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Hepatic Drug Metabolism, Uremic Toxins and Bacterial Composition Over Chronic Kidney Disease Progression

Emily Dee Hartjes
The University of Western Ontario

Supervisor
Dr. Brad Urquhart
The University of Western Ontario

Graduate Program in Physiology and Pharmacology

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Abstract

Uremic toxin retention and an altered gut microbiota are suspected to influence cytochrome P450s (CYPs) contributing to the unpredictable pharmacokinetics in chronic kidney disease (CKD). We aim to characterize dysbiosis and uremia to elucidate associations between CYP expression and CKD progression. Rats fed control or CKD-inducing diet were subsequently sacrificed across five time points over 42 days. CYP expression and activity were compared to alterations in the 1) plasma and liver metabolome and 2) bacterial microbiota. CYP3A2 and CYP2C11, respectively, were downregulated in CKD by ≥76% (p<0.001) simultaneously or slightly premature to CKD onset defined by creatinine. Metabolite profiles were altered before the gut microbiota and gut-derived uremic toxins including indoxyl sulfate, phenyl sulfate and 4-ethylphenyl sulfate correlated with CYP3A2 or CYP2C11. Identified bacterial genera, Turicibacter and Parabacteroides, characterized CKD and require future study. In conclusion, CYP3A2 and CYP2C11 are downregulated prior to dysbiosis but correlate with select uremic toxins.

Keywords

Uremia, gut microbiota, dysbiosis, cytochrome P450, metabolomics, sequencing
Acknowledgments

This project might not have been about chronic kidney disease if Dr. Brad Urquhart had not so eagerly agreed to have me join the lab. Your enthusiasm about research and teaching has made the lab an enjoyable place of discovery for all your students. Your unwavering support and guidance has never once allowed me to feel like I was pressured, alone or unable to achieve something. This is in part due to your presence in and out of the lab, which is not only impressive but extremely helpful when obstacles like a Mass Spec error seem pitted against you. It is also because you value equality, allowing students to form their own opinions and become independent voices in the scientific community. Even though my aspirations are taking me off the academia path, for now, you have taught me more than how to test hypotheses and be successful in research. You have been a role-model of work-life balance, networking skills I never knew possible, and professionalism while still being human. I truly thank you for all the time and effort you have invested in me.

Next, I would like to thank my lab family whom I’ve grown very close with over the last year. As I start a new stage in my life, I realize how truly sad I am to be leaving my closest friends. Each of you have taught me so much about friendship, life and science while supporting an amazing place to express ourselves and feel included while at work. I wish you all the best in finding your passion and continue enjoying what you do.

Tom Velenosi requires a shout-out for trying his best to integrate me into the previously male dominated lab group. Thank you for not just showing me the ropes, but also for truly caring about my work’s integrity, time spent making mistakes and the overall learning experience. As I go on to teach others, I aim to be as thorough in my explanations and as honest about conclusions as you were with me.

Lastly, the support and understanding I received from my family and other-half Kyle Rupay, has allowed me to overcome the unavoidable stresses of graduate school. Simple gestures like making dinner or telling me to stop doubting myself, made my time out of the lab a de-stressing environment. Down-time spent with you made getting back to work, a challenge rather than a chore.
Ultimately, I want to thank all of those who contributed to my time at Western, in and out of research, that have helped me grow, nail down my values and provide direction for my future.
Author Contributions

Study design and experiments including animal husbandry, sample collection, sample preparation, drug metabolizing enzyme quantification, UPLC/MS operation and DNA extraction for bacterial sequencing was performed by the author Emily Hartjes alongside processing metabolomic and sequencing data, statistical analysis and thesis writing. In-house metabolomics R script was designed by Dr. Thomas Velenosi. Validations of qPCR primers, western blotting antibodies and testosterone assay concentrations were conducted previously by Dr. Thomas Velenosi and Dave Feere. Knowledge and mentorship with respect to data processing of bacterial sequence reads, ALDEEx2 analysis and interpretation of microbiota results was provided by Dr. Greg Gloor. Andrew Kucey assisted with animal care and sample collection. Dr. Jeremy Burton and Kait Al provided DNA extraction assistance and materials. Dr. Brad Urquhart assisted in study design, thesis editing and general supervision.
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<td>Acute Kidney Injury</td>
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<tr>
<td>ADE</td>
<td>Adverse Drug Event</td>
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<tr>
<td>ADR</td>
<td>Adverse Drug Reaction</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>ADH</td>
<td>Antidiuretic Hormone</td>
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<td>ABC</td>
<td>ATP Binding Cassette</td>
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<td>ANP</td>
<td>Atrial Natriuretic Peptide</td>
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<td>BCA</td>
<td>Bicinchoninic Acid</td>
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<td>CVD</td>
<td>Cardiovascular Disease</td>
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<td>CKD</td>
<td>Chronic Kidney Disease</td>
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<tr>
<td>CKD-EPI</td>
<td>Chronic Kidney Disease Epidemiology Collaboration</td>
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<tr>
<td>CAR</td>
<td>Constitutive Androstane Receptor</td>
</tr>
<tr>
<td>CONT</td>
<td>Control</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P-450</td>
</tr>
<tr>
<td>POR</td>
<td>Cytochrome P450 Oxidoreductase</td>
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<tr>
<td>DN</td>
<td>Diabetic Nephropathy</td>
</tr>
<tr>
<td>DME</td>
<td>Drug Metabolizing Enzyme</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-Stage Renal Disease</td>
</tr>
<tr>
<td>EOG</td>
<td>Equol-4/7-O-glucouronide</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated Glomerular Filtration Rate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EPS</td>
<td>4-Ethylphenyl Sulfate</td>
</tr>
<tr>
<td>FDR</td>
<td>False Discovery Rate</td>
</tr>
<tr>
<td>FMO</td>
<td>Flavin-containing Monooxygenase</td>
</tr>
<tr>
<td>FMT</td>
<td>Fecal Microbial Transplantation</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular Filtration Rate</td>
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<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
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<td>Growth Hormone</td>
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<td>HNF-4α</td>
<td>Hepatocyte Nuclear Factor Alpha</td>
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<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<td>HMDB</td>
<td>Human Metabolome Database</td>
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<td>HMP</td>
<td>Human Microbiome Project</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic Interaction Liquid Chromatography</td>
</tr>
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<td>IS</td>
<td>Indoxyl Sulfate</td>
</tr>
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<td>IBD</td>
<td>Irritable Bowel Disease</td>
</tr>
<tr>
<td>IPO</td>
<td>Isotopologue Parameter Optimization</td>
</tr>
<tr>
<td>LCA</td>
<td>Lithocholic Acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<tr>
<td>Vmax</td>
<td>Maximal Enzyme Velocity</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>MDRD</td>
<td>Modification of Diet in Renal Disease</td>
</tr>
<tr>
<td>NGS</td>
<td>Next-Generation Sequencing</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>Nf-kB</td>
<td>Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational Taxonomic Unit</td>
</tr>
<tr>
<td>OAT</td>
<td>Organic Anion Transporter</td>
</tr>
<tr>
<td>OPLS-DA</td>
<td>Orthogonal Partial Least Squares Discriminant Analysis</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid Hormone</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisome Proliferator-Activated Receptor Alpha</td>
</tr>
<tr>
<td>PS</td>
<td>Phenyl Sulfate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnan X Receptor</td>
</tr>
<tr>
<td>PCA</td>
<td>Principle Component Analysis</td>
</tr>
<tr>
<td>QTof/MS</td>
<td>Quadrupole Time-of-flight Mass Spectrometer</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin-angiotensin-aldosterone System</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
</tr>
<tr>
<td>RPLC</td>
<td>Reverse Phase Liquid Chromatography</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short-Chain Fatty Acid</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>SLC</td>
<td>Solute Carrier</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SULT</td>
<td>Sulphotransferase</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TMAO</td>
<td>Trimethylamine-N-oxide</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferase</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>VIP</td>
<td>Variable Importance in Projection</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D Receptor</td>
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</table>
Chapter 1

1 Introduction

1.1 Preface

Understanding biological systems is quickly becoming a multidimensional and multidisciplinary effort. With the wealth of technological advances in biological methodology, study of whole-organism physiology has become a reality. The combination of “omics” methods such as metabolomics and sequencing of the microbiome has provided the opportunity to compare whole-system host physiological processes. Our era of public databases and data sharing allows blinded discovery of complex biological fingerprints without requiring the identity of any individual sequence or metabolite. This exploratory and hypothesis-forming science supports big questions about phenotype and disease states with relation to dependent bodily systems such as the gut microbiome and host metabolome (Nicholson et al., 2005). In this thesis, detectable host gut bacteria and metabolomic profiles are characterized and related to altered drug metabolism over the progression of chronic kidney disease (CKD).

1.2 Renal Physiology

The kidney is an essential organ responsible for excretion of harmful biological waste products while simultaneously maintaining water, electrolyte, nutrient and pH balance (Reese et al., 2011). Nephrons, the functional units of the kidney, filter water, urea and all other molecules excluding large proteins and red blood cells at the glomerulus. Immediately following filtration, reabsorption back into the bloodstream begins in the proximal tubule where active transport of sodium stimulates the reabsorption of water, chloride, glucose and amino acids (Reese et al., 2011). Most water reabsorption occurs in the descending limb of the loop of Henle, effectively concentrating the urine. Further reabsorption of sodium and chloride ions occurs in the water impermeable ascending limb. The distal tubule performs fine adjustments in salt and ion concentrations by activation of
the renin-angiotensin-aldosterone system (RAAS) (Reese et al., 2011). In short, macula densa cells detect decreased sodium levels of the filtrate in the distal tubule and signal to juxtaglomerular cells of the afferent or efferent arteriole to produce renin. Renin released into the bloodstream sends a signalling cascade through angiotensinogen, angiotensin I, angiotensin II and finally aldosterone to increase the reabsorption of sodium at the distal tubule. The filtrate then reaches the collecting duct where it may be further influenced by antidiuretic hormone (ADH) or atrial natriuretic peptide (ANP) depending on the persisting imbalance of either nutrients or arterial pressure, respectively (Reese et al., 2011). Urea, a small molecule responsible for removing harmful ammonia is recycled from the collecting duct filtrate and used to increase the osmolarity at the earlier descending loop of Henle allowing for more water to be reabsorbed (Reese et al., 2011). After the urea helps reabsorb water, it returns to the final filtrate and is excreted in the urine. Besides maintaining water, pH and electrolyte balance, the kidney eliminates toxic metabolites, controls blood pressure and produces renin, erythropoietin, prostaglandins and activated vitamin D (Reese et al., 2011).

1.3 Chronic Kidney Disease

1.3.1 Prevalence

Chronic kidney disease (CKD) is a progressive and irreversible loss of kidney function heavily associated with age, obesity and diabetes (Levey et al., 2003; O’Hare et al., 2007; Zhou et al., 2008; Kopple, 2010; Hahr & Molitch, 2015). An estimated three million Canadians (12.5%) are affected by renal insufficiency or complete renal failure but inconsistencies in identifying earlier stages of CKD suggest prevalence may be even higher (Jha et al., 2013; Arora et al., 2013). In 2015, the number of Canadians aged 65 years or older (16.1%) was higher than those aged 0-14 for the first time in history, and projections suggest this will increase to 20.1% by 2024 (Statistics Canada, 2015). In addition to an aging population, a staggering 24.8% of Canadians over 18 years of age have a body mass index classified as obese which increased by 17.5% from 2003 (OECD, 2011). As a result, the prevalence of insulin resistance in the form of diabetes mellitus, the most common cause of CKD, is also expected to increase (Hahr & Molitch, 2015; Wouters et al., 2016).
Thus, the national health care burden of CKD is extensive and projected to increase along with these associated demographics (Manns et al., 2007). Alongside Canada, 16% of the global population is affected by CKD in other aging or obese countries such as Taiwan, USA, Japan, Portugal, Belgium and South Korea (Jha et al., 2013). More recently, developing countries such as Nepal, Sri Lanka, Mexico and many locations in India are experiencing prevalent CKD because of insufficient health care and access to renal therapies (Abraham et al., 2016).

1.3.2 Causes & Comorbidities

There is no singular blanketing CKD pathophysiology due to the many causes and comorbidities across individuals. Kidney damage is most commonly mediated by hypertension or diabetic nephropathy although acute kidney injury (AKI), lupus nephritis or genetic variations such as polycystic kidney disease may translate into CKD later in life (Levey et al., 2003).

Hypertension is both a stimulus and consequence of CKD. Persistently increased blood pressure can induce thickening and subsequent narrowing of the arterioles entering the glomerulus. This decreases the glomerular pressure reducing the amount of filtrate entering the proximal tubule and sequentially decreases urine output (Hall et al., 2014). A healthy kidney experiencing reduced glomerular pressure produces renin to retain sodium and water to increase blood volume and heart rate. The injured kidney will also activate the RAAS system but this increases the blood flow through narrow arteries promoting fluid retention (Sereno et al., 2016). Thus, an increased fluid volume worsens glomerular pressure, further contributing to hypertension and leading to edema, cardiovascular stress and heart disease (Tedla et al., 2011). Additionally, lack of blood flow to the kidney can cause glomerulosclerosis or hardening of the capillaries required for filtration. This triggers ischemia and eventually nephron death (Sugiyama et al., 1996; Tedla et al., 2011).

Diabetic nephropathy (DN) is an anticipated outcome for 40% of diabetics (Gross et al., 2005; Hahr & Molitch, 2015). Although controversial, some research suggests that strictly maintaining glucose levels reduces the rate at which pathologies such as mesangial cell expansion and proliferation, podocyte death and glomerulosclerosis manifest. However,
other factors such as hormone regulation and inflammation are also suspected to have a role in DN onset (Schena, 2005). DN can be organized into four severity classes, the final being glomerulosclerosis which eventually leads to nephron death (Tervaert et al., 2010).

1.3.3 Detection & Progression

CKD is an irreversible disease because following nephron death, undamaged nephrons acquire the added filtration load in a condition called glomerular hyperfiltration (Kopple, 2010). This usually occurs in early stages of CKD when the kidney has reduced but adequate function. Unfortunately, overworked nephrons eventually succumb to glomerulosclerosis, vascular sclerosis and tubulointerstitial fibrosis responsible for the transition into late-stage CKD.

To eliminate variance caused by multiple pathologies, the National Kidney Foundation has clinically defined CKD as a progressive loss of renal function by measuring glomerular filtration rate (GFR). The GFR is the volume of filtrate produced by glomeruli over time and surface area expressed in units of ml/min/1.73m². Normal GFR ranges between 120-130 ml/min/1.73m², but this decreases with age by about 1ml/min/1.73m² per year (Levey et al., 2003). An estimated GFR (eGFR) is arguably the least invasive and most effective method of CKD assessment. It relates patient age, sex, race and body mass with a measurement of the disease marker creatinine by a common eGFR calculation. Creatinine is a normal by-product of non-enzymatic creatine degradation that remains relatively constant from day to day (Wyss & Kaddurah-Daouk, 2000). Creatinine remains the biomarker of choice because of its unhindered filtration through the glomerulus and minimal reabsorption (Lopez-Giacoman, 2015). However, creatinine has limited accuracy not only because it is dependent on diet and muscle mass but it is also secreted via the proximal tubule. In severe CKD, hypersecretion of creatinine can exaggerate the estimate of GFR (Shemesh et al., 1985). Examples of eGFR calculations are the Modification of Diet in Renal Disease (MDRD) equation and Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation, the latter of which is newer and has received more support for its improved accuracy over the MDRD method (Stevens et al., 2010; Arora et al., 2013).
Changes in GFR can be preceded by proteinuria which is known to indicate CKD risk earlier than GFR, especially in diabetic patients (Gross et al., 2005). Proteinuria is the amount of albumin or any other protein normally unable to escape the bloodstream that is allowed to enter the urine due to structural epithelial malfunctions at the glomerulus (Satchell & Tooke, 2008). Albuminuria is solely the loss of albumin. Microalbuminuria is considered moderately increased and macroalbuminuria severely increased levels of albumin (Hahr & Molitch, 2015). Albuminuria is commonly expressed as a ratio of albumin to creatinine where a level under 30mg/g is normal, 30-200mg/g is indicative of early to moderate CKD and a level over 200mg/g is indicative of late-stage CKD (Levey et al., 2003).

Kidney disease has five clinically identified stages defined by the National Kidney Foundation (Levey et al., 2003) (Figure 1.1). Stage 1 represents kidney damage detectable by albuminuria but expressing a normal GFR >90ml/min/1.73m². Stage 2 is kidney damage with GFR between 60-89ml/min/1.73m². To be categorized as having moderate CKD, a patient must have a GFR <60ml/min/1.73m² or kidney damage that has persisted for over three months. This definition can also be classified as Stage 3 with a GFR between 30-59ml/min/1.73m². Severe CKD begins in Stage 4 with a GFR between 15-29ml/min/1.73m² and requires extensive pharmaceutical interventions to combat comorbidities such as hypertension, diabetes and cardiovascular diseases (CVD). End-stage renal disease (ESRD) specifically refers to patients requiring renal replacement therapies such as dialysis or kidney transplantation to sustain life and generally include Stage 5 CKD patients with kidney failure or a GFR <15ml/min/1.73m². Dialysis modalities include hemodialysis or peritoneal dialysis where the blood of a patient is filtered through a dialysis membrane simulating the filtering function of a kidney (Luo et al., 2011).
**Figure 1.1.** Prevalence and clinical stages of CKD. A) Canadian and worldwide prevalence of all CKD and CKD stages 1-5. B) Description, GFR and albuminuria guidelines with respect to CKD stages 1-5. # Prevalence estimate cannot be reported due to low sample size and variability (coefficient of variation > 33.3%); ≈ 0.1% world population. Adapted from (Levey et al., 2003; Smink et al., 2012; Arora et al., 2013; Hill et al., 2016).
1.3.4 Clinical Manifestations & Uremia

Nephrone loss causes a host of nutrient imbalances that can prove fatal if left untreated. Metabolic acidosis and hypocalcaemia will cause bone decalcification and osteodystrophy if left unchecked (KDIGO Work Group, 2009). Even more dangerous is the imbalance of potassium, sodium and water that can lead to hyperkalemia, peripheral edema, hypertension and heart disease (KDIGO Work Group, 2009). The leading cause of death in CKD is CVD inflicted by hypertension and largely unknown mechanisms that may be related to vitamin D deficiency, anemia, uremia, dyslipidemia, low-grade inflammation or hormone imbalances (Gansevoort et al., 2013). Currently, there is no cure for CKD and therapeutics focus on the treatment of comorbidities with the aim of delaying disease progression into ESRD.

Uremic syndrome however, remains a clinically challenging symptom to alleviate and drives the progression of CKD into ESRD (Meyer & Hostetter, 2007). Uremic syndrome manifests when harmful molecules called uremic toxins are permitted to circulate in the plasma at abnormally high concentrations regardless of dialysis interventions (Lisowska-Myjak, 2014). Uremia is the pathological environment of the blood plasma in CKD patients experiencing retention of urea and other organic compounds due to a loss of renal clearance (Meyer & Hostetter, 2007). The state of uremia has been associated with the activation of the immune response and gut microbial alterations (Vitetta & Gobe, 2013), cardiovascular events (Ito & Yoshida, 2014), bone toxicity (Barreto et al., 2009a), neurological disorders (Bugnicourt et al., 2013) and a host of other manifestations affecting every organ of the body (Vanholder & De Smet, 1999; Lisowska-Myjak, 2014). As of 2012, concentrations of 88 uremic toxins were identified as abnormal in CKD patients and can be sorted into three classes dependent on molecular size and protein binding (Duranton et al., 2012). Free water-soluble metabolites such as creatinine and urea have the lowest molecular mass (<0.5kDa) and are largely removable by dialysis. Middle molecules range from 0.5-60kDa and include small proteins, hormones and cytokines. Protein-bound uremic toxins are often of low molecular weight but are bound to larger protein carriers in circulation. Dialysis membranes will not allow the removal of albumin; thus, uremic toxins bound to albumin are especially difficult to remove from the plasma. Out of the over 88 recognized uremic
toxins, 25% are protein-bound and have proven clinically challenging to eliminate with traditional dialysis strategies (Duranton et al., 2012).

1.3.5 Animal Models of CKD

Models of CKD in rats include 5/6th nephrectomy, diabetic nephropathy and adenine-induced CKD. The most common 5/6th nephrectomy method surgically removes the top and bottom thirds of one kidney and the entire contralateral kidney effectively simulating the loss in kidney function although potentially inducing post-surgery stress (Ali et al., 2013). Diabetic nephropathy models of diabetes-induced kidney disease usually use the administration of streptozotocin to cause pancreatic β cell death. Additionally, the diabetic nephropathy model although most exemplary of human CKD caused by diabetes, requires a timely experimental period to successfully cause CKD that may not occur simultaneously across all animals. The newer adenine-induced model of CKD is a less invasive orally administered model suggested to provide a more consistent rate of CKD onset than the 5/6th nephrectomy (Terai et al., 2008). The adenine model of CKD also shows the best promise as a progressive CKD model suitable for temporal evaluation due to its timely onset in comparison with the diabetic model. Additionally, the adenine model has been used to study alterations in DMEs of the liver in the past, providing a clear example of severe CKD after 42 days of oral administration (Feere et al., 2015; Velenosi et al., 2016). Mechanistically, adenine and its metabolite 2,8-dihydroxyadenine precipitate when concentrated at the kidney. This mechanically damages the kidney tubules comparable to that inflicted by kidney stones and is thus a tubular model of kidney damage (Engle et al., 1996; Morishita et al., 2011; Succar et al., 2017).

1.4 Hepatic Physiology

Where the kidney is made up of functional units, the liver is made up of cells lining hepatic sinusoids organized around hepatic veins. Parenchymal hepatocytes make up the majority of the liver volume alongside non-parenchymal sinusoidal endothelial, stellate and Kupffer cells which together are responsible for the multitude of hepatic functions (Porth, 2011).
Select roles of the liver are important with respect to CKD and kidney physiology. Firstly, the liver is responsible for the continuation of the RAAS signalling pathway by production of angiotensinogen, the hormone stimulated in response to the release of renin by the kidney (Reese et al., 2011). Secondly, the liver’s ability to produce carrier proteins synthesized with the purpose of transporting signalling molecules is relevant in CKD. Albumin, a highly versatile carrier protein responsible for much of the endogenous and xenobiotic transport within the plasma, is the most abundant plasma protein produced by the liver (Berg et al., 2012). Since it cannot pass through the glomerulus, albumin can retain bound molecules in the plasma, preventing their excretion via the kidney and is particularly exaggerated in CKD (Meyer & Hostetter, 2007). The liver is also known for its major roles in metabolism. The liver not only handles lipid, carbohydrate and amino acid synthesis and degradation but also xenobiotic metabolism (Porth, 2011). An endogenous example is the catabolic degradation of amino acids by aminotransferases which release ammonia that is excreted as urea through the kidney (Berg et al., 2012). Next, exogenous metabolism by specific enzymes produced in the liver will be explicitly discussed.

### 1.5 Drug Metabolizing Enzymes

Approximately 75% of all exogenous molecules require metabolic transformation into active metabolites or waste products before being excreted (Williams et al., 2004). The functional units are drug metabolizing enzymes (DMEs) and can be categorized into three groups. Phase I and II DMEs are responsible for the direct biotransformation of endogenous and exogenous molecules usually by increasing hydrophilicity to ease distribution and excretion. The cytochrome P450 superfamily dominates Phase I and are found ubiquitously, but concentrated in the intestine, lungs, kidney and most abundantly in hepatocytes. Phase II DMEs consist of multiple conjugating enzyme superfamilies such as sulfotransferases (SULT), UDP-glucuronosyltransferases (UGT) and glutathione S-transferases (GST) amongst others. Phase III comprises membrane transport proteins belonging to the ATP binding cassette (ABC) family or solute carrier (SLC) family (Xu et al., 2005). Together, DMEs build the framework for drug absorption, distribution, metabolism and excretion (Sheweita, 2000; Williams et al., 2004).
1.5.1 Cytochrome P450s

CYP enzymes are heme-containing membrane-bound proteins found primarily in the endoplasmic reticulum of cells. The common mono-oxygenase is essential for accepting an oxygen moiety in a process where electrons, provided by an NADPH-dependent P450 oxidoreductase (POR), are required for CYP function (Johnson & Stout, 2005). The enzymatic goal is to transform lipid-soluble molecules via oxidation into more water-soluble products. However, the promiscuous substrate-binding nature of CYP enzymes mean a single CYP enzyme is capable of multiple reactions utilizing various forms of oxidation and reduction (Gad, 2009). The substrate specificity is so broad that ~75% of all hepatic xenobiotic metabolism is mediated by CYP enzymes (Williams et al., 2004; Wienkers & Heath, 2005).

There are 57 CYP enzyme genes identified in humans categorized into 18 families determined by their amino acid composition. For example, the enzyme CYP3A4 is broken down into four components: CYP referring to the cytochrome P450 superfamily, gene family #3, subfamily A, and individual isoform #4. CYPs can also be organized with respect to the number of drugs or endogenous molecules they are capable of metabolizing. Subfamilies CYP1, CYP2 and CYP3 predominately metabolize xenobiotics while most other families primarily carry out specific endogenous metabolism of hormones, vitamins and bile acids (Gad, 2009).

Out of the 57 CYP genes, the CYP3A family, CYP2C9, CYP2D6, CYP2C19 and the CYP1A family are responsible for ~95% of all CYP mediated drug metabolizing activity (Wienkers & Heath, 2005). Specifically, single enzymes CYP3A4 and CYP2C9 are responsible for the metabolism of ~43% of all clinically used drugs, thereby making them well-studied in the literature and the focus of this work (Zanger & Schwab, 2013). To study human CYP enzymes, gene sequence, evolutionary divergence and gene clustering information is used to determine the orthologous pairs between human and non-human species. Therefore, CYP3A4 and CYP2C9 genes are usually represented as CYP3A2 and CYP2C11 in rat models or Cyp3a11 and Cyp2c29/Cyp2c37 in mouse models, respectively (Nelson et al., 2004; Pan et al., 2016).
1.5.2 Induction & Regulation

CYP enzyme expression and activity is not only determined by a number of factors including genetic polymorphisms, sex and age, but also by continually fluctuating bioavailability of substrate (Zanger & Schwab, 2013). Evidence for genetic polymorphisms of CYP3A4 are slowly emerging, but remain elusive in comparison to CYP2C9 which has multiple single nucleotide polymorphisms (SNPs). The two most common in European ancestry are CYP2C9*2 and CYP2C9*3 while CYP2C9*5 is considered rare (Schwarz et al., 2008). Warfarin, a known CYP2C9 substrate and anticoagulant drug used in CKD, must be administered at a fraction of a normal dose if an individual expresses CYP2C9*2 or CYP2C9*3 to avoid drug toxicity and adverse effects like bleeding (Gong et al., 2011; Klein & Zanger, 2013; Vear et al., 2014). Females tend to have higher levels of CYP3A4 than males, but age slowly decreases activity in both sexes (Sotaniemi et al., 1995). CYP2C9 is relatively static across sexes but increases as juveniles mature (Yang et al., 2010; Zanger & Schwab, 2013; Vear et al., 2014).

Substrate bioavailability regulates the expression and activity of CYPs by utilizing a plethora of induction pathways. Specifically, CYP3A4 and CYP2C9 can be regulated at the transcriptional level by substrate induction of either pregnane X receptor (PXR) or constitutive androstane receptor (CAR) (Tirona et al., 2003; Chen et al., 2005). PXR and CAR are nuclear receptors containing both a ligand-binding and DNA-binding domain. Upon ligand-binding, PXR or CAR translocate into the nucleus to form a heterodimer with retinoid X receptor (RXR) before binding to the proximal promotor region of the CYP3A or CYP2C gene to upregulate its production (Kliewer et al., 2002). To optimize CYP2C9 induction, hepatocyte nuclear factor 4 alpha (HNF-4α) is also required to bind to a promotor of the CYP2C9 gene (Jover et al., 2009). HNF-4α, along with a list of other constitutive elements promote or inhibit CYP3A4 expression in the absence of inducers (Honkakoski & Negishi, 2000; Tirona et al., 2003; Zanger & Schwab, 2013). In addition to the PXR/CAR induction mechanisms, peroxisome proliferator-activated receptor alpha (PPARα) (Thomas et al., 2013), glucocorticoid and vitamin D receptor (VDR) pathways (Wang et al., 2013) are slowly being recognized as influencers of CYP3A4 and CYP2C9 expression (Zanger & Schwab, 2013). More recently, microRNA (miRNA) has shown to
influence CYP3A4 directly and indirectly through nuclear receptors PXR, HNF-4α and VDR (Pan et al., 2009; Wei et al., 2014). Besides miRNA, mechanisms of post-transcriptional or post-translational regulation of CYP3A4 and CYP2C9 are largely understudied.

Disease states and associated inflammation are suspected to alter DMEs via changes in levels of hormones, regulatory cytokines and other metabolites. Specifically, disease modulation of CYP enzymes is usually a downregulation of activity or expression and occurs by alteration of basal and inducible transcriptional mechanisms. Inflammation activates the NF-κB pathway and inhibits RXR, thus acting as an antagonist for PXR, CAR and other transcription factors responsible for CYP induction (Jover et al., 2002; Zhou et al., 2006; Morgan, 2009). Models of diabetes mellitus suggest a unique induction of CYP3A4 activity and PXR expression by the increased levels of free fatty acids present in the serum (Kim & Novak, 2007; Hu et al., 2014). A model of nutritionally induced obesity in mice and rats exhibited increased liver triacylglycerol levels as well as decreased liver Cyp3a11 and Cyp2c29, although nuclear factors and inflammatory factors were unchanged (Yoshinari et al., 2006). The accumulation of normally excreted metabolites is a common theme in the suspected cause of disease-associated CYP regulation (Fisher et al., 2009).

Most drugs are substrates for CYP enzymes (Williams et al., 2004; Wienkers & Heath, 2005). Pharmaceutically, this creates the dangerous potential for drug interactions. Rifampicin is a classic example of a PXR ligand that induces both CYP3A4 and CYP2C9 expression (Goodwin et al., 1999). A drug co-administered with rifampicin requiring CYP3A4 or CYP2C9 metabolism will be extensively metabolized resulting in decreased plasma concentration and reduced efficacy (Lynch et al., 2007). In the case of a prodrug such as losartan used to treat hypertension, activation will be inhibited if the CYP enzyme responsible for the activation is inhibited by a co-administered drug (Lynch et al., 2007). This same idea can apply to dietary molecules that influence CYPs. A clinically relevant example is the CYP3A4 inhibition by grapefruit juice. When grapefruit juice is co-administered with drugs dependent on CYP3A4 metabolism, the result is abnormally high bioavailability (Bailey et al., 1998). Drug interactions in combination with the multiple influences of CYP expression result in high intra- and inter-individual variability in
pharmacokinetics. The clinical outcome can be drug toxicity in the form of adverse drug reactions (ADR) or drug ineffectiveness. This will be discussed in the context of CKD in section 1.5.4.

1.5.3 Altered Drug Metabolism in CKD

It is evident that renal drug clearance is hindered by CKD due to a reduced GFR. However, it was not until 2009 that non-renal, hepatic drug clearance became clinically relevant for kidney disease patients in the Guidance for Industry and not until 2011 that KDIGO (Kidney Disease: Improving Global Outcomes) identified non-renal clearance as a usually forgotten aspect of pharmacokinetic studies necessary for dose recommendations (Matzke et al., 2011). Reviewed by Nolin and colleagues in 2008, eleven drugs known to be metabolized by hepatic CYP3A4 showed altered clinical pharmacokinetics in CKD patients (Nolin et al., 2008). More recently, eight drugs extensively metabolized by CYP3A4 and another three drugs metabolized via CYP2C9 presented reduced non-renal clearance in CKD (Ladda & Goralski, 2016). Aliskiren, carvedilol, erythromycin and telithromycin are a few CYP3A4 xenobiotic substrates that are commonly administered and exhibit altered pharmacokinetics in CKD patients (Ladda & Goralski, 2016). CYP2C9 is the major metabolizer of warfarin, a drug commonly used in CKD as a blood thinner to reduce the risk of stroke or atrial fibrillation associated with cardiovascular diseases. A 50% reduction in warfarin metabolism in CKD is suspected to be caused by inhibited or decreased CYP2C9 (Nolin et al., 2008). This is further supported by Gong and colleagues who established that renal function is a determinant of warfarin dosing although warfarin is excreted through non-renal pathways suggesting altered DMEs are responsible for warfarin pharmacokinetic changes in CKD (Gong et al., 2011). Over 75 drugs exhibit reduced non-renal clearance in CKD patients (Yeung et al., 2014). However, it remains largely undecided whether levels of DMEs are altered in humans and responsible for the altered pharmacokinetics seen in CKD (Nolin, 2015). Early human studies utilized the erythromycin breath test to identify impaired CYP3A4 metabolism in ESRD patients (Dowling et al., 2003; Nolin et al., 2006). Simply, radiolabeled carbon dioxide end-product is measured from the breath of subjects given a known dose of radiolabeled erythromycin, a known CYP3A4 specific substrate. When further studies showed DME transporters also
influence erythromycin disposition *in vitro*, the breath test was scrutinized for its indication of CYP3A4 activity and blamed for subsequent contradictory results (Frassetto *et al.*, 2007; Ladda & Goralski, 2016). Interestingly, using a specific probe drug suggests human CYP3A4 activity may not be significantly changed in CKD (Nolin *et al.*, 2009; Thomson *et al.*, 2015). Moreover, the most recent meta-analysis study suggested there was only modest downregulation of human CYP3A4 in comparison to other CYP enzymes over CKD progression (Yoshida *et al.*, 2016). However, extensive animal models of severe CKD show severely decreased gene expression, protein expression and enzymatic activity of CYP enzymes including CYP3A2 and CYP2C11 as well as Phase II and DME transporters (Leblond *et al.*, 2001; Velenosi *et al.*, 2012; Ladda & Goralski, 2016). Therefore, the hypothesis remains that altered pharmacokinetics in humans with CKD stems from altered DME expression or activity. Research today focuses on the mechanisms regulating CYPs in animal models of CKD to further elucidate their function with respect to disease.

1.5.4 Pharmacy & Outcomes in CKD

Altered CYP function is particularly worrisome in CKD patients because this population is elderly, receiving polypharmacy and potentially on dialysis (Sharif-Askari *et al.*, 2014; Ladda & Goralski, 2016). These factors increase the risk of a CKD patient experiencing an adverse drug event (ADE) or ADR inflicted by a medical intervention, usually by the administration of a drug (Bates *et al.*, 1995; Edwards & Aronson, 2000; Munar & Singh, 2007). ADEs are often due to inappropriate dosing that either enhances the activity of the drug and exceeds the intended therapeutic range or fails to meet the therapeutic range potentially reducing efficacy and allowing the symptom to persist.

As there is no cure for CKD, co-morbidities that exist in moderate to severe CKD are primarily treated with medications. In total, patients require an average of 7-12 drugs daily to manage associated comorbidities and the likely causes of CKD (Talbert, 1994). In a study of 512 CKD patients, the risk of ADR was increased when patients were taking over 8 medications, of which anticoagulant drugs including warfarin caused the highest incidence (Sharif-Askari *et al.*, 2014). Secondly, if the patient is on dialysis, a drug’s
pharmacokinetics can differ pre- and post-dialysis, further complicating the appropriate dose (Velenosi & Urquhart, 2014). Thirdly, most CKD patients are over 65 years of age and are likely to be taking additional medications aside from those required to treat kidney disease specific comorbidities. As such, the elderly population alone increases the risk of ADEs (Sharif-Askari et al., 2014; Davies & O’Mahony, 2015). Thus, dosing considerations in each CKD patient requires an individual drug plan and continuous monitoring (Corsonello et al., 2005; Matzke et al., 2011). Ultimately, the combination of unavoidable polypharmacy and unpredictable drug metabolism is the suspected driver of ADEs and ADRs seen in CKD patients (Manley et al., 2005).

It is therefore important to assess the probable causes of pharmacokinetic alterations in CKD. In the following sections, suspected factors contributing to changes in DME regulation in CKD will be discussed including the intestinal microbiome and uremia.

### 1.6 The Microbiome

The microbiome is the entire community of bacterial species and their corresponding genes that co-exist within the human host gastrointestinal (GI), urogenital and respiratory systems as well as on the exterior surfaces of the skin (Cho & Blaser, 2012; Ursell et al., 2012). Compared to the human genome, the microbiome is approximately 150 times larger and contains many genes that aid in symbiotic functions humans are incapable of carrying out on their own (Qin et al., 2010). Bacterial acquisition occurs in infants during and immediately following birth (Lloyd-Price et al., 2016). During childhood through to adulthood the microbiome is continually shaped in response to a regular diet and environmental insults. These are largely associated with geography and race/ethnicity, and by two years of age, a “core microbiome” or stable abundance of each major bacterial group is established (Huttenhower et al., 2012; Ursell et al., 2014; Lloyd-Price et al., 2016). However, the microbiome is far from static and small short-term changes can occur within hours of a dietary alteration, largely not affecting the core microbiome (David et al., 2013).
1.6.1 Microbiota Methodology

A decade ago, bacterial species analysis was performed primarily by culture and PCR methods limited by the knowledge of individual bacterial species and associated primers. However, there are thousands of species residing in a single fecal sample and a large proportion of bacteria are unculturable with current techniques making it increasingly difficult to assess the bacterial composition or “microbiota” as a complete community (Stewart, 2012). Culture-independent methods like Sanger sequencing, although developed in the 1970s (Sanger et al., 1977) was a long and laborious process and was rarely utilized for sequencing the microbiota (Hiergeist et al., 2015). Around 2005, the first high-throughput next generation sequencing (NGS) techniques improved the cost and efficiency of sequencing, promoting an interest in the microbiome (Gloor et al., 2010; Hiergeist et al., 2015). However, in addition to high error rates per-read, the first NGS methods (e.g. pyrosequencing) remained expensive and unattainable for many research groups (Gloor et al., 2010). Within the last 10 years NGS methods such as Illumina MiSeq have provided the ability to assess the microbiota with higher throughput and accuracy at an attainable cost (Caporaso et al., 2012; Ramezani et al., 2015). Phylogenetic microarrays are another popular method of bacterial sequencing and both NGS and phyloarrays involve a reference gene. Usually the 16S small ribosomal subunit rRNA gene is used due to its combination of both highly conserved regions used for primer design applicable across all bacterial species and the adjacent variable regions utilized for species identification (Janda & Abbott, 2007). Unlike phyloarrays that are limited to the known 16S sequences applied to the plate, NGS methods allow all 16S genes within a sample to be sequenced regardless of previous identification (Vaziri et al., 2012). This is important because unidentified bacteria are necessary to understand species richness and diversity indices that are otherwise skewed without them.

Although methodology is constantly improving, gene sequencing results still heavily rely on the sequencing method used. In this work, the Illumina MiSeq platform was used to perform paired-end sequencing of the 16S rRNA gene focusing on the V4 variable region for maximum fecal bacteria coverage (Gloor et al., 2010). Through a process called bridge amplification, clusters of DNA are formed on a flow-cell within the sequencer.
Sequencing-by-synthesis and dye-terminated primer extension uses fluorescent signals to indicate what nucleotide is being added along the amplicon as it is made. Final instrument outputs are called reads and after data processing are matched with their most likely bacterial sequence. The final product is called an operational taxonomic unit (OTU) and generally needs to match the expected bacterial sequence with 97% accuracy (Cho & Blaser, 2012; Ursell et al., 2012). OTUs are quantifiable and used to understand the relative abundance of each species and diversity indices of a sample. Today, large scale endeavours such as the Human Microbiome Project (HMP) and the European project (MetaHIT) provide the foundation for bacterial databases (Turnbaugh et al., 2007; Cho & Blaser, 2012; Huttenhower et al., 2012).

Due to the vast number of bacteria at varying levels of identification and the large proportion yet to be identified, bacterial classification is usually presented as the most confirmed tier of the taxonomy ladder. Taxa are arranged in the ecological order of kingdom, phylum, class, order, family, genus, species followed by strain if applicable. Nomenclature for kingdom through genus taxa are capitalized and only species or strain names are italicized (e.g. Escherichia coli; E. coli).

1.6.2 Gut Microbiota & Host Physiology

The gut microbiota is the collection of bacteria residing in the lumen or mucosal layer of the upper to lower intestine and comprises up to 5000 bacterial species (Nieuwdorp et al., 2014; Ramezani et al., 2015). Ninety percent of the residential bacteria are dominated by phyla Firmicutes and Bacteroidetes and further split into five most prevalent genera Bacteroides, Clostridium, Lactobacillus, Escherichia and Bifidobacterium (Nicholson et al., 2005; Eckburg et al., 2005; Huttenhower et al., 2012; Goodrich et al., 2014; Mafra & Fouque, 2015). It is suggested with some controversy that global intestinal enterotypes or common diversity profiles exist in the human population (Arumugam et al., 2011; Knights et al., 2014; Moeller & Ochman, 2014). It is agreed however, that different species reside in the upper GI tract than those found in the colon and most are obligate anaerobes responsible for digestion, immune functions and gut physiology (Eckburg et al., 2005; Turnbaugh et al., 2007). Gut bacteria found in the lumen elicit the metabolism of dietary
fibres, sugars, alcohols and carbohydrates unable to be metabolized by the host (Nieuwdorp et al., 2014). Dietary fibre and some carbohydrates are largely indigestible until bacterial fermentation to short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate (den Besten et al., 2013). Commensal, or harmless co-existing bacteria, residing in the intestinal mucosal layer also support immune function of the host by ultimately inhibiting intestinal wall inflammation and subsequent bacterial translocation that could cause infection. Some bacteria produce anti-microbial peptides to fend off pathogenic microbes while others stimulate immune cell maturation of the host (Belkaid & Hand, 2014; Yip et al., 2015). SCFAs produced by bacteria limit the proliferation of effector CD4+ T cells and stimulate macrophage suppression of pro-inflammatory molecules (Felizardo et al., 2016).

Gut bacteria also determine host physiology. Studies utilizing germ-free mice raised in sterile environments, show reduced intestinal angiogenesis and ensuing malformed villi (Belkaid & Hand, 2014).

Ties between gut bacteria and host physiology spark questions regarding health and disease. Healthy fluctuations in symbiotic gut bacterial abundances are difficult to assess as most research focuses on deleterious disease states. In a large cohort of 1106 human stool samples, the largest influencers of gut composition were medications, blood parameters (e.g. red blood cell count, uric acid, hemoglobin), bowel properties (e.g. Bristol stool score, time since previous relief), health and diet (Falony et al., 2016). Dysbiosis is the altered bacterial composition associated with a non-infectious disease state (Lloyd-Price et al., 2016). Many diseases including inflammatory bowel disease, obesity, cardiovascular disease, asthma and more recently cancer exhibit dysbiosis (Cho & Blaser, 2012; Carding et al., 2015; Lloyd-Price et al., 2016). Often the probable cause of dysbiosis is indistinguishable from its added clinical manifestations (Vanholder & Glorieux, 2015). Luckily, a dignified method of bacterial replacement, usually in the form of fecal microbial transplantation (FMT), is used to assess the deleterious effects of diseased bacterial compositions by giving dysbiotic bacterial culture to a healthy individual (Manichanh et al., 2010). FMT gained popularity after multiple studies showed inducible adiposity when intestinal bacteria from obese mice were introduced to lean counterparts (Turnbaugh et al., 2008). In humans, FMT from healthy, lean individuals into obese individuals improved peripheral insulin sensitivity (Vrieze et al., 2012). Even increased susceptibility to
atherosclerosis was seen in mice receiving FMT from mice with atherosclerosis-induced dysbiosis (Gregory et al., 2015). Thus, the gut microbial community is capable of driving disease states.

1.6.3 Dysbiosis in CKD

In the 1106 stool sample cohort described above, one of the largest associated factors of altered bacterial composition was GFR and thus it comes as no surprise that CKD patients exhibit a differing bacterial composition from healthy individuals (Falony et al., 2016). Actinobacteria, Firmicutes (especially subphylum Clostridia), and Proteobacteria phyla of CKD patients contain changes in specific bacterial genera when assessed using phylogenetic microarray (Vaziri et al., 2012). Similar to the microarray results, bacterial sequencing showed altered Actinobacteria, Bacteroidetes, Firmicutes (Clostridia), Proteobacteria and Verrucomicrobia in ESRD patients (Wong et al., 2014). Unfortunately, the only study assessing early renal decline in humans showed bacterial alterations occurred solely in the Clostridiales order within the phylum Firmicutes (Barrios et al., 2015). Rat feces show similar overall composition to those of humans with Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria among the phyla with the highest abundances (Vaziri et al., 2012). When subjected to severe CKD, rat models show decreases in both Firmicutes and Bacteroidetes and decreased overall diversity (Vaziri et al., 2012). Obesity, a risk factor for CKD, is shown to have decreased levels of Bacteroidetes when compared as a proportion to Firmicutes in obese mice (Ley et al., 2005). A summary of specific gut bacterial alterations in CKD has been reviewed (Ramezani et al., 2015).

Although still unclear, most studies suggest the altered gut composition is a result of CKD pathophysiology; however, once established, the altered gut composition has a supportive role in CKD progression. CKD patients experience reduced gut motility, dietary changes, and a host of pharmaceutical insults which all have explainable impacts on the host gut microbial composition. For example, clinicians may recommend the removal of some fruits, vegetables or fibre from the diet to combat hyperkalemia which is one contributing factor to the reduced abundance of SCFA-producing bacteria in CKD (Wong et al., 2014;
Vaziri, 2016). Less obvious is the uremia-associated gut wall inflammation suspected to effect bacterial changes in CKD patients. The gut wall integrity is compromised in CKD shown by gap-junction alterations likely brought on by inflammation (Lau et al., 2016). The gut wall then becomes permeable to uremic toxins including urea, a substrate for bacteria with urease activity, which converts urea to ammonia. Ammonia is spontaneously transformed into ammonium hydroxide increasing the luminal pH and further damaging the gut lumen (Felizardo et al., 2016; Vaziri, 2016). This increase in pH naturally selects for species capable of surviving in alkaline environments exhibited by Wong and colleagues who demonstrated that bacteria established in ESRD patients favoured a uremic environment (Vaziri et al., 2013; Wong et al., 2014).

Alternatively, manifestations including systemic ramifications of dysbiosis are evident in CKD by endotoxin and bacterial DNA fragments found in the bloodstream. Not only would a leaky gut lumen explain dysbiosis, but also elucidate the parallel movement of bacteria into the blood-stream presented as endotoxemia in CKD and CVD patients (Feroze et al., 2012; Lau et al., 2016). Appealing to the idea of targeting dysbiosis as a therapy, pro- and prebiotics are being investigated as novel dysbiosis treatments (Koppe et al., 2015). A recent clinical trial demonstrated positive effects in reducing select uremic toxins in pre-dialysis CKD patients after a 6 week symbiotic therapy comprised of pre- and probiotics (Rossi et al., 2016). Although minor improvements have been suggested in overall CKD health, pro- or prebiotic treatment with intent to improve DME function has yet to be studied.

1.6.4 Gut-Derived Uremic Toxins

Gut-derived uremic toxins are uremic toxins produced by the commensal gut community under disease circumstances. An elegant study of germ-free versus conventionally raised mice first established that gut bacteria were required to produce host plasma metabolites such as indoxyl sulfate (IS) and phenyl sulfate (PS) (Wikoff et al., 2009). Out of the over 88 uremic toxins identified in CKD, IS and PS are two highly retained, protein-bound, gut-derived uremic toxins difficult to remove via dialysis (Duranton et al., 2012). Thereafter, a feasible connection between uremia and the altered gut microbiota in CKD was formed
In addition to indole and phenol metabolites, other by-products of bacterial metabolism include branched-chain fatty acids, SCFAs, ammonia, choline, hydrogen sulfide, amines [e.g. trimethylamine-N-oxide (TMAO)] mercaptanes and carbon dioxide (Macfarlane & Macfarlane, 2012).

Indoxyl sulfate begins as dietary tryptophan, transformed into indole by tryptophanase possessing gut bacteria including species within the genera Bacteroides, Bifidobacterium, Clostridium, Lactobacillus and Parabacteroides (Zhang & Davies, 2016). Indole circulates in the plasma until it is shuttled to the liver to be hydroxylated by CYP enzymes and sulfated by SULTs before returning to the plasma as indoxyl sulfate (Figure 1.2). Phenyl sulfate begins as dietary tyrosine before being sulfated in the liver. Bacteria capable of phenol metabolism belong to the Bacteroides, Bifidobacterium, Lactobacillus, Enterobacter, and Clostridium genera (Ramezani et al., 2015). Both IS and PS are normally excreted via the proximal kidney tubule, but are retained if kidney function is inhibited by reduced GFR or CKD-associated inhibition of transporters (Niwa, 2013). In a study of bacterial functionality in human ESRD, 12 of 19 microbial families with an increased abundance compared to healthy individuals possessed urease activity. Another four possessed the ability to produce indole. Two of four families that showed a decrease due to ESRD were capable of SCFA production (Wong et al., 2014). This data highlights the influence of CKD-induced uremia on the gut through altering the luminal environment and selecting for uremic toxin-producing bacteria, ultimately furthering disease progression through added uremic toxin production (Figure 1.2). Although biological manifestations of each gut-derived uremic toxin are unique and still being elucidated, IS has been of particular interest due to its association with overall mortality and multiple comorbidities through mechanisms of endothelial dysfunction, renal and cardiac fibrosis, immune activation and regulation of various other proteins with functions across all organ systems (Barreto et al., 2009b; Vanholder et al., 2014; Ramezani et al., 2015). With uremic toxins like IS so heavily linked to CKD progression and comorbidities, therapeutic options like pro- and prebiotics are suspected to improve the gut microbiota and systematically reduce uremia (Koppe et al., 2015).
1.6.5 Metabolomics

Metabolomics is the study of the entire metabolic profile of a biological matrix utilizing high-throughput diagnostic tools such as mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy. Endogenous as well as exogenous metabolites including uremic toxins, food metabolites, hormones and other small molecular weight molecules can be studied by metabolomics. The field is indispensable for understanding the final outputs of cellular processes and provides a picture of cellular mechanisms when analyzed alongside gene expression. In this work, metabolomics using MS coupled with advanced chromatography tools were used to measure metabolites present in plasma and liver homogenates.

In short, MS involves the ionization of a sample to obtain a mass-to-charge (m/z) ratio at a specific retention time unique to each molecule within a sample. To effectively measure many metabolites in a complex biological sample, ultra-performance liquid chromatography (UPLC) is often used in conjunction with MS to first separate metabolites by hydrophobicity before entering the mass spectrometer for ionization. The electrospray ionization (ESI) utilized in this work, uses high voltage to form an aerosol from a liquid sample. The aerosol is the point at which the molecules of the sample are ionized either in positive or negative ionization mode determined by either the addition or removal of a hydrogen ion, respectively. Whether a molecule ionizes better in positive or negative mode is determined by its molecular composition so generally both modes are used to capture as many metabolites as possible. After ionization, the metabolites travel parallel and in the center of four quadrupoles where brief alterations in the applied current alters the movement of the metabolites travelling through them. The mass of the metabolite ultimately determines if it reaches the other end of the quadrupole without being pushed or pulled out of the center. Systematically, by altering the window of current applied to the quadrupole, each m/z ratio is scanned at high mass accuracy. Following the quadrupole, the metabolites enter the collision cell where the parent metabolite may be broken into smaller fragments for additional information during identification later in analysis. In this work, the MS was run using the MS® Waters method that continuously switches from MS to MS/MS data acquisition using a low collision energy for production of parent molecules,
and ramping high collision energy, for production of fragments. The parent molecule is also called function 1 and the fragmentation data is called function 2. These fragments then enter the final time-of-flight (TOF) chamber which further separates the fragments by mass but using the time it takes for the ionized molecule to hit a detector after accelerating all molecules with the same force. Thus, the measured time required for the metabolite to reach the detector is dependent on the mass of the molecule. Ultimately this provides the m/z ratio, retention time and fragmentation pattern for each detected metabolite within a sample.

Using this instrument, both targeted and untargeted metabolomics can be conducted. Untargeted metabolomics refers to detection and measurement of metabolites within a sample without defining metabolites of interest prior to the experiment and is particularly useful for understanding the uremic milieu of CKD (Zhao, 2013).

Due to the large metadata output from metabolomics analysis, complex statistical analysis must be utilized (Saccenti et al., 2014). Principle component analysis (PCA) is a common multivariate method of displaying samples with respect to their relative metabolite compositions in a map-like format for visual verification of differing compositions. PCA was also used to visualize patterns of OTUs from the microbiota sequencing data. Metabolite features such as the accurate mass, retention time and fragmentation pattern captured via MS can be used to identify the metabolite when referenced to an online database such as the Human Metabolome Database (HMDB; www.hmdb.ca/) (Wishart et al., 2013).

1.7 Mechanistic DME Regulation in CKD

The mechanisms suggested for CYP downregulation in CKD are minimally understood, but have implicated uremia and uremic toxins, bacterial gut alterations, hormone alterations and associated inflammation. The next subsections break down two mechanistic pathways of DME alteration in CKD, uremia (section 1.7.1) and dysbiosis (section 1.7.2).
1.7.1 Uremia & DMEs

Since the discovery of uremic toxins, uremia has been considered a top candidate for the primary influencer of CYP enzyme manipulation in CKD because multiple mechanisms spanning pre-transcription to direct inhibition have been suggested (Velenosi et al., 2014; Volpe et al., 2014; Nolin, 2015). In addition to IS and PS, uremia provides a vast number of metabolites, many of which are unsuccessfully removed by dialysis and inflict pathological effects on other organs including the liver and cardiovascular systems (Niwa, 2013; Lin et al., 2015). The proposed mechanisms of uremia on DME expression include i) reduced nuclear receptor binding and epigenetic histone deacetylation (Velenosi et al., 2014), ii) NF-κB modulation via inflammatory cytokines or parathyroid hormone (PTH) (Michaud et al., 2006, 2008) and iii) direct inhibition by various uremic toxins (Guévin et al., 2002; Sun et al., 2004; Barnes et al., 2014; Volpe et al., 2014). However, uremic toxins may not be the sole regulator of DMEs. Preliminary studies using intestinal adsorbent AST-120 in rats with severe CKD show effective removal of uremic toxins without recovery of CYP3A2 or CYP2C11 function (Velenosi, 2015). In addition, most of the mechanistic studies described above, do not sufficiently provide support for 100% of the observed DME alteration.

1.7.2 Bacteria & DMEs

The altered gut composition of CKD is suggested to influence DME regulation. Two mechanisms are described here. The first is through increased production of uremic toxins. In short, increases in uremic toxin-producing bacteria are suspected to increase the load of uremic toxins, which are responsible for DME downregulation through mechanisms relating to the increased concentration of uremic toxins themselves (see section 1.7.1). Thus, removal of uremic-toxin producing bacteria may lessen the strain of uremia in CKD (Koppe et al., 2015), but this notion has yet to be evaluated in the context of DME alteration.

It is also possible that bacterial species are required for DME regulation independent of uremia. When bacterial species are lost due to CKD, associated dysbiosis may result in
faulty DME regulation. This mechanism was first hypothesized after recognizing the antibiotic ciprofloxacin diminished the intestinal diversity and impacted CYP expression (Toda et al., 2009). However, at the time, it was unclear if this decreased CYP expression was due to the drug acting as a direct inhibitor of CYP expression or killing the necessary bacteria for CYP function. Shortly thereafter, germ-free mice lacking gut bacteria showed reduced drug metabolizing Cyp3a11 and Cyp2c29 expression; however the microbiota was recovered upon colonization with gut bacteria from conventionally raised mice (Toda et al., 2009; Claus et al., 2011). An extensive gene expression study further showed that germ-free mice without gut bacteria have 87% downregulation of Cyp3a11 but unaffected Cyp2c29 (Selwyn et al., 2015).

Displacement of commensal bacteria in a dysbiotic state may remove the normally applied suppression of the inflammatory response. Essentially, bacterial SCFAs are implicated with many immunomodulatory effects including T cell inactivation, downregulation of TNFα/β, IL-6 and IL-1β, and inhibition of macrophage NF-κB nuclear translocation required for its activation (Lührs et al., 2002). As previously demonstrated, inflammation through activation of the NF-κB pathway can block DME expression via inducible PXR regulation (Gu et al., 2006). Mechanistically, molecules derived from bacteria like lithocholic acid (LCA) promote PXR activation responsible for CYP induction (Staudinger et al., 2001; Toda et al., 2009). Thus, one of the ways dysbiosis might affect drug metabolism is through loss of beneficial bacteria or beneficial molecules produced by bacteria required for DME induction.

It is important to note however, that dysbiosis is suspected to be caused by deleterious effects of uremic toxins on the gut wall. This highlights the proposed positive feedback manifestation of dysbiosis and uremia in CKD (Figure 1.2) (Vanholder & Glorieux, 2015). Assessment of both dysbiosis and uremia along the progression of CKD is suspected to shed light onto which factor may be primarily influential in DME alteration.
Figure 1.2. Pathophysiological summary of uremia and dysbiosis in CKD. A) Pathway of uremic toxins: IS, PS, p-cresol sulfate and TMAO. B) The cyclic gut-kidney relationship. Uremic toxins and associated inflammation are suspected to impede the integrity of the gut lumen allowing urea and other toxins to cross the leaky intestinal barrier and increase the luminal pH promoting growth of uremic-toxin-producing bacteria and overall dysbiosis. This promotes further production of uremic toxins associated with disease progression and CKD comorbidities. Images were modified from Servier Medical Art (www.servier.co.uk/medical-art-gallery).
1.8 Hypothesis & Objectives

1.8.1 Rationale

Currently the literature demonstrates non-renal drug clearance is altered in CKD. It is less clear why proteins such as DMEs are altered. Suggested mechanisms include alterations of the gut microbiota or uremic toxins. Thus, studies are needed to link gut-derived uremic toxins (e.g. IS) and gut bacteria with DMEs. Human DME orthologs have been studied in rat models of CKD but these studies focus only on severe or late-stage CKD leaving a gap in our understanding of when CKD factors and DME expression changes (Leblond et al., 2001; Velenosi et al., 2012; Ladda & Goralski, 2016). Additionally, very few studies have looked at both bacterial alterations and metabolomics within one CKD cohort, none of which have focused on DMEs (Ursell et al., 2014; Nallu et al., 2016). To our knowledge, no studies have assessed the effect of CKD on hepatic DMEs over disease progression in attempt to understand when uremic toxins are changing relative to DME alterations. Additionally, this thesis will be the first to encompass metabolomics, 16S bacterial sequencing and DME analysis over CKD progression.

1.8.2 Hypothesis & Objectives

Although both the uremic and microbial environments are altered in CKD patients, their roles in DME regulation remain unclear. The pathophysiological factors of uremia and dysbiosis have never been tested temporally over the progression of CKD. The basic principle of causality dictates that for a factor (e.g. uremia or dysbiosis) to be causative, it must exist or apply the effect before the outcome (e.g. DME regulation) in time (Otero et al., 2014). Elucidating the series of events that occur over CKD progression may provide support for current hypotheses, which suggest uremic toxins or bacterial alterations are causal factors in DME regulation. Alternatively, results may fail to support current hypotheses by finding DME changes occur before the suspected causal factors. Thus, this thesis aims to reveal the timing in which each pathophysiological factor changes in the attempt to support the hypothesis that uremic toxins and/or bacterial alterations are mechanistically involved in the regulation of DMEs in CKD. Specifically, we aim to
characterize plasma and liver uremic toxins, the gut microbial composition and DME fluctuation over the progression of an adenine-fed rat model of CKD. We hypothesize that adenine-induced CKD will cause uremia and gut microbial changes detectable prior to the anticipated downregulation of CYP3A2 and CYP2C11 DMEs.
Chapter 2

2 Materials & Methods

2.1 Animal Model & Study Design

Animal work was approved by the Western University Animal Care Committee and experiments were conducted in accordance with the Canadian Council on Animal Care (Appendix A). Sixty-six male Wistar rats (initially 150g) were obtained from Charles River Laboratories, Inc. (Wilmington, MA) and randomized into six groups defined by time of sacrifice (day 0, 3, 7, 14, 28 and 42). Each time point consists of a minimum six control and six CKD rats along with six control rats on day 0. Rats were housed in a conventional animal care facility with a 12h light cycle. Rats were paired with a same-group cage mate to minimize coprophagy alterations of the gut microbiota. All rats were acclimated for seven days prior to starting either 0.5% adenine supplemented Teklad 22/5 Rodent Diet (Harlan Laboratories Inc., Madison, WI) for CKD rats or standard chow pair fed to match caloric intake for control rats (Prolab® RMH 3000, LabDiet, St. Louis, MO). Water was provided *ad libitum* to both groups. Body weights were monitored daily (Table 3.1) until sacrifice via isoflurane anesthetization followed by decapitation. Blood was collected in heparinized tubes. Liver was snap-frozen in liquid nitrogen. Caecal samples were obtained on a sterile, single culture swab (BD, Sparks, MD) touched to an open incision at the enlarged proximal end of the rat caecum. All samples were stored at -80°C until further analysis excluding the right kidneys which were stored in 10% formalin until histological embedding.

2.2 Disease Markers & Histology

Conventional CKD markers urea (mmol/L) and creatinine (µmol/L) were measured in rat plasma using standard methods by the Pathology and Laboratory Medicine group (PaLM, London, ON; www.lhsc.on.ca/palm/). Kidney tissue and histological images were prepared as previously described (Feere et al., 2015). Briefly, kidneys were fixed in 10% formalin
(Anachemia Canada Co., Montreal, QC) before undergoing tissue slide preparation at the Department of Pathology (Western University, ON). Light microscopy and photographs of prepared haematoxylin and eosin (H&E) stained slides were obtained under identical exposure, saturation and contrast on a Leica DM1000 light microscope paired with a Leica DFC295 colour camera and Leica Application Suite v3.8.0 software.

2.3 Real-Time PCR

In preparation for quantitative (real-time) PCR, total mRNA was extracted from rat liver using TRIzol reagent (Life Technologies, Burlington, ON) following the manufacturers protocol before quantification and purity testing using a Nanodrop 2000 (ThermoScientific, West Palm Beach, FL). One µg of total RNA underwent reverse transcription (Bio-Rad C1000) for cDNA synthesis using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). Amplified cDNA was diluted 1:40 before running 5µL in triplicate on a 384-well plate with 7µL mastermix containing primers and PerfeCTa SYBR green fastmix (Quanta Biosciences, Gaithersburg, MD). Rat primers for CYP3A2 (forward) \[5'\-GCTCTTGATGCGTAAAGATTTG-3'\] and (reverse) \[5'\-ATCACA GACCTTGGCCACTCCTT-3'\] and CYP2C11 (forward) \[5'\-CCCTGGAGACTTTTGGGATGGGC-3'\] and (reverse) \[5'\-AGGGGCACCTTTGCTCTTCCTC-3'\] (Invitrogen) were previously validated by cycle threshold (Ct) and melt-curve analysis (Velenosi et al., 2014). β-Actin was used as the housekeeping gene (forward) \[5'\-ACGAGGCCAGAGCAAGA-3'\] and (reverse) \[5'\-TTGGTTACAATGCGGTGTTCA-3'\], samples were run on a Bio-Rad CFX384 Real-Time System (Hercules, CA, USA) and relative RNA expression was assessed through the ΔΔCt method (Livak & Schmittgen, 2001; Rao et al., 2013).

2.4 Western Blotting

2.4.1 Microsomal Isolation & BCA Assay

Hepatic microsomal fractions were prepared by differential centrifugation followed by Pierce BCA assay as previously described (Feere et al., 2015). Briefly, approximately 1/6 of each rat liver was homogenized using a T10 Basic Ultra-TURRAX (Sigma Aldrich, St.
Louis, MO) on ice in 1mL of buffer [1.15% KCl + 1mM ethylenediaminetetraacetic acid (EDTA)] for 5 min. Samples were centrifuged at 4°C in an ultracentrifuge (Beckman Coulter Optima L-90K, Fullerton, CA), first at 9000×g for 20 min to collect the supernatant which was further spun at 105,000×g for 1 hour. Total microsomal pellet was suspended in 500µL storage buffer (0.1M potassium phosphate buffer + 20% glycerol, pH=7.4) and subsequently separated into two aliquots of approximately equal amounts before storing at -80°C. A Pierce BCA Protein Assay kit (Thermo Scientific) was used to assess total protein content from diluted microsomal fractions (1:100) in a 96-well plate. Concentrations of 1000, 500, 250, 125, 62.5, 0 µg/mL protein standard (Sigma Aldrich) provided a sufficient standard curve to measure all samples by chemiluminescence on a plate reader (SpectraMax-M5). The original microsomal fraction was aliquoted to 5µg/µL total protein and stored at -80°C until western blot analysis.

2.4.2 Gel Electrophoresis & Blotting

Western blot analysis was performed as previously depicted with minor alterations (Feere et al., 2015). Electrophoresis was optimized on a 15-well 10% polyacrylamide resolving gel with 4% stacking gel containing 0.1% sodium dodecyl sulfate (SDS) and polymerized using ammonium persulfate and tetramethylethlenediamine (TEMED) (Bio Basic Inc, Markham, ON). A total of 4µL loading volume contained 4.8µg of protein per well. The samples were diluted in sample buffer (10% SDS, 26% glycerol and 0.5% bromophenol blue) and reduced with beta-mercaptoethanol at 80°C for 20 min. The gel was run at 60V for 20 min followed by 120V for 45mins in Bio-Rad Running Buffer. Protein was transferred to a nitrocellulose membrane for 1.5 hours with 120V in Bio-Rad Transfer Buffer. The membrane was washed with PBS + 0.1% tween (PBS-T) before blocking with PBS-T + 5% skim milk powder + 0.6% bovine serum albumin (BSA) for 1 hour followed by three washes of PBS-T. Primary CYP3A2 rabbit anti-rat antibodies (Millipore, Temecula, CA) were diluted 1:8000 with PBS-T + 0.6% BSA. CYP2C11 mouse anti-rat antibody (Millipore, Temecula, CA) was diluted 1:5000 with PBS-T + 5% skim milk powder. β-Actin housekeeping primary antibody, conjugated to horseradish peroxidase (HRP), was made in mouse (Sigma Aldrich), diluted 1:50000 with PBS-T + 0.6% BSA and made fresh before each use. After 4°C overnight incubation with primary antibody and
subsequent PBS-T washes, secondary antibody (rabbit or mouse) linked to HRP (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:10000 dilution in PBS-T + 0.6% BSA were incubated with the blots for no longer than 1 hour. Imaging was performed on the Bio-Rad VersaDoc Imaging System (Hercules, CA) and accompanying QuantityOne (v4.6.3) software after final washing steps and 1 min incubation of 1 mL chemiluminescent agent Luminata Forte western blot HRP substrate (Millipore, Billerica, MA). After imaging of CYP3A2, the blot was washed, stripped of protein (Restore Western Blot Stripping Buffer, ThermoScientific, Rockford, IL) and washed with PBS-T once more before the application of the β-Actin or CYP2C11 primary antibody before repeating the washes and imaging steps. Densitometry data was normalized to β-actin, presented as a percent of day 0 control samples and standardized across blots by a communal pooled sample run on every blot. Western blot analysis was completed in duplicate for both CYP3A2 and CYP2C11.

2.5 Enzymatic Activity

Enzymatic activity was analyzed by incubating microsomal fractions with testosterone, a known substrate of CYP3A2 and CYP2C11 (Chovan et al., 2007). The respective products 6βOH-testosterone and 16αOH-testosterone were measured via mass spectrometry (MS) in a 96-well plate assay adapted from (Feere et al., 2015). In a final volume of 75µL, 0.2mg/mL microsomal protein and reaction buffer (50 mM potassium phosphate with 2 mM MgCl2 pH 7.4) was incubated with 1µL testosterone (Steraloids Inc., Newport, RI) substrate at concentrations of 12.5, 25, 75, 200 and 400µM for 10 min at 37°C. Reaction volume is completed by incubation with 1mM NADPH (Sigma Aldrich) and shaken at 37°C for exactly 20 min before sequestration using 225µL ice-cold acetonitrile with 80ng/mL flurazepam as an internal standard (Cerilliant, Round Rock, TX). Plates were shaken, centrifuged at 4000×g for 10 min, supernatant diluted 1 in 5 with milliQ water and stored at 4°C no longer than 24 hours until analysis. Enzymatic products were separated on an ultra-performance liquid chromatography (UPLC) Phenomenex Kinetex phenyl-hexyl column (1.7µm particle size, 50mm × 2.1 mm) maintained at 40°C in a Waters ACQUITY UPLC I-Class System (Milford, MA). Mobile phase flow was set to 0.5 ml/min and consisted of UPLC-grade water (A) and acetonitrile (B) both containing 0.1% formic acid with a gradient as follows: 0–0.5 mins, 25% B; 0.5–2 mins 25–35% B; 2–2.5 mins
35–80% B; 2.5–3.5 mins held at 80% B; 3.5 mins 25% B. Analytes were detected using quadrupole time-of-flight mass spectrometry (UPLC-QToF/MS) on a Waters Xevo™ G2S-QToF/MS and Waters ACQUITY I-Class UPLC with parameters as previously described (Feere et al., 2015). The positive mode mass-to-charge ratio (m/z) for 6βOH-testosterone and 16αOH-testosterone (m/z = 305.2117) were quantified using standard curves of purchased 6βOH-testosterone (0.04–50μM) and 16αOH-testosterone (0.09–100μM) (Steraloids Inc., Newport, RI) using QuanLynx v4.1 software. Michaelis-Menten curves generated with GraphPad Prism (v5.0 for Windows; GraphPad Software Inc., San Diego, CA) were used to find the maximum enzymatic reaction rate (V_max), Michaelis-Menten constant (K_m) and average intrinsic clearance (V_max/K_m) for each group.

2.6 Untargeted Metabolomics

2.6.1 Sample & Batch Preparation

Plasma and liver biological matrices each run on two different chromatography columns totaling four metabolomics runs. The first column, a Waters ACQUITY UPLC HSS T3 (1.8μm particle size, 100 mm × 2.1 mm) reverse-phase liquid chromatography (RPLC) column best encompasses hydrophobic molecules while the second hydrophilic interaction liquid chromatography (HILIC) column, a Waters ACQUITY BEH Amide (1.7μm particle size, 100 mm × 2.1 mm), best separates hydrophilic molecules. Plasma and liver samples were prepared as previously described (Velenosi et al., 2016) by adding 3:1 ice-cold acetonitrile with 2.5μM chlorpropamide internal standard (Sigma Aldrich) to 100μL of rat plasma or 200mg ± 5mg homogenized liver sample. Samples were vortexed, incubated at -20°C for 20 min then centrifuged at 14,000×g for 5 min. Supernatant was either diluted 1:5 in water for RPLC or directly injected for HILIC. Sample injection order was randomized and a quality control sample made from pooled samples was run every ten injections to monitor instrument drift. All samples were run in a single batch for each biological matrix and column.
2.6.2  Chromatography & Mass Spectrometry

Both RPLC and HILIC columns were maintained as previously stated (Velenosi et al., 2016) at 45°C and mobile phase flow set to 0.45 ml/min consisting of UPLC-grade water (A) and acetonitrile (B), both containing 0.1% formic acid. A gradient of 0–2 min 60% B; 2–6 min 85% B; 6–8 min 99% B; 8–10 min 1% B was used. The HILIC column followed a gradient of 0–0.5 mins 99% B; 0.5–6 mins 99–50% B; 6–8 mins 50–30% B; 8–8.5 mins 30–99% B. Samples were run separately in succession for both positive and negative electrospray ionization (ESI) modes on the UPLC-QTof/MS instrument described in section 2.5. Mass spectrometer method consisted of capillary voltage 2kV, cone voltage 40V, source temperature at 150°C, desolvation gas flow 1200L/h at 600°C, and cone gas flow 50L/h. Data acquisition was conducted in centroid mode using the MS² method as described in section 1.6.5, with 0.05s scan time over a range of 50-1200 m/z with collision energy of 0V for MS (function 1) and ramped from 15V-50V for MS/MS (function 2) fragmentation. For mass accuracy, lockmass solution leucine-enkephalin (500ng/mL) was used as the lockmass and set at a flow rate of 10µL/min, measured every 10s and averaged over 3 scans. Data was collected by MassLynx v4.1 software (Waters). In anticipation of detecting previously identified uremic toxins IS, PS and 4-ethylphenyl sulfate, a standard curve of these metabolites was added to the front and back of both RPLC runs in plasma and liver.

2.6.3  Data Processing

Data processing for each run and ESI mode was performed separately in R studio (v3.2.3). MassLynx data files were converted from raw to mzData files using convert.waters.raw package v1.0 (github.com/stanstrup/convert.waters.raw). Pooled samples were used to find the optimal peak picking parameters, retention time corrections and grouping parameters simultaneously with the isotopologue parameter optimization (IPO) package v1.0.0 (github.com/rietho/IPO/blob/master/vignettes/IPO.Rmd). Minimum peak width ranged from 2 to 5 seconds, maximum peak width ranged from 10 to 20 seconds and a static ppm of 15 were used as starting parameters for the IPO process. The resulting IPO parameters unique for each mode were inputted into the XCMS package v1.50.1 (Smith et al., 2006) to pick appropriate peaks, integrate the area under the curve and replace zero values for all
samples within the dataset. The CAMERA package v1.32.0 was used to annotate possible isotopes and adducts (Kuhl et al., 2012). XCMS and CAMERA packages were used to integrate positive and negative ESI modes before normalizing to internal standard chlorpropamide and applying a threshold of 30% variability of the quality control. Through an in-house R script positive and negative modes were combined to make one dataset ready for statistical software with masses identified in both modes retained in the mode with greater intensity (Urquhart Laboratory, unpublished).

2.6.4  Metabolite Identification

The accurate monoisotopic mass (m/z) and fragmentation spectrum of each metabolite was used to identify potential metabolite matches within the METLIN, MassBank or Human Metabolome Database (HMDB) (Wishart et al., 2013). This was carried out by first searching the parent m/z ratio in the corresponding ionization mode across all possible adducts with a molecular weight tolerance of ±0.01Da. The MOL file for each suspected metabolite was downloaded from the HMDB website and uploaded using MassFragment, a MassLynx software add-on, to compare expected fragmentation patterns with the experimental fragmentation pattern. Fragmentation profiles of metabolites representing the most experimental fragments were purchased for identity confirmation.

A categorical system of metabolite identification has been adapted from previously defined levels 1 through 4 (Salek et al., 2013). To confirm a suspected metabolite’s identity, a purchased standard was run and compared to a sample known to have the unidentified signal. If the retention time, accurate mass and fragmentation pattern matched across the experimental sample and purchased standard, it was considered “level 1 identified”. Level 2 identified metabolites exhibited HMDB and METLIN database matches with delta ≤ 5ppm and conformation to at least one of the following criteria: a matching online database fragmentation spectrum, or matching m/z ratio in a publication where it was similarly identified. Level 2 identification is typically used when analytical standards are unavailable or the cost prohibits level 1 identification. Level 3 identified metabolites required only a HMDB and METLIN match with delta ≤ 5ppm. Metabolites with multiple matches, zero matches or deltas ≥ 5ppm were considered unknown.
Metabolite standards were obtained as follows: indoxyl sulfate from Gold Biotechnology (Olivette, MO), phenyl sulfate and 4-ethyl phenyl sulfate were synthesized as previously described (Velenosi et al., 2016), allantoin, L-carnitine, 2,8-dihydroxyadenine and equol 4/7 glucuronide were purchased from Toronto Research Chemicals (Toronto, ON), creatinine from Sigma Aldrich and pantothenic acid from Supelco (Bellefonte, PA).

2.7 Gut Microbial Sequencing

2.7.1 Illumina Sequencing

DNA was extracted from caecum swabs using the PowerSoil-96 Well DNA isolation kit from MoBio using convenience modifications of the Earth Microbiome Project protocol (Gilbert et al., 2014). Unique barcoded primers named 515F and 806R (Caporaso et al., 2011; Gilbert et al., 2014) amplified the V4 variable region of the 16S rRNA gene. A total of 12 forward and 24 reverse primers barcodes were used to provide 288 unique primer combinations. The primers are as follows: forward primer [5’-ACACTCTTTCCCTACACGACGCTCTTCCGATCTnnnn(8)GTGCCAGCMGCCGCGGTAA-3’] and reverse primer [5’-CGGTCTCGGATCTTCCGATCTTTCCTCGATCTnnnn(8)GGACTACHVGGGTWTCTAAT-3’] where the 5’ end is the Illumina adaptor sequence, the nnnn indicates four random nucleotides, (8) represents one of the 36 barcoded sequences eight nucleotides in length and the 3’ end is the primer region for V4 (Supplementary Table 1. Appendix B) (Gloor et al., 2010). Amplification was carried out in 42µL total volume with 20µL primer mix (3.2pmol/µL per primer), 20µL GoTaq Hot Start Mastermix (Thermo Scientific) and 2µL template DNA then run for 2 min at 95°C followed by 25 cycles of 1 min at 95°C; 1 min at 52°C and 1 min at 72°C excluding a final elongation. Barcoded PCR products were quantified with a Qubit dsDNA assay kit on a Qubit 2.0 (Life Technologies), normalized by amount of DNA, pooled then purified with a PCR clean-up column. The cleaned DNA is amplified once more with primers OLJ139 [5’AATGATACGGCACGAGACAGATCTACTACCTCTCTCGAGAC3’] and OLJ140 [5’CAAGCAGAAGACCGATACGAGATCGGTCTCGGCGATTCTGCTGAAC3’] (Majerczyk et al., 2010) before paired-end, high-throughput
sequencing on the Illumina MiSeq platform at the London Regional Genomics Centre (LRGC, lrgc.ca, London, ON).

2.7.2 Data Processing

Paired reads, each 220bp long were processed with the Illumina_SOP protocol accessed through Github with minor convenience revisions (https://github.com/ggloor/miseq_bin). After demultiplexing, raw reads were first overlapped with a minimum 30 nucleotides using Pandaseq (v2.5) (Masella et al., 2012) then filtered with in-house Perl and UNIX scripts to ensure exact barcode matching and primer matching with up to two allowable mismatches (Gloor et al., 2010). OTUs were clustered using the uSearch (v7.0.1090) script (Edgar, 2010) at 97% identity and the most abundant sequence was applied for annotation via the mothur script (Schloss et al., 2009) to search the Silva 16S rRNA gene reference database (Silva.nr_v119) (Quast et al., 2013; Yilmaz et al., 2014). In mothur, a bootstrap cut-off of 70% was used for taxonomical identification and redundancy. A total of 1199 OTUs were retained across all samples (Supplementary Table 2. Appendix B). Due to study time-period differences, only days 3, 14, 28 and 42 could be used for sequencing analysis. In R studio (v3.2.3) the zCompositions (v1.0.3-1) package (Martín-Fernández et al., 2003) was used for zero-replacement before data was centre-log ratio (clr) transformed for compatibility with downstream univariate and multivariate statistics (Gloor & Reid, 2016; Gloor et al., 2016b).

2.8 Statistical Analysis

2.8.1 Disease Markers, Real-Time PCR, Western Blotting & Enzymatic Activity Assay

DME measurements and disease markers urea and creatinine are presented as mean ± SEM and analyzed by 2-way ANOVA paired with Sidak’s multiple comparisons test. *p < 0.05 compared to matching day control indicates significance.

2.8.2 Untargeted Metabolomics

Principal component analysis (PCA) was used to evaluate the initial separation between CKD and control over time for each of the four analytical runs using MassLynx software
and the EZInfo v2.0 package (Umetrics, Umeå, Sweden). Placement of each sample within a PCA plot is determined by the entire detectable metabolite composition within each sample with relation to all other samples. Samples within close proximity are considered “clustered” and represent samples with similar compositions. Data for each of the four runs was Pareto scaled to remove emphasis on metabolites with large magnitude of variance. Outliers were removed utilizing a Hotelling’s analysis with a maximum allowable $T^2$ range of 35 (Wiklund, 2008). Pooled samples are confirmed to show little variance and a clear separation is identified between control and condition before applying statistics. Each statistical method: multivariate, univariate and correlations to each DME, was performed independently on the same metabolomics dataset for each of the four runs with the aim to isolate metabolites related to CYP alterations. Each of the 1] multivariate, 2] univariate and 3] correlation analyses are described below.

1] Multivariate analysis was performed on all four runs, on all 5 days in comparison with same day control. EZInfo was used to generate orthogonal partial least squares discriminant analysis (OPLS-DA) of the original PCA (Figure 2.1). OPLS-DA, unlike PCA, is a supervised comparison between known groups. To assess multivariate OPLS-DA sufficiency, each comparison received goodness of fit values $R^2$ and $Q^2$. $R^2$ is the goodness of fit estimated for the model and $Q^2$ is the level of prediction accuracy the model infers if the experiment were to be repeated. The values are dependent and thus can be expressed as a ratio ($R^2/Q^2$) with the expectation that $R^2/Q^2 < 2$ or $Q^2 > 0.5$ (Triba et al., 2015). The metabolites characteristic of treatment were then plotted as an S-plot with axis of “$p(\text{corr})[1]$” representing the treatment difference and “$p[1]$” representing the magnitude of the metabolite’s influence. Strict thresholds were applied (VIP > 0.8; $p(\text{corr})[1] > 0.4$ or < -0.4) by finding the variable importance in projection (VIP) and the $p(\text{corr})[1]$ axis as a general measure of magnitude and difference between treatments (Farrés et al., 2015) (Figure 2.1). Only metabolites that met or exceeded the S-plot thresholds on two or more consecutive time points were retained for comparison with univariate and correlative analyses.

2] Univariate analysis on the original metabolomics datasets were used to assess the relative concentration of each metabolite over time and course of the disease. Open access
online software MetaboAnalyst 3.0 was used to perform a p-value corrected (FDR = 0.05) independent 2-way ANOVA on each metabolite via the “Time Series” and “Two-factor independent samples” applications (Wishart et al., 2013). No additional data filtering, normalization or transformation was performed. Pareto scaling was applied for consistency. Significance (p<0.05) was required for both “Time” and “Disease” to retain the metabolite for comparison with multivariate and correlative analyses.

3) Spearman correlations were conducted between each metabolite and the mRNA, protein or enzymatic activity levels of each enzyme. Spearman correlation analysis of metabolomics results have been conducted in the past to compare metabolites to other metabolites (Camacho et al., 2005), metabolites to bacterial abundances (Theriot et al., 2014; McMillan et al., 2015), and metabolites to gene expression (Bartel et al., 2015; Auslander et al., 2016). Original processed metabolomics dataset for each run was matched by sample to the corresponding DME dataset and a correlation coefficient (r) obtained before comparison to the final multivariate and univariate subsets. R-values were manually filtered with high stringency (r > 0.65 or r < -0.65) since more than 50% of the dataset satisfied p<0.0001. After filtering, correlation analysis provided the smallest subset of the three analysis methods. Metabolites on this list that did not also satisfy univariate analysis were removed from the correlation subset. Metabolites that did not satisfy multivariate analysis are indicated but retained to capture biologically relevant changes independent of magnitude.
Figure 2.1. Example of multivariate analysis workflow utilizing OPLS-DA and S-plots. A PCA is made using EZInfo software (1), comparisons are chosen for OPLS-DA, an OPLS-DA plot of sample distribution is made and $R^2$ and $Q^2$ thresholds are applied (2). Successful OPLS-DA plots are further analyzed by observing metabolite contributions towards either condition by S-plot (3) where VIP and $p(\text{corr})[1]$ thresholds are applied. From the S-plot, a list of $m/z$ ratios and retention times (4) in addition to fragmentation pattern are searched within online databases such as HMDB in attempt to identify the metabolite.
2.8.3 Caecal Microbiota

Multivariate PCA was performed in EZInfo as described for untargeted metabolomics except no scaling was utilized to emphasize large variations. Although applied infrequently in the microbiology field (Hong et al., 2011; Stewart et al., 2015), R² and Q² values are important for understanding the variability in the PCA and were calculated for consistency with metabolomics analysis. To obtain univariate differences between CKD and control groups, the effect size and overlap for each bacterial taxonomic group was calculated for each time point individually using the R package ALDEx2 (v1.2.0) (bioconductor.org/packages/release/bioc/html/ALDEx2.html) (Fernandes et al., 2014; Gloor et al., 2016a). Severe thresholds were applied to both effect size (> 1.5 or < -1.5) and overlap (< 6.5%) for each bacterial abundance (Macklaim et al., 2013; Halsey et al., 2015; Gloor et al., 2016a). OTUs that met or exceeded the thresholds were graphed and significance was defined as satisfying the effect size and overlap thresholds with 95% confidence. Where applicable, genera were manually searched for species and strain information by Targeted Loci Nucleotide BLAST application through NCBI (blast.ncbi.nlm.nih.gov/Blast.cgi).
Chapter 3

3 Results

3.1 Model Validation

First, I validated the adenine-fed CKD model by measuring plasma CKD markers urea and creatinine alongside kidney histological analysis across all time points. CKD markers urea and creatinine both showed significant increase in CKD rat plasma by day 14 (Figure 3.1.A-B). This increase continued to a 9-fold and 11-fold difference between CKD and control for urea and creatinine, respectively, on day 42. Kidney histology showed enlarged tubules, inflammatory cells and fibrosis by day 14 through to day 42 (Figure 3.1.C-H). Animal weights did not change between groups (Table 3.1).
Figure 3.1. Assessment of CKD in Wistar rats orally administered 0.5% adenine over 42 days. (A) Plasma urea (mM) and (B) serum creatinine (µM) concentrations of control and CKD rats presented as mean ± SEM. *p < 0.05 when compared to matching day control; n ≥ 6. H&E stained rat kidney sections from day 42 control (C) and CKD days 3 (D), 7 (E), 14 (F), 28 (G) and 42 (H). Arrows indicate enlarged nephron tubules and areas of fluid retention. Inflammatory cells and areas of atrophy are evident on days 14, 28 and 42.
Table 3.1. Weight in grams of control and CKD rats over 42 days.

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>Control</th>
<th>CKD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>206.5 ± 1.4 (n=41)</td>
<td>207.1 ± 1.8 (n=36)</td>
</tr>
<tr>
<td>Day 3</td>
<td>188.6 ± 1.1 (n=41)</td>
<td>186.1 ± 1.5 (n=36)</td>
</tr>
<tr>
<td>Day 7</td>
<td>201.0 ± 1.5 (n=24)</td>
<td>202.4 ± 1.9 (n=26)</td>
</tr>
<tr>
<td>Day 14</td>
<td>227.3 ± 2.0 (n=18)</td>
<td>227.9 ± 3.3 (n=19)</td>
</tr>
<tr>
<td>Day 28</td>
<td>257.3 ± 2.6 (n=12)</td>
<td>258.3 ± 5.0 (n=13)</td>
</tr>
<tr>
<td>Day 42</td>
<td>274.5 ± 8.4 (n=6)</td>
<td>254.7 ± 12.7 (n=7)</td>
</tr>
</tbody>
</table>
3.2 Hepatic CYP3A2 & CYP2C11 mRNA Expression over CKD Progression

Having confirmed the adenine-fed rats were developing CKD, the mRNA expression of DMEs CYP3A2 and CYP2C11 were assessed in the liver. CYP3A2 mRNA expression was minimally decreased on day 3, recovered on day 7, then declined substantially by day 14 (-83%, p<0.001) which persisted to day 42 (-99%, p<0.001) (Figure 3.2.A). CYP2C11 mRNA expression was unchanged on day 3 but largely increased in the control group on day 7 leaving CKD rats well below normal (-76%, p<0.001) (Figure 3.2.B). On day 14 (-84%, p<0.001), day 28 (-96%, p<0.001), and day 42 (-98%, p<0.001) the CKD CYP2C11 mRNA expression remained low in comparison to control.

3.3 Hepatic CYP3A2 & CYP2C11 Protein Expression over CKD Progression

To ascertain whether protein expression was changed with respect to mRNA expression, CYP3A2 and CYP2C11 protein quantification was carried out by western blotting. Decreases in CYP3A2 protein levels were seen on day 14 (-63%, p<0.001), day 28 (-86%, p<0.001) and day 42 (-85%, p<0.01) (Figure 3.2.C). CYP2C11 protein quantification also shows depletion in CKD but starting on day 7 (-42%, p<0.05) through to day 42 (-83%, p<0.001) (Figure 3.2.D). A non-specific CYP3A2 band was not quantified.

3.4 Hepatic CYP3A2 & CYP2C11 Enzymatic Activity over CKD Progression

Sequentially, CYP3A2 and CYP2C11 enzymatic activity was assessed from liver microsomes. CYP3A2 intrinsic activity in CKD rats decreased on day 3, recovered on day 7 and fell again 3.6-fold by day 14, 13-fold by day 28 and nearly 14-fold lower than controls by day 42 (Figure 3.2.E). The intrinsic activity of CYP2C11 showed a 4.6-fold difference between CKD and control as early as day 7 and up to 12.8-fold difference by day 42 (Figure 3.2.F). $V_{\text{max}}$, $K_m$ and intrinsic clearance are summarized (Table 3.2).
Figure 3.2. Relative mRNA expression, protein expression and enzymatic activity levels of CYP3A2 and CYP2C11. CYP3A2 (A) and CYP2C11 (B) mRNA expression and protein expression, CYP3A2 (C) and CYP2C11 (D), with representative western blots. Values were relative to β-actin represented as the mean ± SEM, normalized to control day 0 and arbitrarily defined as 100%. A non-specific band in CYP3A2 immunoblot was not quantified. Enzymatic activity of CYP3A2 (D) and CYP2C11 (E) in control and CKD rats represented as the mean intrinsic clearance $V_{\text{max}}/K_m$ [(pmol/min/mg protein)/µM] of testosterone metabolite ± SEM. *$p < 0.05$ when compared to matching day control; n ≥ 6.
Table 3.2. CYP3A2 and CYP2C11 enzymatic activity over CKD progression. 6βOH-testosterone (CYP3A2) and 16αOH-testosterone (CYP2C11) production of liver microsomes measured following incubation with NADPH and testosterone. *p<0.05 compared to matching day control; n ≥ 6.

### 6βOH-testosterone (CYP3A2)

<table>
<thead>
<tr>
<th></th>
<th>V$_{max}$</th>
<th>K$_m$</th>
<th>Intrinsic Clearance (V$_{max}$/K$_m$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CKD</td>
<td>Control</td>
</tr>
<tr>
<td>Day 0</td>
<td>35938±2841</td>
<td>35938±2841</td>
<td>29.5±9.02</td>
</tr>
<tr>
<td>Day 3</td>
<td>56184±5640</td>
<td><strong>26425±2097</strong></td>
<td>40.1±14.46</td>
</tr>
<tr>
<td>Day 7</td>
<td>49912±1806</td>
<td>42396±4911</td>
<td>40.2±5.218</td>
</tr>
<tr>
<td>Day 14</td>
<td>38223±2797</td>
<td>12804±1983</td>
<td>49.4±12.34</td>
</tr>
<tr>
<td>Day 28</td>
<td>61702±3069</td>
<td>10102±1289</td>
<td>66.6±10.43</td>
</tr>
<tr>
<td>Day 42</td>
<td>45706±2266</td>
<td><strong>7849±1297</strong></td>
<td>58.5±49.46</td>
</tr>
</tbody>
</table>

### 16αOH-testosterone (CYP2C11)

<table>
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<tr>
<th></th>
<th>V$_{max}$</th>
<th>K$_m$</th>
<th>Intrinsic Clearance (V$_{max}$/K$_m$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CKD</td>
<td>Control</td>
</tr>
<tr>
<td>Day 0</td>
<td>17983±1210</td>
<td>17983±1210</td>
<td>14.2±4.56</td>
</tr>
<tr>
<td>Day 3</td>
<td>25892±2353</td>
<td>15453±1195</td>
<td>21.8±8.28</td>
</tr>
<tr>
<td>Day 7</td>
<td>41420±2488</td>
<td>27114±3431</td>
<td>14.3±4.09</td>
</tr>
<tr>
<td>Day 14</td>
<td>51824±2556</td>
<td><strong>17521±2406</strong></td>
<td>16.9±3.75</td>
</tr>
<tr>
<td>Day 28</td>
<td>72358±2064</td>
<td><strong>16236±1548</strong></td>
<td>20.1±2.45</td>
</tr>
<tr>
<td>Day 42</td>
<td>62571±3147</td>
<td><strong>6658±513</strong></td>
<td>16.2±3.71</td>
</tr>
</tbody>
</table>

*aV$_{max}$, K$_m$ and intrinsic clearance (V$_{max}$/K$_m$) were obtained by Michaelis-Menten kinetics.*
3.5 Plasma & Liver Metabolomics

Having demonstrated changes in mRNA, protein levels and enzymatic activity in the CYP enzymes, I next performed an untargeted metabolomics analysis by MS to assess changes in metabolite composition. Principle component analysis (PCA) clearly separated CKD and control for both rat plasma and liver samples (Figure 3.3). Early disease stages are arbitrarily defined as day 3-14 and late stages days 28 and 42. A loadings biplot for each run shows the position of each loading/metabolite (Figure 3.4). OPLS-DA plots were generated for each of the twenty comparisons across all four runs. OPLS-DA plots comparing control versus CKD at each time point for the plasma RPLC run provide an example for the other three runs (Figure 3.5). R² and Q² parameters were used to accompany the interpretation of OPLS-DA plots and R²/Q² ratio < 2 represent results with high reproducibility between control and CKD on each day (Table 3.3). Metabolites in rat plasma were well separated from control as early as day 3 when using RPLC (Figure 3.3.A). Liver RPLC showed far less separation between control and CKD before day 28 (Figure 3.3.B). The HILIC column showed separation back to day 7 except for poor Q² values on day 14 in both plasma and liver samples (Figure 3.3.C-D).
Figure 3.3. Unsupervised principle component analysis (PCA) plots of rat plasma (A) and liver (B) metabolome separated by RPLC. PCA of plasma (C) and liver (D) metabolome separated by HILIC. Each point is either control (●), early stage CKD defined by day 3, 7 and 14 (■), or late stage CKD defined by days 28 and 42 (■). Each axis is either the first [1], second [2] or third [3] principle component showing the two components representing the largest variation between groups and displayed as a percentage of component contribution. Placement of each sample is determined by the metabolite composition within each sample and clustered samples share similar compositions. Data is centered and Pareto-scaled. Select rat samples were removed as outliers (A) no outliers, (B) a day 3 and day 42 control, (C) a day 28 CKD sample, and (D) a day 7 control sample.
Figure 3.4. Loadings biplot of rat plasma (A) and liver (B) metabolome separated by RPLC and plasma (C) and liver (D) metabolome separated by HILIC. Each point is either a sample control (■), early stage CKD defined by day 3, 7 and 14 (■), late stage CKD defined by days 28 and 42 (■), or a metabolite loading (●). Metabolites PS (i), EPS (ii) and IS (iii) are indicated. Each axis is either the first [1], second [2] or third [3] principle component showing the two components representing the largest variation between groups and displayed as a percentage of component contribution. Data is centered and Pareto-scaled. Select rat samples were removed as outliers (A) no outliers, (B) a day 3 and day 42 control, (C) a day 28 CKD sample, and (D) a day 7 control sample.
Figure 3.5. OPLS-DA plots generated from the PCA of rat plasma separated by RPLC for day 3 (A), day 7 (B), day 14 (C), day 28 (D) and day 42 (E). Each point is a control (■) or CKD (■) sample. Each axis is the first [1] and second [2] principle component representing the largest variation between supervised groups and displayed as a percentage of component contribution.
Table 3.3. Multivariate OPLS-DA parameters $R^2$ and $Q^2$. $R^2$ and $Q^2$ values for plasma and liver metabolomics using RPLC and HILIC across all time points.

<table>
<thead>
<tr>
<th></th>
<th>Rat Plasma</th>
<th>HILIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RPLC</td>
<td>HILIC</td>
</tr>
<tr>
<td><strong>Day 42</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comp No.</td>
<td>$R^2$</td>
<td>$Q^2$</td>
</tr>
<tr>
<td>[1]</td>
<td>0.9859</td>
<td>0.9175</td>
</tr>
<tr>
<td>[2]</td>
<td>0.9859</td>
<td>0.9493</td>
</tr>
<tr>
<td>[3]</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 28</strong></td>
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</tr>
<tr>
<td>Comp No.</td>
<td>$R^2$</td>
<td>$Q^2$</td>
</tr>
<tr>
<td>[1]</td>
<td>0.9982</td>
<td>0.6410</td>
</tr>
<tr>
<td>[2]</td>
<td>0.9982</td>
<td>0.8176</td>
</tr>
<tr>
<td>[3]</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 14</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comp No.</td>
<td>$R^2$</td>
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</tr>
<tr>
<td>[1]</td>
<td>0.8940</td>
<td>0.5438</td>
</tr>
<tr>
<td>[2]</td>
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</tr>
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<td>[3]</td>
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<table>
<thead>
<tr>
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<th>Rat Liver</th>
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<tbody>
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<td>HILIC</td>
</tr>
<tr>
<td><strong>Day 42</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comp No.</td>
<td>$R^2$</td>
<td>$Q^2$</td>
</tr>
<tr>
<td>[1]</td>
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</tr>
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</tr>
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<td>[3]</td>
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<td><strong>Day 28</strong></td>
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<tr>
<td>Comp No.</td>
<td>$R^2$</td>
<td>$Q^2$</td>
</tr>
<tr>
<td>[1]</td>
<td>0.9691</td>
<td>0.7830</td>
</tr>
<tr>
<td>[2]</td>
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<td>0.9284</td>
</tr>
<tr>
<td>[3]</td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Comp No.</td>
<td>$R^2$</td>
<td>$Q^2$</td>
</tr>
<tr>
<td>[1]</td>
<td>0.8357</td>
<td>0.0070*</td>
</tr>
<tr>
<td>[2]</td>
<td>0.8357</td>
<td>0.2953©</td>
</tr>
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<td>[3]</td>
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<tr>
<td>Comp No.</td>
<td>$R^2$</td>
<td>$Q^2$</td>
</tr>
<tr>
<td>[1]</td>
<td>0.9973</td>
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</tr>
<tr>
<td>[2]</td>
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<tr>
<td>[3]</td>
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</tr>
<tr>
<td>[3]</td>
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<td>0.7510</td>
</tr>
</tbody>
</table>

* Values indicate poor predictability. ©Abnormal values, considered poor predictability. Comp No. = principle component number.
3.6 DME & Uremic Toxins

After establishing changes in the metabolomic profile by PCA, metabolites were correlated to CYP mRNA, protein and enzymatic activity after satisfying multivariate and univariate analysis. 204 \( m/z \) ratios were identified across all four runs that correlated with either CYP3A2 or CYP2C11 (Supplementary Table 3. Appendix B). Of these 204 \( m/z \) ratios, 9 metabolites were identified at identification level 1 using purchased standards. These metabolites include: Allantoin, L-carnitine, creatinine, 2,8-dihydroxyadenine, equol-4/7-O-glucuronide, 4-ethylphenyl sulfate, IS, pantothenic acid (vitamin B5) and PS (Table 3.4). IS, PS and 4-ethylphenyl sulfate concentrations across both plasma and liver samples using RPLC were quantified via standard curve. All three metabolites show significantly increased concentrations (\( p<0.0001 \)) on days 28 and 42 for both plasma and liver tissue (Figure 3.6).
**Table 3.4.** Metabolites classified level 1 from CKD and control rat plasma and liver untargeted metabolomics.

<table>
<thead>
<tr>
<th>ID Level</th>
<th>Mass Error (ppm)</th>
<th>Mass (m/z)</th>
<th>Retention Time (min)</th>
<th>Empirical Formula (Adduct)</th>
<th>Identity</th>
<th>Matrix</th>
<th>Column</th>
<th>ESI Mode</th>
<th>CYP3A2 Correlation (r value)</th>
<th>CYP2C11 Correlation (r value)</th>
<th>Satisfies Multivariate Analysis</th>
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</thead>
<tbody>
<tr>
<td>1 2</td>
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<td>54.57</td>
<td>C4H6N4O3 (M+Na)</td>
<td>Allantoin</td>
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<td>RPLC</td>
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<tr>
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<td>0.56</td>
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<td>Allantoin</td>
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<td>RPLC</td>
<td>(-)</td>
<td>-0.7284</td>
<td>-0.7920</td>
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<tr>
<td>1 1</td>
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<td>C7H15NO3 (M+H)</td>
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<td>RPLC</td>
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*aMass error was obtained using the 4th decimal place m/z from the Human Metabolome Database (HMDB).

*bP = Plasma; L = Liver. Metabolites satisfying univariate analysis and Spearman correlation to same-sample CYP3A2 or CYP2C11 data with correlation coefficients (r value) listed. 2-way independent ANOVA was conducted via MetaboAnalyst v3.0 (Wishart et al., 2013) using FDR<0.05 to correct for multiple comparisons and satisfaction required p<0.05 across both “Time” and “Disease”.

C Multivariate analysis required VIP > 0.8 and 0.4 < p(corr)[1] < -0.4 indicating adequate separation by OPLA-DA and S-plot.
Figure 3.6. Quantitative analysis of metabolites IS, PS and EPS. Plasma IS (A), PS (C), EPS (E) (µM) and liver IS (B), PS (D) and EPS (F) (pmol/mg liver tissue) concentrations obtained via untargeted metabolomics. Results are presented as mean ± SEM, *p < 0.0001 when compared to same day control; n≥6.
3.7 Caecal Microbiota

To understand if the gut microbiota was changing in parallel with metabolite changes, next-generation Illumina sequencing was used to assess the bacterial composition of the caecum. Following data processing of Illumina sequencing reads, 1199 bacterial OTUs were identified (Supplementary Table 2. Appendix B). Unsupervised PCA analysis separated caecum samples primarily by time, regardless of disease state along the first principle component (Figure 3.7.A). The PCA also clearly separated CKD and control groups at day 28 and 42 on the second principle component (day 28: \( R^2 = 0.97 \); \( Q^2 =0.71 \) and day 42: \( R^2 = 0.98 \); \( Q^2 =0.70 \)) (Figure 3.7.B). The ALDEx2 R package (Fernandes et al., 2014; Gloor et al., 2016a) was used to compare the relative bacterial abundances responsible for the observed clustering on each day. Using effect size and overlap statistical thresholds, relative abundance of each OTU was tabulated and assessed for trends (Table 3.5). Only two bacterial OTUs changed between control and CKD on two or more consecutive days with respect to effect size and overlap. The first OTU was from the phylum Firmicutes and genus Turicibacter and was significantly higher in CKD rats compared to control animals on days 14, 28 and 42 (Figure 3.8.A) with an increasing trend associated with disease progression. The second OTU from phylum Bacteroidetes and genus Parabacteroides showed a significant decrease in control rats over time, but CKD rats did not show a similar decrease on days 28 and 42 (Figure 3.8.B).
Figure 3.7. Unsupervised principle component analysis (PCA) of control and CKD rat caecum bacterial sequences coloured by (A) day 0 (■), 3 (■), 14 (■), 28 (■) and 42 (■) or by (B) treatment, CKD (■) or control (■). Data is centered without scaling.
Table 3.5. Relative abundances of caecal bacteria. Taxonomy of bacteria with relative abundance effect sizes > 1.5 or < -1.5 and overlap of < 6.5% across days 3, 14, 28 and 42 obtained using R v3.2.3 package ALDEx2 v1.2.0; n ≥ 6 per group.

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<th>Genus</th>
<th>Effect Size</th>
<th>Overlap (%)</th>
<th>OTU #</th>
<th>% Identity</th>
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<tr>
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*Positive effect sizes indicate bacteria with higher relative abundance in control while negative values indicate bacteria with a higher relative abundance in CKD. Bolded values indicate bacteria with -2 < effect size > 2 and overlap < 0.03%. *Clostridium_sensu_stricto_.1.
Figure 3.8. Average relative abundance of genus Turicibacter (A) and genus Parabacteroides (B) displayed as an average relative abundance ratio of all OTUs ± 95% confidence interval using R v3.2.3 package ALDEx2 v1.2.0. *Effect size < -1.5 and overlap < 6.5% compared to same day control; n ≥ 6.
Chapter 4

4 Discussion

The pharmacokinetics of many drugs are unpredictably altered in CKD and these patients are susceptible to ADEs (Corsonello et al., 2005; Manley et al., 2005). Hepatic DMEs such as CYP3A4 and CYP2C9 are responsible for the non-renal drug clearance of nearly half of all marketed pharmaceuticals. It is therefore suggested that DMEs play a role in the altered pharmacokinetics of CKD. Although studies on the downregulation of CYPs in CKD patients are ongoing, animal models clearly identify downregulation associated with loss of renal clearance and subsequent retention of uremic toxins where mechanistic studies currently suggest a wide array of pathways from pre-transcriptional regulation through to direct inhibition of DMEs by uremic toxins, inflammatory factors and hormones (Guévin et al., 2002; Sun et al., 2004; Michaud et al., 2006, 2008; Barnes et al., 2014; Velenosi et al., 2014; Volpe et al., 2014; Yeung et al., 2014). Uremic toxins are also suggested to change the environment of the gut, altering the relative abundance of gut bacteria favouring uremic-toxin producing microbes and creating a state of dysbiosis in CKD patients (Wong et al., 2014; Felizardo et al., 2016; Vaziri, 2016). Further, metabolites or toxins produced by the altered microbiota may fuel the cycle of CKD progression and potentially DME downregulation. However, the pathophysiological factors of uremia and dysbiosis had yet to be tested temporally. In this thesis, uremia and dysbiosis have been characterized over CKD progression to identify potential causes of DME downregulation. Metabolite and bacterial profiles were compared to the expression and activity of CYP3A2 and CYP2C11 in an attempt to support my hypothesis that uremic toxins and/or bacterial alterations are temporally associated with changes in DME regulation.

4.1 Conclusions

4.1.1 CKD Characterization

As expected, urea and creatinine levels increased non-linearly in response to adenine-induced CKD (Kobayashi et al., 2014; Feere et al., 2015). Both disease markers urea and
creatinine confirmed CKD induction by day 14 which increased in severity through to the end of the study and matched with kidney histology (Figure 3.1). Pathohistological features including enlarged nephron tubules, areas of fluid retention, inflammation, atrophy and adenine deposits were seen in diseased kidneys after day 14 as observed in previous studies using this model (Ali et al., 2013). Body weights were unchanged between groups, due to the validity of the pair-feeding method (Figure 3.2).

4.1.2 DMEs over CKD Progression

CYP3A2 and CYP2C11 DMEs were both negatively impacted by CKD on all three levels of detection as previously observed (Hashimoto et al., 1997; Velenosi et al., 2012). The enzymatic activity and protein levels mirror what is seen in mRNA expression suggesting that DMEs are influenced at a transcriptional level, influencing the production of protein and ultimately enzyme activity. CYP3A2 has stable expression in control rats while in CKD, levels decrease starting on day 14. These observations suggest a constitutive factor required for expression is being removed, inhibited or downregulated. Although further studies are required, this could be mediated by the increase in uremic toxins discussed in section 4.1.4 (Wright et al., 1997). In contrast, CYP2C11 shows an increase in control rats as early as day 7 but a failure to increase in CKD rats. The increase in control CYP2C11 has been noted previously in healthy male rat juveniles where it is suspected to reflect the increase of endogenous substrate testosterone during puberty (Wright et al., 1997; Yun et al., 2010). The testosterone levels in male Wistar rats are known to increase starting at 25 days of age and peaking at 60 days of age (Ghanadian et al., 1975). The estimated age of our rats is 60 days of age on day 28 of the study which aligns with our observed peak in CYP2C11 mRNA on day 28 (Figure 3.2.B). This may suggest we are capturing the end of puberty and testosterone peak where CKD may be inhibiting the testosterone peak in these adolescent rats. Additionally, CKD is known to impact male patients by causing hypogonadism associated with decreased levels of testosterone further suggesting a hormonal role in CYP regulation (Carrero et al., 2011). CYP2C11 is also influenced by alterations in the normally cyclic levels of growth hormone (GH) where continuous GH release or complete loss of GH production will both downregulate CYP2C11 (Kaufhold et al., 2002). Interestingly, one-third of pediatric CKD patients experience GH insensitivity.
and stunted stature (Cayir & Kosan, 2015; Akchurin et al., 2017). It is therefore possible that the rat CYP2C11 downregulation is due to insufficient levels of substrate testosterone or irregular production of GH. However, testosterone was not identified as a correlated metabolite and GH was not targeted for analysis.

Perhaps the differing trends seen between CYP3A2 and CYP2C11 are attributed to nuclear receptor differences. CYP2C11 is shown to be less dependent on HNF-4α induction, and CKD-induced receptor binding inhibition is less extensive for CYP2C11 than it is CYP3A2 (Honkakoski & Negishi, 2000; Velenosi et al., 2014; Feere et al., 2015). Alternatively, it is possible that the inactivation of shared nuclear receptor PXR or reduced receptor binding capacity of RNA polymerase II is more severe for CYP2C11; however, this has yet to be tested (Mikamo et al., 2003; Velenosi et al., 2014; Feere et al., 2015).

Enzymatic activity effectively produced the same trend as mRNA and protein results for both enzymes. However, CYP3A2 $K_m$ showed a significant increase between CKD and control on days 28 and 42. The increased $K_m$ suggests the concentration of substrate testosterone required to obtain the same half-maximal velocity ($V_{\text{max}}/2$) has increased. This could be explained by i) the upregulation of another enzyme with substrate specificity for testosterone, or more likely, ii) an alternate CYP3A2 substrate associated with CKD days 28 and 42 was bound to CYP3A2 prior to microsome isolation and had a stronger binding affinity than testosterone (Berg et al., 2012). This occurred in another study using 4-hydroxylation of triazolam (Toda et al., 2009) although the reason was unexplained. Overall, we can conclude that both CYPs studied herein are negatively regulated in CKD at the transcriptional and expression levels, and CYP2C11 changes occur earlier than those of CYP3A2.

4.1.3 Metabolome over CKD Progression

Untargeted metabolomics analyzed by PCA showed that plasma RPLC allowed for the greatest magnitude of separation between control and CKD rats when compared to plasma HILIC separation. By PCA visualization, plasma samples showed greater separation in earlier stages of disease (days 3-14) than the liver samples. Unlike the plasma samples, liver metabolites were better differentiated with the HILIC column. This suggests CKD
first inflicts a uremic environment in the plasma before infiltrating the liver, which is the physiological expectation since the reduced GFR in CKD directly increases uremic toxin concentration at the glomerulus before the blood is cycled through the liver. This also tells us that uremic changes overlap with the early changes in CYP3A2 and CYP2C11, supporting the hypothesis that uremic toxins are involved with DME regulation.

4.1.4 Uremic Toxins Correlated with DMEs

Metabolites from each run were subjected to correlation analysis with CYP3A2 or CYP2C11 mRNA, protein or enzymatic activity levels. Of the 204 m/z ratios retrieved, 8 of the 9 identified at level 1 classification were seen to increase with CKD progression [allantoin, creatinine, 2,8-dihydroxyadenine, pantothenic acid (vitamin B5), IS, PS, equol-4/7-O-glucuronide and 4-ethylphenyl sulfate]. L-carnitine was the only level 1 metabolite that showed a positive correlation with CYP downregulation and thus decreased over CKD progression.

Allantoin and creatinine are non-protein bound metabolites successfully eliminated by dialysis (Meyer & Hostetter, 2007). This makes them potentially less harmful than their protein-bound counterparts, and in general, the implications of non-protein bound uremic toxins with relation to drug metabolism or altered pharmacokinetics has largely been overlooked. Allantoin is an expected component of uremia because it is the primary elimination product of uric acid and increases are observed in the plasma and urine of CKD rats induced by 5/6th nephrectomy or adenine (Akiyama et al., 2012; Zhao et al., 2013a, 2013b). Here we also find allantoin in rat liver, suggesting further infiltration due to advanced uremia. Allantoin is generally not produced by humans because we lack uricase activity although allantoin has been detected in CKD patients (Toyohara et al., 2010; Niwa, 2013). Allantoin in humans is suggested to reflect the presence of uric acid-converting reactive oxygen species; however, it could provide evidence for uric acid translocation into the gut. Bacteria in our gut possess uricase activity and are capable of producing allantoin, although studies are needed to confirm this hypothesis (Zhao et al., 2013a; Wong et al., 2014).
As discussed (see section 1.3.3), creatinine is a product of creatine and the most common marker of glomerular function used as a clinical tool to calculate eGFR. CYP3A2 and CYP2C11 both correlate with creatinine and urea, the two disease markers defining CKD in this model. Since creatinine increases with respect to CKD and DMEs correlate with CKD onset, creatinine by default will correlate with DME downregulation. However, creatinine involvement with DME regulation has been overlooked and little is known about its pathophysiological impact in CKD. Studies have established that of all creatinine in a patient experiencing renal failure, a variable 16-66% undergoes metabolic clearance (Wyss & Kaddurah-Daouk, 2000). Interestingly, up to 68% of creatinine cleared by metabolism is hypothesized to be converted back to creatine by creatinase-possessing bacteria of the gut, suggesting creatinine translocation across the gut lumen (Wyss & Kaddurah-Daouk, 2000). The creatine can then i) return to the plasma via enteric cycling explaining raised levels of creatine in CKD patients, ii) or continue to be metabolized by gut bacteria to end-products 3,1-methylhydantoin or glycolate (Wyss & Kaddurah-Daouk, 2000). Alternatively, creatinine can undergo oxidation to methylguanidine or methylurea of which methylguanidine has been identified as a harmful uremic toxin, progressing CKD when administered to rats (Wyss & Kaddurah-Daouk, 2000). Ultimately, more information on the toxic or adverse effects of creatinine and its degradation products need to be established with respect to DME regulation in CKD (Storm et al., 2013).

2,8-Dihydroxyadenine, the adenine metabolite responsible for the induction of CKD, is also associated with CYP3A2 and CYP2C11. 2,8-Dihydroxyadenine induces CKD in a mechanistic, concentration-dependent manner and thus, DMEs correlating with CKD onset are expected to correlate with 2,8-dihydroxyadenine (Terai et al., 2008). Since DMEs are downregulated in other models of CKD, it is unlikely that 2,8-dihydroxyadenine is impacting DME regulation directly (Leblond et al., 2002; Velenosi et al., 2014; Feere et al., 2015).

Pantothenic acid (vitamin B5) is an essential nutrient found in many foods, including the provided rat chow, that is metabolized into coenzyme A, a molecule necessary for healthy biochemical synthesis and energy metabolism (Berg et al., 2012). However, pantothenic acid has rarely been associated with toxic effects nor has any tolerable upper dosage been
established (Institute of Medicine, 1998; Kelly, 2011). An elegant study of organic anion transporter 1 (Oat1) suggests pantothenic acid requires Oat1 transport since it is accumulated in the plasma of Oat1 knockout mice (Wikoff et al., 2011). Additionally, administration of probenecid, a drug known to inhibit Oat transporters, increases plasma pantothenic acid levels (Kelley, 1975; Niwa, 2013). The proposed inhibition of OATs in CKD is associated with high-affinity uremic substrates, effectively incapacitating OAT function and likely causing the increase of pantothenic acid by inhibiting its movement though excretory pathways (Nigam et al., 2015). Pantothenic acid has not been studied as a modulator of DMEs possibly because it is considered non-toxic. However, studies are required to confirm the presence of seemingly non-toxic endogenous metabolites like pantothenic acid are not modulating DMEs.

Gut derived uremic toxins that have prospect for being involved in DME downregulation from this study include IS, PS, 4-ethylphenyl sulfate, equol-4/7-O-glucournide and products of L-carnitine metabolism. IS and PS are two highly retained gut-derived uremic toxins (Wikoff et al., 2011; Leong & Sirich, 2016) both found in CKD patients and animal models (Itoh et al., 2012; Zhao et al., 2013a; Velenosi et al., 2016). IS and PS are linked to CKD comorbidities such as CVD (Hung et al., 2017) and have been associated with uremic influences of drug metabolism both through transcriptional regulation (Guévin et al., 2002), and IS as a direct inhibitor of CYP activity (Volpe et al., 2014). Thus, IS and PS found in this study support previously described roles in modifying CYP regulation in CKD.

Equol-4/7-O-glucouronide (EOG) and 4-ethylphenyl sulfate (EPS) are both soy-derived uremic toxins seen in animal models fed a soy-based chow (Kikuchi et al., 2010; Velenosi et al., 2016). The rat chow in this experiment used 22% crude protein content of which daidzein and genistein aglycone equivalents (ranging from 350 to 650 mg/kg) were present. These isoflavones undergo bacterial transformation into equol derivatives such as EOG and EPS in the gut. EOG has only been found in animal models of CKD (Velenosi et al., 2016) where EPS has been detected in dialysis patients, although without significant changes compared to healthy individuals (Itoh et al., 2012). Interestingly, EPS has a similar structure to p-cresyl sulfate, a metabolite usually found concomitant with IS and repeatedly
found in CKD (Itoh et al., 2013; Zhang & Davies, 2016). Due to the similar structure, the function of EPS is suspected to act similarly to p-cresyl sulfate which has been associated with deleterious human liver microsomal CYP3A4 regulation by direct inhibition (Barnes et al., 2014; Zhang & Davies, 2016; Gryp et al., 2017). It has yet to be assessed whether EPS can also directly inhibit the regulation of CYP3A2 or CYP2C11 in rats.

L-Carnitine is a required nutrient primarily obtained through the diet in meat, eggs and protein-rich foods including soy (Klaassen & Cui, 2015). In contrast to an increase in L-carnitine observed in the heart and kidney of adenine-induced CKD rats (Velenosi et al., 2016), here we see a decrease in L-carnitine in the plasma in conjunction with downregulated CYPs in the liver. This could suggest a removal of L-carnitine as it is transformed into TMAO as seen previously (Koeth et al., 2013). L-Carnitine and choline are the precursors to TMA, a bacteria by-product that is converted to TMAO in the liver by flavin-containing monooxygenases (FMO) (Koeth et al., 2013). TMAO is associated with increased risk of developing atherosclerosis and thus, of increasing interest as a harmful uremic toxin in CKD and CVD (Koeth et al., 2013; Aron-Wisnewsky & Clément, 2015). TMAO detection needs a sensitive MS method sometimes referred to as target enhancement, usually utilizing a triple quadrupole MS rather than a QTof which is better for sensitivity (Heaney et al., 2016). Therefore, TMAO was missed by our untargeted methods because the sensitivity was insufficient (Heaney et al., 2016). Neither L-carnitine nor TMAO have been tested as DME regulatory factors but have been mentioned in relation to drug metabolism and provide potential candidates for future studies (Selwyn et al., 2015).

The identification and correlation of these uremic toxins support their involvement in DME regulation. However, as discussed in section 1.7.1, administration of AST-120 in CKD, reduces uremic toxin levels (Kikuchi et al., 2010), but CYP3A2 expression is not recovered (Velenosi, 2015). This protection by AST-120 suggests factors other than uremic toxins may be involved. The quantified IS, PS and EPS concentrations over CKD progression observed in this thesis adds support to this idea (Figure 3.6). Severe concentrations (over 150µM for IS) are not observed until following day 28, well after we observe a decrease in CYPs. Preliminary studies using Huh7 human hepatoma cells show that protein-bound
IS at concentrations of 180±20µM were required to reduce CYP3A4 expression by 50% (Velenosi, 2015). In addition, a similar study found that concentrations of IS required to downregulate CYP3A4 activity were above the abnormal range seen in CKD patients, which was defined as the highest ever recorded IS level in a CKD patient at >940µM (Vanholder et al., 2003; Volpe et al., 2014). Although the studies differ dramatically, collectively they suggest that other hypotheses such as immunological factors, PTH or undiscovered pathways might be initiating DME downregulation, and it is not until severe stages of CKD that uremic toxins contribute to the dramatic reduction in CYP expression seen here and by others (Velenosi et al., 2012). In conclusion, it is temporally plausible that uremic toxins contribute to DME downregulation, but other factors may be involved prior to uremic toxin influences.

Interestingly, of the metabolites found by correlation to CYP3A2 or CYP2C11, the five metabolites that are potentially associated to DME downregulation in the literature are all gut-derived uremic toxins. This supports the need to understand the changes in the gut microbial environment and assess if bacterial alterations are impacting the presence of these toxins.

4.1.5 Microbiome over CKD Progression

The gut microbiota, sampled by caecum swab, was phylogenetically analyzed using 16S sequencing. Multivariate analysis showed the microbiota was most significantly influenced by time and secondarily by disease state. This correlation suggests that the microbiota changes caused by CKD induction are less profound than age-associated bacterial changes. Additionally, in comparison to the metabolomic PCA, microbial clustering with respect to disease state was poor and showed little to no separation before day 14. This observation suggests the uremic environment in the plasma and the liver are altered well before dysbiosis occurs. Interestingly however, the metabolite concentrations of IS, PS and EPS all dramatically increase after day 28 when changes are simultaneously observed in the gut microbiota. This lends support to the idea that uremia may be driving the change in gut microbial abundance through a damaged gut wall (Figure 4.1) (Magnusson et al., 1991; Felizardo et al., 2016; Vaziri, 2016). The late and dramatic increase in gut-derived uremic toxins also suggests dysbiosis contributes in the cycle of worsening uremia, likely adding
to the uremic milieu by increasing bacteria capable of uremic toxin production (Wong et al., 2014; Vanholder & Glorieux, 2015; Felizardo et al., 2016). Referencing the KEGG results of Wong and colleagues, all bacterial families significantly changed on days 28 and 42 contained strains capable of producing at least one of the following genes: urease, tryptophanase, phosphotransbutyrylase or butyrate kinase, although the bacteria are inconsistently characteristic of control or CKD rats (Wong et al., 2014). The sole bacterial genus significantly changed due to disease state prior to day 14 resided in the bacterial order Clostridiales, which matched the findings of Barrios and colleagues who sequenced the gut microbiota of 855 people and correlated only bacteria from the Clostridiales order with eGFR in early renal decline (Barrios et al., 2015).

Although interesting to examine the individual days for OTUs differing due to CKD, only two bacterial genera, Turicibacter and Parabacteroides, were significant on two or more consecutive days, best correlating with DME trends. Turicibacter was the most consistently changed bacteria, changing as early as day 14 through to day 42 with an increasing trend as CKD progressed. Identifying the genus Turicibacter in CKD animals is a novel finding. All Turicibacter are gram-positive, strictly anaerobic, rod-shaped bacteria of which very little is known. Turicibacter was first identified and named in 2002 by the University of Zürich, Switzerland where the bacteria were found in the blood of a febrile 35-year-old male with acute appendicitis (Bosshard et al., 2002). Shortly thereafter, another febrile patient diagnosed with acute appendicitis had Turicibacter cultured from their blood; this time a 79-year-old female in Sweden (Bosshard et al., 2002). In 2007, Turicibacter was associated with pouchitis in ulcerative colitis patients which is a complication of proctocolectomy (Falk et al., 2007). We also know Turicibacter sp. are found in healthy human feces from a FMT study where human feces were transplanted into colons of germ-free rats to ascertain whether rats should be used as a transplantation model (Licht et al., 2007). Turicibacter was one such bacteria that survived two weeks after transplantation via stool gavage.

Only 4 strains, all within the same species sanguinis, have been published to date: MOL361 (Bosshard et al., 2002), PC909 (Cuív et al., 2011), ZCY83 (Cao et al., 2015), H121 (Auchtung et al., 2016). A BLASTn search of our Turicibacter sequence matched the
MOL361 species with 100% identity (NR_028816.1). Turicibacter is anaerobic and therefore, considerably difficult to culture. Of the strains successfully cultured, none have tryptophanase activity suggesting these bacteria are not involved in producing indole derivatives such as IS (Bosshard et al., 2002). The Erysipelotrichaceae family of which Turicibacter belongs does contain strains capable of butyrate and phosphotransbutyrylase activity, although this has yet to be confirmed in the Turicibacter genus (Wong et al., 2014). Assuming all rats were exposed to Turicibacter for the study duration, we suggest our findings indicate CKD animals are more susceptible to gut colonization by Turicibacter.

The BLASTn results for the Parabacteroides genus OTU suggested 99% sequence identity to two stains of the species distasonis: strain ATCC 8503 (NR_074376.1) and JCM 5825 (NR_041342.1). In 2006, Bacteroides distasonis was reclassified as Parabacteroides distasonis and thus, all subsequent information pertains to either classification (Sakamoto & Benno, 2006). Parabacteroides is a gram-negative, anaerobic, non-spore-forming genus. P. distasonis is present in the human gut and has been identified via PCR in fecal samples long before the existence of next-generation sequencing methods (Franks et al., 1998). P. distasonis is classified in the KEGG pathway database as an opportunistic pathogen capable of anaerobic infection (Xu et al., 2007). In a study of gut microbiota in Crohn’s disease, P. distasonis was more abundant in the control group, contradictory to expected findings (Mondot et al., 2011). More recently, components of P. distasonis have shown improvement of irritable bowel disease (IBD) in mice and have been suggested as a potential therapeutic for reduction of inflammation in IBD (Kverka et al., 2011). However, analysis of bacteria capable of generating phenol and indole compounds found P. distasonis proficient at producing p-cresol (Gryp et al., 2017) and IS (Zhang & Davies, 2016). In general, it seems P. distasonis is potentially both harmful or beneficial depending on translocation, relative abundance and physiological state of the host. Our results show a unique trend where CKD rats have a stable level of Parabacteroides and controls slowly reduce the abundance of this genus after 28 days. Given the multitude of associations with disease, Parabacteroides may be taking advantage of the dysbiotic state in CKD when it is normally removed in controls by other healthy bacteria as a part of the progression in age-associated microbial changes.
This data shows that DME regulation in CKD is initially mediated by changes independent of an altered gut microbiota; however, late and dramatic increases in both uremic toxins and the bacterial environment suggest the gut microbiota may influence DMEs in severe CKD. It also remains possible that in this late stage CKD, gut microbes are influencing DMEs through uremic-independent methods such as inflammatory factor production as suggested by germ-free studies that also see downregulation of CYPs (Toda et al., 2009; Claus et al., 2011; Selwyn et al., 2015).

4.1.6 Summary

In conclusion, global plasma and liver alterations of the metabolome over disease progression provide support for uremic toxins playing a role in DME downregulation. Alternatively, the early detection of DME downregulation and late surge of gut-derived uremic toxin concentrations suggest other factors are involved in DME regulation in early stages of CKD (Figure 4.1). A temporal association was established between severe CKD, caecal dysbiosis and increase in gut-derived uremic toxins IS, PS and EPS. This association supports the positive-feedback loop of uremia and dysbiosis suspected to drive severe CKD (Figure 4.1).
Figure 4.1. Temporal associations of uremia and dysbiosis with CYP3A2 and CYP2C11 expression over CKD progression. Days (3 through 42) refer to rat study time points carried out in this thesis. CKD was characterized by urea and creatinine beginning on day 14 and correlating with the decrease in CYP3A2 expression. CYP2C11 expression decreased as early as day 7. Although plasma uremia may be involved as early as day 7 indicated by untargeted multivariate analysis, quantified uremic toxins were significantly increased only on days 28 and 42. Similarly, gut bacterial dysbiosis was detectable on days 28 and 42 supporting the hypothesis of a positive-feedback cycle involving uremia and the gut microbiota. This study suggests there are likely other factors influencing DMEs in early stages of CKD. Images were modified from Servier Medical Art (http://www.servier.co.uk/medical-art-gallery).
4.2 Limitations

The greatest limitation of this study is that causative conclusions cannot be made from vast cross-sectional data such as those obtained from sequencing or metabolomics. Results from these discovery-based methods instead provide whole-system comprehension of complex mechanistic processes and allows for discovery of previously unknown biological factors that drive future experiments assessing causation.

4.2.1 Animal Model & Study Design

Utilizing in vivo animal models is accompanied with limitations when used to understand human physiology. Human CYP expression is comparable to rat and mouse CYP expression through orthologous enzymes that largely perform the same tasks, yet some substrates are species-specific indicating the enzyme activities are not identical to their human orthologs (Nelson et al., 2004; Pan et al., 2016). Secondly, the induction of CKD is achievable through different methods. Adenine-induced CKD is a less invasive orally administered model in comparison to the more common 5/6th nephrectomy model and although not linear, it is suggested to provide a more consistent rate of CKD onset than 5/6th nephrectomy (Terai et al., 2008). Applying a steady rate of CKD onset was important for our temporal study design. Conceptually however, the adenine model has been questioned as a CKD model because it could also represent an acute kidney injury (AKI) model. Adenine metabolites precipitate when concentrated, mechanically damaging the kidney tubules comparable to kidney stones (Engle et al., 1996; Morishita et al., 2011; Succar et al., 2017). Though separately defined in nephrology for clinical diagnosis, AKI is a risk factor for CKD and CKD is a risk factor for AKI, and thus, the adenine model continues to be used for either condition (Chawla et al., 2014). The adenine model also reduces the amount of food ingested during the first days of adenine-induction, requiring that pair-feeding be established. This is especially important for CYP expression analysis since CYPs are influenced by short-term fasting (Lammers et al., 2015). Lastly, it is unknown how adenine and its metabolites will affect the gut microbial environment apart from CKD. When ingested, adenine absorption is proposed to occur within the small intestine, earlier in the intestinal tract than the caecal sampling site (Salati et al., 1984). It
is noteworthy that the adenine metabolite 2,8-dihydroxyadenine was only detected in the plasma and not in the liver of CKD rats suggesting 2,8-dihydroxyadenine is unlikely to be impacting the liver.

4.2.2 Time Point Inclusion

Initial results from this study evaluated only rats sacrificed on day 0, 3, 14, 28 and 42 and suggested CYP expression and uremic toxin levels were changing between days 3 and 14. In the attempt to obtain as much information about the transition between early and late CKD, a second study included rats subjected to identical conditions for 7 days. Upon completion of the second study, preliminary CYP3A2 mRNA expression results showed day 0 control rats had decreased expression in comparison with the other control groups and essentially increased after 3 days (Figure 4.2). It was later identified that DME expression is impacted by short-term fasting (Lammers et al., 2015). Thus, a third study compared only control ad libitum fed animals with animals receiving the CKD pair-fed amount of control food. After 72 hours, it was found that DME levels of animals receiving reduced feed were equivalent to levels observed in day 3 control rats. Therefore, day 0 rats from the final study were carried onward as the control day 0 group for the remainder of the investigation (Figure 4.2).
Figure 4.2. Short-term fasting effects on CYP3A2 mRNA expression. A) Preliminary CYP3A2 mRNA expression data comparing rats fed ad libitum to rats pair-fed lower food volumes matching those consumed by CKD rats. Values are expressed as the mean ± SEM relative to housekeeping gene β-actin and normalized to the ad libitum group. *p<0.05 using a one-way ANOVA with Holm-Sidak’s multiple comparisons test. B) CYP3A2 mRNA expression results before and after accounting for short-term fasting effects by waiting to sacrifice 72 hours after pair-feeding is initiated.
However, this time sensitivity impacted bacterial sampling. All animals from which bacteria are sampled, ideally must eat and live in the same area and be exposed to all experiences that may introduce foreign bacteria to the same extent if the sample size is small and bacterial differences are expected to be detectable. Ideally, particularly in animal studies, this means the animals should all be bought at the same time in one shipment if being compared together. Since the animals from day 7 and day 0 were obtained from the supplier at different times than the original study, the gut microbiota variability was too large to assess as a whole and therefore removed from bacterial analysis, reducing the number of time points available for evaluation.

4.2.3 “Omics” Method Limitations

Although broadly encompassing, both the Illumina sequencing and untargeted metabolomics methods are limited. Even though the 16S rRNA gene is currently the gene of preference, it is highly conserved, limiting its resolution and accuracy to the genus level (Poretsky et al., 2014). Also, the development of short, overlapping pair-end reads enabled the use of 16S gene sequencing on the MiSeq platform given only a small section of the 16S gene required sequencing (Poretsky et al., 2014). However, this requires that out of the nine variable regions (V1-V9), only a select few can be covered by the read length capabilities of the MiSeq (Caporaso et al., 2012). It has been identified that using different variable regions (V1 through V6) results in different subsets of identifiable bacteria (Chakravorty et al., 2007; Youssef et al., 2009). Therefore, the V4 variable region was chosen based on the most bacteria that could be detected and likely present within a caecal sample (Caporaso et al., 2011).

Similarly, untargeted metabolomics can identify a vast number of metabolites in a single sample, but each metabolite is only detectable if the combination of sample preparation, UPLC column, chromatography and MS configuration are satisfactory for that metabolite. As an example, TMAO is likely present in our rat plasma samples, but is not detected because the MS method needs to be adjusted for sensitivity (Heaney et al., 2016). To obtain as many metabolite masses as possible, two columns of differing hydrophobicity and both positive and negative ionization modes were used.
4.2.4 Statistics

In addition to method limitations, analysis methods were also limiting. Little continuity in genera abundance across time points was primarily caused by small sample size of <7 per group. The inter-individual variability, in addition to machine and sampling variability, effectively masked small changes caused by condition and required broad shifts in bacterial abundance to prove significance (Poretsky et al., 2014; Gloor et al., 2016a). Without increasing sample size, this could be somewhat circumvented by using a sequencing method with less error and greater read depth (e.g. Illumina HiSeq), potentially providing more bacterial associations (Caporaso et al., 2012).

Correlative analysis between metabolites and DME levels was the second-best option after a univariate model capable of acquiring an independent, FDR corrected, 2-way ANOVA with multiple comparisons for each time point between CKD and same day control. Although an independent, FDR corrected 2-way ANOVA is obtainable via MetaboAnalyst v3.0, p-values do not indicate on which days the significance is occurring. To answer the question of when the metabolite is changing over time, multiple comparison analysis using Sidaks multiple comparisons test is generally applied to the 2-way ANOVA. However, MetaboAnalyst v3.0 is incapable of multiple comparison testing and alternative software including GraphPad are unable to hold the vast amount of information present in a metabolomics dataset. It is also possible that a custom R script be written to obtain this same goal. However, for this study, there were limited options for identifying at what day each metabolite was changing and related to CYP3A2 (day 14) and CYP2C11 (day 7) expression. Alternatively, Spearman correlation analysis was used to identify metabolites that exhibit a similar trend to DME changes over CKD progression. Unfortunately, this correlation analysis assumes that metabolite concentrations influencing DMEs are directly proportional to DME levels, potentially limiting the results.

4.3 Future Studies

This thesis has emphasized the need for research around DMEs in early stages of CKD. Specifically, it needs to be elucidated if DMEs are truly changed in humans with CKD, apart from animal models. Alternatives to the erythromycin breath test are essential to
understanding human CYP3A4 regulation. In the meantime, DME studies can improve the rodent-to-human physiological gap by employing humanized mouse models of CYP3A4 or CYP2C9 along with other DMEs or transporters (Becker & Hewitson, 2013; Ladda & Goralski, 2016).

Although it is established that dialysis can effectively change the bioavailability of drugs in humans, it has yet to be determined if dialysis changes DME levels (Atkinson & Umans, 2009). In theory, if only protein-bound uremic toxins are affecting drug pharmacokinetics, dialysis will not improve DME levels. An in vitro study looked at CYP expression and activity following the implementation of pre-dialysis versus post-dialysis serum in rat hepatocytes and showed a decrease in DMEs after only pre-dialysis serum suggesting uremic toxins involved in DME downregulation can be removed via dialysis (Michaud et al., 2008). Additional information on what metabolites are being removed, perhaps through MS methods, would help researchers identify metabolites worth targeting for causation analysis. In vivo models of dialysis have been established and would provide an interesting avenue for studying DME levels before and after dialysis (Mortier et al., 2002; Zareie et al., 2005).

It would also prove helpful if the list of mechanistic causes of DME downregulation were assessed together to understand the extent to which each mechanism is contributing to the entire downregulation of DMEs. For example, one could introduce suspected uremic toxins such as IS, PS, EPS, EOG and TMAO to human cells in vitro or human liver microsomes ex vivo and subsequently test for direct inhibition of human CYP3A4 or CYP2C9 expression alongside nuclear receptor binding alterations and detection of PTH and inflammatory factors.

Studies are needed to assess the implications of specific bacteria on disease states. It is unclear if Turicibacter is normally found in the gut at very low abundance or a foreign bacterium infiltrating a weak host. It is also unknown if this bacterium is pathological or commensal in nature. It would be beneficial to confirm that CKD is not inducible via bacterial alterations such as infection with Turicibacter. This assessment could be accomplished by FMT to introduce CKD microbiota, potentially from human CKD
patients to germ-free mice to see if i) the mouse acquires CKD regardless of initial kidney damage, and ii) if the presence of CKD-associated bacteria can cause DME downregulation in the absence of uremia. In conjunction, caecal or fecal metabolomics would provide information about what metabolites reach the gut lumen and aid in dysbiosis.

4.4 Relevance & Conclusions

In general, future CKD therapies should be targeted not only to improve disease comorbidities and prolong the progression into ESRD, but also improve drug disposition to ensure the pharmaceuticals received are providing the highest efficacy attainable while avoiding drug toxicity. Emerging therapies still directed towards improving pathophysiology and uremia include uremic toxin removal (e.g. AST-120) and pro- or prebiotics to stimulate the reversal of dysbiosis (Ranganathan et al., 2006; Koppe et al., 2015; Rossi et al., 2016; Yamamoto et al., 2016). Currently there is no prospect for FMT to benefit CKD patients, although it has proved largely successful for C. difficile infections and theoretically translatable to CKD (Persky & Brandt, 2000; Al Khodor & Shatat, 2016). Our results suggest that future therapeutic research for DME regulation be targeted in the areas of uremic toxin removal as a priority over the reestablishment of the gut microbiota because our results suggest uremic toxins provide a more likely candidate for early stage DME alteration.

Ultimately, understanding the effects of uremia and bacteria with respect to CKD can help assess whether uremic toxins or the gut microbiota are potential therapeutic targets in DME regulation. Whether it be adsorption of uremic toxins or combating bacterial dysbiosis, providing emphasis for one method may drive future research in the direction of greatest efficacy. On a larger scale, knowledge of how DMEs fluctuate with respect to metabolites and bacteria could lead to improved clinical testing, dosing and prevention of adverse reactions.
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Appendices

Appendix A: Ethics Approval

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 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 #2009-058.

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.
5. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 4 Years

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<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>Wistar</td>
<td>200-300g, male</td>
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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.

cc. Approved Protocol - B. Urquhart, W. Lagerwerf
Approval Letter - B. Urquhart, W. Lagerwerf
Appendix B: Supplementary Information

**Supplementary Table 1.** Table of barcoded primers used for Illumina sequencing. Refer to excel file (.xlsx) titled “Barcoded Primers for Illumina Sequencing”.

**Supplementary Table 2.** Table of 1199 operational taxonomic units identified from Illumina sequencing. Refer to excel file (.xlsx) titled “Final OTU Table from Illumina Sequencing”.

**Supplementary Table 3.** Table of 204 m/z ratios found by untargeted mass spectrometry from CKD and control rat plasma and liver samples and RPLC or HILIC chromatography. Refer to excel file (.xlsx) titled “Final Metabolite Table from UPLC-MS”. Metabolites satisfying univariate analysis and Spearman correlation to same-sample CYP3A2 or CYP2C11 mRNA, protein or enzyme activity data. Spearman correlation coefficients (r value) are listed. 2-way independent ANOVA was conducted via MetaboAnalyst v3.0 using FDR<0.05 to correct for multiple comparisons and satisfaction required p<0.05 across both Time and Disease. Multivariate analysis required VIP > 0.8 and 0.4 < p(corr)[1] < -0.4 indicating adequate separation by OPLA-DA and S-plot. Mass error was obtained using the 4th decimal place m/z from the Human Metabolome Database (HMDB). Italicized suspected metabolites refer to a group of plausible metabolites of similar structure. For definitions of identification levels refer to section 2.6.4.

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Curriculum Vitae

Emily Dee Hartjes

Post-secondary Education and Degrees:

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<tr>
<td>BSc. Honours in Biochemistry and Molecular Biology; Specialization in Medical Sciences</td>
<td>Trent University</td>
<td>Peterborough, ON</td>
<td>Canada</td>
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<tr>
<td>MSc. in Physiology &amp; Pharmacology</td>
<td>University of Western Ontario</td>
<td>London, ON</td>
<td>Canada</td>
<td>2015-2017</td>
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Honours and Awards:

- 2015 - Ontario Graduate Student Award
- 2016 - Canadian Institutes of Health Research
- 2016 – Physiology & Pharmacology Research Day Poster Award Winner
- Western Graduate Program Funding 2015-2017

Related Work Experience

Teaching Assistant
The University of Western Ontario, London, ON
Department of Physiology & Pharmacology
2015 Physiology 3140A - Cell Physiology
2016 Physiology 3140A - Cell Physiology

Manuscript:


Presentations:


James (Yong) Lim, Emily Hartjes, Brad Urquhart. Characterization of the Metabolome and Renal Tubular Cisplatin Disposition in Cisplatin Induced Acute Kidney Injury. American Society of Nephrology, New Orleans, Louisiana, USA, November 2-5, 2017. (Upcoming Poster)

Nicholas Tonial, Emily Hartjes, Jean-Francois Thibodeau, Chet Holterman, Eldjonai Kamto, Lyne Gagnon, Brad L. Urquhart. Effect of chronic kidney disease on expression of Cyp3a11, Cyp2c37, Cyp2d22 and Oatp1b2 in C57BL/6 mice. American Society of Nephrology, New Orleans, Louisiana, USA, November 2-5, 2017. (Upcoming Poster)