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Erythropoietin and Chronic Kidney Disease Alter Hepatic Expression of Cytochrome P450 Enzymes and Drug Transport Proteins

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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Erythropoietin and Chronic Kidney Disease Alter Hepatic Expression of Cytochrome P450 Enzymes and Drug Transport Proteins

by

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Department of Physiology and Pharmacology
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The University of Western Ontario
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Abstract

Chronic kidney disease (CKD) is a progressive disease involving the irreversible loss of kidney function. Patients with CKD require concurrent dosing of many medications to manage their disease and associated comorbidities. Pharmacokinetic studies in patients with CKD and previous studies using animal models of CKD have demonstrated changes in hepatic drug metabolism and transport. Our studies aim to examine the effects that end-stage renal disease (ESRD) and continuous dosing of recombinant human erythropoietin (EPO) have on hepatic cytochrome P450 (P450) drug metabolizing enzymes and drug transport proteins. We used an adenine-fed rat model of CKD to demonstrate that EPO decreases expression and function of hepatic CYP3A2. Additionally, P450 and drug transport protein expression were evaluated in cadaveric liver samples from ESRD patients. Human ESRD liver samples displayed significantly different mRNA expression hepatic transport proteins OATP1B1, OATP1B3, breast cancer resistance protein and P-glycoprotein, relative to control liver samples ($p < 0.05$). Our results suggest that, in the human CKD patient, changes in drug disposition are likely a result of changes in hepatic drug transport protein expression.

Keywords – chronic kidney disease, cytochrome P450, drug metabolism, drug transport, end-stage renal disease, erythropoietin
Co-Authorship Statement


Chapter 3: David A. Feere, Melisa L. Gaspar, Thomas J. Velenosi and Bradley L. Urquhart. The Effect of End-Stage Renal Disease on Hepatic Expression of Cytochrome P450 Drug Metabolizing Enzymes and Transport Proteins in Human Liver Samples. Thomas Velenosi aided with experimental techniques. Melisa Gaspar was involved in mRNA quantification and assisted in the preparation of liver microsomes and microsomal metabolism. Dr. Urquhart supervised the project and aided in experimental design.
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Many people have been instrumental in supporting me throughout my time before and during university. Thank you to my parents for supporting my pursuits in sports, music and, most of all, my academic adventures in science – one of these days I will explain what DNA is. To my brother, I know patience does not come easily, but thank you for trying to put up with my science rants and being everything a little brother should be. My grandparents are also deserving of a big thank you for all the support and interest they show in my life. I also cannot forget to thank all of my friends, including fellow Phys/Pharm grad student and former roommate, Blommer, you’re a real firecracker of a person.

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### Abbreviations, Symbols and Nomenclature

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<th>Description</th>
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<tr>
<td>ABC</td>
<td>ATP-Binding Cassette</td>
</tr>
<tr>
<td>$^{14}\text{C}$</td>
<td>Carbon-14</td>
</tr>
<tr>
<td>$^{14}\text{CO}_2$</td>
<td>Radioactive Carbon Dioxide</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-Binding Cassette</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl Hydrocarbon Receptor</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Transaminase</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate Transaminase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast Cancer Resistance Protein/ABCG2</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive Androstane Receptor</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
</tr>
<tr>
<td>CORR</td>
<td>Canadian Organ Replacement Register</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated Glomerular Filtration Rate</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>EPOR</td>
<td>Erythropoietin Receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal Regulated Kinases</td>
</tr>
<tr>
<td>ESA</td>
<td>Erythropoiesis Stimulating Agent</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-Stage Renal Disease</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FXR</td>
<td>Farnesoid X Receptor</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular Filtration Rate</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
</tr>
<tr>
<td>HK-2</td>
<td>Human Kidney 2 Immortalized Cell Line</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-Hydroxy-3-Methyl-Glutaryl-CoA</td>
</tr>
<tr>
<td>HNF-4α</td>
<td>Hepatocyte Nuclear Factor 4 Alpha</td>
</tr>
<tr>
<td>JAK2</td>
<td>Janus Kinase 2</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X Receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MRP2</td>
<td>Multidrug Resistance-Associated Protein 2/ABCC2/cMOAT</td>
</tr>
<tr>
<td>MRP3</td>
<td>Multidrug Resistance-Associated Protein 3/ABCC3/cMOAT2</td>
</tr>
<tr>
<td>OAT</td>
<td>Organic Anion Transporter</td>
</tr>
<tr>
<td>OATP</td>
<td>Organic Anion Transporting Polypeptide</td>
</tr>
<tr>
<td>OCT</td>
<td>Organic Cation Transporter</td>
</tr>
<tr>
<td>P450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein/ABCB1/MDR1</td>
</tr>
<tr>
<td>PI3K/Akt</td>
<td>Phosphatidylinositol 3 Kinase/Akt</td>
</tr>
<tr>
<td>pK$_a$</td>
<td>Acid Dissociation Constant</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisome Proliferator-Activated Receptor Alpha</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome Proliferator-Activated Receptor Gamma</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X Receptor</td>
</tr>
<tr>
<td>RXRa</td>
<td>Retinoid X Receptor Alpha</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute Carrier</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>SLCO</td>
<td>Solute Carrier Organic Anion</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>STAT5</td>
<td>Signal Transducer and Activator of Transcription 5</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D Receptor</td>
</tr>
</tbody>
</table>
1.0 Introduction
1.1 Chronic Kidney Disease

Chronic kidney disease (CKD) is a prevalent disorder in Canada and worldwide accounting for 3 to 6% of total healthcare expenditure by country (Jha et al., 2013). The prevalence of CKD has increased over the past 20 years and moved from 27th to 18th on the list of global causes of death. This rate of movement as a cause of death is second only to HIV/AIDS (Lozano et al., 2012). Chronic kidney disease is a growing healthcare concern as it is associated with prevalent comorbidities that cause and enhance the progression of CKD. Conversely, CKD causes changes in renal and non-renal drug clearance that complicate the pharmacological treatment and management of kidney disease and the associated comorbidities.

1.1.1 Causes and Progression

Chronic kidney disease is a progressive and irreversible disorder that is characterized by a persistent decrease in kidney function over a time period of greater than three months. It is estimated that the prevalence of CKD in many parts of the world, such as Canada, China, the United States and the United Kingdom, is between 10 and 15% in the adult population (Anandarajah et al., 2005; Arora et al., 2013; Coresh et al., 2003; Zhang et al., 2007). Patients with CKD often have many associated health conditions and comorbidities, in addition to declining kidney function. The main causes and risk factors for CKD are hypertension and diabetes; although, other factors such as obesity, are also associated with the development and progression of CKD (Chen et al., 2004;
Okada et al., 2013; Tozawa et al., 2003). As the prevalence of these comorbidities has increased over previous decades, it is not surprising that the prevalence of CKD has also increased (KEEP, 2002; Lago et al., 2007). According to the Kidney Foundation of Canada (2013), the leading causes of CKD are diabetes and hypertension which account for 35 and 16% of cases, respectively (KFOC, 2013).

Kidney function is measured using equations that estimate glomerular filtration rate (GFR). The various formulas used to calculate GFR may include variables such as age, mass, serum creatinine, gender and ethnicity. Currently, the most widely used equation is from the Modification of Diet in Renal Disease Study Group (Levey et al., 1999). This equation incorporates four variables to calculate GFR – serum creatinine, age, gender and ethnicity. Normal kidney function is generally defined as having an estimated GFR of 90 to 120 mL/min/1.73m² with no proteinuria or evidence of structural abnormalities of the kidney. Diagnosis of CKD is based on structural or functional abnormalities of the kidney that cause albuminuria, greater than 30 mg of albumin per gram of creatinine, or by an estimated glomerular filtration rate (eGFR) below 60 mL/min/1.73m² for a period of time greater than 3 months (Hallan et al., 2012; KEEP, 2002). As renal damage in CKD is irreversible and progresses over time, patients are grouped into five stages of increasing severity and progress through the stages as eGFR decreases (Table 1.1; (KEEP, 2002). Initial stages of CKD are associated only with normal or near normal estimates of eGFR and structural
Table 1.1 Clinical stages of chronic kidney disease as defined by estimated glomerular filtration rate

<table>
<thead>
<tr>
<th>Stage of CKD</th>
<th>eGFR (mL/min/1.73m²)</th>
<th>Additional Diagnosis Parameters, Therapy or Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>≥90</td>
<td>normal or slightly diminished GFR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>evidence of kidney damage by pathology, abnormal imaging or blood or urine tests</td>
</tr>
<tr>
<td>Stage 2</td>
<td>60-89</td>
<td>mild decrease in GFR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>evidence of kidney damage by pathology, abnormal imaging or blood or urine tests</td>
</tr>
<tr>
<td>Stage 3</td>
<td>30-59</td>
<td>moderate decrease in GFR</td>
</tr>
<tr>
<td>Stage 4</td>
<td>15-29</td>
<td>severe decrease in GFR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>preparation or initiation of renal replacement therapy</td>
</tr>
<tr>
<td>Stage 5</td>
<td>&lt;15</td>
<td>failure of kidney function</td>
</tr>
<tr>
<td></td>
<td></td>
<td>termed end-stage renal disease (ESRD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Renal replacement therapy required</td>
</tr>
</tbody>
</table>

Adapted from the Kidney Early Evaluation Program (KEEP, 2002) of the National Kidney Foundation of the United States.
abnormalities or pathologies that cause proteinuria. However, by stage 3 CKD, changes begin to appear in cellular function and metabolism (Parmar, 2012). The reduction in GFR at this stage leads to the inability to clear metabolic waste products from the blood and is known as uremia (Meyer et al., 2007). The condition of uremia is known to cause physiological changes that affect neurological function, basal metabolic rate and glucose and amino acid homeostasis (Depner, 2001; Meyer et al., 2007). As GFR decreases, the uremia worsens in later stages of CKD as metabolic waste produced accumulates to higher concentrations in the blood (Meyer et al., 2007). Ultimately, some patients reach the fifth, and final, stage of CKD, end-stage renal disease (ESRD).

1.1.2 End-Stage Renal Disease and Renal Replacement Therapy

End-stage renal disease is the most severe stage of CKD and, not surprisingly, is associated with the greatest systemic side effects (KEEP, 2002). The severity of kidney damage in ESRD represents the functional expiration of the natural kidney and necessitates renal replacement therapy to support the life of the ESRD patient. For the treatment of ESRD, renal replacement therapies include hemodialysis, peritoneal dialysis and renal transplantation (Horl et al., 1999; Tucker et al., 1991). In most cases, ESRD patients in Canada and other developed countries undergo hemodialysis treatment three times per week to mechanically filter the blood and manage otherwise life-threatening uremia (Woods et al., 1997).
Hemodialysis involves removing blood from the ESRD patient, pumping it through a dialyzer, and returning the filtered blood to the patient. Standard hemodialysis treatment is performed in a clinical setting by healthcare professionals for three or four hours three times per week (Tucker et al., 1991). The goal of hemodialysis is to remove water and accumulated solutes from the CKD patient. In contrast, peritoneal dialysis can be completed at home, by the patient, in one of two ways 1) automated peritoneal dialysis or 2) continuous ambulatory dialysis (Covic et al., 2010). Both forms of peritoneal dialysis involve the initial surgical implantation of a catheter to allow for the introduction, and removal, of fluid into the peritoneal cavity. Automated peritoneal dialysis is usually performed during the night, for approximately six hours, while sleeping. The other form of peritoneal dialysis, continuous ambulatory dialysis, involves keeping two liters of fluid in the peritoneal cavity at a given time and exchanging the fluid multiple times per day (Tucker et al., 1991). The fluid, dialysate, used in peritoneal dialysis is hyperosmotic to allow for the removal of much of the excess water in these patients.

Many studies have compared the long-term prognosis and quality of life between hemodialysis and peritoneal dialysis patient groups (Keshaviah et al., 2002; Purnell et al., 2013; Tucker et al., 1991). According to the Canadian Organ Replacement Register (COOR), as of 2008, 48% of ESRD patients were receiving hemodialysis, 41% were living with a functional transplant and 11% were being treated with peritoneal dialysis (CORR, 2011). Despite these statistics, when educated on dialysis modalities, more patients prefer peritoneal
dialysis; however, physicians choose hemodialysis over peritoneal dialysis for most patients (Woods et al., 1997). It may be likely that the preference for modality by physicians may be due to a belief of hemodialysis being better for the management of CKD. Contrary to this, studies conducted that control for age and comorbidities, such as diabetes and cardiovascular disease, show similar survival rates, and no significant differences in prognosis, between patients of hemodialysis and peritoneal dialysis (Keshaviah et al., 2002). Despite patient preference and similar survival rates, it is interesting that physicians still favour hemodialysis, a modality that may be more inconvenient for patients (Davies et al., 2014; Tucker et al., 1991). The role of peritoneal dialysis continues to be relatively small despite a considerable cost advantage; treating a patient by peritoneal dialysis costs about two-thirds of that by hemodialysis (Coyte et al., 1996).

To date, successful renal transplant is the only cure for CKD/ESRD and is the only treatment capable of increasing GFR in CKD patients (Horl et al., 1999). Rates of transplant in ESRD patients depend on country and type of healthcare system. Countries with public healthcare systems, such as Canada, have the highest rate of ESRD patients treated by transplant (Horl et al., 1999). Worldwide, approximately 23% of ESRD patients are living with a functional transplant and the rate in North America is slightly higher at 30% (Grassmann et al., 2005). The five year survival of transplant recipients is approximately 85% (Womer et al., 2009). As expected, many studies and systematic reviews report that quality of life, survival and long-term prognosis among transplant recipients is
greater than their dialysis-treated counterparts (Purnell et al., 2013).

Transplantation is even an advantageous treatment modality in the elderly. A study by Oniscu et al. (2004) elegantly showed that transplant recipients over the age of 60 have 90 and 130% increases in life expectancy and 5-year survival, respectively, relative to their counterparts treated by hemodialysis. The benefit for transplant is clear, the only risk relative to dialysis is a 5-fold increase in mortality the first 30 days post-transplant (Oniscu et al., 2004).

1.2 Complications in Chronic Kidney Disease

1.2.1 Associated Comorbidities

Most patients with CKD have one or more pre-existing condition at the time of CKD diagnosis. Not surprisingly, the top two causes of CKD, diabetes and hypertension, are two common comorbidities in CKD patients.

Rates of hypertension increase with stage of CKD and hypertension is reported in almost 80% of patients in stages 3-5 of CKD (Jha et al., 2013; Rao et al., 2008).

Hypertension is the most common comorbidity in patients with CKD and uncontrolled hypertension hastens the progression of kidney disease. As CKD progresses, and GFR decreases, the fluid retention and increased resistance of the vasculature makes managing hypertension more difficult (Botdorf et al., 2011). Ultimately, the increasing blood pressure, declining kidney function, and associated vascular stiffening, are major risk factors for cardiovascular disease.
As cardiovascular disease is the leading cause of death in CKD patients, strict monitoring and management of hypertension is required in CKD to maintain blood pressure targets of 130 mmHg and 80 mmHg for systolic and diastolic pressures, respectively (Chobanian et al., 2003; KEEP, 2002). Patients are prescribed angiotensin receptor blockers or angiotensin converting enzyme inhibitors as a first line of therapy. Many ESRD patients also require secondary treatment with diuretics to maintain adequate blood pressure control (Chobanian et al., 2003).

Similar to hypertension, diabetes is a common comorbidity in CKD patients and, if not properly managed, is able to enhance the progression of renal failure. Markers of glomerular and tubular damage are elevated in patients with diabetes and are associated with proteinuria (Nauta et al., 2011). Glycemic control recommendations in CKD patients are identical to guidelines for diabetic patients without kidney disease. The National Kidney Foundation (2007) recommends a hemoglobin A\textsubscript{1c} target of < 7% in CKD patients with diabetes. Patients with diabetes, especially type II diabetes, are often managed on oral hypoglycemic agents with or without insulin. However, special consideration of dosing adjustment is required, and some oral hypoglycemic agents must be avoided, for patients at or beyond stage 3 CKD (Lalau et al., 2014). Specifically, the use of metformin in CKD is thought to be beneficial if adverse effects are avoided; however, due to the risk of lactic acidosis, metformin is often avoided in patients with renal impairment (Lalau et al., 2014). Further complicating treatment of
diabetes is that CKD patients have a decreased clearance of insulin and reduced renal gluconeogenesis (Gerich et al., 2001; Gin et al., 1994).

Hypertension, diabetes and CKD are all independent risk factors for cardiovascular disease (McCullough et al., 2011). These coexisting diseases in CKD patients complicate disease management and control of risk factors for major cardiovascular outcomes. Patients with CKD, especially ESRD, are more likely to suffer myocardial infarction, stroke and premature death than non-CKD patients (Shastri et al., 2010). Cardiovascular disease is the leading cause of death in CKD patients and accounts for about half of all mortality. This occurs as the prevalence of cardiovascular disease in CKD is higher and develops at a younger age than in patients without kidney disease. Chronic kidney disease patients are at a 4, 5 and 10-fold higher risk for the development of left ventricular hypertrophy, ischemic heart disease and heart failure, respectively, than the general population (Sarnak et al., 2003). The main contributor to cardiovascular disease is dyslipidemia – elevation of plasma triglycerides and cholesterol. Statins, HMG-CoA reductase inhibitors, are the main line of treatment for the reduction of low-density lipoproteins to manage risk for cardiovascular disease in the general population. Statin effectiveness, as shown in the AURORA trial using rosvastatin, has been investigated in ESRD patients and, despite a significant lowering of low-density lipoprotein cholesterol concentration in plasma, statins appear to be ineffective in reducing CVD-associated morbidity and mortality in this group of patients (Fellstrom et al., 2009; Wanner et al., 2005). Despite these findings, statins appear to have some benefit in earlier stages of CKD. More
recently, emerging evidence suggests that the anti-cholesterolemia drug ezetimibe may be beneficial in the management of CVD among CKD patients (Morita et al., 2014). Ezetimibe acts at the luminal brush border membrane of intestinal enterocytes to inhibit cholesterol uptake by the Niemann-Pick C1-Like 1 transport protein. In addition to known cholesterol-lowering effects of ezetimibe, novel findings on renal protective effects have been reported (Morita et al., 2014). Despite modern pharmacological drugs and the attempts to manage kidney disease and associated comorbidities, cardiovascular disease remains the leading cause of death in patients with CKD (Go et al., 2004).

Anemia, unlike hypertension, diabetes and cardiovascular disease, frequently develops in CKD patients later in their course of disease and as a direct result of their kidney failure. Anemia becomes a concern in patients with stage 4 and 5 CKD (Hayat et al., 2008). General causes of anemia include cobalamin and folic acid deficiency, iron deficiency and decreased erythropoietin (EPO) production. Decreased renal production of EPO is the main cause of anemia in CKD patients as the kidneys lose the ability to properly perform normal endocrine function. Anemia in CKD is associated with complications, such as ventricular hypertrophy, hospitalizations and mortality (Kovesdy et al., 2006). Additionally, anemia is associated with poor cardiovascular outcomes, which, as previously mentioned, are a concern and major cause of morbidity and mortality in CKD patients (Ezekowitz et al., 2003).

Due to the many disorders, aside from kidney failure, that must be managed concurrently with CKD, these patients are often prescribed more than
seven drugs simultaneously (Talbert, 1994). Many ESRD patients have a medication burden that exceeds this and daily pill administration may be higher than 10 or 12 (Manley et al., 2004). Irrespective of changes in kidney function, drug interactions alone are a safety risk for patients receiving concurrent dosing of this many medications.

1.2.2 Management of Anemia

Anemia is a major comorbidity caused by renal failure as the kidney loses endocrine function. The progression of CKD and CVD associated with hypoxia is a major cause of mortality in untreated CKD patients. The advancement of erythropoiesis-stimulating agents has dramatically increased quality of life and prognosis in CKD patients.

Anemia is defined as hemoglobin levels < 12g/L for females and < 13 g/dL for males and post-menopausal females (Hayat et al., 2008). Anemia worsens over time as eGFR decreases in CKD patients (Hayat et al., 2008). Therefore, patients with CKD, especially ESRD patients, require administration of recombinant human erythropoietin (EPO), normally produced by the peritubular cells of the kidney, in order to signal for sufficient differentiation of erythrocyte progenitors in the bone marrow (Lacombe et al., 1988). This allows patients to maintain normal blood hematocrit required for sufficient oxygen delivery throughout the systemic circulation (Babitt et al., 2012).

The first recombinant human EPO was produced 25 years ago (Eschbach et al., 1989). Known as epoetin alfa, this first generation erythropoiesis
stimulating agent (ESA) has been of clinical benefit in the management of CKD, especially among ESRD patients (Hayat et al., 2008). Second generation ESA darbopoetin is also used in the management of anemia with the benefit of once weekly administration, due to a longer half-life, relative to as many as three doses per week of epoetin alfa (Hayat et al., 2008). More recently, drugs to treat anemia have been developed that target the regulation of normal EPO production by inhibiting the regulatory enzyme prolyl hydroxylase. The appropriately termed prolyl hydroxylase inhibitors are able to prevent degradation of transcription factors, known as hypoxia inducible factors (HIFs), involved in upregulation of EPO transcription (Bernhardt et al., 2010).

1.2.3 Regulation and Actions of Erythropoietin

Erythropoietin is synthesized by peritubular cells of the adult kidney and by hepatocytes in the fetal liver. Although the primary EPO synthesis site changes in utero, the adult liver is still able to synthesize EPO (Bernhardt et al., 2010). However, the level of EPO synthesis by the adult liver is not sufficient to maintain hematocrit levels required for sufficient systemic oxygen delivery (Bondurant et al., 1986; Fried, 1972).

Erythropoietin gene expression is regulated by hypoxia-inducible factors (HIFs), which form heterodimers using an oxygen-sensitive α subunit, 1α or 2α, and a constitutively expressed HIF-β subunit (Bernhardt et al., 2010). These heterodimers can then act as transcription factors in the nucleus and affect expression of target genes such as EPO and genes involved in iron homeostasis.
Hypoxia inducible factor α subunits are constitutively expressed; however, under normoxic conditions, prolyl-hydroxylase enzymes hydroxylate HIF-1α at proline residues (Noguchi et al., 2008). The result of hydroxylation is recognition and ubiquitination of HIF-1α by Von-Hippel-Lindau E3 ubiquitin ligase and subsequent proteasomal degradation (Ohh et al., 2000). Under low oxygen tension, HIF-1α and HIF-2α are not hydroxylated and are able to act as transcription factors, after heterodimerizing with the β-subunit, aryl hydrocarbon receptor nuclear translocator, to regulate genes involved in response to hypoxia (Noguchi et al., 2008). Among these genes, EPO is regulated by the HIF heterodimer and hepatocyte nuclear factor 4α (HNF-4α) which stabilize the promoter enhancer complex to increase gene transcription (Noguchi et al., 2008).

Erythropoietin signaling is mediated by the erythropoietin receptor (EPOR) and its associated Janus kinase 2 (Jak2) intracellular kinase (Grebien et al., 2008). Classically, EPO signaling through EPOR causes Jak2-mediated phosphorylation of intracellular targets, specifically signal transducer and activator of transcription 5 (STAT5), to increase differentiation of red cell progenitors (Grebien et al., 2008). However, more recent evidence has uncovered additional, non-hematopoietic, roles for EPO including neuroprotection, cardiac angiogenesis and modulation of wound healing (Arcasoy, 2008; Galeano et al., 2006; Lipsic et al., 2006). Data supporting EPO involvement in drug metabolism or transport is limited to recent findings which demonstrate EPO is able to increase gene expression of efflux transport proteins.
in an indirect, unknown, manner dependent on liver X receptor (LXR) in murine macrophages (Lu et al., 2010). Other than this study, the potential role of EPO in regulating activation of nuclear receptors, drug transport proteins or drug metabolizing enzymes remains unknown.

1.3 Altered Drug Pharmacokinetics in CKD

For some time it has been known that the pharmacokinetics of drugs is altered in CKD. Specifically, the renal excretion of drugs was the first pharmacokinetic parameter found to be altered in CKD due to decreased GFR. However, more recent findings have highlighted that changes in drug absorption, distribution, metabolism and non-renal clearance are also altered in CKD.

1.3.1 Drug Metabolism and Transport

Most clinically used drugs are small, relatively lipophilic molecules given to patients by oral administration. Drug distribution to tissues depends on physical and chemical properties – size, partition coefficient and $pK_a$ – and affinity as a substrate for passive drug transport proteins and active efflux proteins.

Drug transport proteins play an important role in pharmacokinetics and, most obviously, in absorption and excretion of substrates (Ho et al., 2005). However, drug transport proteins are also important for permitting drug entry into target tissues, tissues that mediate toxicity, and tissues, such as the liver, where metabolism of the drug may occur (DeGorter et al., 2012). Additionally, drug
transport proteins are also a crucial component of the blood-brain barrier to protect the brain from exposure to xenobiotics. Generally, drug transporters can be classified as uptake transporters or efflux transporters.

Drug uptake transporters have broad substrate specificity and are involved in the movement of substrates from the intestinal lumen to the enterocyte, or from the blood into cells such as hepatocytes of the liver. In human liver, many drugs enter hepatocytes via organic anion transporting polypeptides (OATPs) of the solute carrier organic anion (SLCO) gene subfamily (Ho et al., 2005). These proteins belong to the solute carrier family (SLC) and additional uptake transporters of the SLC family include organic cation transporters (OCTs) and organic anion transporters (OATs).

Members of the SLCO subfamily, specifically, OATP1B1, OATP1B3 and OATP2B1 are highly expressed in the liver and of importance when considering drug uptake by hepatocytes (Kalliokoski et al., 2009). These transport proteins are expressed on the sinusoidal membrane of hepatocytes and have broad specificity for drugs and endogenous compounds such as bile acids, conjugated hormone metabolites and amphipathic molecules (DeGorter et al., 2012). The most studied of the OATPs is OATP1B1. Hepatic expression of OATP1B1, OATP1B3 and OATP2B1 is relatively high and responsible for the uptake of drugs used in the treatment of cancer, hypercholesterolemia, hypertension, allergies, bacterial infection and tuberculosis (Kalliokoski et al., 2009). The broad substrate specificity of OATPs is also associated with affinity for a number of ligands that are capable of inhibiting OATP function (Ho et al., 2005). Ultimately,
uptake transporters permit the passive uptake of drugs, down a concentration gradient, into hepatocytes where drug molecules, or metabolites, may be substrates for enzyme-mediated drug metabolism or biliary elimination via ATP-dependent efflux proteins.

Largely due to lipophilic structure, approximately 75% of all commonly used pharmaceutical drugs require metabolism to be cleared from the body (Wienkers et al., 2005). Of the large number of drugs eliminated by metabolism, approximately 75% are primarily metabolized by the cytochrome P450 (P450) superfamily of monooxygenase enzymes. Cytochrome P450 mediated metabolism of xenobiotics occurs largely in tissues of high expression – liver, intestine and, to a lesser extent, the kidney. The high expression in the intestine and liver are determinants of oral drug bioavailability. The extent of first-pass metabolism varies by drug depending on P450 enzyme substrate specificity. In general, the P450 enzymes involved in drug metabolism are promiscuous to various xenobiotic and endogenous substrates due to broad substrate specificity. These enzymes exist to protect organisms, specifically mammals and humans, from harmful natural xenobiotic compounds by forming hydrophilic metabolites that can then be excreted from the body (Guengerich et al., 2011).

Cytochrome P450 enzymes are hemoproteins localized to the smooth endoplasmic reticulum of cells (Neve et al., 2010). These are phase I drug metabolizing enzymes and largely act by adding an oxygen or hydroxyl moiety to a parent drug molecule to form a metabolite. The metabolites of P450-catalyzed reactions tend to be less pharmacologically active and more polar than the
original drug compound (Iyer et al., 1999). In many cases, the addition of an oxygen or hydroxyl group allows the phase I drug metabolite to be conjugated by phase II drug metabolizing enzymes. Phase II drug metabolism usually involves the addition of large, polar moieties, and includes reactions of glucuronidation, sulfation and acetylation, to increase water solubility and increase renal elimination of drugs (Iyer et al., 1999).

Although 57 P450 enzymes have been identified in the human, five isozymes are responsible for over 90% of oxidative drug metabolism – CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Guengerich et al., 2011; Wienkers et al., 2005). The majority of drug metabolism in humans, and other mammals, comes from the CYP2C and CYP3A isozymes and accounts for 30 and 24% of all drug metabolism, respectively (Zanger et al., 2013).

Hepatic expression of drug metabolizing enzymes in humans is altered in some disease states. Data using cadaveric human liver samples has shown decreased expression and function, using microsomal metabolism of midazolam, of CYP3A4 in patients with diabetes (Dostalek et al., 2011). Similarly, expression and function data from cadaveric human livers of patients with varying stages of non-alcoholic fatty liver disease have shown significant changes in CYP1A2, CYP2C9, CYP2E1 and CYP3A4 expression (Fisher et al., 2009). To date, no data exists on the hepatic expression or in vitro function of human P450s from CKD patients.

After being transported into the liver, and after metabolism within the hepatocyte, drugs and drug metabolites may be pumped out of the hepatocyte by
efflux transporters. Efflux transport proteins are able to move drugs against a concentration gradient and, thus, require energy to do so (Beringer *et al.*, 2005). Most of these transporters use primary active transport, with ATP as an energy source, and are appropriately termed as belonging to the family of ATP-binding cassette (ABC) transporters.

In the liver, most drug efflux transporters are localized to the canalicular, apical, membrane of the hepatocyte and move substrates from the cytosol of the hepatocyte to the bile canaliculus (DeGorter *et al.*, 2012). This movement of drugs represents biliary clearance where drugs, and drug metabolites, may be stored in the gallbladder prior to being released into the intestinal lumen and, ultimately, excreted in the feces. It is here where some drugs may undergo enterohepatic recycling (Nolin *et al.*, 2008). Substrates excreted in the bile can avoid elimination from the body by being reabsorbed while in the intestinal lumen. Often this occurs as a result of deconjugation, such as removal of glucuronides by bacterial glucuronidase in the gut, to convert a drug metabolite back to the parent molecule. Important drug transporters involved in pumping drugs into bile include ABCB1/P-glycoprotein, ABCC2/MRP2 and ABCG2/BCRP (DeGorter *et al.*, 2012). Not all hepatic drug efflux proteins are located apically. Some transport proteins are located on the sinusoidal membrane and are capable of moving drug substrates from the hepatocyte to the blood. Most multidrug resistance-associated proteins (MRPs) are located on the sinusoidal membrane, such as ABCC3/MRP3 (Konig *et al.*, 1999).
It is important to consider that drug transport protein expression patterns differ by tissue. Additionally, the localization, of efflux proteins especially, on apical vs. basolateral membranes also differs by tissue (DeGorter et al., 2012). These differences in expression, and localization, are responsible for a large part of the variability in distribution pharmacokinetics between different drugs (Ho et al., 2005). A summary of some clinically relevant hepatic drug transporters is shown schematically in Figure 1.1. As with drug metabolizing enzymes, no study, to date has examined the hepatic expression of drug transport proteins in liver samples from human patients with CKD.

1.3.2 Chronic Kidney Disease and Drug Transport

Several animal expression studies have demonstrated altered hepatic, renal, intestinal, and brain drug transporter mRNA and protein expression in rat models of CKD (Naud et al., 2012; Naud et al., 2007; Naud et al., 2008).

Hepatic drug transporter protein expression is significantly increased for P-gp, and decreased for OATP2, in a rat 5/6 nephrectomized model of CKD (Naud et al., 2008). Similarly, primary rat hepatocytes incubated with serum from CKD rats has shown the same changes in P-gp and OATP expression.

Intestinal expression data has demonstrated decreased protein expression of P-gp, MRP2 and MRP3 in rats with CKD (Naud et al., 2007). The same study was able to show decreases in P-gp and MRP2 in the human Caco-2 cell line.
Figure 1.1 Schematic representation of hepatic drug transport proteins of interest in this study. Solid arrows represent direction of active, ATP-dependent, substrate transport; dashed arrows represent passive direction of substrate movement. Figure adapted from the International Transporter Consortium (Giacomini et al., 2010).
when incubated with 10% serum from CKD rats relative to controls. Transport assays, using rhodamine-123 as a probe for P-gp activity, have demonstrated increased apical to basolateral flux in cells incubated for 24 hours with 10% control or CKD rat serum (Naud et al., 2007). This difference was abolished when cells were incubated with cyclosporine A, a P-gp inhibitor, in addition to rhodamine-123.

Kidney transporter mRNA and protein expression data has shown significant increases in MRP2, MRP3, MRP4, OATP2 and OATP3 in CKD rats (Naud et al., 2011). Conversely, mRNA and protein expression of renal P-gp, OATP5 and multiple OATs are significantly decreased in CKD rats, relative to control. These results were then replicated for MRP2 and MRP4 increases, and P-gp decrease, in vitro by incubating human kidney cells (HK-2) with serum from CKD rats (Naud et al., 2011). Similarly, incubation with the uremic toxin indoxyl sulfate in human kidney cell lines has been shown to decrease OATP4C1 expression (Akiyama et al., 2013). Most recently, the effect of CKD on drug transporters has been studied in rat brain tissue. Protein and mRNA expression of passive uptake transporters OAT3, OATP2 and OATP3 were significantly decreased in CKD rats relative to control (Naud et al., 2012). Similarly, expression of active efflux transporters P-gp, BCRP, MRP2 and MRP3 was significantly decreased in 5/6 nephrectomized rats. A significant increase in in vivo accumulation, in the brain, of $^{14}$C-benzylpenicillin has also been demonstrated in rats with CKD (Naud et al., 2012). A substrate for OAT3 and
MRPs, the accumulation of $^{14}$C-benzylpenecillin suggests MRP down-regulation or inhibition of function in the blood-brain barrier of rats with CKD.

Based on pharmacokinetic data, there is evidence to suggest CKD mediates changes in drug transport in the human. Fexofenadine has been used in pharmacokinetic studies to demonstrate altered drug transport in ESRD (Nolin et al., 2009). Fexofenadine is a non-specific transporter substrate used as an indicator of P-gp and OATP function. A pharmacokinetic study in ESRD patients by Nolin (2009) demonstrated an increased exposure to fexofenadine, shown by a significant, 3-fold increase in area under the plasma concentration time curve. The results of this study suggest decreases in intestinal P-gp and hepatic OATP expression are responsible for the significant increase in exposure to fexofenadine shown in ESRD patients. Similarly, a human pharmacokinetic study by Joy et al. (2014) demonstrated increased exposure to fexofenadine in glomerulonephritis and CKD patients, relative to controls. To date, this is the best data that exists on drug transporters in CKD, no study has examined the expression of drug transporter mRNA or protein in human liver samples of patients with any stage of CKD.

1.3.3 Chronic Kidney Disease and Drug Metabolism

Previous studies using rat models have demonstrated decreased hepatic, intestinal and renal CYP enzyme expression in CKD (Leblond et al., 2001; Leblond et al., 2002; Naud et al., 2011; Velenosi et al., 2012). Additionally, our lab has also shown decreased CYP2C and CYP3A expression and microsomal
metabolism of probe substrates in moderate and severe rat models of CKD (Velenosi et al., 2012).

Summarizing the data from rat models of CKD, hepatic CYP2C11 and CYP3A mRNA and protein expression are known to be decreased along with a roughly 50% decrease in total hepatic P450 content. Despite down-regulation in the CKD model, CYP3A mRNA and protein expression in the rat was shown to be reversible with intraperitoneal injection of known inducers dexamethasone and phenobarbital (Leblond et al., 2001). Hepatic protein expression of rat CYP1A2, CYP2C6, CYP2D and CYP2E1 are known to not change in CKD relative to controls (Leblond et al., 2001).

Assessment of metabolism in the live rat CKD model has been attempted using probe substrates. \textit{In vivo} use of the erythromycin breath test, as a probe for CYP3A activity, in control and CKD rats also demonstrated decreased erythromycin metabolism, in CKD rats relative to control. Aminopyrine breath tests, for CYP2C metabolism, were also performed \textit{in vivo} to show decreased activity in CKD rats (Leblond et al., 2000). The drawbacks of using these breath tests are the lack of CYP specificity, for aminopyrine metabolism, and dependence on drug transport proteins for erythromycin as a CYP3A probe (Frassetto et al., 2007; Kurnik et al., 2006).

Additionally, \textit{in vitro} assessment of hepatic drug metabolism has been assessed using hepatic microsomes. Erythromycin was the first substrate used to show decreased hepatic microsomal metabolism by CYP3A in rats with CKD (Leblond et al., 2001). Previous data from our lab has also used rat liver
microsomes to show decreased metabolism of tolbutamide, midazolam and testosterone by CYP2C11, CYP3A and both CYP2C11 and CYP3A isozymes, respectively (Velenosi et al., 2012).

Similar to the liver, intestinal mRNA and protein expression of CYP3A is significantly decreased in a rat model of CKD. Additionally, CYP1A1 expression is decreased in the intestine as well, and unlike the liver, CYP2C expression is not different between control and CKD groups (Leblond et al., 2002). Renal CYP1A, but not CYP3A, expression is also decreased in the 5/6 nephrectomized rat model of CKD (Naud et al., 2011). Important to note is analysis on the kidney expression of CYPs in the 5/6 model is based on the remaining 1/6 of the kidney after surgical induction of CKD.

Despite compelling data in rat models, human pharmacokinetics remains controversial, especially when compared to drug transport, regarding the alteration of CYP function in CKD patients (Zhang et al., 2009). In particular, Zhang et al. (2009) reported that 6 of 13 CYP3A substrates show altered pharmacokinetics in patients with renal impairment, whereas 7 substrates do not. Evidence from Nolin et al. (2006), using ESRD patients, demonstrated that hemodialysis acutely increases metabolic activity of CYP3A4. This study used the $^{14}$C-erythromycin breath test to show a 27% increase in $^{14}$CO$_2$ post-dialysis in the patients. These findings suggested that uremic molecules are capable of directly inhibiting CYP function, as the 2-hour time interval between dialysis and the breath test was too short to cause a change in CYP expression. These results also appeared to be consistent with in vivo rat studies showing decreased
CYP3A metabolism by the erythromycin breath test (Leblond et al., 2000). However, it was later realized that erythromycin is not an ideal in vivo probe substrate for CYP3A metabolism as its metabolism is affected by OATP-mediated uptake and efflux by transporters P-gp and MRP2 (Franke et al., 2011; Frassetto et al., 2007; Kurnik et al., 2006).

More recently, a study on CYP3A metabolism has used midazolam as a probe substrate. There was no difference in oral midazolam pharmacokinetic parameters between ESRD patients, the day after regular hemodialysis, and healthy controls (Nolin et al., 2009). Another pharmacokinetic study also failed to show differences in CYP3A4 and CYP2C9 function using probe substrates erythromycin and flurbiprofen, respectively (Joy et al., 2014).

To date, no study has examined the effect of CKD on the expression, or function of CYP enzymes in human liver samples.

### 1.4 Nuclear Receptors

Drug metabolizing enzymes and drug transport proteins are highly regulated at the level of transcription. This transcriptional regulation occurs through the actions of xenosensing nuclear receptors (Urquhart et al., 2007). These receptors have the ability to act as receptors and transcription factors. The ligand-binding domain of a nuclear receptor allows for interaction with substrates (Urquhart et al., 2007). For type I nuclear receptors, localization is within the cytosol and, upon ligand binding, these cytosolic nuclear receptors are able to shed co-repressors and chaperone proteins in order to translocate to the
nucleus. Alternatively, type II nuclear receptors are localized to the nucleus in the ligand absent state. In either case, ligand bound nuclear receptors may heterodimerize, in the case of PXR and RXR, or homodimerize, in the case of HNF-4α, in the nucleus prior to binding response elements in promoter regions of DNA (Urquhart et al., 2007).

1.4.1 Regulation of Cytochrome P450 Enzymes

Cytochrome P450 drug metabolizing enzymes, with few exceptions, are regulated by nuclear receptors. Cytochrome P450 enzymes are also influenced by genetic polymorphisms including single nucleotide polymorphisms (SNPs). Many SNPs are loss of function polymorphisms and can affect P450 activity indirectly, by decreasing expression through altered mRNA splicing, or directly by changing amino acid sequence and altering quaternary protein structure (Zanger et al., 2013). Additionally, CYP2D6 is affected by gain of function polymorphisms affecting copy number variation.

A relatively large number of nuclear receptors, in various combinations, are responsible for the regulation of human P450 isozymes. For the two most abundant hepatic P450 isozymes, CYP2C9 and CYP3A4, pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are the main regulators of transcription (Urquhart et al., 2007). Pregnane X receptor is a major regulator for most P450 enzymes involved in drug metabolism and responds to a broad range of ligands (Smutny et al., 2013). In addition to ligand interactions, post-translational modifications of PXR can also affect its action as a major regulator.
of P450s (Pavek et al., 2014). Cytochrome P450 1A is primarily regulated by aryl hydrocarbon receptor (AhR) and responds to various hydrocarbon and aromatic molecules (Pavek et al., 2014). Hepatocyte nuclear factor 1α is responsible for the regulation of CYP2E1, which mediates the metabolism of alcohol and a limited number of drugs (Liu et al., 1995). Unlike the majority of P450s, human CYP2D6 has no known inducers and is not regulated by any nuclear receptor (Zuber et al., 2002). Instead, CYP2D6 expression and function vary depending on copy number variation and dozens of distinct alleles that give rise to four phenotypes termed ultrarapid, extensive, intermediate and poor metabolizers (Schaeffeler et al., 2003).

1.4.2 Regulation of Drug Transport Proteins

Similar to P450 enzymes, drug transporter expression and function is highly variable as these proteins are regulated by xenosensing nuclear receptors and both transporters and nuclear receptors are subject to genetic variability in the form of SNPs (Kalliokoski et al., 2009; Tirona, 2011). Highly characterized in OATP1B1, molecular and clinical data have implicated SNPs as being associated with significantly decreased uptake activity (Tirona et al., 2001). Although less data exist, evidence suggests that a large number of SNPs exist and also cause decreased substrate affinity, and associated activity, in OATP1B3 and OATP2B1 (Kalliokoski et al., 2009).

The uptake transporter OATP1B1 expression is regulated, at the level of transcription, by liver X receptor (LXR), farnesoid X receptor (FXR) and pregnane
X receptor (PXR) (Meyer Zu Schwabedissen et al., 2010; Tirona, 2011). Similarly, OATP1B3 is known to be regulated by FXR (Tirona, 2011). P-glycoprotein is regulated in a similar manner to CYP3A, with constitutive androstane receptor (CAR), PXR, and to a lesser extent vitamin D receptor (VDR), involved in inducing P-gp expression (Geick et al., 2001). Nuclear receptors CAR, PXR and peroxisome proliferator-activated receptor (PPARα) are also involved in the regulation of MRP2, MRP3 and BCRP expression (Hoque et al., 2012; Tirona, 2011). Ultimately, the majority of hepatic transport proteins involved in drug disposition are regulated by similar combinations of nuclear receptors PXR, CAR, FXR (Tirona, 2011).

Hepatic transporter expression has been highly studied relative to other tissues and variability in hepatic uptake transporters OATP1B1 and OATP1B3 have been found to be 21 and 7-fold different, respectively, between individuals (Ho et al., 2006). Similarly, efflux transporters, such as P-gp and MRP2, have shown 55 and 365-fold differences in hepatic expression, respectively (Meier et al., 2006; Schuetz et al., 1995).

1.4.3 Differences Between Human and Rat Regulation

The rat is often used as an animal model of human disease due to having a short generation time, relatively low cost and ethical considerations. Specific to drug metabolism, P450 and drug transport protein orthologs are similar, but not identical to human isoforms. The rat orthologs for human CYP1A2 and CYP2E1
are the most similar, among drug metabolizing CYPs, regarding inducibility and substrate function and are identically named to the human isoforms (Zuber et al., 2002). In contrast, the rat has two orthologs for human CYP3A4, CYP3A1 and CYP3A2, and demonstrates differences in inducibility. Despite these differences, rat CYP3A isozymes still display affinity for many substrates of human CYP3A4. These orthologs produce the same metabolites for many characteristic substrates, such as midazolam and testosterone. Additionally, the most abundant CYP subfamily in rat liver drug metabolism is CYP2C, specifically CYP2C11, whereas human CYP3A4 is the most important isoform for human metabolism (Zuber et al., 2002). The rat CYP2D6 ortholog, CYP2D1, is similar to the human CYP2D6 in terms of substrate affinity; however, the rat CYP2D1 is unique in regulation as it is inducible (Gonzalez et al., 1987). The human CYP2D6 activity is known to be dependent on allele inheritance and copy number variation.

Similar to CYP enzyme differences, nuclear receptors in the rat display some differences to the human. Differences in ligand binding and DNA promoter region binding, with data coming mostly from CYP3A, account for altered inducibility of P450s and transporters between the human and the rat (Tirona et al., 2004; Tirona et al., 2003a). For example, although amino acid sequence is >90% identical between human and rat, differences in amino acid sequence of ligand-binding domains account for specificity for rifampin activation in human PXR but not rodent PXR (Tirona et al., 2004). Conversely, the rodent form of PXR shows an increased ability to induce CYP3A after exposure to pregnenolone.
16α carbonitrile than the human isoform of PXR (Xie et al., 2000). These differences have implications for the PXR mediated inducibility of genes, such as CYP3A and P-gp, in the rat compared to the human. Additionally, nuclear receptors, such as HNF-4α, have differing binding site locations in the rat and human. Highly expressed in the liver of humans and rats, HNF-4α is responsible for the regulation of rodent CYP3A by binding to the proximal promoter region of CYP3A1 and CYP3A2. However, the human proximal promoter region for CYP3A4 does not have a HNF-4α binding site (Tirona et al., 2003a). Instead, CYP3A4 is dependent on PXR and CAR-mediated upregulation of transcription with HNF-4α playing a role in coordinating the binding of these two nuclear receptors at a distal enhancer region.

1.5 Animal Models of Chronic Kidney Disease

1.5.1 Remnant Kidney Model (5/6 Nephrectomy)

To date, the majority of drug metabolism studies in animal models of CKD have utilized the remnant kidney model of CKD in the Norwegian rat (Leblond et al., 2001; Leblond et al., 2000; Naud et al., 2008; Velenosi et al., 2012). The 5/6 nephrectomy as a model of CKD was first described in the pig, and shortly thereafter, the rat (Ravnskov et al., 1975; Shimamura et al., 1975). General protocols include anesthesia followed by removal of the superior and inferior thirds of the left kidney. After a week of recovery the right kidney is removed (Terai et al., 2008). Control rats undergo sham laparotomies a week apart, like the 5/6 nephrectomized rats, and receive the same anesthetic. After 6 to 12
weeks the CKD rats show significant elevations, relative to controls, in plasma creatinine and blood urea nitrogen. These are used as markers of kidney disease and increase approximately 3 to 5-fold in the CKD rat model relative to control (Leblond et al., 2000).

The remnant kidney model in the Sprague-Dawley strain of Norwegian rat has been used in studies evaluating cytochrome P450 and drug transport protein expression in the liver and intestine (Leblond et al., 2000; Naud et al., 2007; Naud et al., 2008; Velenosi et al., 2012).

Despite being widely used, the remnant kidney model possesses many drawbacks for studying drug metabolism and drug transport. First, the surgical removal of the majority of renal tissue does not accurately represent how the vast majority of human CKD patients develop their kidney disease. The removal of renal tissue also makes analysis of expression of renal drug metabolizing enzymes and transporters very unreliable and difficult, as tissue is limited. Additionally, the surgical procedure is very difficult and variable (Terai et al., 2008). This leads to a large percentage of 5/6 nephrectomized rats dying, and a small percentage of rats fail to develop sufficient renal failure. Additionally, the variability in remaining renal function makes plasma creatinine and blood urea nitrogen more variable than in other models of CKD (Terai et al., 2008). The time to CKD is usually 12 weeks post-surgical removal of renal tissue. This is also much longer than the adenine-fed model of CKD where most studies use 4 to 6 weeks to develop comparable levels of plasma markers for CKD (Terai et al., 2008).
1.5.2 Adenine-Fed Model

The adenine-fed model of CKD is widely used in mice and rats for the study of aortic calcification, hyperphosphatemia and CKD-induced cardiovascular damage (Diwan et al., 2013; Terai et al., 2008). The induction of CKD in a high adenine diet is usually achieved using a normal chow diet supplemented with 0.5 to 0.75% adenine w/w. Alternatively, direct oral dosing by gavage can be used to induce CKD using 1 mL per rat of 100mg/mL adenine sulfate solution once daily for 12 days (Terai et al., 2008).

The generation of CKD is dependent on the production of 2,8-dihydroxyadenine (Terai et al., 2008). This metabolite is concentrated in the kidneys during blood filtration where it precipitates and damages renal nephrons and glomeruli and leads to an inflammatory response characterized by increased interstitial cell nuclei (Diwan et al., 2013). After 3 to 4 weeks, the damage is irreversible as the chronic severe inflammation leads to fibrosis (Diwan et al., 2013).

An advantage of the adenine model includes a potentially more severe renal failure. Previously, our lab was only able to generate 5-fold increases in plasma markers of CKD using the 5/6 nephrectomy model. The severity is easier to control depending on dosing of adenine and, more importantly, the variability in CKD severity between rats on the same diet is lower in the adenine-induced model compared to the 5/6 nephrectomy (Terai et al., 2008). Additionally, the 4 to 6 weeks to generate the model is advantageous and few, if any, animals are
The adenine diet allows researchers to study processes in the kidneys as they remain intact.

The adenine-fed model of CKD is likely a better model for comparison to human CKD; however, drawbacks of using the adenine-model do exist. The animals consume less food than control animals, especially during the first week of the diet, leading to a slower weight gain (Terai et al., 2008). Therefore, food must be weighed and limited in the control group to account for this difference. This decrease in food consumption slows the weight gain of rats but can be overcome using the oral gavage protocol for adenine administration. Oral gavaged rats eat normal chow at the same rate as control rats (Terai et al., 2008).

1.6 Objectives and Hypotheses

There are two main projects in this thesis relating to CKD and hepatic expression of drug metabolizing enzymes and drug transport proteins.

The first objective is to determine the role EPO plays in regulating hepatic drug metabolizing enzymes in the presence, and absence, of CKD. Evaluating the potential role of EPO is important because it has been shown to activate nuclear receptors, is commonly used in the ESRD patient and its effect on human hepatic drug metabolism is unknown. This was achieved by designing an experiment using a rat model of CKD. We chose to use an adenine-fed rat model of CKD and treat rats with recombinant human EPO. Our hypothesis for this
experiment is that treatment with EPO will restore the decreased hepatic CYP2C
and CYP3A expression and function that occurs after initiating CKD in male
Wistar rats.

The second objective in this thesis is to determine the changes in hepatic
expression of drug metabolizing enzymes and drug transport proteins in liver
samples of patients with ESRD relative to controls. We hypothesize that there will
be decreases in hepatic drug metabolizing enzyme and drug transporter mRNA
and protein expression in ESRD livers.
1.7 References


KFOC (2013). Facing the Facts.


2.0 The Effect of Erythropoietin on Hepatic Cytochrome P450 Expression and Function in an Adenine-Fed Rat Model of Chronic Kidney Disease
2.1 Introduction

Chronic kidney disease (CKD) is a progressive, irreversible loss of renal function characterized by decreasing glomerular filtration rate (GFR). Rates of CKD in the population are increasing largely due to increasing prevalence of associated comorbidities such as diabetes and hypertension. Patients with CKD are commonly prescribed more than seven drugs concurrently, with even higher prescription rates among dialysis patients, to manage their CKD and comorbidities (Manley et al., 2004; Talbert, 1994). Drug dosing in CKD is complicated by altered pharmacokinetic profiles, involving reduced renal and non-renal clearance of drugs. These pharmacokinetic alterations and the heavy medication burden result in high rates of adverse drug events in patients with CKD (Manley et al., 2004; Nolin et al., 2009).

Non-renal clearance of most drugs largely involves drug metabolism. In fact, the majority of prescribed drugs require some degree of metabolism prior to elimination from the body. The primary site of drug metabolism is the liver, and these reactions are primarily mediated by cytochrome P450 enzymes (Wienkers et al., 2005). CYP3A4/5 is responsible for the metabolism of 30-50% of all clinically used drugs with the CYP2C subfamily accounting for metabolism of approximately 24% of drugs. (Nolin et al., 2003a; Wrighton et al., 1996; Zanger et al., 2013). Previous studies using animal models of moderate and severe CKD
have shown decreased hepatic expression and function of CYP2C, the most abundant P450 in rat liver, and CYP3A (Leblond et al., 2001; Leblond et al., 2000; Velenosi et al., 2012).

Erythropoietin (EPO) is a glycoprotein hormone produced by the interstitial cells of the kidney (Lacombe et al., 1988). Normally produced by the healthy kidney in response to hypoxia, the primary action of EPO is to control proliferation and differentiation of erythroid progenitor cells. Accordingly, EPO is commonly used to treat anemia associated with CKD and is also used in the treatment of other anemia related disorders such as those encountered during chemotherapy, treatment of HIV and rheumatic disease. Specific to CKD, hemodialysis patients require doses of recombinant human EPO three times per week to maintain blood hematocrit required for sufficient tissue oxygenation (Eschbach, 2002).

Although investigated for decades in the regulation of red blood cell production, other non-hematopoietic roles of EPO have been recently discovered. The non-hematopoietic effects of EPO signaling have been largely investigated in the protection of cells from ischemia and reperfusion injury in the central nervous system, heart, kidney and liver. The direct effects of EPO are mediated by interaction at the surface of cells, including hepatocytes, which express the erythropoietin receptor (Pinto et al., 2008). Further, recent evidence also suggests that EPO-mediated signaling is able to activate transcription factors including the nuclear receptor liver X receptor (LXR) (Lu et al., 2010; Pinto et al., 2008). Nuclear receptor activation by EPO is of particular interest as enzymes and transporters involved in drug metabolism and disposition are transcriptionally
regulated by xenosensing nuclear receptors. Specifically, CYP3A4 expression is regulated by pregnane X receptor (PXR) and both CYP3A and CYP2C enzymes are regulated by hepatocyte nuclear factor 4α (HNF-4α) (Ibeanu et al., 1995; Tirona et al., 2003b). Despite these findings and the increasing number of CKD patients receiving EPO to treat anemia, the effect of continuous EPO administration on the enzymes involved in drug metabolism in the presence, or absence, of CKD has not been directly investigated.

The objective of this study was to examine the effect of erythropoietin on P450 expression and function in the presence, and absence, of CKD. We utilized an adenine-fed rat model of CKD combined with treatment of recombinant human EPO to evaluate the impact on hepatic expression and activity of CYP2C and CYP3A drug metabolizing enzymes. We hypothesize that the dosing of EPO will partially restore CKD-mediated down-regulation of P450 expression and activity.
2.2 Materials and Methods

Chemical Reagents and Drugs

Adenine was obtained from Amresco (Solon, OH). Testosterone, 6βOH-testosterone and 16αOH-testosterone were purchased from Steraloids Inc. (Newport, RI). Carbamazepine was purchased from Cerilliant (Round Rock, TX). Epoetin alfa (EPREX®) was obtained from Janssen-Ortho (Markham, ON).

Experimental Model

Thirty-two male Wistar rats, weighing 150 g, were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Animals were acclimated for 4 days on a 12h light cycle with standard rat chow and water ad libitum. All animal protocols and procedures were approved by the Western University Animal Care Committee and conducted in accordance with the Canadian Council on Animal Care.

Adenine-Induced Chronic Kidney Disease

After acclimation, rats were assigned to a normal chow diet (n=16) or a chow diet supplemented with 0.7% adenine (n=16) for 6 weeks. Rats were pair-fed, food was weighed daily and control rats were fed the same mass of food per day as their adenine-fed counterparts. After 4 weeks on the adenine or control diet, rats received either 150 U/kg epoetin alfa, or saline, in a volume of 0.25 mL per 100 g body weight, by intraperitoneal injection every other day for 2 weeks prior to sacrifice. The resulting treatments created four groups of equal number
(n=8) – chow-fed saline injected rats (control), chow-fed EPO injected rats (EPO),
adenine-fed saline injected rats (CKD) and adenine-fed EPO injected rats (CKD
EPO). After the 6-week protocol, rats were sacrificed under isoflurane anesthesia
by decapitation. Blood was collected and hematocrit, plasma markers of CKD
(creatinine and urea) and liver enzymes (AST and ALT) were determined by the
London Laboratory Services group by standard methods (London, ON).

**Histology**

Fresh liver and kidney samples were fixed in 10% formalin obtained from
Anachemia (Rouses Point, NY). Tissue processing, sectioning and staining were
completed by the Department of Pathology (Western University, Canada).
Samples were dehydrated using ethanol, cleared with xylene and embedded in
paraffin wax. Sections (5 µm) of the liver and kidney were cut, using a microtome,
and mounted on slides before being rehydrated and stained with hematoxylin and
eosin (H&E). Slides were visualized using an Olympus SZX16 microscope and
Qcapture Pro software version 6.0 by QImaging (Redwood City, CA).

**Real-Time PCR Analysis**

Hepatic total RNA was extracted using TRIzol Reagent from Life
Technologies Inc. (Burlington, ON) according to manufacturer’s protocol. RNA
concentration and quality were determined using a NanoDrop spectrophotometer
from Thermo Scientific (West Palm Beach, Florida). cDNA was synthesized from
1 µg total RNA using Quanta Biosciences qScript cDNA Supermix (Gaithersburg,
MD). Relative mRNA was quantified by real-time PCR with Quanta Biosciences
PerfeCta SYBR Green Fastmix. Specific primers were designed for CYP1A2,
CYP2C11, CYP2D1, CYP3A2, PXR, RXRα, CAR, AhR and HNF-4α using NCBI Primer-Blast. Gene expression was normalized to β-actin using the ΔΔCT method (Livak et al., 2001). Relative mRNA levels were analyzed compared to the control treatment group that received normal-chow and saline injections.

**Hepatic Microsome Isolation**

Liver microsomes were isolated by differential centrifugation as described previously (Velenosi et al., 2012). Liver tissue was rinsed in 0.9% NaCl solution and homogenized in 1.15% KCl containing 1mM EDTA. Tissue homogenate was centrifuged at 9000g for 20 min at 4°C. The resulting supernatant was centrifuged at 105 000g for 60 min at 4°C. The microsome pellet was resuspended in 250 µL of 100 mM potassium phosphate buffer containing 20% glycerol at pH 7.4. Total protein concentration was quantified using Pierce BCA protein assay from Fisher Scientific (Waltham, MA) and microsomes were aliquoted and stored at -80°C until use.

**Determination of Total Cytochrome P450 Content**

Microsomal protein was used to determine total cytochrome P450 content using spectral analysis after reduction of P450 using carbon monoxide and sodium dithionite (Omura et al., 1964).

**Western Blot Analysis**

Microsomes were used for the determination of CYP3A2 and CYP2C11 protein expression using western blot analysis. Twenty micrograms of microsomal protein were loaded per well in a 10% polyacrylamide gel containing 0.1% SDS and electrophoresed for 60 min at 120 V. Proteins were transferred to
a nitrocellulose membrane for 60 min at 150 V and immunoblots were incubated at room temperature with primary antibodies for 90 minutes prior to being washed with PBS with 0.1% tween and incubated with secondary antibodies linked to horseradish peroxidase for 60 minutes. Primary antibodies used were polyclonal rabbit anti-rat CYP1A, dilution 1:5000, Detroit R&D Inc. (Detroit, MI), monoclonal mouse anti-rat CYP2C11, dilution 1:5000, Detroit R&D Inc., polyclonal rabbit anti-rat CYP2D, dilution 1:5000, Detroit R&D, monoclonal rabbit anti-rat CYP3A2, dilution 1:10000, Millipore (Billerica, MA), and monoclonal mouse anti-β-actin-peroxidase, dilution 1:50000, Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase-linked secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Luminata Forte western HRP substrate was obtained from Millipore (Billerica, MA). Immune blots were imaged on a Bio-Rad VersaDoc Imaging System (Berkeley, CA) and band intensity was quantified by densitometry using Bio-Rad Quantity One 1D analysis software.

**Microsomal Metabolism and Analysis by UPLC-PDA**

Metabolic activity of CYP3A and CYP2C in hepatic microsomes was determined using the probe substrate, testosterone, as previously described (Velenosi et al., 2012). Buffered reactions, using 50 mM potassium phosphate with 2 mM MgCl₂ (pH 7.4), were conducted with 1 mg/mL hepatic microsomal protein in a final volume of 250 µL. Metabolism was determined previously to be linear at 10 minutes for the formation of testosterone metabolites. All reactions were pre-incubated with probe substrates for 10 minutes at 37°C and initiated by the addition of 1 mM NADPH (final concentration). Final substrate concentrations
of testosterone used in the reaction were 50, 100, 400 and 1000 µM. Reactions were terminated by adding 50 µL of ice-cold acetonitrile followed by a 15 minute incubation on ice and centrifugation to pellet precipitated protein. Metabolites 6βOH-testosterone (CYP3A) and 16αOH-testosterone (CYP2C) were extracted by solid-phase extraction, using carbamazepine as an internal standard, followed by UPLC-PDA as described previously (Velenosi et al., 2012). In brief, solid-phase extraction was completed using C18, Strata-X polymeric reverse phase 33 µm cartridges from Phenomenex (Torrance, CA). Analytes were passed across the packing and washed with 1 mL of nano-pure water followed by 1 mL of 50:50 methanol:water. Analytes were eluted into clean glass test tubes with 1 mL of methanol with 0.1% triethylamine and 0.1% trifluoroacetic acid. Eluent was dried in a nitrogen evaporator with a water bath at 40°C. Dried eluent was reconstituted in mobile phase and injected on a Phenomenex Kinetex C18 column (1.7 µm particle size, 50 x 2.1 mm). The column temperature was constant at 40°C in a Waters ACQUITY UPLC H-Class system. Mobile phase flow was constant at 0.8 mL/min and consisted of 5 mM KH₂PO₄ (pH 3.0), acetonitrile and methanol under gradient conditions previously described (Velenosi et al., 2012). A Waters ACQUITY UPLC PDA detector was used to detect testosterone and metabolites (245 nm) and carbamazepine (290 nm) for quantification.

Chromatin Immunoprecipitation

ChIP was performed on rat liver tissue using previously published methods (Sohi et al., 2011). In brief, tissue was homogenized and DNA cross-linked by 1%
formaldehyde before being sheared by sonication. Sonicated chromatin was dilute, aliquoted and stored at -80°C. Samples were pre-cleared with protein A/G agarose and incubated with 5 µg of antibody per aliquot, unless stated, of RNA Pol II, 1 µg per aliquot, Millipore; PXR, Santa Cruz or HNF-4α, Santa Cruz, overnight at 4°C. Separate aliquots of sample chromatin were incubated with the same amount, and same host species of normal IgG, Millipore, to determine non-specific binding. Protein A/G agarose beads, Santa Cruz, were used to collect antibody, antigen and associated chromatin. Beads were washed and then eluted with SDS buffer. Antibody collected chromatin and sample inputs were heated to 65°C to reverse crosslinks. Proteinase K, purchased from Bio Basic (Markham, ON), was added for 1h at 45°C and then DNA was extracted and precipitated prior to being reconstituted in Tris-EDTA buffer. Primers were designed, using NCBI primerblast, to amplify 100-200 bp regions of genomic DNA around known or putative binding sites of proteins of interest in the promoter region of CYP2C11 or CYP3A2. Real-time PCR was used to quantify RNA Pol II, PXR, HNF-4α and STAT5 promoter region binding.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism (version 5.0). A Michaelis-Menten model was used to fit the formation of testosterone metabolites. Statistical differences between control and treated rats were assessed by ANOVA followed by Tukey’s post-hoc test. Results are expressed as mean ± SEM and a $p < 0.05$ was considered significant.
2.3 Results

Plasma Biochemistry and Body Weight

Hematocrit was significantly increased in the EPO and CKD EPO groups \( (p < 0.001) \), and significantly decreased in the CKD group \( (p < 0.01) \) relative to control. Plasma creatinine levels were 9.3 and 8.5-fold higher in CKD and CKD EPO, respectively, when compared to control \( (p < 0.001) \) (Table 2.1). CKD and CKD EPO groups had a 10.8 and 7.1-fold higher plasma urea concentration compared to control, respectively \( (p < 0.001) \). Plasma liver enzymes aspartate transaminase (AST) and alanine transaminase (ALT) were measured to demonstrate adenine diets and EPO injections did not cause hepatocellular injury (Table 2.1). Plasma liver enzyme levels in our study were within reference limits and comparable to previously published reports based on gender and body weight (Zhang et al., 2004). Additionally, examination using H&E staining showed normal liver histology in all treatment groups (Figure 2.1). Kidney damage shown by histology demonstrates damaged, enlarged renal tubules and increased interstitial cell nuclei consistent with previous characterization of the adenine-fed model of kidney disease (Figure 2.1) (Diwan et al., 2013). No histological differences were observed in the liver of EPO, CKD or CKD EPO groups relative to control. Spectral analysis of total cytochrome P450 content in liver microsomes showed no decrease between control and EPO treated groups. Consistent with the 5/6 nephrectomy rat model of CKD used previously (Leblond et al., 2001), a 52% decrease in hepatic total P450 content was demonstrated in CKD and CKD
Table 2.1 Physical characteristics and biochemistry of control, EPO, CKD and CKD EPO rats. Data presented are mean ± SEM

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>EPO</th>
<th>CKD</th>
<th>CKD EPO</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>248.5 ± 8.2</td>
<td>249.0 ± 9.7</td>
<td>223.3 ± 18.1</td>
<td>232.6 ± 11.7</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>47.2 ± 0.5</td>
<td>67.3 ± 0.7***</td>
<td>36.7 ± 2.3**†††</td>
<td>65.9 ± 2.3***</td>
</tr>
<tr>
<td>Plasma Creatinine (µmol/L)</td>
<td>21.4 ± 1.3</td>
<td>25.9 ± 0.6</td>
<td>199.3 ± 24.4****†††</td>
<td>180.8 ± 18.2****†††</td>
</tr>
<tr>
<td>Plasma Urea (mmol/L)</td>
<td>6.7 ± 0.4</td>
<td>7.8 ± 0.7</td>
<td>72.3 ± 13.2****†††</td>
<td>47.8 ± 2.5****†††</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>99.6 ± 11.1</td>
<td>87.3 ± 8.1</td>
<td>69.1 ± 8.7</td>
<td>61.3 ± 5.1*</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>33.1 ± 2.1</td>
<td>33.0 ± 1.9</td>
<td>8.1 ± 2.2*****</td>
<td>8.1 ± 2.2*****</td>
</tr>
<tr>
<td>Hepatic Total P450 Content (nmol/mg protein)</td>
<td>0.71 ± 0.05</td>
<td>0.65 ± 0.07</td>
<td>0.35 ± 0.03****†††</td>
<td>0.35 ± 0.04****†††</td>
</tr>
</tbody>
</table>

* $p < 0.05$ relative to control
** $p < 0.01$ relative to control
*** $p < 0.001$ relative to control
† $p < 0.05$ relative to EPO
‡‡‡ $p < 0.001$ relative to EPO

58
Figure 2.1 Histology using hematoxylin and eosin (H&E) staining of kidney (A-D) and liver (E-H) tissue from control (A,E), EPO (B,F), CKD (C,G) and CKD EPO (D,H) treated rats. Asterisks (*) highlight enlarged nephrons with atrophy of associated tubular cells. Arrows highlight increased interstitial cell nuclei, a characteristic of kidney damage. Liver histology of all groups is consistent with normal hepatic structure.
EPO rats relative to control \((p < 0.001)\) (Table 2.1). There were no significant differences in body weight between groups.

**Nuclear Receptor mRNA Expression**

CKD EPO rats showed a 49% decrease in constitutive androstane receptor (CAR) hepatic mRNA expression compared to control \((p < 0.05;\) Fig. 2.2). CKD and CKD EPO rat groups had a, 48% and 41% decrease, in PXR hepatic mRNA expression, respectively \((p < 0.01;\) Fig. 2.2). There were no significant differences in the hepatic mRNA expression of aryl hydrocarbon receptor \((\text{AhR}; p = 0.24)\), retinoid X receptor \(\alpha\) \((\text{RXR}\alpha; p = 0.10)\) or HNF-4\(\alpha\) \((p = 0.26)\) between treatment groups and control.

**CYP1A2, CYP2C11, CYP2D1 and CYP3A2 mRNA and Protein Expression**

CYP2C11 mRNA expression was decreased by 97% and 98% for CKD and CKD EPO rats, respectively, relative to control \((p < 0.001)\) (Fig. 2.3B). Similarly, hepatic CYP3A2 mRNA expression was significantly decreased, relative to control, by 98% and 99% in CKD and CKD EPO rats, respectively. Hepatic CYP3A2 mRNA expression was also decreased by 55% in EPO treated rats compared to control \((p < 0.001;\) Fig. 3D). No differences were seen in hepatic CYP1A2 \((p = 0.94;\) Fig. 2.3A) or CYP2D1 \((p = 0.46;\) Fig. 2.3C) mRNA expression relative to control.

Similar to mRNA expression, hepatic protein expression of CYP2C11 was significantly decreased by 93% and 96% in the CKD and CKD EPO groups relative to control \((p < 0.001;\) Fig. 2.4B). Hepatic CYP3A2 protein
Figure 2.2 mRNA expression of hepatic nuclear receptors constitutive androstane receptor (CAR), pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR), retinoid X receptor alpha (RXRα) and hepatic nuclear factor 4 alpha (HNF-4α). Expression is normalized to housekeeping gene β-actin and shown as relative to percentage of control expression (black) for treatment groups EPO (red), CKD (blue) and CKD EPO (orange). Analysis was performed in triplicate and results shown are mean ± SEM, n=8. * p < 0.05 compared with control, ** p < 0.01 compared with control, *** p < 0.001 compared with control; † p < 0.05 compared with EPO.
Figure 2.3 mRNA expression of hepatic CYP1A2 (A), CYP2C11 (B), CYP2D1 (C) and CYP3A2 (D) in control, EPO, CKD and CKD EPO treated rats. Expression is normalized to β-actin and shown as a percentage of control. Results are mean ± SEM, n=8. * p < 0.05 compared with control, ** p < 0.01 compared with control, *** p < 0.001 compared with control; † p < 0.05 compared with EPO, †† p < 0.05 compared with EPO and ††† p < 0.05 compared with EPO.
Figure 2.4 Protein expression of hepatic CYP1A (A), CYP2C11 (B), CYP2D (C) and CYP3A2 (D) in control, EPO, CKD and CKD EPO treated rats. Protein bands are standardized to β-actin and expressed as relative densitometry units with control bands arbitrarily defined as 100%. Western blots were performed in duplicate with representative blots shown. Results shown are mean ± SEM, n=8. *** p < 0.001 compared with control and ††† p < 0.05 compared with EPO.
expression was significantly decreased by 81%, 98% and 98% in the EPO, CKD and CKD EPO groups, respectively, relative to control ($p < 0.001$; Fig. 2.4D). No statistical differences were seen for CYP1A ($p = 0.70$; Fig. 2.4A) or CYP2D ($p = 0.65$; Fig. 2.4C) protein expression relative to control.

**Hepatic CYP2C11 and CYP3A-Mediated Drug Metabolism**

We evaluated the function of CYP2C11 and CYP3A, using testosterone as a probe substrate, in rat liver microsomes. Testosterone is specifically metabolized to 16αOH-testosterone by CYP2C11. Formation of 16αOH-testosterone by CYP2C11 in CKD and CKD EPO rat liver microsome samples was below the limit of detection for most substrate concentrations; therefore, Michaelis-Menten values could not be calculated and statistical analysis could not be performed. CYP2C11 $V_{\text{max}}$ was substantially higher in control and EPO groups, $1880.8 \pm 241.6$ and $1669.2 \pm 317.9$ pmol min$^{-1}$ mg protein$^{-1}$, respectively, than the unquantifiable metabolite formation in CKD and CKD EPO groups (Fig. 2.5A).

The formation of 6βOH-testosterone is specifically catalyzed by CYP3A enzymes. EPO $V_{\text{max}}$ for CYP3A-mediated formation of 6βOH-testosterone was significantly decreased by 32% relative to control ($p < 0.05$; Table 2.2). Further significant decreases in $V_{\text{max}}$ of 51% and 61% were seen in CKD and CKD EPO rats, respectively ($p < 0.01$; Fig. 2.5B). Michaelis-Menten values for the formation of 16αOH-testosterone and 6βOH-testosterone are summarized in Table 2.2.
Table 2.2  Michaelis-Menten kinetic values for testosterone in control, EPO, CKD and CKD EPO rat liver microsomes

Data presented are mean ± SEM

<table>
<thead>
<tr>
<th></th>
<th>16α-OH Testosterone</th>
<th>6β-OH Testosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_m ) ( \mu M )</td>
<td>( V_{max} ) pmol min(^{-1}) mg protein(^{-1})</td>
</tr>
<tr>
<td>Control</td>
<td>148.2 ± 22.9</td>
<td>1880.8 ± 241.6</td>
</tr>
<tr>
<td>EPO</td>
<td>121.6 ± 16.5</td>
<td>1669.2 ± 317.9</td>
</tr>
<tr>
<td>CKD</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>CKD EPO</td>
<td>n/d</td>
<td>n/d</td>
</tr>
</tbody>
</table>

n/d, not detectable

* \( p < 0.05 \) relative to control

** \( p < 0.01 \) relative to control

*** \( p < 0.001 \) relative to control
Figure 2.5 Michaelis-Menten plots of the formation of 16αOH-testosterone (A) and 6βOH-testosterone (B) after incubation of rat liver microsomes with 1mM NADPH and testosterone concentrations of 50, 100, 400 and 1000 µM. Formation of 16αOH-testosterone in CKD and CKD EPO liver microsomes was at or below the limit of detection for most concentrations. Experiments were performed in duplicate and results are presented as mean ± SEM, n=8.
Chromatin Immunoprecipitation of RNA Pol II, PXR, HNF-4α

To explore the effects of EPO and CKD on transcriptional regulation of CYP2C11 and CYP3A2, ChIP and quantitative real-time PCR was utilized to quantify binding of RNA Pol II and nuclear receptors, HNF-4α and PXR, to the promoter region of genes. RNA Pol II recruitment to the promoter region of CYP2C11 was decreased by 72% and 78%, relative to control, in CKD and CKD EPO groups, respectively ($p < 0.05$; Fig. 2.6A). Similarly, RNA Pol II binding to the promoter of CYP3A2 was decreased by 88% and 90% in CKD and CKD EPO groups, respectively, compared to control ($p < 0.001$; Fig. 2.7A). Additionally, RNA Pol II binding to the CYP3A2 promoter was decreased by 52% in the EPO group relative to control ($p < 0.01$; Fig. 2.7A).

HNF-4α binding to the promoter region of both CYP2C11 and CYP3A2 was also assessed using ChIP. There was a 54% and 56% decrease in HNF-4α binding in the promoter region of CYP2C11 for CKD and CKD EPO groups, respectively, but this failed to reach significance (Fig. 2.6B). In contrast, significant decreases in HNF-4α binding were seen in the promoter region of CYP3A2 with decreases in HNF-4α binding of 62%, 71% and 90%, relative to control, in the EPO, CKD and CKD EPO treatment groups, respectively ($p < 0.05$; Fig. 2.7B). Additionally, PXR binding to the CYP3A2 promoter showed 71%, 88% and 90% decreased binding, relative to control, in EPO, CKD and CKD EPO groups, respectively ($p < 0.001$; Fig. 2.7C).
Figure 2.6 Chromatin immunoprecipitation (ChIP) in hepatic tissue to quantify the binding of RNA pol II (A) and HNF-4α binding (B) to the promoter region of CYP2C11 for control (black), EPO (red), CKD (blue) and CKD EPO (orange) rats. Results are standardized to input DNA and expressed as a percentage of control. Samples immunoprecipitated with normal mouse IgG (A) or rabbit IgG (B) are shown as IgG (white). Results are mean ± SEM, n=8. *p < 0.05 relative to control.
Figure 2.7 Chromatin immunoprecipitation (ChIP) in hepatic tissue to quantify the binding of RNA pol II (A), HNF-4α (B) and PXR (C) to the promoter region of CYP3A2 for control (black), EPO (red), CKD (blue) and CKD EPO (orange) rats. Results are standardized to input DNA and expressed as a percentage of control. Samples immunoprecipitated with normal mouse IgG (A) or rabbit IgG (B and C) are shown as IgG (white). Results are mean ± SEM, n=8. * p < 0.05 relative to control, ** p < 0.01 compared with control and *** p < 0.001 compared with control.
2.4 Discussion and Conclusions

Several previous reports have described the decreased hepatic expression and function of CYP2C11 and CYP3A2 in rats with CKD using the 5/6 nephrectomy or ligation models of disease (Guevin et al., 2002; Leblond et al., 2001; Leblond et al., 2000; Velenosi et al., 2012). To our knowledge, this is the first study to use the adenine-fed model of CKD to study the impact of renal failure on drug metabolism. The 5/6 nephrectomy is the best characterized rodent model of CKD. Despite its widespread utility, the 5/6 nephrectomy models the consequence of CKD (i.e. nephron loss) but does not completely mimic the progression of CKD. The adenine-fed CKD model was used in this study for several reasons: 1) it is a less invasive model with lower rates of mortality, 2) there is lower variability in creatinine and urea and 3) it produces pathological changes that are consistent with progressive chronic kidney disease. (Diwan et al., 2013; Hewitson et al., 2009; Terai et al., 2008). To ensure that the adenine diet model did not also mediate liver toxicity we confirmed that adenine-fed rats had normal liver histology. In addition plasma biochemistry results support that the adenine-fed model of CKD and the dosing of EPO do not alter the morphology or affect the integrity of liver tissue.

Our first objective was to compare changes in drug metabolizing enzyme expression and function in the adenine fed model of CKD with known P450 changes in the 5/6 nephrectomy model. Our data clearly demonstrate that the adenine-fed model of CKD causes pronounced decreases of hepatic CYP2C11
and CYP3A2 expression and function, consistent with previously published work using the 5/6 nephrectomy model of CKD (Guevin et al., 2002; Leblond et al., 2001; Velenosi et al., 2012). Our next objective was to evaluate our hypothesis that EPO would partially restore expression of P450s downregulated by CKD. We hypothesized EPO would restore P450 expression in CKD for two reasons. First, recent findings demonstrate that EPO is able to activate nuclear receptors known to be involved in the regulation of drug transport proteins involved in drug disposition (Lu et al., 2010; Meyer Zu Schwabedissen et al., 2010; Naik et al., 2006). Second, induction of CYP3A6, the rabbit ortholog of human CYP3A4, was observed in a hypoxia study using isolated rabbit hepatocytes. The authors showed the involvement of plasma mediators (Fradette et al., 2002) and convincingly showed, using anti-EPO antibodies, that part of the CYP3A6 induction observed was due to the actions of EPO (Fradette et al., 2002). Despite these previous findings and contrary to our hypothesis, we observed no restoration in CKD-mediated down-regulation of P450 enzymes. In fact, our results demonstrate that two weeks of IP EPO injections significantly decreases CYP3A2 mRNA expression, leading to a corresponding decrease in protein expression and conversion of the CYP3A probe substrate testosterone to the 6βOH-testosterone metabolite. Further, we demonstrate that treatment with EPO decreases PXR and HNF-4α binding to the CYP3A2 promoter and that this decrease in binding is concurrent with a decrease in RNA polymerase II binding. Although EPO has been shown to activate other nuclear receptors, such as LXR,
our data demonstrate that it mediates decreased activity of hepatic PXR and HNF-4α.

EPO treatment is targeted at increasing the proliferation and differentiation of erythrocytes in order to treat anemia. Erythropoiesis requires iron, for heme synthesis, as the prosthetic heme group is required for the formation of hemoglobin. As cytochrome P450 drug metabolizing enzymes are also hemoproteins, one potential explanation of our data is that EPO administration simply depletes or limits the iron or heme pool. To investigate this possibility, we performed spectral analysis of liver microsomes to quantify total P450 content. There is no significant difference in hepatic total P450 content between control and EPO treated groups indicating that EPO itself does not simply decrease the iron or heme pool available for P450 synthesis. A significant decrease in total hepatic P450 content was observed in CKD and CKD EPO groups; however, this finding was expected and is consistent with previous studies using the 5/6 nephrectomy model of CKD (Leblond et al., 2000). Iron deficiency is known to significantly decrease plasma iron, total liver iron and liver ferritin (Dhur et al., 1989). However, iron deficiency has been shown in many studies to not affect total hepatic microsomal protein or P450 content (Catz et al., 1970; Dhur et al., 1989). Further, limiting heme has only been shown to decrease P450 induction by phenobarbital, not basal expression, in knockout mice unable to synthesize heme (Jover et al., 2000). Additionally, the rats in our study were not subject to iron restriction; thus, it is unlikely that changes in P450 protein content were influenced by utilization of iron for erythropoiesis. Although we did not measure
iron in our study, a previous study in rats with CKD receiving EPO did not display any changes in serum iron concentration (Srai et al., 2010).

Previous reports demonstrate an upregulation of hepatic CYP3A6 in rabbits exposed to hypoxia (Fradette et al., 2002; Kurdi et al., 1999). One of the primary consequences of hypoxia is the stabilization of hypoxia inducible factor (HIF) alpha subunits. HIF-1α and HIF-2α heterodimerize with HIF-1β and bind to HIF responsive elements in target genes such as EPO to upregulate their transcription. Indeed, increased synthesis of EPO is consistently observed in hypoxic conditions. Fradette et al. elegantly demonstrate that anti-EPO antibodies partially abrogate the induction of CYP3A6 under hypoxic conditions. Although our data appear to contradict this finding, it must be noted that there are marked species differences in the transcriptional regulation of P450s and our study did not directly investigate the effect of hypoxia. Inline with our observations, the expression and function of drug metabolizing enzymes, including CYP3A4, are decreased after exposure to hypoxia in differentiated human HepaRG cells (Legendre et al., 2009). In that study, hypoxia-mediated down-regulation of CYP3A4 was shown to involve HIF-1α. Although implicated in the down-regulation of CYP3A, HIF-1α involvement in that study was thought to be acting by an indirect, and unknown, mechanism (Legendre et al., 2009). Despite being an in vitro experiment using a hepatic cell line, it is possible that this unknown mechanism involves EPO signaling. Hypoxia, caused by low oxygen tension or anemia secondary to bilateral nephrectomy or hemorrhage,
has been previously shown to increase hepatic EPO production (Bondurant et al., 1986; Fried, 1972).

Based on the previous work by Legendre et al. (2009) on the effect of hypoxia on CYP3A4 in HepaRG cells, our data suggests that EPO could be the next step in the elusive mechanism that links hypoxia and HIF activation to decreased CYP3A expression. Although constitutively expressed, HIF alpha subunits are extremely sensitive to oxygen and, in the presence of oxygen, are hydroxylated, recognized for ubiquitination and subsequently proteosomally degraded (Jaakkola et al., 2001). Conversely, under hypoxic conditions HIF-1α and HIF-2α act as transcription factors to activate many genes including activating the transcription of EPO as shown in states of low oxygen tension (Lacombe et al., 1988; Wang et al., 1993). The relationship between HIFs and EPO, and corresponding evidence of decreases in CYP3A4 and CYP3A2, respectively, suggests that EPO is largely responsible for hypoxia-mediated changes in the expression of CYP3A. Our data support that exposure to EPO causes decreased binding of PXR, HNF-4α and RNA polymerase II to the promoter region of CYP3A2.

The EPO receptor is expressed on many non-hematopoietic tissues including liver. Upon ligand binding, EPO can activate three major intracellular signaling pathways: 1) the Janus kinase 2 (Jak2) pathway, 2) the extracellular signal regulated kinases (ERK) pathway and 3) the phosphatidylinositol 3 kinase/Akt (PI3K/Akt) pathway. Activation of the Jak2 pathway activates signal transducer and activator of transcription 5 (STAT5) by phosphorylation (Gouilleux
et al., 1995; Parganas et al., 1998). Phosphorylated STAT5 is able to enter the nucleus and act as a transcription factor in the promoter region of genes both as an activator and a suppressor of transcription (John et al., 1999). We attempted to characterize STAT5 binding to putative sites in the CYP3A promoter but have been unable to confirm binding to these sites (data not shown). Activation of the ERK1/2 pathway has been shown to interact with phosphorylated CAR, preventing its dephosphorylation and retaining it in the cytoplasm (Osabe et al., 2011). PXR translocation to the nucleus has also been shown to be negatively impacted by phosphorylation (Lichti-Kaiser et al., 2009). Therefore, it is possible that EPO induced ERK activation plays a role in retaining nuclear receptors in the cytoplasm and therefore decreasing CYP3A2 transcription. The last cell signaling pathway activated by EPO is the PI3K/Akt pathway. Previous studies have shown that downstream kinases in this pathway phosphorylate and negatively regulate the transcriptional activity of PXR (Lichti-Kaiser et al., 2009; Pondugula et al., 2009). Finally, a recent study has shown that EPO activates the LXR in macrophages (Lu et al., 2010). The LXR has been shown to transcriptionally repress the activity of both PXR and CAR, which provides another potential explanation of the molecular mechanism linking EPO to decreased CYP3A2 expression in our study (Handschin et al., 2002; Pondugula et al., 2009). It is clear that EPO mediates its non-hematopoietic effects via many distinct molecular pathways and that further work is needed to isolate the specific mechanism associated with EPO induced decreases in nuclear receptor binding and CYP3A expression.
In conclusion, this study demonstrates that EPO decreases the expression and function of hepatic CYP3A2 by decreasing the binding of the nuclear receptors PXR and HNF-4α. To our knowledge, this is the first study that has evaluated the effect of EPO on drug metabolism. As the principal enzyme for the metabolism of many drugs, knowledge of the impact of EPO on CYP3A expression and function are important for patient safety and appropriate drug dosing. Greater understanding on how EPO impacts hepatic P450 expression *in vitro* and *in vivo*, will be needed before evaluating the pharmacokinetics of individual drugs to optimize drug dosing in patients receiving recombinant human erythropoietin.
2.5 References


3.0 The Effect of End-Stage Renal Disease on the Expression of Hepatic Drug Metabolizing Enzymes and Transport Proteins
3.1 Introduction

Chronic kidney disease (CKD) is a progressive condition characterized by a continual decline in renal function. Patients with CKD are classified in stages based on estimated glomerular filtration rate (eGFR) with the fifth, and final stage being end-stage renal disease (ESRD). Diagnosis of ESRD includes an eGFR of less than 15 mL/min/1.73m$^2$, representing roughly 10-15% of complete kidney function. Patients with ESRD require renal replacement therapy, usually achieved using hemodialysis, in order to filter the blood and prolong life. In addition to hemodialysis, most patients are prescribed more than 7 medications to manage their ESRD and associated comorbidities including diabetes, hypertension and atherosclerotic disease (Manley et al., 2004; Stevens et al., 2010; Talbert, 1994).

Alterations in renal and non-renal drug clearance complicate pharmacological treatment of comorbidities in ESRD patients (Sun et al., 2006). Although CKD dosing guidelines are based on creatinine clearance, these patients experience increased rates of adverse drug events, such as increased drug exposure and toxicity, compared to patients with normal renal function (Bennett, 1988; Cardone et al., 2010; Manley et al., 2005). In the case of ESRD patients on renal replacement therapy, eGFR cannot be used to determine drug dosing as many ESRD patients are anuric. Increased drug exposure in patients with ESRD is mediated by alterations in renal and non-renal clearance of drugs. In particular, recent evidence suggests that aside from decreased renal drug
excretion, hepatic and intestinal drug metabolism and transport are altered in patients with ESRD (Nolin et al., 2009; Sun et al., 2010b).

Studies using animal models of CKD have extensively shown down-regulation of expression and function of hepatic cytochrome P450 (P450) drug metabolizing enzymes (Leblond et al., 2001; Naud et al., 2008; Velenosi et al., 2012). These enzymes are known to be involved in the majority of phase I drug metabolism reactions and typically render drug molecules more hydrophilic, and structurally suitable for conjugation, to increase elimination from the body (Wienkers et al., 2005). Additionally, many disease states have been shown to affect the hepatic P450 expression in the human (Dostalek et al., 2011; Fisher et al., 2009). However, results from human ESRD patient pharmacokinetic data using probe substrates for P450-mediated metabolism remain controversial. Pharmacokinetic data has shown that hemodialysis can acutely improve hepatic clearance of erythromycin (Nolin et al., 2006). However, more recent data, looking at the clearance of oral midazolam, suggests that CYP3A4 activity in ESRD patients is not different from control patients (Nolin et al., 2009).

Further, the study by Nolin et al. (2009) also evaluated the impact of kidney disease on hepatic and intestinal drug transporters. Their data suggests that differences exist in the function of drug transport proteins in the ESRD patient relative to control counterparts without kidney disease. The impact of CKD has also been shown, in animal models, to alter expression levels drug transport proteins in the liver and other tissues (Naud et al., 2012; Naud et al., 2007; Naud et al., 2008). However, the contradicting data and lack of data on
non-renal drug clearance in ESRD patients has led to the FDA recommending this as an area for increased study (Zhang et al., 2009). Specifically, additional ESRD pharmacokinetic studies and studies characterizing the effect of renal impairment on the expression of drug metabolizing enzymes and drug transport proteins in non-renal tissue are required for a better understanding of how drug disposition changes during kidney disease and how this impacts pharmacotherapy of ESRD patients.

In this study, we compare hepatic expression levels of P450 drug metabolizing enzymes and drug uptake and efflux transport proteins between ESRD and control patients using cadaveric human liver samples. We hypothesise that differences in hepatic expression of drug disposition genes do exist and that these differences will follow the findings of pharmacokinetic studies, which implicate decreases in drug transporter protein expression as the principle cause for differences in non-renal drug clearance in ESRD patients.
3.2 Materials and Methods

Chemical Reagents and Drugs

Testosterone and 6βOH-testosterone were obtained from Steraloids Inc. (Newport, RI). Carbamazepine and flurazepam were purchased from Cerilliant (Round Rock, TX). Tolbutamide and chlorzoxazone were purchased from Sigma-Aldrich (St. Louis, MO). Metabolites 4OH-tolbutamide and 6OH-chlorzoxazone was purchased from Toronto Research Chemicals (Toronto, ON). Pentoxifylline was generously provided by Dr. David Freeman.

Procurement of ESRD and Control Human Liver Tissue

Liver samples (~100 gram) were procured from recently deceased subjects by the National Disease Research Interchange (NDRI) (Philadelphia, PA). Samples in the control group represent patients with no evidence of kidney disease. Samples in the ESRD group were procured from patients who were on hemodialysis. All samples were procured within 8 hours from the time of death and were rinsed in saline before freezing in liquid nitrogen. Patient demographic information including age, gender, race, co-morbidities and medication information was also collected.

Real-Time PCR Analysis

Total RNA was extracted from human liver homogenate, according to the manufacturer’s instructions, using TRIzol Reagent purchased from Life Technologies Inc. (Burlington, ON). RNA concentration and quality were
measured using a Thermo Scientific NanoDrop UV/Vis spectrophotometer (Wilmington, DE). Reverse-transcription to synthesize cDNA was accomplished using 1µg of total RNA and qScript cDNA supermix from Quanta Biosciences Inc. (Gaithersburg, MD) in a total volume of 20 µL. Real-time PCR, to determine relative mRNA expression, was performed using Quanta Biosciences PerfeCta SYBR Green Fastmix. Specific primers, for target gene cDNA, were designed using NCBI primer-blast. Gene expression was normalized to β-actin using the ΔΔCT method (Livak et al., 2001).

Hepatic Microsome Isolation
Differential ultracentrifugation was used for the isolation of human liver microsomes (HLMs) as described previously (Velenosi et al., 2012). Liver tissue was washed in 0.9% NaCl solution and homogenized, on ice, in a solution of 1.15% KCl and 1mM EDTA. Liver homogenate was centrifuged at 9000g for 20 minutes at 4°C. The pellet was discarded and the supernatant was centrifuged at 105 000g for 60 minutes at 4°C. The microsomal pellet was suspended in 250 µL of 100 mM potassium phosphate buffer (pH = 7.4) with 20% glycerol. Total protein concentration was quantified using Pierce BCA protein assay (Fisher Scientific, Whitby, ON) and HLMs were aliquoted and stored at -80 °C until use.

Determination of Total P450 Content
Microsomal protein was used to determine total cytochrome P450 content using spectral analysis after reduction using carbon monoxide and sodium dithionite
Sample absorbance was detected using UV-vis spectrophotometry.

**Western Blot Analysis**

Human liver microsomes were used for the determination of hepatic protein expression of selected CYP isoforms involved in drug metabolism. Twenty micrograms of microsomal protein was loaded per well in a 10% polyacrylamide gel containing 0.1% SDS. Samples were electrophoresed for 60 minutes at 140 V. Proteins were transferred to a nitrocellulose membrane for 90 minutes at 120 V. Immunoblots were blocked overnight at 4 °C with 5% skim milk and 0.6% BSA. Probing with primary antibodies occurred for 90 minutes under conditions optimized for each antibody. Primary antibodies used for drug metabolizing enzymes were polyclonal rabbit anti-human CYP1A2 in 0.6% BSA, dilution 1:5000 (Detroit R&D Inc., Detroit, MI), monoclonal rabbit anti-human CYP2C9, dilution 1:10000 (Abcam, Cambridge, MA), polyclonal rabbit anti-human CYP2D6 in 2% BSA, dilution 1:5000 (Detroit R&D Inc., Detroit, MI), polyclonal rabbit anti-human CYP2E1 in 0.6% BSA, dilution 1:5000 (Detroit R&D Inc., Detroit, MI) and monoclonal rabbit anti-human CYP3A4, dilution 1:10000 (Abcam, Cambridge, MA). Loading control antibody used to standardize gene of interest expression was primary monoclonal mouse anti-β-actin-peroxidase, dilution 1:50000 (Sigma-Aldrich, St. Louis, MO). HRP-linked secondary antibodies, goat anti-rabbit and goat anti-mouse, were used at a dilution of 1:10000 for 60 minutes and were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Luminata
Forte western HRP substrate was obtained from Millipore (Billerica, MA) and immunoblots were imaged on a VersaDoc Imaging System (Bio-Rad, Hercules, CA) and band intensity was quantified by densitometry using Quantity One 1D analysis software (Bio-Rad, Hercules, CA).

**Hepatic Microsomal Metabolism of Substrate Drugs**

Metabolic activity of CYP2C9 and CYP3A4 in HLMs was determined using probe substrates tolbutamide and testosterone, respectively, as previously described (Velenosi *et al.*, 2012). Activity of CYP2E1 was assessed using chlorzoxazone as a probe substrate. Reactions were tested for linear rate of metabolite formation by varying incubation time, protein concentration and substrate concentration. Experiments were conducted for 30 minutes for the formation of 4OH-tolbutamide (CYP2C9), 50 minutes for the formation of 6OH-chlorzoxazone (CYP2E1) and 25 minutes for the formation of 6βOH-testosterone (CYP3A4/5).

All reactions were performed in a final volume of 250 µL in 50 mM potassium phosphate solution (pH = 7.4) with 2 mM MgCl₂, and contained 1 mg/mL (2mg/mL for chlorzoxazone metabolism) hepatic microsomal protein. Reactions were pre-incubated for 5 minutes with the probe substrate at 37 °C. Reactions were initiated by the addition of 50 µL of 5 mM NADPH for a final concentration of 1 mM. Reactions were terminated by the addition of 50 µL of ice-cold acetonitrile followed by a 15 minute incubation on ice to precipitate protein. After incubation on ice, microcentrifuge tubes were centrifuged for 5 minutes at 20000g to pellet precipitated protein.
Metabolite Analysis by UPLC-PDA

Metabolites 4OH-tolbutamide (CYP2C9), 6OH-chlorzoxazone (CYP2E1) and 6βOH-testosterone (CYP3A4) were extracted by solid-phase extraction using internal standards flurazepam, pentoxifylline and carbamazepine, respectively. Solid-phase cartridges (C18, Strata-X Polymeric Reverse Phase 33 µm) were obtained from Phenomenex (Torrance, CA). Metabolites 4OH-tolbutamide and 6βOH-testosterone were analyzed by UPLC-PDA as described previously (Velenosi et al., 2012). Samples containing pentoxifylline and 6OH-chlorzoxazone were passed across the solid phase cartridges under vacuum pressure < 50mmHg. Cartridges were washed with 1 mL of nano-pure water followed by 1 mL of 25% methanol/water. Samples were eluted into clean glass test tubes with 1 mL of methanol containing 0.1% trifluoroacetic acid and 0.1% triethylamine. The eluent was dried in an Organomation N-EVAP (Berlin, MA) at 40°C and reconstituted in mobile phase prior to injection on a Kinetex C18 column (1.7 µm particle size, 50 mm length x 2.1 mm diameter; Phenomenex) for separation of analytes. Columns were maintained at 40°C in a Waters ACQUITY H-Class UPLC. Mobile phase flow was constant at 0.8 mL/min and was initially comprised of 93% 5 mM KH$_2$PO$_4$ with 0.1% TEA (pH 3.0) and 7% acetonitrile, 93:7. A linear gradient was run starting at time zero to a mobile phase of 80:20 phosphate buffer/acetonitrile over 2 minutes. The 80:20 ratio was maintained constant for an additional minute followed by one minute of 20:80 (phosphate buffer/acetonitrile) to wash the column prior to re-equilibration for one minute at
initial conditions. Detection of 6OH-chlorzoxazone and chlorzoxazone was achieved using an ACQUITY UPLC PDA detector (Waters) at 287 nm for quantification.

**Data Analysis and Statistical Procedures**

Statistical analysis was performed using GraphPad Prism (version 5.0). Data on mRNA and protein expression are shown as box and whisker plots using Tukey’s outlier test. Testing for normality and variance was performed using D’Agostino & Pearson normality test and F-test, respectively. Statistical differences between ESRD and control liver samples were assessed by unpaired T-test with $p < 0.05$ considered significant. Means with unequal variance were analyzed by unpaired T-test with Welch’s correction. Data not normally distributed was assessed for differences using the Mann-Whitney U non-parametric test. The formation of probe substrate metabolites at varying concentrations of substrate was fit using a Michaelis-Menten model.
3.3 Results

Control and ESRD Liver Sample Demographics

For liver samples obtained through the NDRI, no significant differences were seen in ESRD group patients’ age, body weight or BMI relative to controls. Additionally, ESRD liver and control liver samples were obtained from 7 males and 3 females and 8 males and 3 females, respectively (Table 3.1).

Nuclear Receptor mRNA Expression

ESRD liver samples showed a significant 67.6% decrease in FXR mRNA expression relative to control (p < 0.05). Additionally, ESRD livers had significantly decreased mRNA expression of HNF-4α, 33.7% of control; RXRα, 30.2% of control; and PPARγ, 38.1% of control, relative to control livers (p < 0.01) (Figure 3.1). No significant differences in were seen in LXRα or PXR mRNA expression between control and ESRD liver samples.

Cytochrome P450 mRNA and Protein Expression

An 80.3% decrease in CYP1A2 mRNA expression was seen in ESRD livers relative to control (p < 0.05; Figure 3.2A). A 77.1% decrease was observed in CYP2E1 mRNA expression in ESRD livers relative to control (p < 0.01; Figure 3.2E). ESRD livers also had a 66% decrease in mRNA expression of CYP3A4; however, this decrease failed to reach significance (P = 0.11). The relative hepatic mRNA expression of other phase I drug metabolizing enzymes CYP2C9, CYP2C19 and CYP2D6 was not different between ESRD and control groups.

No significant differences were seen in P450 protein expression between ESRD livers and control (Figure 3.3). Protein expression of CYP2E1 in ESRD
**Table 3.1** Patient demographics and liver sample total P450 content
Values represent mean ± SEM

<table>
<thead>
<tr>
<th></th>
<th>Control (n=11)</th>
<th>End-Stage Renal Disease (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>70.9 ± 2.9</td>
<td>74.8 ± 1.7</td>
</tr>
<tr>
<td>Gender</td>
<td>8M/3F</td>
<td>7M/3F</td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>63.5 ± 4.9</td>
<td>76.5 ± 7.9</td>
</tr>
<tr>
<td>BMI</td>
<td>21.9 ± 1.6</td>
<td>25.8 ± 2.3</td>
</tr>
<tr>
<td>Hepatic P450 Content (nmol/mg protein)</td>
<td>0.125 ± 0.023</td>
<td>0.182 ± 0.031</td>
</tr>
</tbody>
</table>
Figure 3.1 Box and whisker plots of relative mRNA expression of nuclear receptors involved in regulation of genes involved in drug disposition in control (blue) and ESRD (red) cadaveric human liver samples. Black dots represent statistical outliers; control n=11, ESRD n=10. * $p < 0.05$ relative to control and ** $p < 0.01$ relative to control.
Figure 3.2 Box and whisker plot of relative P450 mRNA expression in control (blue) and ESRD (red) liver samples for CYP1A2 (A), CYP2C9 (B), CYP2C19 (C), CYP2D6 (D), CYP2E1 (E) and CYP3A4 (F). Black dots represent statistical outliers; control n=11, ESRD n=10. ** p < 0.01 relative to control.
Figure 3.3 Box and whisker plots, and representative blots, of relative P450 protein expression in control (blue) and ESRD (red) liver samples for CYP1A2 (A), CYP2C9 (B), CYP2D6 (C), CYP2E1 (D) and CYP3A4 (E). Black dots represent statistical outliers; control n=11, ESRD n=10.
livers was 64.6 ± 13.9% vs. 100 ± 10.2% in control livers (p = 0.052); however, this decrease failed to reach statistical significance (Figure 3.3E).

**Total P450 Content and Human Liver Microsomal Metabolism**

Mean total P450 content of human liver samples was not significantly different between groups (Table 3.1). Liver metabolite formation was expressed relative to total P450 content. Full enzyme kinetics for the metabolism of known P450 probe substrates was determined in human liver microsomes and displayed as Michaelis-Menten plots (Figure 3.4). ESRD liver samples had a significant 71% decrease in the maximal enzyme velocity (V<sub>max</sub>) of CYP2E1, relative to controls, for the formation of 6OH-chlorzoxazone (Table 3.2). No significant differences in V<sub>max</sub> were seen between control and ESRD liver samples for the formation of 4OH-tolbutamide or 6βOH-testosterone by CYP2C9 and CYP3A4, respectively. No significant differences were seen for K<sub>m</sub> values, between ESRD and control liver samples, for the formation of selected metabolites. Michaelis-Menten kinetic parameters for the formation of specific metabolites are presented in Table 3.2.

**Drug Transporter mRNA Expression**

ESRD hepatic mRNA expression showed a significant change relative to control, in passive uptake transporters SLCO1B1/OATP1B1 (decreased; 26.6% of control) and SLCO1B3/OATP1B3 (increased; 239.6% of control), respectively (p < 0.05) (Figure 3.5). Canalicular efflux transporter mRNA expression for ABCB1/P-gp and ABCG2/BCRP was significantly decreased in ESRD livers, by
Table 3.2 Michaelis-Menten kinetic values and intrinsic clearance of probe substrates in control (n=11) and ESRD (n=10) liver samples
Values represent mean ± SEM

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>End-Stage Renal Disease</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td></td>
<td>µM</td>
<td>pmol/min/nmol P450</td>
</tr>
<tr>
<td>4OH-tolbutamide</td>
<td>98 ± 23</td>
<td>3429 ± 847</td>
</tr>
<tr>
<td>6OH-chlorzoxazone</td>
<td>430 ± 30</td>
<td>1161 ± 338</td>
</tr>
<tr>
<td>6βOH-testosterone</td>
<td>295 ± 80</td>
<td>595 ± 159</td>
</tr>
</tbody>
</table>

* $p < 0.05$ relative to control
Figure 3.4 Michaelis-Menten plots of in vitro metabolism performed using human liver microsomes from control (blue) and ESRD (red) cadaveric liver samples using probe substrates. Plots represent the formation of metabolites at varying substrate concentrations for 4OH-tolbutamide, CYP2C (A); 6OH-chlorzoxazone, CYP2E1 (B) and 6β-OH testosterone, CYP3A4/5 (C).
Figure 3.5 Box and whisker plot of relative drug transporter mRNA expression in control (blue) and ESRD (red) liver samples for OATP1B1 (A), OATP1B3 (B), OATP2B1 (C), MRP2 (D), MRP3 (E), P-gp (F) and BCRP (G). Black dots represent statistical outliers; control n=11, ESRD n=10. * $p < 0.05$ relative to control and ** $p < 0.01$ relative to control.
72.9% and 42.0%, respectively, relative to control ($p < 0.05$). No significant differences were seen in the mRNA expression of ABCC2/MRP2, or ABCC3/MRP3, in ESRD relative to control liver samples.

3.4 Discussion

To date, this is the first study to evaluate the expression of drug transport protein and drug metabolizing enzyme expression in cadaveric liver samples of patients with ESRD. Previous work, using animal models of CKD, to evaluate hepatic P450s have shown decreases in expression and function of CYP2C and CYP3A (Leblond et al., 2001; Velenosi et al., 2012). However, results from clinical pharmacokinetic studies using probe substrates of P450 function, such as midazolam for CYP3A, in patients with CKD undergoing hemodialysis have yielded unexpected results (Nolin et al., 2009; Vinik et al., 1983). Our data demonstrate that, unlike rodent models of CKD, which show drastic decreases in expression of CYP2C and CYP3A, hepatic P450 expression and function in the human ESRD patient may be largely unaffected. Interestingly, our study is the first to report a decrease in expression of CYP2E1 of ESRD patients. A previous study evaluating CYP2E1 function in CKD patients, using pharmacokinetics of chlorzoxazone as a probe for CYP2E1 activity, demonstrated no difference from controls in chlorzoxazone AUC after oral dosing (Nolin et al., 2003b). However, this study did also question the validity of chlorzoxazone as a phenotypic probe of CYP2E1 activity in CKD patients due to an impaired metabolic ratio of
chlorzoxazone to its metabolite, hydroxychlorzoxazone. The impact of impaired renal function on the clearance of many pharmacokinetic probe substrates or metabolites makes the functional characterization of many P450 isozymes, including CYP1A and CYP2D, difficult in the CKD patient (Kevorkian et al., 1996; Tang et al., 1994). This functional characterization is difficult both because of the inability to rely on measuring metabolite clearance in the urine, but also because of dialysis in the ESRD patient.

Dialysis in patients with renal disease has been demonstrated to have an effect on drug metabolizing enzyme expression. Hemodialysis has been demonstrated to acutely increase hepatic CYP3A4 metabolic activity in the ESRD patient (Nolin et al., 2006). A possible explanation for the findings of drug metabolizing enzyme expression and function increasing after dialysis is that this process clears uremic toxins that are responsible for down-regulating and inhibiting function of various P450s (Sun et al., 2004). Other studies have shown that uremic serum from ESRD patients may decrease CYP3A4 expression, but not directly inhibit function, in vitro (Tsujimoto et al., 2013). Despite this, a recent publication has shown uremic toxins directly inhibiting human microsomal metabolism of CYP3A4 (Volpe et al., 2014). This study also failed to demonstrate an effect on human CYP3A4 microsomal metabolism in the presence of uremic serum from ESRD patients. Complicating the possibility that dialysis improves removal uremic toxins involved in down-regulating certain P450 isoforms is that some uremic toxins are not removed during dialysis. Indeed, indoxyl sulfate, a uremic toxin shown to inhibit CYP3A4 activity in vitro, is highly
protein bound and its accumulation is highly related to a lack of residual renal function in the ESRD patient on dialysis (Huang et al., 2012; Sun et al., 2004). The various contradictory findings highlight the uncertainty surrounding the potential impact of specific uremic toxins, and uremic serum, on drug metabolizing enzyme expression and function. Our results displayed no differences in expression, and a high variability of hepatic expression, for CYP3A4 and other major P450s involved in human hepatic drug metabolism. This study is consistent with others reporting high inter-individual variability, especially in CYP3A4, in hepatic expression of P450 isozymes (Koch et al., 2002).

Further complicating the understanding of how drug metabolism may be affected in kidney disease are that some new drugs metabolized by a specific P450 isoform are affected by renal impairment, whereas other drugs metabolized by the same isoform, are not (Zhang et al., 2009). This study looked at new drugs approved for use after the publication of renal impairment guidelines for drug dosing by the FDA. These nonrenally eliminated drugs were grouped based on P450 isoform involved in metabolism. This included groups of drugs primarily metabolized by CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Of these new molecular entities, roughly 50% of drugs in each group demonstrated altered pharmacokinetics, and 50% did not (Zhang et al., 2009). Ultimately, the interplay between drug uptake, metabolism and efflux is complex and CKD-mediated alterations in transporters or enzymes have impacts on drug pharmacokinetics and, in turn, drug dosing in the ESRD patient.
Our results suggest that altered expression of hepatic drug transport proteins may be a more important determinant when considering the differences in non-renal clearance of drugs observed in ESRD patients relative to patients with normal renal function. This finding is consistent with previous pharmacokinetic data finding a decrease in fexofenadine clearance, but not midazolam, in ESRD patients (Nolin et al., 2009). That study proposed that reduced P-gp and OATP expression could be the cause of reduced fexofenadine clearance. Our data support this explanation as we observed decreased hepatic mRNA expression of P-gp and OATP1B1 in ESRD samples.

Interestingly, our human expression data, and previous human pharmacokinetic reports in the literature, suggest that animal models of CKD may not be representative of what is occurring to expression of drug disposition genes in the human. Although this discrepancy could be a result of differences in the severity of CKD between dialyzed patients and animal models, this is likely not the case as the majority of animal model data demonstrates large CKD-mediated decreases in hepatic and intestinal P450 expression and function (Leblond et al., 2001; Leblond et al., 2002; Velenosi et al., 2012). In contrast, our data does not suggest that mild decreases in expression exist, especially for the CYP2C and CYP3A isoforms known to be down-regulated in the animal model of CKD. Although more work is required to confirm our findings, our data imply that the 75% reduction in expression of CYP2C and CYP3A seen in animal models of CKD are not consistent with what happens in the liver of the human ESRD patient.
Several limitations exist in our study. At this time, we have only confirmed mRNA levels of expression for drug transport proteins. These results will have to be confirmed at the level of protein expression to further support the finding of decreased hepatic expression of OATP1B1, P-gp, BCRP, and increased expression of OATP1B3, in liver samples of ESRD patients on hemodialysis. Currently, we also have not evaluated the functional activity of CYP1A2 and CYP2D6 using probe substrates phenacetin and dextromethorphan, respectively. The activity of these P450 isoforms will need to be evaluated using mass spectrometry as we were unable to detect metabolites for these probe substrates by UPLC-PDA. Furthermore, our study had a relatively small sample size and used cadaveric liver samples to determine expression and function. As these samples were collected from different centers across North America, it is likely that there was variation in the timing of collection and storage of liver samples. Additionally, our samples displayed a lower total P450 content than the 0.2-0.5 nmol P450/mg protein typically seen in freshly isolated human liver microsomes (Guengerich et al., 2009). Thus, it is possible that denaturation of P450 to P420 did occur prior to receiving our samples. Despite these concerns, previous studies, looking at differences in P450 expression and function in diabetes and non-alcoholic fatty liver disease, have used cadaveric liver samples with a similar sample size (Dostalek et al., 2011; Fisher et al., 2009). Finally, the samples were not genotyped and it is unknown if expression, or functional, differences between samples could be attributed to inter-individual genetic differences, such as single
nucleotide polymorphisms of target genes analyzed or elements, such as nuclear receptors, involved in the regulation of these genes.

In summary, we present the novel finding of altered transporter expression in cadaveric liver samples from ESRD patients. As the first study to use tissue from ESRD patients to quantify expression of genes involved in drug metabolism, future studies will be required to confirm our findings and evaluate the expression of genes involved in drug disposition in extra-hepatic tissue of ESRD patients. Ultimately, there is a need for future evaluation of how uremia can cause changes in hepatic drug metabolism and transport. Pharmacokinetic studies, using patients with varying degrees of renal impairment, are also required to help guide appropriate dosing in order to optimize pharmacotherapy, and avoid adverse drug events, in the CKD patient.
3.5 References


4.0 Discussion

4.1 Chronic Kidney Disease and Drug Clearance

Past research has demonstrated that patients with CKD, especially ESRD patients, have alterations in renal and non-renal clearance of drugs and drug metabolites (Nolin et al., 2009; Nolin et al., 2003a; Sun et al., 2010a). Additionally, based on the many medications taken by the CKD patient to manage kidney failure and associated comorbidities, these patients are at an increased risk for adverse drug events (Manley et al., 2005). Data from our research suggest that EPO, a drug given to manage the complication of anemia in CKD, may also play a role in the transcriptional regulation of genes involved in drug disposition.

In CKD patients, the impaired drug clearance can result in drug accumulation, leading to increased systemic exposure to drugs. Increased exposure to drugs due to decreased renal clearance of drugs is the most widely studied, and most obvious, consequence of drug use in the CKD patient (Nolin et al., 2008). However, more recently, the effect of altered non-renal clearance has been an area of intense investigation as CKD patients experience increased exposure to drugs, such as fexofenadine, that are cleared through biliary excretion (Nolin et al., 2009). Thus, for the safety of the patient, it is necessary to adjust the dosage of many drugs in order to prevent adverse effects related to drug toxicity (FDA, 1998).
The safety of drug dosing in patients with renal impairment has been identified as an important area of study by the United States Food and Drug Administration (FDA, 1998). The FDA guideline for industry recommends pharmacokinetic characterization of drugs in the CKD patient in order to provide appropriated dosing adjustments, even when clearance is mediated by non-renal pathways. It is important to note that dosage adjustment may not only be required to limit drug exposure, but also to avoid accumulation of drug metabolites that may be active or toxic (FDA, 1998). The FDA guideline also highlights the importance of using control populations, for pharmacokinetic studies, that are not young healthy volunteers. As the average CKD patient tends to be older, it is important to consider the demographic of the CKD patient when selecting control patients for studies on drug metabolism and clearance.

In addition to altered renal and non-renal clearance of drugs, the use of pharmacotherapeutics in CKD patients is further complicated by dialysis treatment of the ESRD patient. Generally, drugs with a high volume of distribution, and drugs with a large amount of non-renal clearance will not be greatly affected by dialysis (FDA, 1998). In contrast, drugs with a small volume of distribution can be largely affected and different modalities of dialysis impact the clearance of some drugs differently than other modalities. Ultimately, patients with a higher severity of renal impairment, especially ESRD patients, are the most difficult to dose due to the effects of dialysis and the large alterations in renal and non-renal drug clearance.
### 4.2 Conclusions

We are the first group to use the adenine-fed rat model of CKD to evaluate changes in P450 expression. The adenine diet appears to cause a consistent kidney disease without high variability in plasma markers. Additionally, the adenine model of CKD demonstrated results consistent with the 5/6-nephrectomy model in terms of kidney disease markers and down-regulation of specific P450 isoforms in the liver (Leblond et al., 2001; Velenosi et al., 2012). Despite previous findings showing LXR-dependent activation of efflux transporters, our data demonstrate that EPO is not able to activate other nuclear receptors, such as PXR, required to restore CKD-mediated down-regulation of P450 expression (Lu et al., 2010). In the rat, EPO decreases hepatic function of CYP3A secondary to decreased expression, both at the level of mRNA and protein. These findings are associated with decreases in RNA polymerase II recruitment and decreased promoter region binding of regulatory nuclear receptors PXR and HNF-4α. Erythropoietin classically signals through its cell surface receptor, EPOR, and activates JAK2 phosphorylation of intracellular signaling protein STAT5 (Gouilleux et al., 1995; Parganas et al., 1998). Initially, we believed phosphorylated STAT5 may be down-regulating transcription as it has the ability to activate or suppress transcription depending on the target gene (John et al., 1999). We were unable to confirm STAT5 to putative binding sites in the CYP3A promoter. Therefore, it is likely that STAT5 is not directly down-regulating the transcriptional expression of CYP3A in the rat. Additionally, our data demonstrating no difference in mRNA expression of nuclear receptors by EPO.
indicates that down-regulation of nuclear receptor expression is also not a likely mechanism for decreased PXR, HNF-4α or RNA Pol II binding to the CYP3A promoter.

It is evident that differences in drug metabolism and transport exist between rodent models of CKD and the ESRD patient. To our knowledge, no cases in humans have ever reported describing changes in drug metabolism after or during chronic use of EPO. However, this does not rule out the possibility that EPO can affect drug disposition in the human. Our data from human ESRD patients demonstrate that expression of hepatic drug transporters is changed in CKD. It is possible that changes in uptake and efflux transporter expression could be a result of the effects of EPO and/or CKD (e.g. uremia). Erythropoietin has been shown to activate expression of ABC efflux transporters ABCA1 and ABCG1 in an LXR dependent manner (Lu et al., 2010). Liver X receptor has also been shown to negatively regulate CYP3A4 in the brain, and positively regulate CYP7A, involved in cholesterol hydroxylation for the formation of bile acids (Handschin et al., 2002; Tirona et al., 2005). Perhaps most importantly for systemic drug disposition, LXR is a major regulator of OATP1B1 (Meyer Zu Schwabedissen et al., 2010).

Our human liver samples displayed different expression levels of mRNA for some hepatic uptake and efflux transporters. These changes could be clinically relevant as OATP1B1 is responsible for the hepatic uptake of HMG-CoA reductase inhibitors (Tomita et al., 2013). Further, these drugs, statins, act within the hepatocyte and OATP1B1 expression determines the disposition of statins to
this target tissue. Additionally, rosuvastatin is cleared from the body by BCRP-mediated efflux into the bile. Thus, the ESRD patient may experience decreased efficacy and clearance of statins, especially rosuvastatin (Tomita et al., 2013). Decreased expression levels of hepatic OATP1B1 could also impact the ESRD patient taking angiotensin II receptor antagonists, ACE inhibitors or ezetimibe (Kalliokoski et al., 2009). The prevalence of hypertension and dyslipidemia in ESRD make these changes in drug disposition important, as they are involved in the treatment of co-morbidities. Additionally, the endogenous substrates of OATP1B1 – bilirubin, bile acids, glucuronidated and sulfated steroid hormones, and thyroid hormones – may also have altered transport in the ESRD patient (Kalliokoski et al., 2009). The alterations in hepatic drug transporter expression found in our study could have implications for the disposition and clearance of other commonly used drugs. In particular, ESRD-mediated changes in P-gp expression, an efflux transporter with a broad substrate affinity could alter the non-renal clearance of many pharmacologically relevant drugs.

4.3 Future Research

Based on our findings in the rat, the future direction of EPO involvement in altering expression of drug disposition genes should incorporate drug transport proteins. As hepatic drug transport proteins are regulated by similar nuclear receptors as CYP3A, it would be important to evaluate any potential changes in uptake or efflux transporter expression. After identifying all drug disposition genes affected by EPO, future studies could attempt to characterize the pathway
that leads to decreased expression of target genes. We could not demonstrate direct phosphorylated STAT5 inhibition of CYP3A; therefore, it is likely that STAT5 is acting indirectly or, possibly, EPO is signaling through a different pathway than the EPOR-JAK2-STAT5 classical pathway. Alternatively, EPO, as a circulating hormone, may be acting on extra-hepatic tissue to indirectly affect the liver. Therefore, confirmation of a direct effect of EPO on hepatocytes would need to first be evaluated. After this, if EPO is able to directly affect hepatocytes, confirmation of signaling through the EPOR-JAK2-STAT5 pathway could first be demonstrated. To confirm the role of this pathway, in vitro inhibitors of JAK2 could be used in conjunction with EPO to determine if EPO-mediated down-regulation of CYP3A is abolished. If this does not eliminate the effect, EPO may be signaling through alternative pathways. Previous evidence has shown the ability of EPO to signal through the extracellular signal regulated kinases (ERK) pathway and the phosphatidylinositol 3 kinase/Akt (PI3/Akt) pathway (Shi et al., 2010). As our data has demonstrated decreased CYP3A, but not CYP2C, under constant exposure to EPO, PXR may play a critical role in the effect seen. We were unable to demonstrate PXR binding to CYP2C promoter region through ChIP; therefore, this difference may explain why CYP3A was the only P450 affected by EPO. Interestingly, PXR activity is known to be affected by the ERK and PI3/Akt pathways as PXR can be phosphorylated and retained in the cytoplasm to prevent nuclear translocation and DNA binding (Lichti-Kaiser et al., 2009; Pondugula et al., 2009). Therefore, it remains possible that EPO signaling may cause phosphorylation of PXR. To determine this, western blot or mass
spectrometry could be used to quantify phosphorylated PXR. Alternatively, immunofluorescence could be utilized to examine the localization of PXR to determine its accumulation in the cytosol. Regardless, future research should focus on human cell lines due to the differences in regulation between rat and human P450s and drug transport proteins.

The CYP3A isoforms have been the most widely studied and compared P450s between species. Rat CYP3A1/2 and human CYP3A4 show differences in regulation, particularly by PXR. Sequence differences account for species-specific activation and explain how the classical ligands rifampin and pregnenolone-16α-carbonitrile are specific to activation of human PXR and rat PXR, respectively (Tirona et al., 2004). Additionally, the rat CYP3A is regulated by proximal promoter region binding of HNF-4α; however, the human CYP3A has no HNF-4α binding site in the proximal promoter (Tirona et al., 2003b). Tirona et al. (2003) were able to demonstrate that human HNF-4α is responsible for the coordination of PXR and CAR mediated induction of CYP3A4 expression. Due to this difference in CYP3A regulation between the rat and the human, experimental confirmation would be required to determine if EPO dosing is able to affect CYP3A4 expression. Generally, data in the rat should only be used to support investigation into in vitro study using human cells. The exception to this would be the potential of using humanized mice. Many types of transgenic and knockout CYP3A and PXR mice exist (Cheng et al., 2011). In particular, the PXR knockout mouse with double transgene insertion of human PXR and CYP3A4 may likely be the most appropriate animal model for this study.
Erythropoietin effects should next be studied, ideally, in vitro using primary human hepatocytes. Primary human cells would be beneficial for the study because they provide the most physiologically relevant expression of P450s and drug transporters. Alternatively, immortalized cells lines are often used as an in vitro model of hepatocytes for studying drug metabolism. Commonly employed for drug metabolism studies, Huh7 cells, after growing these cells for four weeks post-confluence, can be used as a good physiological model of CYP3A4 expression. However, drawbacks of Huh7 use is hyperphysiological expression of OATP1A and various HNFs and low expression of many P450s and OATP1B1 (Sivertsson et al., 2010). Additionally, the use of differentiated HepaRG cells, especially for the study of OATP transporters and BCRP may be a better model of primary human hepatocytes than Huh7 cells (Kanebratt et al., 2008). Both Huh7 and HepaRG cells are superior cells lines to model drug metabolism than HepG2 cells (Kanebratt et al., 2008; Sivertsson et al., 2010). Ultimately, human primary hepatocytes and cell lines may be beneficial for the study of EPO because of the differences that exist between human and rat regulation, specifically of CYP3A. In addition, increased inducibility of CYP3A and assessment of transporter function can be assessed by sandwich culture of hepatocytes (Swift et al., 2010). A valuable in vitro method, sandwich culture of hepatocytes prevents the depolarization of hepatocytes and allows for more ideal study of canalicular efflux transporters and can differentiate between sinusoidal and canalicular efflux (Swift et al., 2010).
Additionally, as drug transporters OATP1B1, OATP1B3, BCRP and P-gp show different expression patterns in ESRD patients, relative to control, future studies *in vitro* would favour the use of primary human hepatocytes as P-gp is not expressed at physiological levels in immortalized cell lines (Kanebratt *et al.*, 2008). Experiments could focus on how the expression of these drug transport proteins change when incubated with uremic serum from CKD patients. For example, recent findings have demonstrated that uremic serum from dialysis patients decreases mRNA expression of OATP1B1 in Hep3B cells (Tsujimoto *et al.*, 2012). Alternatively, individual accumulated solutes not cleared in the CKD patient, uremic toxins, could be used at disease-relevant concentrations for treatment of cells to examine changes in expression.

A need for greater clinical pharmacokinetic data is also required for indirectly determining changes in drug transport protein expression and examining drug disposition *in vivo*. This area of study is limited by the lack of specific *in vivo* probe substrates for transporter function. An ideal probe substrate would be transported by a single transporter, not undergo P450 mediated metabolism and have no urinary excretion. Unfortunately, due to the broad substrate specificity of drug transporters and metabolizing enzymes, the vast majority of substrates have overlapping specificity. Therefore, challenges with probe substrate specificity will make characterization of changes to *in vivo* transport difficult in CKD.
4.4 Summary

Ultimately, the effect of continuous EPO administration down regulating the expression of hepatic CYP3A in the rat is a novel finding. However, the effect of EPO on the expression of drug metabolizing enzymes and drug transport proteins in the human is unknown. Additionally, the expression study on cadaveric human liver samples suggest that, unlike animal models of CKD, human patients with kidney failure may experience altered non-renal clearance of drugs primarily as a result of altered expression of drug transport proteins. Further research in the human is required to better understand drug disposition in ESRD patients in order to optimize drug dosing effectiveness and avoid drug toxicity to minimize the number of adverse drug events in the CKD patient.
4.5 References


KFOC (2013). Facing the Facts.


Appendix A – Animal Ethics Approval

September 10, 2006

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

Dear Dr. Urquhart:

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

has been approved by the University Council on Animal Care. This approval is valid from September 10, 2009 to September 30, 2010. The protocol number for this project is #2009-056.

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. The application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.

ANIMALS APPROVED FOR 4 Years

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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.

c.c. Approved Protocol - B. Urquhart, W. Lagerwer
Approval Letter - B. Urquhart, W. Lagerwer
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Manuscripts


Presentations and Conferences


Feere DA, Velenosi TJ, Gaspar ML, Urquhart BL. Expression and Function of Hepatic Drug Metabolizing Enzymes and Transport Proteins in Livers of Patients with End-Stage Renal Disease (ESRD). American Society of Nephrology. Atlanta, Georgia. November 11, 2013. [poster]
