer and Hostetter, 2012). The majority of gut-derived uremic toxins are also protein-bound solutes that are less efficiently dialyzed and therefore, remain highly concentrated in patients on dialysis (Niwa and Ise, 1994; Lesaffer et al., 2000). Gut-derived aromatic amino acid metabolites, such as indoxyl sulfate and p-cresyl sulfate, have been extensively studied in CKD (Vanholder et al., 2003; Duranton et al., 2012). Indoxyl sulfate and p-cresyl sulfate play a significant role in cardiovascular disease, which is the leading cause of death in patients with CKD accounting for 45% of mortalities (Collins et al., 2010; Cao et al., 2015; Wang et al., 2015). Indoxyl sulfate concentration is correlated with aortic calcification and vascular stiffness in patients with CKD (Barreto et al., 2009). High plasma levels of unbound p-cresyl sulfate increases the risk of cardiovascular and all-cause mortality in hemodialysis patients (Wu et al., 2012). Other gut-derived metabolites identified in animals have also been shown to contribute to
cardiovascular disease and through the generation of pro-atherosclerotic metabolites (Wang et al., 2011; Meyer and Hostetter, 2012).

AST-120 is a spherical carbon adsorbent that has been used in Japanese CKD patients since 1991 to remove the precursors of uremic toxins produced in the gut. This compound is formulated to bind small molecules (100 to 10,000 Da) while larger molecules such as digestive enzymes and hormones are left unbound (Schulman et al., 2015). Adsorption of gut-derived uremic precursors to AST-120 decreases circulating levels of these toxins. In clinical trials, AST-120 has been shown to mitigate symptoms of uremia in CKD (Akizawa et al., 1998; Schulman et al., 2015).

A number of studies have quantified individual uremic toxin levels in patients with CKD. In 2003, the EuTox compiled mean and median serum or blood levels of 90 uremic toxins using meta-analysis (Vanholder et al., 2003). Subsequent updates were published in 2007 and 2012, adding 14 and 56 uremic toxins, respectively (Vanholder et al., 2007; Duranton et al., 2012). This is an effective method for determining mean patient uremic toxin levels; however, the uremic toxins reported are a result of targeted analysis, which can limit the identification of novel toxins. Recent advances in mass spectrometry allow for identification of novel uremic toxins using an untargeted metabolomics approach.

Metabolomics is the study of all metabolites generated in a biological system. Thousands of metabolites can be detected using untargeted metabolomics without prior knowledge of their composition (Beger, 2013). In the setting of CKD, metabolomics can provide uremic signatures directly associated with decreased renal function through plasma analysis (Rhee, 2015). Recently, clinical and animal studies have begun to identify plasma uremic toxins using untargeted metabolomics (Rhee et al., 2010; Sharma et al., 2013; Niewczas et al., 2014).

Plasma uremic toxin levels have been correlated with CKD complications, most notable are the cardiovascular effects including endothelial dysfunction and heart failure (Shibahara and Shibahara, 2010; Cao et al., 2015). Metabolic changes in the kidney are known to occur as a result of kidney damage, and the accumulation of uremic toxins has been shown to further kidney injury (Zhao et al., 2012; Zhao et al., 2014). However,
effects of accumulating toxins on metabolic changes in other major organs such as the heart and liver, have not been assessed. Therefore, the purpose of this study was to determine the metabolic changes in plasma, liver, heart and kidney tissue as well as the metabolic axis between these matrices in rats with CKD. We also assessed the plasma and tissue metabolic effects of removing gut-derived uremic toxins using AST-120 to further understand the gut contribution to the uremic condition in both plasma and tissues in CKD.
5.2 Methods

5.2.1 Chemicals and Reagents

Harlan 8640 Teklad 22/5 rodent diets were used for both control and 0.7% adenine supplemented diets (Madison, WI). Indoxyl sulfate was obtained from Gold Biotechnology (Olivette, MO). P-cresyl glucuronide and equol 7-glucuronide were purchased from Toronto Research Chemicals (Toronto, ON, Canada) and β-glucuronidase from Helix pomatia (G-7051), pyrocatechol, p-cresol and 4-ethylphenol were obtained from Sigma-Aldrich (St. Louis, MO). Flurazepam was purchased from Cerilliant (Round Rock, TX). Isatin was purchased from Alfa Aesar (Ward Hill, MA). P-cresyl sulfate, phenyl sulfate and 4-ethyl phenyl sulfate were synthesized as previously described (Feigenbaum and Neuberg, 1941). AST-120 was a kind gift from the Kureha Corporation (Tokyo, Japan).

5.2.2 Animal Models

A total of 30 male Wistar rats were obtained from Charles River Laboratories, Inc. (Wilmington, MA) for this study. Animal care and experimental protocols and procedures were approved by the Western University Animal Care Committee and conducted in accordance with the Canadian Council on Animal Care (Appendix A). Rats initially weighed 150 g and after 5 days of acclimatization were divided into two groups: control (n=10) and CKD (n=20). The CKD group received 0.7% adenine supplemented into rat chow ad libitum for 5 weeks to induce kidney disease. Food was weighed daily for the CKD group and pair-fed as standard chow to control animals. After 5 weeks, the CKD group was equally divided and the diet was changed to standard chow (CKD, n=10) or standard rat chow supplemented with 8% AST-120 (CKD+AST-120, n=10) for an additional 3 weeks. All animals were weighed daily throughout the study. Three rats from the CKD and CKD+AST-120 groups experienced significant weight loss and were euthanized prior to the end of the study. At the conclusion of the study, control (n=10), CKD (n=7) and CKD+AST-120 (n=7) rats were sacrificed and blood was obtained in heparinized tubes to acquire plasma. Liver, heart and left kidneys were removed, snap-frozen in liquid nitrogen and stored at -80°C. Plasma creatinine and urea were determined.
by the London Laboratory Services Group (London, ON, Canada) using standard methods.

5.2.3 Sample Preparation

Plasma samples were thawed to room temperature and protein was precipitated with the addition of ice-cold acetonitrile (3:1, acetonitrile:plasma). Liver, heart and kidney tissue (100 mg) were homogenized in 250 µL of ice-cold acetonitrile. All samples were placed on ice for 20 minutes and centrifuged at 14,000 g for 5 minutes. The supernatant from tissue samples was diluted to 80% acetonitrile. Quality control samples were generated for each matrix by creating a pooled sample. Acetonitrile used for protein precipitation contained isatin (5 µg/mL) and flurazepam (25 ng/mL) as internal standards.

5.2.4 Chromatographic Separation and Mass Spectrometry

Samples were transferred to vials and 1 µL was injected in triplicate for each vial. Injections were randomized to reduce error associated with instrument drift and quality control samples were run every 6 injections. Metabolites were separated using a Waters ACQUITY UPLC HSS T3 column (1.8 µm particle size, 100 mm X 2.1 mm). The column was maintained at 45°C in a Waters ACQUITY UPLC I-Class system (Waters, Milford, MA). The mobile phase flow was set to 0.45 ml/min and consisted of water (A) and acetonitrile (B), both containing 0.1% formic acid. The UPLC conditions were as follows: 0-2 mins, 1-60%B; 2-6 mins 60-85%B; 6-8 mins 85-99%B; 8-10 mins 99-1%B. Mass spectrometry was performed using a Waters Xevo™ G2S-QToFMS. Metabolites were measured separately in positive and negative ESI modes. Capillary voltage and cone voltage were set at 2 kV and 40 V, respectively. The source temperature was 150°C. The desolvation gas flow was set to 1200 L/h at 600°C and the cone gas flow was 50 L/h. The data was acquired in centroid mode using an MS<sup>E</sup> method, which allows for both MS and MS/MS fragmentation in a single run. Functions 1 and 2 of the MS<sup>E</sup> method acquired data with a 0.1 s scan time in the range of 50-1200 m/z. Collision energy was set to 0V and ramped from 15-50V for functions 1 and 2, respectively. Function 3 acquired locksspray to ensure mass accuracy. Leucine-enkephalin (500 ng/mL) was used as the lockmass set at a flow rate of 10 µL/min, measured every 10 s and averaged over 3 scans.
5.2.5 Data Analysis

5.2.5.1 Multivariate Data Analysis

Multivariate analysis of LC-MS data was achieved by Waters Markerlynx with EZinfo 2.0 (Umetrics, Umeå, Sweden) software packages. Peak intensities were normalized to total marker intensity in Markerlynx and subsequently transferred to EZinfo. Pareto scaling was used to dampen the selection of features with the highest variance. EZinfo was used for principal component analysis (PCA) of plasma, liver, heart and kidney, as well as orthogonal partial least squares discriminant analysis (OPLS-DA) between control and CKD rats for each matrix.

5.2.5.2 Univariate Data Analysis

XCMS Online (Tautenhahn et al., 2012) (https://xcmsonline.scripps.edu) was used for univariate data analysis. Raw data files were initially converted to mzData files and the lockmass was removed using massWolf 4.3.1 and the chemhelper package in R version 3.2.0. Function 1 MS data was uploaded to XCMS Online and the data were processed as a multigroup experiment using the default UPLC with G2S MS parameters. Briefly, the centWave method was used for feature detection (m/z tolerance = 15 ppm, min peak width = 2 s, max peak width = 25 s). The Obi warp method was used for retention time correction (profStep = 0.5) and chromatograms were aligned using the following parameters: mzwid = 0.1, minfrac = 0.5, bw = 2. Metabolite features were statistically analyzed using one-way ANOVA with Tukey’s post-hoc test. Isotopes and adducts were annotated using CAMERA (m/z absolute error = 0.015, ppm = 5) and arranged into feature groups. Features for each biological matrix were filtered for isotopes. The feature with the maximum intensity in each feature group and present in at least 80% of pooled sample injections was chosen for statistical analysis. Highly significant metabolites were selected to assess differences between biological matrices in control, CKD, and CKD+AST-120 rats (p < 0.01, q < 0.001, maximum intensity > 1500).

Pairwise metabolite differences between control and CKD rats that were common across all biological matrices were determined using metaXCMS (Patti et al., 2012). Highly
significant metabolites for each tissue were aligned using a 20 second retention time tolerance and a 0.01 m/z tolerance.

### 5.2.6 Metabolite Identification

Metabolites that were considered highly significant between control and CKD rats from multivariate and univariate analysis were searched in METLIN and Human Metabolome Database (HMDB) metabolomics databases. Fragmentation patterns for each metabolite were compared to putative database compound fragmentation using MassFragment®. Compound standards were purchased or synthesized, and retention time and fragmentation pattern were compared to confirm the metabolite identity. Pyrocatechol sulfate was identified by incubation of plasma with β-glucuronidase from *Helix pomatia*, which has ≥10,000 units/g sulfatase activity. The sulfatase product fragmentation pattern was confirmed as pyrocatechol to a purchased standard.
5.3 Results

5.3.1 Validation of Experimental Model

The extent of CKD was assessed by plasma creatinine and urea levels (Table 5.1). Creatinine levels were 8.0-fold and 5.8-fold higher in CKD and CKD+AST-120 groups, respectively, compared to controls \((p < 0.05)\). CKD and CKD+AST-120 rats had a 6.8 and 5.1-fold increase in plasma urea levels compared to controls. There were no significant differences in weights between each group.

5.3.2 Principal Component Analysis and the Removal of Metabolites by AST-120 in Biological Matrices

To determine the metabolic changes in CKD with and without the administration of AST-120, samples were analyzed by ultra performance liquid chromatography with quadropole time-of-flight mass spectrometry (UPLC-QToFMS) in positive and negative ESI modes. All samples, including pooled samples, were evaluated by multivariate analysis. Pooled sample injections demonstrated reproducibility and were clustered in the center of principal component analysis plots (Supplementary Figure C3 and C4, Appendix C). The first component in plasma negative mode PCA did not show clustering of experimental groups. When using second and third components, CKD rat plasma samples appeared as a separate cluster compared to control and CKD+AST-120 groups (Fig. 5.1A). The control and CKD+AST-120 samples were also grouped together in this analysis. The majority of metabolites were increased in CKD relative to control and CKD+AST-120 groups. Using XCMS Online multigroup experiment, 26 metabolites were significantly increased in CKD plasma samples compared to control. Of those 26 metabolites, 24 were significantly reduced by the administration of AST-120 to rats with CKD (Fig. 5.1C). The PCA for liver metabolites in negative mode also demonstrated clustering of CKD samples; however, CKD+AST-120 showed some separation from control samples (Fig. 5.1B). Further analysis revealed that AST-120 removed 60.7% of metabolites that were significantly increased in CKD rat livers (Fig. 5.1D). Positive ESI did not show marked differences between groups for plasma and liver samples. The
Table 5.1. Physical and biochemical characteristics of control, CKD and CKD+AST-120 rats.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CKD</th>
<th>CKD+AST-120</th>
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<tbody>
<tr>
<td>Body Weight (g)</td>
<td>277 ± 12</td>
<td>297 ± 18</td>
<td>258 ± 23</td>
</tr>
<tr>
<td>Serum Creatinine (µM)</td>
<td>23 ± 1</td>
<td>181 ± 22*</td>
<td>130 ± 17*</td>
</tr>
<tr>
<td>Serum Urea (mM)</td>
<td>6.6 ± 0.5</td>
<td>44.9 ± 5.6*</td>
<td>33.6 ± 5.8*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM.

* $p < 0.05$ compared to control.
A. Plasma

B. Liver

C. 26 Metabolites ↑ CKD
   24 Metabolites ↓ AST-120

D. 107 Metabolites ↑ CKD
   65 Metabolites ↓ AST-120
Figure 5.1 Plasma and liver tissue principal component analysis and metabolites increased in CKD and decreased by AST-120 in negative ESI mode.

Control (■), CKD (●) and CKD+AST-120 (▲) negative ESI mode principal component analysis of plasma (A) and liver (B) tissue in rats. Triplicate injections are shown. Metabolites significantly increased in CKD compared to control (represented as whole pie) and significantly decreased in CKD+AST-120 compared to CKD (represented as shaded pie) for plasma (C) and liver (D) tissue. Metabolites were considered significantly altered using one-way ANOVA (p < 0.01, q < 0.001, and maximum intensity > 1500) and Tukey’s post-hoc test.
A. Heart

B. Kidney

C. 56 Metabolites ↑CKD
   36 Metabolites ↓AST-120

D. 131 Metabolites ↑CKD
   72 Metabolites ↓AST-120
Figure 5.2 Heart and kidney tissue principal component analysis and metabolites increased in CKD and decreased by AST-120 in negative ESI mode.

Control (■), CKD (●) and CKD+AST-120 (▲) negative ESI mode principal component analysis of heart (A) and kidney (B) tissue in rats. Triplicate injections are shown. Metabolites significantly increased in CKD compared to control (represented as whole pie) and significantly decreased in CKD+AST-120 compared to CKD (represented as shaded pie) for heart (C) and kidney (D) tissue. Metabolites were considered significantly altered using one-way ANOVA (p < 0.01, q < 0.001, and maximum intensity > 1500) and Tukey’s post-hoc test.
metabolic profile in heart tissue was distinctly different between control and CKD rats with complete separation of samples in the PCA for both negative and positive ESI modes (Fig. 5.2A and 5.3A). CKD+AST-120 rat samples were clustered between control and CKD groups. AST-120 significantly decreased 58 of 92 metabolites in heart tissue that were increased in CKD (Fig. 5.2C and 5.3C). Metabolites between control, CKD and CKD+AST-120 kidney tissue were mutually exclusive in the PCA for negative ESI mode showing the greatest separation between all matrices analyzed (Fig 5.2B). In positive ESI mode, control and CKD components were well separated with slight overlap between CKD and CKD+AST-120 groups. Kidney tissue had the largest number of significantly increased metabolites in CKD compared to control. However, AST-120 reduced less than 40% (90 of 233) of metabolites that were significantly increased in CKD compared to control kidneys (Fig. 5.2D and 5.3D).

5.3.3 Orthogonal Partial Least Squares Discriminant Analysis of Control and CKD Biological Matrices.

The metabolites responsible for the differences between control and CKD rats were assessed by OPLS-DA in plasma, liver, heart and kidney. Plasma metabolites in control and CKD samples were well described by OPLS-DA (R²(Y) = 0.92) with high predictive ability (Q²(Y) = 0.75). The majority of metabolites in plasma samples were increased in CKD relative to control. Many of these metabolites were aromatic amino acid derivatives including phenyl sulfate, indoxyl sulfate, p-cresyl sulfate, 4-ethylphenyl sulfate, hippuric acid and p-cresol glucuronide (Fig. 5.4E and Table 5.2). Tissue samples from rats with CKD also had high levels of aromatic amino acid derivatives. Few metabolites were significantly higher in control plasma samples compared to CKD. However, many fatty acids including palmitic acid, oleic acid, linoleic acid, arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were present at higher levels in control livers. CKD rat livers were described by increases in equol sulfate and taurocholic acid compared to control livers along with a similar aromatic amino acid derivative profile as CKD plasma in OPLS-DA (Fig. 5.4F, R²(Y) = 0.98, Q²(Y) = 0.87). In negative ESI mode, heart metabolites in the control group were defined by lysophosphatidylcholines and lysophosphoethanolamines (Fig. 5.4G, R²(Y) = 0.97, Q²(Y) = 0.89). Heart
**Figure 5.3** Principal component analysis and metabolites increased in CKD and decreased by AST-120 in positive ESI mode.

Control (■), CKD (●) and CKD+AST-120 (▲) positive ESI mode principal component analysis of heart (A) and kidney (B) tissue in rats. Triplicate injections are shown. Metabolites significantly increased in CKD compared to control (represented as whole pie) and significantly decreased in CKD+AST-120 compared to CKD (represented as shaded pie) for heart (C), kidney (D). Metabolites were considered significantly altered using one-way ANOVA (p < 0.01, q < 0.001, and maximum intensity > 1500) and Tukey’s post-hoc test.
Figure 5.4 Orthogonal partial least squares discriminant analysis (OPLS-DA) and S-plots of control and CKD biological matrices in negative ESI mode.

Control (■) and CKD (○) negative ESI mode OPLS-DA of plasma (A) ($R^2(Y) = 0.92$, $Q^2(Y) = 0.75$), liver (B) ($R^2(Y) = 0.98$, $Q^2(Y) = 0.87$), heart (C) ($R^2(Y) = 0.97$, $Q^2(Y)=0.89$), and kidney (D) ($R^2(Y) = 1.00$, $Q^2(Y) = 0.95$) tissue in rats. Triplicate injections are shown. S-plots showing ions that define the CKD group in the upper right quadrant and ions that define control group in the lower left quadrant for plasma (E), liver (F), heart (G) and kidney (H) tissue. Ions with the greatest contribution in separating control and CKD groups are labeled and defined in Table 5.2 and Supplementary Table C2, Appendix C. Gut-derived metabolites that are increased in CKD and shared by all tissues are labeled 1 to 8 (■).
Table 5.2 Summary of metabolites altered in plasma, liver, heart and kidney tissue in rats with CKD compared to control and CKD+AST-120.

<table>
<thead>
<tr>
<th>Ion</th>
<th>t&lt;sub&gt;n&lt;/sub&gt; (min)</th>
<th>Mass (m/z)</th>
<th>Empirical Formula</th>
<th>Mass Error (ppm)</th>
<th>Identity</th>
<th>Tissue</th>
<th>Change compared to Control</th>
<th>Change compared to CKD+AST-120</th>
<th>Metabolite ID Level</th>
<th>Correlation with Plasma Levels (R²)</th>
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*One plasma CKD outlier with a value of 14351% of control for p-cresyl sulfate was removed in the correlation analysis.*
metabolites that were increased in CKD included aromatic amino acid derivatives as well as pantothenic acid and glutathione, which also appeared in positive ESI mode (Fig. 5.4G and 5.5C). Positive ESI OPLS-DA demonstrated increased levels of L-carnitine and L-carnitine derivatives in heart tissue of control rats compared to CKD. Rat kidneys had the greatest separation in negative and positive ESI mode OPLS-DA plots, and showed the best fit and predictive ability compared to all other biological matrices (Fig. 5.4D, $R^2(Y) = 1.0$, $Q^2(Y) = 0.95$, Fig. 5.5B, $R^2(Y) = 0.99$, $Q^2(Y) = 0.92$). CKD kidneys had higher levels of aromatic amino acid derivatives and adrenic acid. Both positive and negative ESI mode demonstrated increased levels of lysophosphatidylcholines and lysophosphoethanolamines in CKD. Control kidneys had higher levels of similar fatty acids seen in control livers including linoleic acid and arachidonic acid. DHA glycocholic acid, L-carnitine and L-carnitine derivatives were also increased in control kidneys compared to CKD.

5.3.4 Common Significant Metabolites

A second order analysis in metaXCMS was used to determine common metabolic differences in plasma, liver, heart and kidney tissue between control and CKD rats. All tissues shared 8 common metabolites, which were significantly increased in CKD compared to control rats (Fig. 5.6A). All 8 metabolites were gut derived uremic toxins and included the aromatic amino acid derivatives: phenyl sulfate, indoxyl sulfate, p-cresyl sulfate, hippuric acid, pyrocatechol sulfate and p-cresol glucuronide as well as 4-ethylphenyl sulfate and equol 7-glucuronide (Fig. 5.7, Table 5.2). These 8 metabolites were significantly decreased in CKD+AST-120 rats compared to CKD. Plasma levels of these metabolites also showed a significant correlation with liver, heart and kidney levels (Table 5.2, Supplementary Fig. C5, Appendix C). In positive ESI mode, L-carnitine and many of the L-carnitine derivatives found to be significantly different between control and CKD rats in pairwise comparisons were shared between heart and kidney tissue (Fig. 5.6B).
**Figure 5.5** Orthogonal partial least squares discriminant analysis (OPLS-DA) and S-plots of control and CKD biological matrices in positive ESI mode.

Control (■) and CKD (●) positive ESI mode OPLS-DA of heart (A) \( R^2(Y) = 0.98, Q^2(Y) = 0.86 \), and kidney (B) \( R^2(Y) = 0.99, Q^2(Y) = 0.92 \) tissue in rats. Triplicate injections are shown. S-plots showing ions that define the CKD group in the upper right quadrant and ions that define control group in the lower left quadrant for plasma (E), liver (F), heart (G) and kidney (H) tissue. Ions with the greatest contribution in separating control and CKD groups are labeled and defined in Supplementary Table C2, Appendix C.
A second-order analysis of significantly different metabolites in negative ESI mode for plasma, heart, liver and kidney tissue (A) and positive ESI mode for heart and kidney tissue (B). Highly significant metabolites ($p < 0.01$, $q < 0.001$, and maximum intensity > 1500) were used for second-order analysis. Second-order analysis parameters included fold-changes $\geq 1.5$ and $p < 0.05$ for Tukey’s post-hoc test. Common metabolites between biological matrices were aligned using a m/z = 0.01 and a retention time = 20 s tolerance. Information about the 8 common metabolites between all biological matrices in negative ESI mode is listed in Table 5.2.

**Figure 5.6** Meta-analysis of significantly different metabolites between control and CKD in biological matrices.
Figure 5.7 Common significantly different metabolites found in all biological matrices.

Plasma, liver, heart and kidney levels of the 8 gut-derived metabolites found to be significantly different in Fig. 5.5A: 4-ethylphenyl sulfate (A), phenyl sulfate (B), indoxyl sulfate (C), p-cresyl sulfate (D), hippuric acid (E), equol 7-glucuronide (F), pyrocatechol sulfate (G) and p-cresyl glucuronide (H). Controls were arbitrarily defined as 100%. Results are means ± SEM; n = 10 (control) and n = 7 (CKD and CKD+AST-120). * p < 0.05 vs control and CKD+AST-120.
5.4 Discussion

In this study, rat plasma, liver, heart and kidney metabolic signatures were evaluated in CKD with and without the administration of AST-120. Rat plasma metabolic changes in CKD have been assessed in previous studies with the use of AST-120 (Kikuchi et al., 2010; Akiyama et al., 2012). Renal metabolic profiles in negative ESI mode have also been evaluated in rats with CKD (Zhao et al., 2013); however, this study is the first to assess tissue metabolic signatures in heart, liver and kidney including the metabolic axis between plasma and tissues. We demonstrate that the uremic condition causes a number of metabolic changes in both plasma and tissues in CKD and that many of these can be mitigated with AST-120.

The accumulation of uremic toxins in plasma and their contribution to the residual syndrome can be deleterious to patients with CKD (Depner, 2001). Non-invasive plasma sampling is crucial for evaluating the uremic condition in patients; however, to further our understanding of the metabolic interaction between plasma and tissues in CKD, tissue metabolic signatures must be directly assessed. A total of 29, 149, 72 and 236 metabolites were significantly altered between control and CKD rats for plasma, liver, heart and kidney, respectively (Fig. 5.6). Only 10 of the 29 metabolites in plasma were also significantly altered in other tissues. This accounted for less than 14% of metabolites that were significantly different between control and CKD rats in liver, heart and kidney tissues. Therefore, tissue metabolic signatures of CKD were weakly represented in plasma samples.

Rats with CKD had reduced kidney function that was not significantly affected by AST-120. The majority of plasma metabolites increased in CKD were reduced by administration of AST-120, suggesting that a number of these metabolites are derived from the gut or produced from the effects of gut-derived uremic toxins on in vivo metabolic processes (Fig. 5.1C). Surprisingly, more than 50% of metabolites were reduced by administration of AST-120 in both liver and heart tissue metabolic profiles (Fig. 5.1D, 5.2C). Therefore, although kidney function was not altered by AST-120, a number of tissue metabolic processes are affected by gut-derived metabolites.
The 8 metabolites that were significantly increased in all biological matrices were also significantly reduced by AST-120 (Fig. 5.6A, 5.7). P-cresyl sulfate, p-cresyl glucuronide, indoxyl sulfate, hippuric acid and phenyl sulfate are known gut-derived uremic toxins (Meyer and Hostetter, 2012). Gut precursors of p-cresyl sulfate, indoxyl sulfate, p-cresyl glucuronide and phenyl sulfate undergo sulfation or glucuronidation, which occurs through phase II drug metabolizing enzymes (Banoglu and King, 2002; Kikuchi et al., 2010). The major site of phase II drug metabolism is in the liver and therefore, hepatic metabolism of gut precursors plays a major role in the accumulation of these toxins. Gut-derived toxins can also affect hepatic drug metabolizing enzymes. Cytochrome P450 3A4 metabolizes approximately 40% of drugs on the market and indoxyl sulfate levels have been correlated with decreased levels of the CYP3A4 endogenous metabolite, 4β-hydroxycholesterol (Zanger and Schwab, 2013; Suzuki et al., 2014). Once in the bloodstream, these uremic toxins are also highly protein bound and both p-cresyl sulfate and indoxyl sulfate renal clearance is mediated by organic anion transporters (OAT) 1 and 3 in the kidney (Miyamoto et al., 2011; Wikoff et al., 2011). Only the free fraction of these uremic toxins can exhibit biological effects or undergo transport into cells. In OAT1 knock-out mice, indoxyl sulfate, p-cresyl sulfate, phenyl sulfate and pantothentic acid accumulate in plasma (Wikoff et al., 2011). Therefore, increased levels of these toxins in both plasma and tissues are likely a result of decreased OAT1 function in the kidneys of rats with CKD.

In this study, we demonstrate for the first time that both p-cresyl sulfate and indoxyl sulfate accumulate in heart tissue and can be mitigated by AST-120 in vivo. P-cresyl sulfate is a gut-derived bacterial metabolite produced from tyrosine. In a prospective clinical study, free p-cresyl sulfate levels predicted cardiovascular mortality and all-cause mortality in dialysis patients (Wu et al., 2012). The effect of p-cresyl sulfate on cardiac toxicity and dysfunction were recently published in rats with CKD suggesting NADPH oxidase activation and ROS as possible mechanisms in p-cresyl sulfate mediated cardiac apoptosis (Han et al., 2015). Indoxyl sulfate is formed from tryptophan and has been shown to cause pro-fibrotic, pro-inflammatory and pro-hypertrophic affects through p38 MAPK, p42/44 MAPK, and NF-κB pathways in both rat cardiac myocytes and fibroblasts in vitro (Lekawanvijit et al., 2010). In patients, indoxyl sulfate has been
associated with the first heart failure event, and removal of indoxyl sulfate by AST-120 has been shown to improve cardiac function (Shibahara and Shibahara, 2010; Cao et al., 2015). Prevention of cardiovascular disease in CKD is essential as these patients are more likely to die of cardiovascular disease than progress to requiring dialysis (Sarnak et al., 2003). P-cresyl sulfate and indoxyl sulfate are increased 361-fold and 438-fold, respectively, in CKD heart tissue compared to control (Fig. 5.7A, B). Therefore, accumulation of these uremic toxins in the heart may provide evidence for potential mechanisms of cardiovascular disease development in CKD.

Pyrocatechol sulfate is a dietary phenolic metabolite that has not been previously described as a uremic toxin (Hanhineva et al., 2015). Equol 7-glucuronide and 4-ethyl phenyl sulfate are also produced from gut bacteria but are specifically derived from soy protein. Standard rat chow includes soy as the main source of protein and the diet used in this study contained daidzein and genistein aglycone equivalents between 350 and 650 mg/kg, which are metabolized by gut bacteria into precursors of equol 7-glucuronide and 4-ethylphenyl sulfate, respectively (Setchell and Clerici, 2010; Yang et al., 2012). These uremic toxins have not been identified in patients with CKD. Intestinal bacteria produce equol in 20-30% of Western populations and 40-60% of Asian populations (Setchell and Clerici, 2010; Rafii, 2015). Consequently, equol may only accumulate in patients who both consume soy protein and have the gut bacteria to convert daidzein to equol. Although all patients with CKD will have high aromatic amino acid derived uremic toxins levels, accumulation of equol 7-glucuronide and 4-ethyl phenyl sulfate demonstrate the importance of diet on the production of uremic toxins in CKD. The 8 common metabolites identified had the highest variable importance in projection (VIP) values defining CKD in plasma and tissue S-plots (Fig. 5.4 and Table 5.2). Therefore, gut-derived uremic toxins provide the most definitive metabolic signatures of both plasma and tissue in CKD. Tissue accumulation of these gut-derived uremic toxins in patients is unknown; however, in this study, we demonstrate that plasma levels of the 8 gut-derived metabolites were highly correlated with tissue levels (Supplementary Figure C5, Appendix C). This suggests that the non-invasive measurement of these 8 gut-derived uremic toxins in plasma may be used as a surrogate for tissue levels in CKD.
In control animals, higher levels of free fatty acids were observed in liver tissue, which were decreased in animals with CKD. These included palmitic acid, oleic acid, linoleic acid, arachidonic acid, EPA and DHA (Fig. 5.4 and Supplementary Table C2, Appendix C). All of these fatty acids are provided in the rat diet. A previous study identified increased palmitic acid levels in feces of rats with adenine induced CKD (Zhao et al., 2012). Therefore, it is possible that absorption of palmitic acid is compromised in rats with CKD. The depletion of both EPA and DHA in kidney tissue of rats with CKD is demonstrated in this study and others (Zhao et al., 2013; Zhao et al., 2014). Although plasma levels of these fatty acids were not detected, patients on dialysis have lower plasma levels of EPA and DHA, which are unaltered in non-dialysis CKD patients (Nakamura et al., 2008). A recent clinical study supplementing CKD patients with omega-3 fatty acids showed increases in specialized pro-resolving lipid mediators, which can inhibit pro-inflammatory cytokine production and potentially decrease low grade inflammation in CKD (Mas et al., 2015). AST-120 recovered free fatty acid levels in animals with CKD (see Supplementary Table C2, Appendix C).

Fatty acids are an important energy source for cardiac tissue, which can be utilized via β-oxidation (Rebouche, 1992). L-carnitine mediated transport of long chain fatty acids into the mitochondria is the rate-limiting step in fatty acid oxidation (Flanagan et al., 2010). In this study, we demonstrate decreased levels of L-carnitine and L-carnitine derivatives in heart tissue from rats with CKD. Cardiac tissue cannot synthesize L-carnitine, which must be obtained through plasma from dietary sources or production in the liver and kidney. L-carnitine is cleared through the dialysis membrane resulting in decreased levels for dialysis patients; however, levels in CKD patients are not correlated with kidney function (Fouque et al., 2006). In humans, 75% of L-carnitine is obtained from the diet (De Vivo and Tein, 1990; Flanagan et al., 2010). The standard rat diet used in this study was not supplemented with L-carnitine. As a result, it is possible that synthesis of L-carnitine is decreased in rats with CKD; however, this effect is not seen in patients due to substantial dietary L-carnitine consumption. AST-120 recovered levels of L-carnitine in CKD rats and therefore gut-derived uremic toxins may affect the synthesis of L-carnitine (see Supplementary Table C2, Appendix C).
In summary, gut-derived metabolic signatures most prominently defined plasma, liver, heart and kidney tissues in rats with CKD. The majority of these compounds were aromatic amino acid metabolites. Increased levels of soy protein metabolites 4-ethyl phenyl sulfate and equol-glucuronide, which have not been described in patients with CKD, emphasize the potential for dietary effects on individual uremic toxin profiles. This study also highlights the gut-plasma-tissue metabolic axis, directly demonstrating the accumulation of gut-derived uremic toxins in multiple major organs of rats with CKD.
5.5 References


Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. *Hypertension* **42**:1050-1065.


6 THE EFFECT OF GUT-DERIVED UREMIC TOXINS ON THE EXPRESSION OF HEPATIC DRUG METABOLIZING ENZYMES IN CHRONIC KIDNEY DISEASE

6.1 Introduction

CKD is the result of a progressive decline in renal function over time. According to the Kidney Early Evaluation Program (KEEP, 2000 to 2010) and the National Health and Nutritional Examination Survey (NHANES, 1996 to 2006), the prevalence of renal failure is estimated to be upwards of 14% in the United States (Saran et al., 2015). The leading causes of CKD are diabetes and vascular disease (primarily hypertension), which are prevalent in 40% and 31% of adults with CKD, respectively (National Kidney Foundation, 2002). Other comorbidities include cardiovascular disease and obesity. According to the United States Renal Data System (USRDS), there has been a 30% increase in diabetic related ESRD from 1994 to 2008 (Collins et al., 2012).

Kidney function is determined based on estimated glomerular filtration rate (eGFR). The National Kidney Foundation divides CKD into 5 distinct stages classified by eGFR (National Kidney Foundation, 2002). Complications generally begin when eGFR is < 60 ml/min per 1.73 m² in stage 3, which contains more than half of all CKD patients and is the fastest growing stage of the disease (Coresh et al., 2007; Saran et al., 2015). CKD progresses when eGFR continues to decline until stage 5 (< 15 ml/min per 1.73m²) or end-stage renal disease (ESRD), where patients require renal replacement strategies such as dialysis or transplantation to sustain life.

As renal function declines, organic waste products begin to accumulate in the blood. Uremia is the condition of increased organic waste products that are retained in CKD (Meyer and Hostetter, 2007; Meyer and Hostetter, 2014). A number of these organic waste products are derived from metabolites produced by bacteria in the colon. These include aromatic amino acid metabolites such as indoxyl sulfate, phenyl sulfate, hippuric acid, p-cresyl sulfate and p-cresyl glucuronide. We have previously identified these gut-derived uremic toxins as the most definitive metabolic signatures of CKD in both plasma and liver, using untargeted metabolomics (Chapter 5). Plasma levels of indoxyl sulfate and p-cresyl sulfate have been correlated with cardiovascular events and implicated in the progression of CKD (Niwa and Ise, 1994; Watanabe et al., 2013; Cao et al., 2015; Wang et al., 2015). Unlike metabolic waste products that are produced by various organs in the
body, gut-derived uremic toxins are produced in an external environment in the colon lumen. Therefore, these metabolites can be targeted for removal through oral dosing of AST-120, a spherical carbon adsorbent. AST-120 has been used in a number of animal and clinical studies to limit the absorption of gut-derived uremic toxins (Inami et al., 2014; Schulman et al., 2015; Yamamoto et al., 2015). Its large surface area and spherical shape allow it to bind small molecules, leaving large molecules such as digestive enzymes unaffected (Niwa et al., 1991; Schulman et al., 2015).

End stage renal disease patients on dialysis have a daily median pill burden of 19, which is considered the highest of any chronic disease (Chiu et al., 2009). The incidence of adverse drug reactions (ADRs) is more prevalent in patients with CKD (Manley et al., 2005). According to the Institute of Medicine, ADRs have become the 4th leading cause of death among hospitalized patients and approximately 2 million ADRs occur each year (Lazarou et al., 1998). In CKD patients, it is estimated that 1 medication problem occurs per 2.7 medication exposures (Manley et al., 2005). Inhibition and downregulation of drug metabolizing enzymes often lead to ADRs through increased systemic drug levels and subsequent toxicity. It is well understood that the renal clearance of drugs is compromised in renal failure; however, a number of studies have demonstrated that kidney disease can also affect non-renal clearance (Leblond et al., 2001; Naud et al., 2008; Nolin et al., 2009; Thomson et al., 2015).

Hepatic drug metabolism is the major route of drug elimination and is primarily mediated by the cytochrome P450 superfamily of oxidizing enzymes. Two major P450 isoforms, CYP3A4 and CYP2C9, metabolize 43% of clinically used drugs (Zanger and Schwab, 2013). Many of these P450 enzymes are tightly regulated by nuclear receptors; specifically, PXR and HNF-4α regulate CYP3A drug metabolizing enzymes (Tirona et al., 2003). We have previously shown that CYP3A and CYP2C drug metabolizing enzyme function and expression are downregulated in rats with moderate and severe CKD (Chapter 3). We have also demonstrated that PXR and HNF-4α nuclear receptor binding and histone H3 and H4 acetylation are decreased in the promoter of CYP3A and that HNF-4α and histone H3 acetylation are decreased in the promoter of CYP2C (Chapter 4).
A number of literature reviews have suggested that uremic toxins cause downregulation of drug metabolizing enzymes in CKD; however, it is unknown which uremic toxin(s) mediate this effect (Nolin et al., 2008; Velenosi and Urquhart, 2014; Yeung et al., 2014). A previous study demonstrated downregulation of CYP2C and CYP3A when treating primary rat hepatocytes with uremic serum from ESRD patients (Michaud et al., 2005). Therefore, the first aim of this study was to identify which uremic toxin(s) cause the down-regulation of hepatic CYP3A in vitro. Gut-derived uremic toxins have been shown to cause a number of deleterious effects in CKD (Meyer and Hostetter, 2012). For this reason, the second aim of this study was to determine if gut-derived uremic toxins down regulate hepatic drug metabolizing enzymes in CKD. We hypothesize that the removal of gut-derived uremic toxins, using AST-120, would recover expression and function of hepatic CYP2C and CYP3A in rats with CKD.
6.2 Methods

6.2.1 In vitro Assessment of Uremic Toxins on CYP3A4 Expression

Human hepatoma Huh7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin and 2 mM L-glutamine. Cells were grown at confluence for 4 weeks prior to treatment to ensure adequate CYP3A4 expression levels (Sivertsson et al., 2010). Huh7 cells were treated with creatinine (2121.6 µM), p-cresyl sulfate (186.1 µM), CMPF (391.7 µM), guanidinosuccinic acid (268.6 µM), hippuric acid (2631.3 µM), indole-3-acetic acid (51.9 µM), indoxyl sulfate (1113.2 µM), methylguanidine (24.9 µM), urea (76.6 mM) and a cocktail of the above uremic toxins at their listed concentration in the above maintenance media without FBS for 24 hours (Vanholder et al., 2003). Indoxyl sulfate and CMPF were obtained from Gold Biotechnology (Olivette, MO) and Cayman Chemical Company (Ann Arbor, MI), respectively. All other toxins were obtained from Sigma-Aldrich (St. Louis, MO). An indoxyl sulfate concentration-response effect on CYP3A4 mRNA expression was generated using a concentration range of indoxyl sulfate found in normal and CKD patients (0 to 1000 µM) with 40 g/L human serum albumin (HSA, Lee Biosolutions, St. Louis, MO)(Duranton et al., 2012). Indoxyl sulfate was incubated in media containing 40 g/L HSA for 3 hours at 37°C to allow for plasma protein binding equilibration prior to cell treatment. Cell viability was assessed using the TACS 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Arp et al., 2005).

6.2.2 Animal Model

Studies were performed in 40 male Wistar rats initially divided into two groups. Each group received a control diet or 0.5% adenine supplemented into rat chow for 5 weeks to induce CKD. After 5 weeks, groups were equally divided and rats received either a control diet or 8% AST-120 supplemented into rat chow for 3 weeks. Control rats in the study were pair-fed the same amount of rat chow that was ingested by CKD rats on the previous day. Upon completion of the study, control, control+AST-120, CKD and
CKD+AST-120 groups each contained 10 animals (Fig. 6.1). Rats were sacrificed 8 weeks after the initiation of the study and liver tissue was harvested, flash frozen in liquid nitrogen and stored at -80°C prior to analysis. Plasma creatinine and urea were determined by the London Laboratory Services Group by standard methods.

6.2.3 Plasma Quantification of Gut-derived Uremic Toxins

Plasma protein was precipitated with ice-cold acetonitrile containing isatin as internal standard in a 3:1 acetonitrile:plasma ratio. Samples were placed on ice for 20 minutes and centrifuged at 14,000 g for 5 minutes. The supernatant was injected onto a Waters Acquity UPLC™ BEH C18 column (1.7 µm, 50 X 2.1 mm) maintained at 40°C in a Waters Acquity UPLC I-Class system. The mobile phase flow was set to 0.5 ml/min and consisted of water (A) and acetonitrile (B), both containing 0.1% formic acid. The UPLC conditions were as follows: 0-0.5 mins, 0%B; 0.5-2.5 mins 0-60%B; 2.5-3.5 mins 80%B; 3.5-4.5 mins 0%B. The total run time was 4.5 minutes. Indoxyl sulfate, p-cresyl sulfate and hippuric acid were detected using a Waters Xevo™ G2S-QToFMS operated in negative electrospray ionization mode. Capillary voltage and cone voltage were set at 0.5 kV and 40 V, respectively. The source temperature was 150°C. The desolvation gas flow was set to 1200 L/h at 600°C and the cone gas flow was 50 L/h. Lockspray was used to ensure mass accuracy. Leucine-enkephalin (500 ng/mL) was used as the lockmass set at a flow rate of 10 µL/min, measured every 10 s and averaged over 3 scans.

6.2.4 RNA Extraction and Real-time PCR Analysis

RNA was extracted from rat livers using TRIzol and cDNA was synthesized from 1 µg of total RNA using qScript cDNA Supermix (Quanta BioSciences, Inc., Gaithersburg, MD). Relative mRNA expression was quantified by real-time PCR using primer sets for CYP2C11 (forward) 5’-CCCTGAGGACTTTTGGGATGGGC-3’ and (reverse) 5’-AGGGGCACCTTTGGCTTCCTCCTC-3’, CYP3A2 (forward) 5’-GCTCTTGATGCGATGTTAAAGATTTG-3’ and (reverse) 5’-ATCACAGACCTTGCCAACCTCCT-3’,
Figure 6.1 Study diet schedule for rats with CKD given AST-120, n = 10.
β-actin (forward) 5′- ACGAGGCCAGAGCAAGA-3′ and (reverse) 5′-TTGGTTACAATGCCGTGTCCA-3′, CYP3A4 (forward) 5′-CAGGAGGAAATTGATGCAGTTTT-3′ and (reverse) 5′-GTCAAGATACTCCCATCTGTAGCAGT3′, and 18S (forward) 5′-GTCAAGATACTCCCATCTGTAGCAGT3′ and (reverse) 5′-CCATCCAATCGGTAGTAGCG-3′. PerfeCta SYBR Green Fastmix (Quanta BioSciences, Inc., Gaithersburg, MD) was used to amplify and quantify cDNA. Gene expression was normalized to β-actin or 18S rRNA by the ΔΔCT method.

6.2.5 Western Blot Analysis

Hepatic microsomal fractions were isolated and used to assess protein expression of CYP2C11 and CYP3A2 by Western blot analysis as previously described (Chapter 3). Briefly, 20 µg of microsomal protein was resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were performed for CYP2C11 (Detroit R&D Inc., Detroit, MI) and CYP3A2 (Millipore, Billerica, MA). Secondary HRP-linked antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin HRP-linked primary antibody was used as an internal control (Sigma, St. Louis, MO). Immune complexes were revealed by horseradish peroxidase (Millipore, Billerica, MA) and band intensity determined by densitometry (Quantity One 1-D Analysis Software on a VersaDoc Imaging System, Bio-Rad, Hercules, CA).

6.2.6 Hepatic CYP3A and CYP2C Microsomal Metabolism

Midazolam and testosterone were used as probe drugs to determine CYP2C11 and CYP3A hepatic microsomal activity, respectively, as previously described with some modifications (Chapter 3). CYP3A metabolizes midazolam to 1’OH-midazolam and 4’OH-midazolam. CYP2C11 metabolizes testosterone to 16α-OH testosterone. Increasing concentrations of midazolam or testosterone were incubated with 0.2 mg/mL microsomal protein at 37°C to generate Michalis-Menten kinetic curves. Reactions were initiated with the addition of 1 mM NADPH. Ice-cold acetonitrile, containing flurazepam as internal standard, was added at 10 and 20 minutes for midazolam and testosterone reactions, respectively, in a 3:1 ratio to stop metabolic activity. Formation of 1’OH-midazolam and
4′OH-midazolam occurs through CYP3A metabolism and formation of 16α-OH testosterone occurs through CYP2C11 metabolism. Linear enzyme activity at these time points was validated prior to Michaelis-Menten experiments. Samples were incubated on ice for 20 minutes, centrifuged at maximum speed, diluted in mobile phase and analyzed by UPLC-QToFMS analysis. Mobile phase consisted of water (A) and acetonitrile (B), both containing 0.1% formic acid, and flow was set to 0.6 ml/min on a Waters Acquity UPLC™ BEH C18 column (1.7 µm, 50 X 2.1 mm) at 40°C. For midazolam metabolites, mobile phase gradient of 0-2.5 mins 20-35%B, 2.5-3.5 mins 80%B and 3.5-4.5 mins 20%B was used. Testosterone metabolites were separated with the following mobile phase parameters: 0-2 mins 20-80%B, 2-3 mins 80%B and 3-4 mins 20%B. Metabolites were quantified by Waters Xevo™ G2S-QToFMS in positive electrospray ionization mode, using the same settings as described above.

6.2.7 Data and Statistical Analysis

One-way ANOVA with Tukey’s multiple comparison tests were used to determine statistical differences between control, control+AST-120, CKD and CKD+AST-120 groups. Dunnet’s post-hoc test was used to compare indoxyl sulfate treatments to baseline in Huh7 cells. Enzyme kinetic data was fitted with a Michaelis-Menten model to define the maximum enzyme velocity (\(V_{\text{max}}\)) and Michaelis constant (\(K_m\)) for midazolam and testosterone probe substrates.
6.3 Results

6.3.1 Indoxyl Sulfate Downregulates Hepatic CYP3A4 Expression in vitro

To investigate the mechanism of CYP3A downregulation in CKD, human hepatoma Huh7 cells were treated with selected uremic toxins as well as a combination of these toxins. As an initial proof-of-concept screen, concentrations of uremic toxins used were the highest found in patients with CKD (Table 6.1) (Vanholder et al., 2003; Duranton et al., 2012). A 24 hour indoxyl sulfate treatment decreased CYP3A4 mRNA expression by 70% in Huh7 cells, which was equivalent to the effect demonstrated by the combination of toxins (Fig. 6.1). Other individual uremic toxins did not affect CYP3A4 mRNA expression. Indoxyl sulfate is a protein bound uremic toxin; therefore, the concentration dependence of this effect was evaluated in the presence of 40 g/L HSA in the culture medium. Treatment with indoxyl sulfate for 48 hours produced a concentration-dependent decrease in CYP3A4 mRNA expression as the concentration was increased from those measured in healthy controls to concentrations in patients with CKD (IC$_{50}$ = 113.0 ± 3.5 µM). A significant decrease in the steady-state levels of CYP3A4 mRNA was demonstrated when Huh7 cells were treated with ≥ 300 µM indoxyl sulfate in the presence of 40 g/L HSA supplemented media (Fig. 6.2A, $p < 0.05$). Huh7 cells treated with indoxyl sulfate in the uremic range resulted in a 21% to 95% decrease in CYP3A4 mRNA expression. Cell viability was unaffected by indoxyl sulfate in media containing HSA after 48 hour treatment at clinically relevant concentrations (Fig. 6.2B).

6.3.2 Plasma Biochemistry and Body Weight

Plasma creatinine was used as a measurement of kidney function. The CKD and CKD+AST-120 group had a ~3.3-fold and ~2.3-fold increase in plasma creatinine and urea levels, respectively, compared to control and control+AST-120 groups (Table 6.2, $p < 0.05$). There were no significant differences in weight between all four groups (Table 6.2). Indoxyl sulfate, p-cresyl sulfate and hippuric acid plasma concentrations were significantly increased in CKD rats compared to control; however, plasma levels of these
Table 6.1 Normal and uremic serum concentrations of selected uremic toxins.

Data are represented as mean ± SD (Duranton et al., 2012; Vanholder et al., 2003).

<table>
<thead>
<tr>
<th>Uremic Toxin</th>
<th>Normal Concentration (µM)</th>
<th>Mean Uremic Concentration (µM)</th>
<th>Fold Increase</th>
<th>Highest Uremic Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>&lt;106.2</td>
<td>1203.5 ± 407.1</td>
<td>11.3</td>
<td>2121.6</td>
</tr>
<tr>
<td>p-Cresyl Sulfate</td>
<td>10.1 ± 6.9</td>
<td>111.1 ± 64.8</td>
<td>11.0</td>
<td>186.1</td>
</tr>
<tr>
<td>CMPF</td>
<td>32.08 ± 13.8</td>
<td>254.2 ± 68.8</td>
<td>7.9</td>
<td>391.7</td>
</tr>
<tr>
<td>Guanidinosuccinic acid</td>
<td>0.17 ± 0.06</td>
<td>37.14 ± 19.40</td>
<td>217.2</td>
<td>268.6</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>&lt;27.9</td>
<td>1379.9 ± 625.7</td>
<td>49.4</td>
<td>2631.3</td>
</tr>
<tr>
<td>Indole-3-acetic acid</td>
<td>0.1 ± 0.1</td>
<td>5000 ± 3200</td>
<td>50.0</td>
<td>51.9</td>
</tr>
<tr>
<td>Indoxyl Sulfate</td>
<td>2.4 ± 1.37</td>
<td>210.9 ± 72.1</td>
<td>88.6</td>
<td>1113.2</td>
</tr>
<tr>
<td>Methylguanidine</td>
<td>&lt;0.1</td>
<td>10.6 ± 6.97</td>
<td>106.0</td>
<td>24.9</td>
</tr>
<tr>
<td>Urea</td>
<td>&lt;6666.7</td>
<td>38333 ± 18333</td>
<td>5.7</td>
<td>76666.7</td>
</tr>
</tbody>
</table>
Figure 6.2 CYP3A4 mRNA expression in Huh7 cells treated with uremic toxins.

CYP3A4 mRNA expression in Huh7 cells treated with 0.1% DMSO, creatinine (2121.6 µM), p-cresyl sulfate (PCS, 186.1 µM), 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF, 391.7 µM), guanidinosuccinic acid (GSA, 268.6 µM), hippuric acid (HA, 2631.3 µM), indole-3-acetic acid (I3AA, 51.9 µM), indoxyl sulfate (IS, 1113.2 µM), methylguanidine (MG, 24.9 µM), urea (76.6 mM) and a cocktail of the above uremic toxins at their listed concentration in the absence of serum albumin for 24 hours. CYP3A4 mRNA expression was standardized to 18S rRNA and controls were arbitrarily defined as 100%. Results are presented as mean ± SEM, n = 3.
Figure 6.3 Huh7 CYP3A4 mRNA expression and cell viability in the presence of increasing indoxyl sulfate (IS) concentrations.

Indoxyl sulfate (IS) concentration-dependent (0-1000 µM) effect on CYP3A4 mRNA expression in Huh7 cells (A). MTT assay for Huh7 cell viability in the presence of 0-5000 µM IS (B). All treatments were performed in media supplemented with 40 g/L HSA for 48 hours. The grey solid area represent normal plasma IS concentrations mean (dashed line) ± 2SD. The grey-checkered area represents mean uremic concentration (dashed line) minus 2SD to the highest individual measured uremic concentration (Duranton et al., 2012). CYP3A4 mRNA expression was standardized to 18S rRNA and controls were arbitrarily defined as 100%. Results are presented as mean ± SEM, n ≥ 3, *p < 0.05 compared to control.
Table 6.2 Rat body weight and plasma biochemistry of 8 weeks after study initiation.

Data are represented as mean ± SEM, n = 10.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control +AST-120</th>
<th>CKD</th>
<th>CKD + AST-120</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (g)</strong></td>
<td>277 ± 12</td>
<td>265 ± 13</td>
<td>280 ± 6</td>
<td>277 ± 5</td>
</tr>
<tr>
<td><strong>Creatinine (µM)</strong></td>
<td>22.8 ± 0.6</td>
<td>23.3 ± 0.7</td>
<td>77.2 ± 8.1*</td>
<td>73.7 ± 6.7*</td>
</tr>
<tr>
<td><strong>Urea (mM)</strong></td>
<td>6.59 ± 0.47</td>
<td>6.55 ± 0.35</td>
<td>14.6 ± 1.61*</td>
<td>15.74 ± 3.46*</td>
</tr>
</tbody>
</table>

*p < 0.05 compared to control
Figure 6.4. Plasma indoxyl sulfate (A), p-cresyl sulfate (B), and hippuric acid (C) levels in control, control +AST-120, CKD, and CKD+AST-120 groups.

Plasma levels were measured at the end of the 8-week study. Results are presented as mean ± SEM, n = 10, *p < 0.05 compared to control.
toxins were not significantly increased in CKD rats given AST-120 compared to controls ($p < 0.05$, Fig. 6.4).

6.3.3 Hepatic CYP2C and CYP3A Enzyme Function

To evaluate hepatic CYP2C and CYP3A enzyme function, microsomal metabolism of the probe drugs, midazolam and testosterone, were used as previously described (Velenosi et al., 2012). Midazolam is converted to 1’OH and 4’OH-midazolam by CYP3A and testosterone is converted to 16αOH-testosterone by CYP2C11. Enzyme kinetics revealed a 65.8% and 60.8% decrease in 1’OH-midazolam Vmax for CKD and CKD+AST-120 groups, respectively, compared to control ($p < 0.05$, Fig. 6.5, Table 6.3). Similarly, 4’OH-midazolam Vmax was also significantly reduced by 86.7% and 84.6% for CKD and CKD+AST-120 groups, respectively, compared to control ($p < 0.05$, Fig. 6.5, Table 6.3). There were no significant differences in Vmax values between control and control+AST-120 groups or CKD and CKD+AST-120 groups. The Vmax values for testosterone metabolism to 16αOH-testosterone were significantly decreased by 67% and 62.5% for CKD and CKD+AST-120 groups, respectively, compared to control ($p < 0.05$, Fig. 6.5, Table 6.3). The Km values were unchanged between all groups for 4’OH-midazolam and 16αOH-testosterone; however, the Km for 1’OH-midazolam was significantly reduced in CKD and CKD+AST-120.

6.3.4 Hepatic CYP2C11 and CYP3A2 Protein and mRNA Expression

CYP3A2 protein expression was decreased 88.7% and 81.7% in CKD and CKD+AST-120 groups, respectively, compared to control (Fig. 6.6). CKD and CKD+AST-120 groups also had a 62.3% and 73.7% decrease in CYP2C11 protein expression respectively, compared to control (Fig. 6.6). CYP3A2 mRNA expression was significantly decreased in CKD rats by 91.4% compared to control; however, AST-120 recovered CYP3A2 mRNA expression by 25% in the CKD+AST-120 group which was not significantly different than control rats (Fig. 6.7). Hepatic CYP2C11 mRNA expression followed protein expression with a 71.6% and 61.7% decrease in CKD and CKD+AST-120 rats, respectively, compared to control (Fig. 6.7).
Figure 6.5 Michaelis-Menten kinetics of hepatic CYP3A and CYP2C drug metabolizing enzymes in control, control +AST-120, CKD and CKD+AST-120 rats.

Michaelis-Menten plots of 1’OH midazolam (A), 4’OH midazolam (B) and 16αOH-testosterone (C) in control (○), control +AST-120 (□), CKD (Δ) and CKD+AST-120 (▽) rats. Analysis was performed in duplicate and data is presented as mean ± SEM, n = 10.
Table 6.3 Michaelis-Menten kinetic values for P450 probe substrates in control, control+AST-120, CKD and CKD+AST-120 rat liver microsomes.

<table>
<thead>
<tr>
<th></th>
<th>1’OH-midazolam</th>
<th>4’OH-midazolam</th>
<th>16α-OH Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{max}$ (pmol min$^{-1}$ mg protein$^{-1}$)</td>
<td>$K_m$ (µM)</td>
</tr>
<tr>
<td>Control</td>
<td>7.62 ± 0.61</td>
<td>68.52 ± 9.66</td>
<td>4.828 ± 2.78</td>
</tr>
<tr>
<td>Control+AST-120</td>
<td>7.10 ± 0.94</td>
<td>56.75 ± 6.37</td>
<td>5.20 ± 0.73</td>
</tr>
<tr>
<td>CKD</td>
<td>2.97 ± 0.59*</td>
<td>23.40 ± 3.02*</td>
<td>4.71 ± 0.43</td>
</tr>
<tr>
<td>CKD+AST-120</td>
<td>3.01 ± 0.82*</td>
<td>26.85 ± 2.45*</td>
<td>4.51 ± 0.62</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM.

* $p < 0.05$ compared to control.
Figure 6.6 Protein expression of hepatic CYP2C11 (A) and CYP3A2 (B) in control, control +AST-120, CKD and CKD+AST-120 rats.

Control bands were arbitrarily defined as 100% and results are presented as mean ± SEM, n = 10, *p < 0.05 compared to control. Representative blots are shown.
Figure 6.7 mRNA expression of hepatic CYP2C11 (A) and CYP3A2 (B) in control, control +AST-120, CKD and CKD+AST-120 rats.

Control expression was defined as 100% and results are presented as mean ± SEM, n = 10, *p < 0.05 compared to control.
6.4 Discussion

In this report, we present an *in vitro* to *in vivo* study design to investigate uremic toxin downregulation of hepatic drug metabolizing enzymes in CKD. A screen of selected uremic toxins provided evidence for indoxyl sulfate downregulation of CYP3A4 in Huh7 cells. Further characterization of this effect was demonstrated with a concentration-dependent decrease in CYP3A4 mRNA expression using indoxyl sulfate concentrations found in patients with CKD. In vivo, removal of indoxyl sulfate and other gut-derived uremic toxins in rats with CKD provided a modest recovery of CYP3A2 mRNA expression. However, enzyme function and protein expression of CYP2C11 and CYP3A2 drug metabolizing enzymes were unaffected by administration of AST-120 to rats with CKD.

Uremic toxins screened in this study were chosen from meta-analysis reviews based on high plasma concentrations in CKD and fold change relative to normal kidney function (Table 6.1). These included the aromatic amino acid metabolites indoxyl sulfate, p-cresyl sulfate, and hippuric acid, which are increased in patient plasma as well as both plasma and liver tissue of animals with CKD. Although indoxyl sulfate, p-cresyl sulfate, and hippuric acid are protein-bound, in the absence of HSA, our initial screen demonstrated no effect of p-cresyl sulfate and hippuric acid on CYP3A4 mRNA expression in Huh7 cells. Indoxyl sulfate, however, caused a pronounced downregulation of CYP3A4 mRNA, which was similar to mRNA levels observed in the treatment combining all toxins. Consequently, this suggests that indoxyl sulfate caused downregulation of CYP3A4 both individually, and in the combined treatment. In the indoxyl sulfate concentration-dependent response, the range of indoxyl sulfate concentrations in normal plasma was defined within two standard deviations of the mean (0-5.09 µM). The range of indoxyl sulfate levels in patients with CKD was described as two standard deviations below the mean and the highest concentration published in a CKD patient (65.6-1113.2 µM). These concentration ranges are mutually exclusive and CYP3A4 mRNA expression was decreased only in the uremic range (Fig. 6.3). The IC$_{50}$ (113.0 µM) was also below the average indoxyl sulfate concentration measured in patients with CKD (210.9 µM, Table 6.1), indicating a physiologically relevant effect. This study is supported by a
previous study in CKD patients, which demonstrated a correlation between plasma indoxyl sulfate concentration and CYP3A4 enzyme function using the endogenous biomarker 4β-hydroxycholesterol (Suzuki et al., 2014). This biomarker is produced from CYP3A4 mediated cholesterol metabolism and is not cleared by the kidney (Diczfalusy et al., 2009). 4β-Hydroxycholesterol also has a 17-day half-life and can assess steady-state CYP3A4 function regardless of dialysis frequency. Although indoxyl sulfate and indole-3-acetic acid plasma concentrations are strongly correlated with eGFR in CKD, indole-3-acetic acid was not correlated with 4β-hydroxycholesterol plasma levels (Niwa and Ise, 1994; Suzuki et al., 2014). Taken together, our in vitro data and the previous clinical study suggest that indoxyl sulfate contributes to the down-regulation of human hepatic CYP3A4 in CKD.

Although we demonstrate CYP3A down-regulation by a single uremic toxin, the in vivo condition of uremia is highly complex. Therefore, to assess the effect of gut-derived uremic toxins, such as indoxyl sulfate, on hepatic CYP3A and CYP2C enzyme function and expression in vivo, we used a rat model of adenine-induced kidney disease and subsequently administered AST-120. Both CKD and CKD+AST-120 groups had similar levels of plasma creatinine, suggesting that AST-120 did not affect renal function following the adenine-induction of CKD (Table 6.2). The use of AST-120 in rodent models of CKD has been previously shown to reduce levels of indoxyl sulfate, p-cresyl sulfate and hippuric compared to CKD rats that do not receive the compound (Shimoishi et al., 2007; Inami et al., 2014). However, in many of these studies, the severe nature of the CKD results in significantly higher levels of indoxyl sulfate, p-cresyl sulfate and hippuric in CKD+AST-120 rats when compared to controls. Therefore, in this study, we developed a model of adenine-induced CKD with less severe kidney dysfunction. Using this model in combination with AST-120, we were able to decrease plasma gut-derived uremic toxin levels similar to those measured in control animals.

Altered hepatic drug metabolism has been described in a number of studies in CKD (Leblond et al., 2000; Leblond et al., 2001; Michaud et al., 2005). Initial studies identified decreased hepatic CYP3A enzyme function using the erythromycin breath test (Leblond et al., 2000; Leblond et al., 2001). More recently, using both midazolam and
testosterone probe drugs, our group demonstrated an inverse exponential correlation between kidney function and CYP2C11 as well as CYP3A2 enzyme activity in rats with CKD (Chapter 3). In the current study, rats with a similar degree of CKD had decreased CYP2C11 and CYP3A2 enzyme function that was unaffected by the administration of AST-120. Protein expression of CYP2C11 and CYP3A2 were also unaffected by AST-120 in rats with CKD; however mRNA levels of CYP3A2 were increased 25% in the CKD+AST-120 group compared to CKD. CYP3A2 mRNA expression is diminished to a greater degree than protein expression and enzyme function are in CKD. This is supported by previous studies, suggesting a transcriptional mechanism of downregulation (Leblond et al., 2001; Michaud et al., 2006; Velenosi et al., 2012; Velenosi et al., 2014). The mechanism of CYP3A2 downregulation has been attributed to decreased PXR, HNF-4α recruitment and histone H3 and H4 acetylation in CKD (Chapter 4). Therefore, removal of gut-derived toxins using AST-120 may moderately recover CYP3A2 mRNA expression through nuclear receptor and epigenetic regulatory mechanisms; however, this effect did not translate into recovered protein expression and enzyme function.

Although many of the same nuclear receptors regulate human and rat CYP3A drug metabolizing enzymes, there are distinct differences in nuclear receptor homology and promoter binding sites between species. CYP3A4 has both proximal and distal nuclear receptor binding regions, unlike CYP3A2, which is only regulated at a proximal binding region (Huss et al., 1999; Tirona et al., 2003). The HNF-4α homodimer is highly conserved and essential for basal transcription of both CYP3A2 and CYP3A4 (Miyata et al., 1995; Tirona et al., 2003). Contrary to rat CYP3A2, which has a proximal HNF-4α binding site, HNF-4α enhances PXR binding to the distal CYP3A4 promoter in humans (Tirona et al., 2003). The DNA binding domain of PXR is also well conserved between rats and humans (95.5% identical); in contrast, the ligand-binding domain amino acid sequence is only 75.9% identical (Jones et al., 2000). As a result, the prototypical human PXR ligand, rifampicin, only moderately activates rat PXR. Conversely, pregnenolone-16α-carbonitrile is a potent activator of rodent PXR and not human PXR (Tirona et al., 2004). Despite the broad substrate specificity of the PXR ligand-binding domain, antagonists have been identified and include polychlorinated biphenyls and azoles (Tabb et al., 2004; Ekins et al., 2007). Accordingly, it is possible that indoxyl sulfate is an
antagonist for human PXR and not for rat PXR. Therefore, CYP3A transcriptional regulation and PXR ligand-binding domain differences may account for the disparity in results between species; however, further study is necessary to support these claims.

Hepatic CYP enzyme expression is also altered during infection and inflammation. Adenovirus infection downregulates hepatic CYP2C11 and CYP3A2 resulting in decreased enzyme activity (Callahan et al., 2005). Lipopolysaccharide treated rats in acute models of inflammation demonstrate decreased function and activity of these enzymes (Cheng et al., 2003). CYP2C11 and CYP3A2 expression are also downregulated as a result of secondary inflammation in ulcerative colitis (Masubuchi et al., 2008). Systemic inflammation occurs secondary to CKD. ESRD patients have increased plasma levels of CRP as well as pro-inflammatory cytokines including TNFα and IL-6, which have been shown to downregulate rat CYP2C11 and CYP3A2 in vitro and in vivo (Morgan et al., 1994; Morgan, 1997; Duranton et al., 2012; Hsu et al., 2014). In ESRD patients, CRP is correlated with alprazolam 4-hydroxylation, which is marker of CYP3A4 activity (Molanaei et al., 2012). Pro-inflammatory cytokines can activate NF-κB, which binds and inhibits the PXR/RXR nuclear receptor heterodimer from transcriptional activation (Gu et al., 2006). Treatment of primary rat hepatocytes with uremic serum results in NF-κB-mediated downregulation of CYP2C11 and CYP3A2 (Michaud et al., 2008). AST-120 has been shown to attenuate systemic inflammation and reduce plasma levels of CRP, TNFα, and IL-6 in rats with CKD (Vaziri et al., 2013). Therefore, elevated circulating levels of other pro-inflammatory cytokines or other pathways activating NF-κB may be the cause of CYP2C11 and CYP3A2 downregulation.

In summary, indoxyl sulfate caused downregulation of CYP3A4 at uremic concentrations in a human hepatoma cell line. Our experiments in Huh7 cells are supported by clinical evidence correlating decreased CYP3A4 function with plasma indoxyl sulfate levels (Suzuki et al., 2014). However, there is more evidence in the literature to suggest that indoxyl sulfate may be one of many factors in the complex uremic milieu that cause downregulation of hepatic CYP2C and CYP3A in CKD. In contrast, removal of gut-derived uremic toxins, including indoxyl sulfate, did not recover function and expression
of CYP2C11 and CYP3A2 in rats with CKD. Therefore, gut-derived uremic toxins do not cause downregulation of hepatic CYP2C11 and CYP3A2 in rats with CKD.
6.5 References


7 DISCUSSION AND CONCLUSIONS
7.1 Summary and Discussion

7.1.1 Chapter 3
Expression and activity of drug metabolizing enzymes are decreased in severe kidney disease, however, only a small percentage of patients with CKD are at the final stage of the disease. The aim of Chapter 3 was to determine the changes in drug metabolizing enzyme function and expression in rats with varying degrees of kidney disease. Sprague-Dawley rats were subjected to surgical procedures that allowed the generation of three distinct models of kidney function: normal kidney function, moderate and severe kidney disease. CKD was developed over 6 weeks and hepatic CYP3A and CYP2C mRNA and protein expression was determined. In addition, enzymatic activity was determined in liver microsomes by evaluating midazolam (CYP3A), testosterone (CYP3A and CYP2C) and tolbutamide (CYP2C) enzyme kinetics. I hypothesized that induction of moderate and severe kidney disease will produce a corresponding decrease in liver CYP2C11 and CYP3A2 function and expression using rat partial nephrectomy models. Both moderate and severe kidney disease were associated with a reduction in CYP3A2 and CYP2C11 expression ($p < 0.05$). Similarly, moderate kidney disease resulted in more than a 60% decrease in enzyme activity ($V_{\text{max}}$) for CYP2C11 and CYP3A compared to controls ($p < 0.05$). Our results demonstrate that CYP3A and CYP2C expression and activity are decreased in both moderate and severe CKD. When the degree of kidney disease was correlated with metabolic activity, an exponential decline in CYP2C and CYP3A mediated metabolism was observed. Our hypothesis was supported by the negative correlation between the degree of CKD and hepatic CYP2C and CYP3A function and expression; however, surprisingly, the negative association was exponential. Therefore, this Chapter suggests that drug metabolism is significantly decreased in earlier stages of CKD, which supports the overall hypothesis of this thesis. The data also imply that patients with moderate CKD may be subject to unpredictable pharmacokinetics.
Patients with CKD require many medications and CYP2C and CYP3A drug metabolizing enzymes play a critical role in determining the pharmacokinetics of almost half of all prescribed medications. These enzymes are transcriptionally regulated by the nuclear receptors PXR and HNF-4α. In Chapter 4, we aimed to determine the transcriptional mechanism of hepatic CYP2C11 and CYP3A2 downregulation in CKD. CKD was induced in rats by 5/6 nephrectomy and we used chromatin immunoprecipitation to determine nuclear receptor and epigenetic mediated differences in the promoter region of the CYP2C and CYP3A genes. Transcriptional activation of PXR is associated with PRMT1 H4R3 asymmetric dimethylation. In this reaction, s-adenosyl methionine is used as a methyl donor to produce H4R3me2 and s-adenosyl homocysteine (SAH) is released (Osborne et al., 2007). SAH is subsequently converted to homocysteine. Both SAH and homocysteine can inhibit PRMT1 methyltransferase activity (Osborne et al., 2007). Homocysteine levels are increased in CKD, which suggested that PRMT1 methyltransferase inhibition may be responsible for decreased P450 transcriptional activation in CKD. I hypothesized that altered drug metabolism in CKD is secondary to decreased nuclear receptor binding to the promoters of CYP3A as a result of histone modulation. RNA Pol II and HNF-4α binding was decreased 76% and 57% in the CYP2C11 and 71% and 77% in the CYP3A2 promoter, respectively ($p < 0.05$). ChIP also revealed a 57% decrease in PXR binding to the CYP3A2 promoter in CKD rats ($p < 0.05$). The decrease in PXR and HNF-4α binding was accompanied by diminished histone 4 acetylation in the CYP3A2 promoter (48%) and histone 3 acetylation in the CYP2C11 (77%) and CYP3A2 (77%) promoter loci for nuclear receptor activation ($p < 0.05$). However, markers of histone methylation, including H4R3me2, were unaltered in the CYP3A promoter. Therefore, PRMT1 methyltransferase inhibition in CKD does not mediate decreased transcriptional activation of CYP3A. Conversely, it is possible that PXR and HNF-4α nuclear receptor binding is inhibited in CKD or that histone deacetylation may be increased in CKD resulting in a transcriptionally silent state of CYP2C11 and CYP3A2. This study suggests decreased nuclear receptor binding and histone acetylation may contribute to the mechanism of drug metabolizing enzyme downregulation and altered pharmacokinetics in CKD. These results support the overall
hypothesis; however, the interplay between altered nuclear receptor binding and histone modulation in CKD remains to be determined.

7.1.3 Chapter 5

Uremia is the accumulation of metabolic waste products that are normally cleared by the kidney in CKD. Many of these waste products are derived from bacteria metabolites in the gut. Accumulation of uremic toxins in plasma and tissue, as well as the gut-plasma-tissue metabolic axis is important for understanding pathophysiological mechanisms of comorbidities in CKD. In Chapter 5, an untargeted metabolomics approach was used to determine uremic toxin accumulation in plasma, liver, heart and kidney tissue in rats with CKD. Rats with CKD were also given AST-120, a spherical carbon adsorbent, to assess metabolic changes in plasma and tissues with the removal of gut-derived uremic toxins. I hypothesized that gut-derived uremic toxins accumulate in both plasma and tissue in CKD. AST-120 decreased >55% of metabolites that were increased in plasma, liver and heart tissue of rats with CKD. CKD was primarily defined by 8 gut-derived uremic toxins, which were significantly increased in plasma and all tissues. These metabolites were derived from aromatic amino acids and soy protein including: indoxyl sulfate, p-cresyl sulfate, hippuric acid, phenyl sulfate, pyrocatechol sulfate, 4-ethylphenyl sulfate, p-cresol glucuronide and equol 7-glucuronide. Gut-derived metabolites also represented the most significant metabolic signatures describing CKD in all tissues supporting the hypothesis. Patients with CKD have increased levels of aromatic amino acid metabolites; however, soy protein metabolites such as 4-ethylphenyl sulfate and equol 7-glucuronide have not been studied in these patients. In the Western world, soy protein is rarely the main protein source for the majority of the population. This suggests that enterotype may determine the accumulation of gut-derived metabolites in CKD. An enterotype is the classification of bacteria in the gut microbiome (Wu et al., 2011). The Bacterioides enterotype is associated with the “Western Diet” of animal protein and saturated fats. In contrast, the Prevotella enterotype is associated with high carbohydrates and agrarian societies (Wu et al., 2011). Therefore, accumulation of gut-derived uremic toxins may be dependent on patient enterotypes. This study highlights the importance of diet and gut-
derived metabolites in the accumulation of uremic toxins and defines the gut-plasma-tissue metabolic axis in CKD.

7.1.4 Chapter 6

The aim of Chapter 6 was to determine which uremic toxins(s) cause downregulation of hepatic CYP2C and CYP3A in CKD. We utilized an *in vitro* to *in vivo* study design with human Huh7 cells and rats with adenine-induced CKD. Initially, uremic toxins that are highly increased in CKD were screened for the potential to cause CYP3A4 downregulation. Indoxyl sulfate was shown to downregulate CYP3A4 at physiologically relevant plasma concentrations. In Chapter 5, we demonstrated that gut-derived uremic toxins accumulate in both plasma and liver of rats with adenine-induced CKD. Therefore, in this Chapter, I hypothesized that the removal of gut-derived uremic toxins would recover expression and function of hepatic CYP2C and CYP3A in rats with CKD. Administration of AST-120 to rats with CKD reduced indoxyl sulfate, p-cresyl sulfate and hippuric acid plasma levels similar to levels measured in control rats. Liver levels are well correlated with plasma levels of these toxins, suggesting that hepatic accumulation is also decreased by AST-120 (Chapter 5). AST-120 recovered CYP3A2 mRNA expression by 25% in rats with CKD; however, AST-120 did not affect CKD mediated decreases in CYP3A2 protein expression or function. CYP2C11 expression and function was also decreased in CKD and unaffected by AST-120 administration. This result was unexpected and not consistent with the *in vitro* data and other studies. Clearly, there are a number of potential uremic mediators that may influence expression of hepatic P450, which cannot be entirely assessed *in vitro*. There are also species differences in the regulation of hepatic P450s, which may account for altered regulation in CKD. Therefore, it is possible that only human CYP3A4 is downregulated by indoxyl sulfate in CKD; however, there is more evidence to suggest that other factors in the complex uremic milieu may be contributing to the downregulation of hepatic CYP2C and CYP3A in CKD. In conclusion, indoxyl sulfate caused CYP3A4 downregulation in Huh7 cells *in vitro*. Conversely, gut-derived uremic toxins do not affect CYP3A and CYP2C function and expression in rats with CKD.
7.2 Models of CKD

In this thesis, a combination of partial nephrectomy and vessel ligation models as well as the adenine model were used to generate varying degrees of CKD. The 2/3 nephrectomy resulted in the most mild form of CKD with a 1.65-fold increase in plasma creatinine levels compared to control (Table 7.1). The 2/3 and 5/6 nephrectomy as well as the use of 0.5% adenine resulted in similar decreases of hepatic CYP2C11 and CYP3A2 enzyme function and expression despite the plasma creatinine levels spanning a 2-fold range. Although the 0.5% adenine model resulted in more severe CKD than the 5/6 nephrectomy according to plasma creatinine, the CYP3A2 mRNA expression levels were higher than 0.5% adenine model. The 2/3 nephrectomy with vessel ligation showed further progression of CKD severity and greater decreases in CYP2C11 and CYP3A2 function and expression. The 0.7% adenine model was the most severe with the greatest increase in plasma creatinine levels.

As mentioned in the introduction (1.9), the adenine model of CKD is more consistent than the use of partial nephrectomy models and does not require surgical expertise. The degree of CKD is also adenine dose-dependent, allowing for more control over the severity of CKD by varying the amount of adenine in the rat diet. Although different models of CKD were utilized, the degree of hepatic CYP2C11 and CYP3A2 downregulation was consistent with the degree of CKD, regardless of the experimental model.
Table 7.1 Hepatic CYP2C11 and CYP3A2 enzyme function and expression in rats with CKD using various experimental models.

<table>
<thead>
<tr>
<th></th>
<th>Plasma Creatinine (µM)</th>
<th>Plasma Urea (mM)</th>
<th>CYP2C11 Function and Expression (% of Control)</th>
<th>CYP3A2 Function and Expression (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vmax (16αOH-testosterone) Protein mRNA</td>
<td>Vmax (1-OH Midazolam) Protein mRNA</td>
</tr>
<tr>
<td>Control (mean of all studies)</td>
<td>23</td>
<td>6.35</td>
<td>100 100 100</td>
<td>100 100 100</td>
</tr>
<tr>
<td>2/3 nephrectomy</td>
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<td>9.1</td>
<td>33.4 59 23</td>
<td>37.7 25 12</td>
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<tr>
<td>5/6 nephrectomy</td>
<td>63</td>
<td>12.0</td>
<td>32 34.4 29.5</td>
<td>22 11.2 2.2</td>
</tr>
<tr>
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<td>14.6</td>
<td>33 37.7 28.4</td>
<td>34.2 11.3 8.6</td>
</tr>
<tr>
<td>2/3 nephrectomy with vessel ligation</td>
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<td>23.35</td>
<td>28.4 32 5</td>
<td>17.8 9 0.4</td>
</tr>
<tr>
<td>0.7% Adenine</td>
<td>181</td>
<td>44.9</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM.

\*p < 0.05 compared to control.
7.3 Therapeutic Implications

Chronic kidney disease is a complex disorder that is associated with multiple comorbidities. The life expectancy of a hemodialysis patient has been estimated to be between 3-5 years. In order to treat co-morbidities and attempt to improve quality of life and life expectancy, patients with kidney disease are prescribed an average of 12 medications, which increases the risk of drug-drug interactions. In addition, it is clear that significant drug accumulation occurs in dialysis patients, which also places patients at an increased risk for adverse medication reactions.

Although it has long been appreciated that hemodialysis patients do not have renal drug excretion capacity, emerging studies have clearly documented that hepatic clearance of many drugs is decreased in kidney disease. Studies in both animal models of kidney disease and clinical pharmacokinetic studies in human dialysis patients have suggested that kidney disease decreases hepatic metabolism. In this thesis, we demonstrate that drug metabolism is decreased in moderate CKD likely as a result of decreased PXR and HNF-4α binding as well as histone acetylation using a rat model. We also provide evidence of the uremic toxin, indoxyl sulfate, mediating downregulation of CYP3A4. Although these studies and others contribute to the possible mechanisms of decreased hepatic drug metabolism in CKD, the specific metabolic pathways that are uniformly altered in human patients with CKD are highly complex and unknown. This is best exemplified by a recent FDA review of new molecular entities, where only approximately 50% of approved molecules metabolized by CYP3A4 and CYP2C9 exhibit altered pharmacokinetics (Zhang et al., 2009). Therefore, pathway specific generalizations regarding the impact of kidney disease on drug metabolism should be avoided and all drugs that may be used in patients with kidney disease should undergo rigorous pharmacokinetic evaluation in this population.

It is evident that regulatory bodies such as the FDA have begun to realize the important changes experienced in non-renal clearance pathways in dialysis patients. Indeed, the guidance document published in 1998 was updated in 2010 to reflect the progress in research investigating pharmacokinetics in kidney disease. Multiple studies have shown that the hemodialysis process itself can impact the inter-dialytic and intra-dialytic...
pharmacokinetics of drugs (Osborne et al., 1986; Amin et al., 1999; Bezzaoucha et al., 2014). Accordingly, clinical pharmacokinetic studies should be used to determine optimal dosing for patients across the spectrum of CKD and those in ESRD who require hemodialysis.

When encountering a dialysis dependent or independent kidney disease patient, clinicians should first search for evidence of drug dosing recommendations in these populations. Indeed, dosage adjustments in renal failure may be found in the product monograph and have been the subject of several peer-reviewed articles (Lam et al., 1997; Gabardi and Abramson, 2005; Munar and Singh, 2007; Brater, 2009; Verbeeck and Musuamba, 2009; Matzke et al., 2011). When specific pharmacokinetic data in kidney disease are not available, the clinician must evaluate all known information on the pharmacokinetics of the drug, what processes are likely to be altered in renal failure (e.g. renal excretion, hepatic clearance) and prescribe a dosage that is supported by available evidence.

7.4 Future Directions

In Chapters 3 and 4, we demonstrate downregulation of hepatic CYP2C and CYP3A drug metabolizing enzymes in moderate CKD and elucidate the possible mechanisms leading to downregulation of these enzymes using rat models of CKD. Rat models have been used in the majority of studies describing hepatic downregulation of CYP2C and CYP3A in CKD. However, there are differences in nuclear receptor homology and binding regions between rodents and humans that need to be addressed. In Chapter 6, indoxyl sulfate mediated CYP3A4 downregulation in human Huh7 cells but had a minimal effect on rat CYP3A2 in vivo suggesting that these enzymes may be regulated differently between species in CKD. Recently, the development of humanized PXR/CAR/CYP3A4 mice facilitate assessment of PXR-mediated induction of CYP3A4 in vivo (Hasegawa et al., 2011). Future studies in humanized mice may be useful for evaluating changes in the CYP3A4 epigenetic environment that occur as a result of CKD.
The endogenous cholesterol metabolite, 4β-hydroxycholesterol, is a marker of CYP3A4 function. A recent study demonstrated a negative correlation between plasma indoxyl sulfate concentrations and 4β-hydroxycholesterol levels (Suzuki et al., 2014). In Chapter 6, we provide evidence of CYP3A4 downregulation when treating Huh7 cells with indoxyl sulfate concentrations found in patients with CKD. Our preliminary work suggests the need to further assess the effects of indoxyl sulfate on hepatic CYP3A4 in a clinical study evaluating 4β-hydroxycholesterol plasma levels before and after prescribing AST-120 to patients with CKD. This study would be challenging to complete, as AST-120 is currently not approved for patient use in Canada.

Finally, uremic toxins are likely responsible for altered function of hepatic CYP2C and CYP3A in CKD. In this thesis, we employed a top-down approach, beginning with the assessment of CYP2C and CYP3A function followed by protein and mRNA expression (Chapter 3). We then assessed changes in transcriptional activation by evaluating nuclear receptor binding as well as epigenetic modifications in the CYP2C and CYP3A promoters (Chapter 4). In Chapter 5, we present the metabolic changes and uremic toxin accumulation in the liver as a result of CKD using untargeted metabolomics. We also provide evidence of indoxyl sulfate mediated downregulation of CYP3A4 (Chapter 6). As a result, future studies are necessary to understand the possible signaling pathways involved in uremic toxin downregulation of CYP2C and CYP3A, including their influence on nuclear receptor activation and epigenetic mediators. The interplay between nuclear receptors and epigenetic mediators also requires further insight in order to understand the sequence of events that result in decreased transcriptional activation in CKD.

7.5 Conclusions

CKD and ESRD patients take many medications to manage the disease along with co-morbidities. It is well known that renal clearance of drugs is compromised in CKD; however, altered non-renal clearance in CKD has more recently been recognized. The majority of drugs are eliminated by metabolism and the focus of this thesis was to
evaluate hepatic drug metabolism in CKD. The first section aimed to assess function, expression and regulation of hepatic drug metabolizing enzymes in CKD. We determined that hepatic CYP2C and CYP3A function and expression were decreased in moderate and severe CKD as a result of decreased nuclear receptor binding and histone acetylation in the promoter of these enzymes.

The second part of this thesis focused on identifying which uremic toxin(s) mediate the downregulation of these CYP2C and CYP3A in CKD. We assessed the accumulation of uremic toxins as well as the metabolic changes that occur in both plasma and liver for rats with CKD using untargeted metabolomics. This resulted in the identification of gut-derived uremic toxins as the most concentrated metabolites present in both plasma and tissues of rats with CKD. AST-120 administered to rats with CKD reduced plasma and liver levels of these toxins confirming that they were gut derived. Indoxyl sulfate, a gut-derived uremic toxin, mediated downregulation of CYP3A4 at concentrations measured in CKD patient plasma; however, removal of gut-derived uremic toxins did not recover expression of CYP2C and CYP3A in rats with CKD. Taken together, these studies provide insight into the mechanisms of altered hepatic drug metabolism in CKD and support the pharmacokinetic evaluation of new drugs in this patient population.
7.6 References


Appendices
Appendix A: Ethics Approval

September 10, 2009

*This is the Original Approval for this protocol*
*A Full Protocol submission will be required in 2013*

Dear Dr. Urquhart:

Your Animal Use Protocol form entitled:
The Effect of Kidney Failure and Kidney Transplantation on the Expression and Activity of Drug Metabolizing Enzymes and Drug Transport Proteins
Funding Agency: UWO Startup/NSERC Applied For

has been approved by the University Council on Animal Care. This approval is valid from September 10, 2009 to September 30, 2010. The protocol number for this project is #2009-055.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
4. If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.

ANIMALS APPROVED FOR 4 Years

<table>
<thead>
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<th>SPECIES &amp; SECT D.5.1 GROUP ID#</th>
<th>STRAIN &amp;/or OTHER SPECIES DETAIL For Rodents, Also Provide Vendor Stock #</th>
<th>AGE or WEIGHT &amp; SEX</th>
<th>4-YEAR TOTAL ANIMAL NUMBER</th>
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<td>780</td>
</tr>
<tr>
<td>As Above ID# 37</td>
<td>Wistar</td>
<td>200-300g, male</td>
<td>120</td>
</tr>
</tbody>
</table>

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol - B. Urquhart, W. Lagerwerf
Approval Letter - B. Urquhart, W. Lagerwerf
Appendix B: Copyright Approval

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10/23/2015

Thomas J Velenosi, BMSc. (Hon.)
PhD Candidate

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Pharmacokinetic considerations in chronic kidney disease and patients requiring dialysis
Expert Opinion on Drug Metabolism & Toxicology 10 (8): 1131-1143.
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Thomas J. Velenosi, Angel Y. N. Fu, Shuhua Luo, Hao Wang, and Bradley L. Urquhart, Down-Regulation of Hepatic CYP3A and CYP2C Mediated Metabolism in Rats with Moderate Chronic Kidney Disease, Drug Metab Dispos August 2012 40:1508-1514; doi:10.1124/dmd.112.045245

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Regards,

Thomas J Velenosi, BMSc. (Hon.)
PhD Candidate
Appendix C: Supplementary Information

**Supplementary Table C1.** UPLC gradient conditions for drug metabolite quantification. Solvents are expressed as mobile phase percentage.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>10 mM KH$_2$PO$_4$ (pH = 3.0)</th>
<th>Acetonitrile</th>
<th>Methanol</th>
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**Supplementary Figure C1** Specificity of anti-rat P450 antibodies tested on recombinant cDNA-expressed P450 enzyme isoforms prepared from the baculovirus-infected insect cell system (BD Supersomes™). Western blots were performed with 2 µg of each Supersome™ preparation.
Supplementary Figure C2  Hepatic mRNA expression of protein arginine methyltransferase 1 (PRMT1) in control rats and rats with CKD. Results are presented as mean ± SEM, n = 6.
**Supplementary Figure C3** Principal component analysis of Control (■), CKD (●), CKD+AST-120 (▲) and pooled samples in negative ESI mode for plasma (A), liver (B), heart (C) and kidney (D) tissue in rats. Triplicate injections are shown.
Supplementary Figure C4 Principal component analysis of Control (■), CKD (●), CKD+AST-120 (▲) and pooled samples in positive ESI mode for heart (A) and kidney (B) tissue in rats. Triplicate injections are shown.
Supplementary Figure C5. Correlations between plasma and tissues for 8 gut-derived uremic toxins.

Plasma levels of 4-ethylphenyl sulfate (A), phenyl sulfate (B), indoxyl sulfate (C), p-cresyl sulfate (D), hippuric acid (E), equol 7-glucuronide (F), pyrocatechol sulfate (G) and p-cresyl glucuronide (H) were correlated to liver ($\circ$), heart (□) and kidney ($\Delta$) levels for control, CKD and CKD+AST-120 groups. Linear regressions (solid lines) are presented with 95% confidence intervals (dashed lines).
### Supplementary Table C2: Summary of metabolites altered in plasma, liver, heart and kidney tissues in rats with CDAD vs. control and CDAD vs. 120 Metabolite ID, Level Change compared to Control Mass Empirical Formula Error Identity Tissue Direction Splot VP Fold P-value Direction Fold P-value

<table>
<thead>
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<th>Ion f2 (m/z)</th>
<th>Mass (ppm)</th>
<th>Mass Error (ppm)</th>
<th>Identity</th>
<th>Tissue</th>
<th>Direction</th>
<th>Splot VP</th>
<th>Fold</th>
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<td>3.01E-08</td>
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<td>246.1712</td>
<td>C12H11N2O4[H+]</td>
<td>2.8</td>
<td>2-methylbutyrylcarnitine or pivaloylcarnitine</td>
<td>Heart</td>
<td>Kidney</td>
<td>∪</td>
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<td>0.26</td>
<td>3.56E-10</td>
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<tr>
<td>56</td>
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<td>424.3431</td>
<td>C25H46NO4[H+]</td>
<td>0.9</td>
<td>Linoleyl or Linoleyl Carnitine</td>
<td>Heart</td>
<td>Kidney</td>
<td>∪</td>
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<td>262.1661</td>
<td>C12H20O9N[H+]</td>
<td>2.7</td>
<td>3-Hydroxyisovaleryl carnitine</td>
<td>Heart</td>
<td>Kidney</td>
<td>∪</td>
<td>6.32</td>
<td>0.42</td>
<td>3.56E-10</td>
</tr>
</tbody>
</table>
Curriculum Vitae

Name: Thomas J Velenosi

Post-secondary	University of Western Ontario
Education and	London, Ontario, Canada
Degrees: 2010-2015 Ph.D.
Pharmacology and Toxicology

The University of Western Ontario
London, Ontario, Canada
2006-2010 BMSc

Honours and Awards:

1. 2015 – ASN Kidney STARS Program Award - American Society of Nephrology Kidney Students and Residents (STARS) program award recipient (travel award).

2. 2015 - Rhoderic Reiffenstein Trainee Presentation Award – Canadian Society of Pharmacology and Therapeutics student oral presentation session.


4. 2014 – Physiology and Pharmacology Graduate Student Council Leadership Award.


10. 2013 – First place in London Health Research Day Platform Presentations

11. 2013 – Graduate Thesis Research Award. (travel award)
13. 2012 – First place in London Health Research Day Platform Presentations

**Related Work**

**Teaching Assistant**

The University of Western Ontario
2011-2015

**Lab Research Assistant**

Supervisor: Mary Jeanne Edgar
Department of Nephrology
University Hospital
London, Ontario
2012-2014

Lab Research Assistant
Supervisor: Dr. Dave Freeman
Department of Medicine – Division of Clinical Pharmacology
University Hospital
London, Ontario
2008-2009

**Publications:**


Presentations:

Invited presentations

Abstracts Presented as Oral Presentations at National and International Conferences

Abstracts Presented as Posters at National and International Conferences


