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Expression of Hepatic Cytochrome P450 Drug Metabolizing Enzymes in Diabetes and Diabetic Nephropathy

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Abstract

The prevalence of diabetes worldwide is rapidly increasing. Polypharmacy, along with a high risk of adverse drug reactions, is common in diabetic patients. Cytochrome P450 (CYP) 3A and 2C drug metabolizing enzymes are reduced in chronic kidney disease (CKD), altering drug pharmacokinetics and contributing to adverse drug reactions. A large fraction of commonly prescribed drugs are metabolized by CYP3A and CYP2C. Approximately 40% of all CKD cases are attributed to diabetic nephropathy (DN) and early DN presents as mild kidney disease. This study aims to evaluate the impact of diabetes and DN on levels and activity of hepatic CYP3A and CYP2C drug-metabolizing enzymes. Diabetes was induced in male C57BL/6 mice and female hPXR/hCAR/CYP3A4 humanized mice using streptozotocin (STZ). Male STZ-treated mice showed no differences in mRNA levels of *Cyp3a11* and *Cyp2c29*, while female STZ-treated mice had significantly decreased mRNA levels of hCAR and *Cyp2e1*. Male C57BL/6 mice had no differences in *Cyp3a11* enzymatic activity, while female hPXR/hCAR/CYP3A4 STZ-treated mice had an increased CYP3A4 enzymatic activity. Small changes in CYP expression and activity in diabetes and DN may lead to altered drug pharmacokinetics. This study highlights the importance of understanding changes in drug disposition to reduce clinically significant adverse drug reactions.

Keywords

Chronic kidney disease, cytochrome P450, diabetes, drug metabolism, diabetic nephropathy, pharmacokinetics

Co-Authorship Statement

Study design, animal husbandry, sample collection, sample preparation, validation of experimental parameters, experimentation, statistical analysis, and thesis writing were performed by the author Cheng Jay Fang. Yong Jin Lim contributed to animal husbandry and sacrifice, sample collection, and UPLC-MS operation. Nicholas Tonial contributed to animal husbandry and sacrifice, sample collection, and glucose tolerance tests. Adrien RaoPeters contributed to sample preparation and UPLC-MS operation. Dr. Brad Urquhart contributed to study design, procurement of equipment and reagents, editing of thesis, and general supervision.

Dedication

This dissertation is dedicated to my parents

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Abbreviations

ACC	Animal Care Committee
ACE	Angiotensin converting enzyme
ACN	Acetonitrile
ADE	Adverse drug event
ADH	Antidiuretic hormone
ADME	Absorption, distribution, metabolism, and excretion
AGE	Advanced glycation end-product
ALT	Alanine aminotransferase
AMDCC	Animal Models of Diabetic Complications Consortium
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
APS	Ammonium persulfate
ARB	Angiotensin receptor blocker
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUC	Area under-the-curve
BCA	Bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
C57BL/6	C 57 black 6
CAR	Constitutive androstane receptor
CCAC	Canadian Council on Animal Care
CCL	Chemokine ligand
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CYP	Cytochrome P450
DCCT	Diabetes Control and Complications Trial
DDI	Drug-drug interaction
DM	Diabetes mellitus
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERα	Estrogen receptor alpha
ESI	Electrospray ionization
ESRD	End stage renal disease
FFA	Free fatty acids
FITC	Fluorescein isothiocyanate
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate

GLUT	Glucose transporter
GST	Glutathione-S-transferase
GTT	Glucose tolerance test
HbA1c	Glycated hemoglobin
HepG2	Hepatocellular carcinoma cell line
HILIC	Hydrophilic interaction liquid chromatography
HLA	Human leukocyte antigen
HNF-4α	Hepatocyte nuclear factor 4 alpha
HRP	Horseradish peroxidase
ICAM	Intracellular adhesion molecule
IRS	Insulin receptor substrate
IS	Indoxyl sulfate
KDIGO	Kidney Disease: Improving Global Outcomes
K_M	Michaelis constant
LBD	Ligand binding domain
LEPR	Leptin receptor
MATE	Multidrug and toxin extrusion
MCP	Monocyte chemoattractant protein
MDRD	Modification in Diet and Renal Disease
mRNA	Messenger RNA
MRP	Multidrug resistance protein
MT	Methyltransferase
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NAT	N-acetyltransferase
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa B
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
PBS-T	Phosphate buffered saline containing Tween 20
PCN	Pregnenolone-16α-carbonitrile
PCR	Polymerase chain reaction
P-gp	Permeability-glycoprotein
PI3-K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PTH	Parathyroid hormone
PXR	Pregnane X receptor
qPCR	Quantitative polymerase chain reaction
QTof	Quadrupole time-of-flight
RAAS	Renin angiotensin-aldosterone system
RAGE	Receptor of advanced glycation end-product
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPLC	Reverse-phase liquid chromatography

RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SGLT	Sodium glucose linked transporter
SNP	Single nucleotide polymorphism
SULT	Sulfotransferase
STZ	Streptozotocin
TEMED	Tetramethylethylenediamine
UACR	Urinary albumin-to-creatinine ratio
UGT	Uridine-5'-diphospho-glucuronosyltransferase
UPLC	Ultra performance liquid chromatography
V_{Max}	Maximal rate of reaction
WR	Working reagent

Chapter 1

1 Introduction

1.1 Diabetes

1.1.1 Classification & Pathogenesis

Diabetes mellitus (DM) is a metabolic disease consisting of hyperglycemia due to defects in insulin production, action, or a combination of both (Kharroubi & Darwish, 2015). The classification of diabetes is difficult, and 10% of patients initially classified may require revision of treatment strategies, especially in younger patients (Cakan, Kizilbash, & Kamat, 2012). For instance, hallmarks of type 1 DM, such as ketosis, are being observed with increased frequency in children with type 2 DM (Nagasaka, Ishikawa, Itabashi, & Kumiko, 1998; Pinhas-Hamiel, Dolan, & Zeitler, 1997). In addition, pancreatic autoantibodies have been detected in patients who display type 2 DM symptoms (Hathout, Thomas, El-Shahawy, Nahab, & Mace, 2001). It is important to accurately distinguish diabetic cases as type 1, type 2, or other classifications due to differences in disease progression, comorbidities, susceptibility, and treatment (Kharroubi & Darwish, 2015).

Type 1 DM affects 5-10% of diabetics, and is relatively uncommon compared to type 2 DM, although its incidence is steadily increasing in both children and adults (Devendra, Liu, & Eisenbarth, 2004; Maahs, West, Lawrence, & Mayer-Davis, 2010). Type 1 DM is characterized by an early onset, and accounts for 80-90% of cases in children and adolescents (Dabelea et al., 2014). The distinguishing feature of type 1 DM is the autoimmune destruction of pancreatic β cells, and is characterized by the absence of insulin secretion (Devendra et al., 2004; Kharroubi & Darwish, 2015). Initiation of this response is not well understood, but is thought to be caused by the interplay of environmental and genetic factors (Devendra et al., 2004; Kharroubi & Darwish, 2015). Environmental factors which have been implicated in type 1 DM include congenital viral infections, exposure to pollutants, improved hygiene in countries with high socioeconomic status, and insulin resistance in early childhood (Canivell & Gomis, 2014). Genetically, human leukocyte antigen (HLA) genes are strongly associated with type 1 DM (Kharroubi & Darwish,

2015). Specifically, HLA-*DR*/*DQ* alleles can be predisposing or protective, and specific corresponding autoantibodies can be found in the plasma of type 1 DM patients (Devendra et al., 2004; Kharroubi & Darwish, 2015).

Type 2 DM affects 90-95% of all diabetics, most of which are adult patients; however, there is an increasing prevalence of type 2 DM in youth (Kharroubi & Darwish, 2015). The main causes of type 2 DM are insulin resistance and defects in insulin secretion (Kharroubi & Darwish, 2015). Insulin resistance is usually present prior to the onset of overt diabetes and following diabetes diagnosis and is associated with sedentary lifestyle, obesity and metabolic syndrome (Lencioni, Lupi, & Del Prato, 2008). Many molecular mechanisms contributing to insulin resistance have been proposed, and include defects in insulin signaling, such as impaired phosphorylation of IRS-1 and decreased PI3-kinase activity (Tripathy & Chavez, 2010). Cumulative evidence indicates that genetic predisposition and environmental factors play synergistic roles in the development of insulin resistance (Kharroubi & Darwish, 2015). In addition, disturbances in glucose and lipid homeostasis place a larger demand for insulin secretion on pancreatic β-cells, which declines over time (Kharroubi & Darwish, 2015). Defects in insulin secretion are associated with β-cell dysfunction and is usually the turning point of insulin dependency in patients (Tripathy & Chavez, 2010). Chronic elevation of free fatty acids (FFA) and glucose are implicated as causes of the impairment in insulin secretion via gluco- and lipotoxicity (Tripathy & Chavez, 2010). Both gluco- and lipotoxicity lead to ER stress, which triggers apoptosis and leads to a shrinkage of β-cell mass (Tripathy & Chavez, 2010).

1.1.2 Prevalence

Diabetes has reached epidemic proportionsin recent decades. Prevalence is an estimated 425 million diabetics worldwide, which is projected to grow to 629 million by the year 2045 (International Diabetes Federation, 2017). The Middle East and North Africa regions have the highest prevalence of diabetes in adult patients (10.9%), while the Western Pacific region has the highest number of adults diagnosed with diabetes (159 million people; Kharroubi & Darwish, 2015). Diabetes is one of the leading causes of global mortality, with 4 million annual deaths, and accounting for over 80% of all premature non-communicable disease deaths (International Diabetes Federation, 2017).

In North America alone, there are over 46 million diabetics; an additional 54 million people have impaired glucose tolerance (IGT) and are estimated to be at risk of developing diabetes (International Diabetes Federation, 2017). Approximately 40% of diabetics between the ages of 20–79 remain undiagnosed (17 million people) (International Diabetes Federation, 2017). In Canada, there is an estimated diabetes prevalence of 9.3% or 3.4 million people, and an additional 5.7 million with prediabetes, where blood glucose is higher than normal but not enough to be considered diabetes (Canadian Diabetes Association, 2015; Kharroubi & Darwish, 2015). Individuals with prediabetes have a high risk of progressing to diabetes (Kharroubi & Darwish, 2015). The per capita health care cost of diabetic patients in Canada has been estimated to be three- to four-fold higher than nondiabetics (Public Health Agency of Canada, 2011). However, the greatest amount of healthcare expenditure on diabetes by any single country is from the United States, spending over 348 billion USD to treat 30 million diabetics (International Diabetes Federation, 2017).

1.1.3 Complications

Prolonged hyperglycemia due to lack of effective insulin can lead to several diabetic complications. Diabetic ketoacidosis is an acute result of untreated hyperglycemia, and may induce coma (Forbes & Cooper, 2013). However, with proper treatment, this outcome can be avoided; more commonly, diabetics suffer from various microvascular and macrovascular complications due to chronically elevated blood glucose levels. Microvascular complications include diabetic nephropathy, neuropathy, and retinopathy, while macrovascular complications include stroke and artery disease (Forbes & Cooper, 2013). The basic underlying mechanism of these complications is hyperglycemia-induced blood vessel damage which manifests as cardio- and cerebrovascular diseases throughout the body (Forbes & Cooper, 2013).

Although hyperglycemia may introduce various diseases, diabetics often have several comorbidities when they are diagnosed, which often complicates treatment. A recent cross-sectional survey indicated that common comorbidities include sleep disturbance, depressive symptoms, and anxiety, and that these are more common than vascular complications of diabetes (Sadosky et al., 2013; Ziegler, Schneider, Boess, Berggren, &

Birklein, 2014). Geriatric patients tend to have especially complicated comorbidities, including dementia and sexual dysfunction, among others (Forbes & Cooper, 2013).

1.1.4 Polypharmacy

Diabetics commonly face polypharmacy, the use of multiple medications used to treat their many co-morbidities. Older patients are even more susceptible, since they often have more comorbidities (Peron, Ogbonna, & Donohoe, 2015). In addition, adults with diabetes are at a greater risk of receiving polypharmacy than those without diabetes (Peron et al., 2015). On average, diabetic patients report using 5 or more medications per day (Indu et al., 2018; Peron et al., 2015). This polypharmacy attempts to treat diabetic complications discussed above, but is exacerbated by drug therapy for diseases other than diabetes, such as obesity, osteoporosis, and anxiety, among others (Peron et al., 2015). In addition, prescribing cascades, in which adverse drug events are misinterpreted as new medical conditions, contributes to polypharmacy (Peron et al., 2015). Polypharmacy is concerning since it is associated with drug-drug interactions (DDI) and adverse drug events (ADE) (Peron et al., 2015). For instance, metformin is associated with cognitive impairment and peripheral neuropathy due to depletion of vitamin B_{12}, and thiazolidinediones increase the risk of heart failure (Moore et al., 2013; Singh, Loke, & Furberg, 2007). In these cases, glycemic control inappropriately exacerbates cardiovascular and neurovascular complications. Given the fact that diabetes is not easily classified, and that patient treatment is complicated by age, disease status, and clinical presentation, the management of diabetes becomes increasingly complicated (Peron et al., 2015). There is a great need for personalized treatment and dosing strategies to reduce drug-drug interactions and adverse drug events in diabetics.

1.2 Diabetic Nephropathy

1.2.1 Kidney Physiology

The kidney is an essential organ of the body responsible for many homeostatic processes. A primary function of the kidney is to filter the blood, reducing build-up of metabolic waste products and maintaining water and ion balance in the body. The primary functional unit of the kidney is the nephron (Lote, 2013). Humans contain anywhere between 200 000 to 2.5 million, and an average of 900 000 to 1 million, nephrons per kidney (Bertram,

Douglas-Denton, Diouf, Hughson, & Hoy, 2011; Nyengaard & Bendtsen, 1992). Mice contain approximately 20 000 nephrons per kidney (Murawski, Maina, & Gupta, 2010; Nyengaard & Bendtsen, 1992). Nephrons are tubular structures that span the kidney cortex and medulla and are surrounded by peritubular capillaries (Lote, 2013). Nephrons can filter, secrete, and reabsorb compounds out of and into blood from these capillaries; these processes are known to reduce or increase the amount of a substance in the body, which includes drugs, toxins, nutrients, and ions (Lote, 2013). Different areas of the nephron perform specific functions. The glomerulus is a network of capillaries surrounded by the Bowman's capsule and is the primary site of filtration where small molecules are filtered (Lote, 2013). Larger molecules such as proteins and erythrocytes are retained in the blood and are not typically filtered at the glomerulus. Bowman's capsule is comprised of specialized epithelial cells called podocytes, which participate in the barrier to filtration (Lote, 2013). These cells are linked to the basement membrane of glomerular capillaries via the protein nephrin (Lote, 2013). The filtrate moves through this filtration barrier into the proximal tubule, where water, ions, glucose, and amino acids are reabsorbed through transcellular (mediated by exchangers and symporters) and paracellular transport (Lote, 2013). In the descending loop of Henle, more water is reabsorbed, while ions are reabsorbed in the ascending loop of Henle which is impermeable to water (Lote, 2013). The distal tubule and collecting duct are responsible for adjusting electrolyte and fluid balance in the filtrate by selectively reabsorbing ions and water in coordination with the renin-angiotensin-aldosterone system (RAAS), and hormones such as antidiuretic hormone (ADH) or atrial natriuretic peptide (ANP) (Lote, 2013).

The kidney is also a very important organ in terms of drug disposition and pharmacokinetics due to its ability to metabolize, excrete and reabsorb drugs. Metabolism in the kidneys is mediated by metabolizing enzymes including cytochrome P450 (CYP) enzymes CYP2B6, CYP3A7, CYP4A11, and several others (Preissner et al., 2013). In addition, the kidney is the primary site of drug elimination, as approximately 32% of the top 200 clinically prescribed drugs in the U.S. are renally eliminated in urine (Morrissey, Stocker, Wittwer, Xu, & Giacomini, 2013). This is largely due to the presence of renal drug transporters located throughout the nephron. Renal transporters such as organic cation transporters (OCT), organic anion transporters (OAT), and organic anion transporting

polypeptides (OATP) are responsible for the basolateral uptake of compounds from peritubular capillaries (Yin & Wang, 2016). P-glycoprotein (P-gp), multidrug resistance protein (MRP), and multidrug and toxin extrusion protein (MATE) are transporter families which are responsible for the apical efflux of compounds into the tubular lumen (Yin & Wang, 2016). In recent studies, these transporters are being recognized as targets for clinically significant DDIs.

1.2.2 Prevalence

Diabetic nephropathy (DN) is one of several complications which may arise from chronically elevated blood glucose in diabetes. DN accounts for 45% of all end-stage renal disease (ESRD) cases and is the leading cause of mortality in type 1 DM (Bjornstad, Cherney, & Maahs, 2014a; Collins et al., 2012). The total prevalence of DN in the U.S. is estimated at 4% of the population, or 12.4 million people (de Boer et al., 2011). The estimated prevalence of DN in Canada is of similar percentage, affecting 3.8% of Canadians, or approximately 1.4 million people (The Kidney Foundation of Canada, 2018). Within diabetics, there is a 20% prevalence of impaired glomerular filtration rate (GFR; $\leq$60 mL/min/1.73 m^2) and albuminuria ($\geq$30 mg/g creatinine) (de Boer et al., 2011). In the past 30 years, the incidence of DN has increased proportionally with the prevalence of diabetes (de Boer et al., 2011). In Canada, the number of total kidney disease cases has grown 36% since 2007, and approximately 40% of these cases are attributed to diabetes (The Kidney Foundation of Canada, 2018). Despite this, ESRD incidence has stabilized, but remains disproportionately high in at-risk populations such as middle-aged African Americans, Native Americans, and Hispanics (de Boer et al., 2011; Tuttle et al., 2014). This disparity is linked to increasing prevalence of obesity and type 2 DM in youth, and the development of diabetic complications in later life (Kharroubi & Darwish, 2015; Tuttle et al., 2014). In diabetics, the relative proportion of DN rises yearly despite increased use of anti-diabetic medications, indicating sub-optimal treatment (de Boer et al., 2011).

The majority of costs of care for people with DN are related to development of cardiovascular diseases and ESRD (Tuttle et al., 2014). The economic burden of ESRD is extraordinarily high for both patients and society: the average personal costs at the transition from late-stage CKD to ESRD in the U.S. (2011) were estimated to be $20 000

(for those covered by Medicare) to $40 000 USD per year per ESRD patient, with $25 billion USD in Medicare spent annually (Tuttle et al., 2014). In Canada, the most common treatment for kidney failure is dialysis, which costs between $56 000 to $107 000 CAD per year, with over $50 billion CAD spent annually (The Kidney Foundation of Canada, 2018). In addition to this, over 75% of dialysis patients received institutional dialysis, which is the most expensive treatment option (The Kidney Foundation of Canada, 2018). Compared to diabetes alone, patients with DN incur significantly higher healthcare costs (Gordois, Scuffham, Shearer, & Oglesby, 2004). Increasing costs are also associated with progression of DN, with annual incremental costs of macroalbuminuria and microalbuminuria compared with normoalbuminuria being +$12 830 and +$3 580 USD per patient, respectively (Zhou et al., 2017). Increasing cost of care for DN patients despite use of glucose-lowering medications over the years is thought to be attributable to a rapidly expanding population of diabetics (de Boer et al., 2011).

1.2.3 Pathogenesis

Similar to other diabetic complications, the driving force causing development of DN in diabetics is chronically elevated blood glucose (Wada & Makino, 2013). This activates various inflammatory pathways which become increasingly central to DN pathogenesis during progression to ESRD (Wada & Makino, 2013). Inflammatory pathways, as well as the accumulation of extracellular matrix (ECM), are involved in DN progression (Wada & Makino, 2013). General DN progression occurs in three stages: glomerular hypertrophy and hyperfiltration, inflammation of glomeruli and tubulointerstitial fibrosis, and reduction in cell number via apoptosis and accumulation of ECM (Wada & Makino, 2013). This progression is similar to that of β-cell death in type 1 and type 2 DM. DN progression is due to activation of three pathways. The general stages of DN as stated above are similar in both type 1 and type 2 DM; however, hyperglycemic insult to the kidneys in type 2 DM patients begins during a later period in life, in which the kidneys may have already suffered the consequences of aging, hypertension, obesity, and dyslipidemia (Ruggenenti & Remuzzi, 2000). This may result in accelerated pathophysiological changes in the renal vasculature in type 2 DM patients with DN, as opposed to the relatively uniform glomerulopathy that type 1 DM patients with DN experience (Ruggenenti & Remuzzi,

2000). General DN progression common to both types of diabetes begins with hyperglycemic insult to the glomerulus, which induces hypertrophy of podocytes and reduction of nephrin, manifesting as proteinuria (Wada & Makino, 2013). Concomitantly, abnormalities develop in intracellular metabolism with activation of polyol and protein kinase C (PKC) pathways (Wada & Makino, 2013). This was first demonstrated in rats treated with streptozotocin (STZ) (a pancreatic β-cell toxin), where DN was ameliorated with administration of an aldose reductase inhibitor (Sugimoto et al., 1997). Continued hyperglycemic insult also causes the formation of advanced glycation end-products (AGE), which binds to its receptor (RAGE) (Wada & Makino, 2013). AGE-RAGE interactions promote inflammation and oxidative stress through various mechanisms which lead to dysfunction of glomerular cells, and are being investigated as a new therapeutic target (Chung et al., 2010). Finally, elevated GFR causes intraglomerular hypertension and the shedding of damaged podocytes in urine (Wada & Makino, 2013). Studies show that use of RAAS blockade can reduce this hyperfiltration and lower proteinuria (Chawla, Sharma, & Singh, 2010).

1.2.4 Detection and Progression

Identification of DN primarily involves two clinical metrics: estimation of GFR and measurement of proteinuria (Tuttle et al., 2014). GFR is normally estimated based on serum creatinine and reported as estimated GFR (eGFR) (Stevens & Levin, 2013). The current standard for estimating GFR is the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation, which estimates GFR based on serum creatinine, age, race, and sex (Levey et al., 2009). The CKD-EPI equation has greater accuracy and precision over previous equations, such as the Modification in Diet and Renal Disease (MDRD) equation, and is more effective at estimating higher GFR (Levey et al., 2009). This is advantageous as the earliest abnormality of DN is glomerular hyperfiltration, generally classified as GFR $\geq$ 90 mL/min/1.73 m^2 (Drummond & Mauer, 2002; Tuttle et al., 2014). Normally, albuminuria is used instead of general proteinuria, as it is a sensitive marker of both DN and cardiovascular risk (Bakris & Molitch, 2014). Albuminuria is commonly measured using urinary albumin-to-creatinine ratios (UACR), which eliminates need for 24-hour urine collection and accounts for variability in hydration (Kim, Kim, &

Kim, 2016). UACR is categorized into stages according to severity: normal to mildly increased albuminuria, <30 mg/g; moderately increased albuminuria, 30-300 mg/g; severely increased albuminuria, >300 mg/g (KDIGO, 2013). The term "normoalbuminuria" refers to the first stage, "microalbuminuria" to the second, and "macroalbuminuria" to the third (Kim et al., 2016). Microalbuminuria manifests during the early stages of DN in addition to elevated GFR (Bakris & Molitch, 2014; Drummond & Mauer, 2002; Tuttle et al., 2014).

The development of DN is associated with poor glycemic control (He, 2016). With hyperglycemia being the main cause of DN, blood glucose is normally heavily monitored in diabetic patients. To this end, glycated hemoglobin (HbA1c) levels are more effective at predicting the likelihood of diabetes as opposed to blood glucose values alone, especially in undiagnosed patients (Rohlfing et al., 2000). An early prospective cohort study testing the diagnosis of diabetes in patients found that standardized screening using HbA1c measurement was effective in predicting diabetes risk (Edelman, Olsen, Dudley, Harris, & Oddone, 2004). Since then, the International Expert Committee, appointed by the American Diabetes Association, has recommended the HbA1c test with a threshold of ≥6.5% being considered diabetes (International Expert Committee, 2009). This agrees with major findings from the Diabetes Control and Complications Trial (DCCT), a large-scale clinical trial conducted in 1993, which found that after an average of 9 years of intensive glycemic control (resulting in a 2% reduction in HbA1c), patients showed 34% and 43% reductions in rates of microalbuminuria in the primary and secondary prevention groups, respectively (The Diabetes Control and Complications Research Group, 1995). In addition, intensive insulin therapy resulting in a mean HbA1c level of 7.1% resulted in 1 of 48 patients developing albuminuria (The Diabetes Control and Complications Research Group, 1993). This was significantly lower than conventional therapy, which resulted in a mean HbA1c of 8.5% and 9 of 54 patients developing albuminuria (The Diabetes Control and Complications Research Group, 1993). These results were corroborated by the Kumamoto study in Japanese subjects, which found that after 6 years, patients administered intensive glycemic control with insulin (HbA1c=7.1%) had a 7.7% progression to microalbuminuria as opposed to 28% in patients with conventional therapy (HbA1c=9.4%) (Ohkubo et al., 1995). After 8 years, these values were 11.5% and 43.5%, respectively; in

addition, progression past microalbuminuria to overt albuminuria was reduced (11.5% in intensive therapy vs. 32% in conventional groups) (Shichiri, Kishikawa, Ohkubo, & Wake, 2000). Based on these results, an HbA1c of <7% is recommended for adults with diabetes to reduce the likelihood of progression to incipient DN (He, 2016).

Progression of DN is also heavily influenced by individual characteristics, such as comorbidities, genetic predisposition, and type of diabetes, making it difficult to establish a predictive timeline of disease progression (Gross et al., 2005; Kim et al., 2016). Current methods of staging clinical progression of DN heavily rely on eGFR and measurement of albuminuria (Kim et al., 2016; Tuttle et al., 2014). For instance, patients classified as having type 2 DM must be screened for DN at the time of diagnosis, since microalbuminuria or even macroalbuminuria is usually already present in these patients (Adler et al., 2003; Molitch et al., 2004). On the other hand, type 1 DM patients tend to be normoalbuminuric up to 5 years post-diagnosis (Molitch et al., 2004). This is complicated by the fact that not all patients with DN progress to later stages if blood glucose and blood pressure are treated vigorously (Caramori, Fioretto, & Mauer, 2000). In addition, patients with microalbuminuria may either stay microalbuminuric or revert to normoalbuminuria, and some patients may display extensive glomerular lesions despite having normoalbuminuria at time of screening (Caramori et al., 2000). A 1980 study found that 80% of microalbuminuric patients progress to macroalbuminuria over 8-14 years, whereas recent studies state that this rate has dropped to 30-45%, possibly due to improvements in blood pressure and glycemic control (Caramori et al., 2000; Mogensen & Christensen, 1984). This highlights the difficulties of identifying DN in its early stages, and the importance for discovery and use of additional early biomarkers of DN in conjunction with eGFR and albuminuria.

The general progression of DN begins with hyperglycemia as the driving force, where diabetics may be asymptomatic for up to approximately 5 years (Gross et al., 2005; Schultz et al., 1999). Figure 1.1 summarizes the progression of DN from diabetes onset. It is estimated that glomerular hyperfiltration and microalbuminuria are present during this time, as determined by eGFR and UACR (Gross et al., 2005; Molitch et al., 2004). Glomerular basement membrane (GBM) thickening and mesangial matrix expansion can

be detected by renal biopsy during this stage, although this is not recommended as a diagnostic test in patients due to its invasive nature (Gross et al., 2005; Caramori et al., 2002; White & Bilous, 2000). Over the next 8-15 years, overt nephropathy develops, and there is a progressive decline in kidney function as measured by a gradual decline in GFR until ESRD occurs (where GFR <15 mL/min/1.73 m^2) (KDIGO, 2013). During this time, microalbuminuria progresses to macroalbuminuria and overt proteinuria (KDIGO, 2013). The kidney phenotype is characterized as widespread microvascular damage and inflammation with the activation of apoptotic and fibrotic pathways, eventually terminating in loss of nephron mass (Wada & Makino, 2013).

Since late stage DN and ESRD are associated with dramatically increased healthcare costs and decline in health status, early detection of DN is important to reduce progression to these stages. This remains a major challenge as early DN symptoms are difficult to detect due to a lack of overt clinical symptoms (Bjornstad, Cherney, et al., 2014a). In addition, detection using modern eGFR equations, namely CKD-EPI, yields discordance when eGFR is measured in the hyperfiltration range (> 90 mL/min/1.73 m^2), and is subject to bias by age, weight, and body type (Bjornstad, McQueen, et al., 2014). There are also limitations of using microalbuminuria as a diagnostic marker. Proteinuria is generally recognized as the first symptom of DN, but this is complicated by the fact that as many as 58% of patients with microalbuminuria can regress to normoalbuminuria (Perkins et al., 2003). In some patients, microalbuminuria may not manifest until later in the hyperfiltration stage when significant glomerular damage has occurred, or may not manifest at all, which indicates reduced reliability as a diagnostic marker compared to conventional eGFR measurement (Barratt & Topham, 2007; MacIsaac et al., 2004).

Current research investigates novel biomarkers of DN, including serum uric acid and insulin insensitivity markers, which may yield more reliable diagnosis in early stages of the disease (Cleland, Fisher, Colhoun, Sattar, & Petrie, 2013; Ficociello et al., 2010). There have also been improvements in eGFR measurement by utilizing novel biomarkers such as cystatin C (along with creatinine) or implementing a feasible method of measuring GFR using previously impractical gold-standard iohexol or inulin clearance (Premaratne et al., 2008; Shlipak et al., 2009). Considering the inflammatory phenotype of DN, more

investigators are using urinary proteomic techniques for the detection of inflammatory cytokines such as IL-6, IL-8, IL-10, and MCP-1 (Donath & Shoelson, 2011; Nauta et al., 2011). These have recently been associated with DN progression, and are preferred due to their non-invasive detection methods (Bjornstad, Cherney, & Maahs, 2014b). The current consensus among research supports the use of novel biomarkers and techniques in conjunction with current standards except where replacements are logical or necessary.

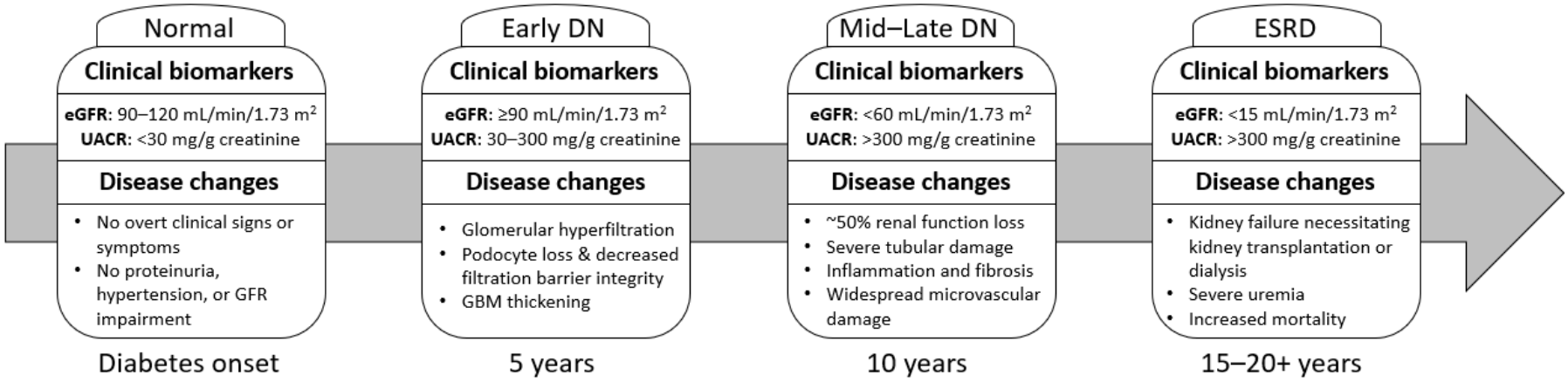

Figure 1.1. Schematic summarizing the approximate timeline of progression of diabetic nephropathy. As nephropathy progresses, there is a gradual decline in estimated glomerular filtration rate and an increase in urinary excretion of albumin normalized to creatinine. Five years after diabetes onset, glomerular hyperfiltration occurs with podocyte shedding and thickening of the glomerular basement membrane. Ten years after diabetes onset, inflammatory and fibrotic pathways cause deposition of extracellular matrix and connective tissue, as well as damage to epithelial and endothelial cells of the tubule. Fifteen years after diabetes onset, kidney function is severely decreased, and patients require transplantation or dialysis to treat severe uremia.

1.2.5 Complications and Treatment

DN accounts for over 40% of chronic kidney disease (CKD) cases, which carries a high disease burden and poor clinical outcomes (Collins et al., 2012; Kendrick & Chonchol, 2013). The main risk associated CKD is the incidence of cardiovascular disease, including coronary heart disease, peripheral artery disease, carotid artery disease, atherosclerosis, and stroke (Go, Chertow, Fan, McCulloch, & Hsu, 2004). Cardiovascular events and death are highly correlated with renal insufficiency, with risk increasing proportionally as GFR declines below 60 mL/min/1.73 m^2 (Go et al., 2004). In addition, patients with severe DN require renal replacement therapy in the form of dialysis or kidney transplantation. This introduces a new suite of problems as DN patients often have a high degree of polypharmacy to treat comorbidities and complications associated with both kidney disease and diabetes. For instance, dialysis patients take on average 12 medications (Manley et al., 2004). Unfortunately, patients with kidney disease battle a combination of these burdens by facing the conventional risks of diabetes, including hyperglycemia and comorbidities, in addition to prescribing cascades and the unpredictable effects of dialysis on drug pharmacokinetics (Peron et al., 2015; Velenosi & Urquhart, 2014).

The primary goal of DN treatment is to delay or prevent the progression to severe CKD and ESRD. To this end, adequate control of metabolic and hemodynamic alterations is required. Glycemic control is the main therapeutic strategy, as several studies, including the 2007 ADVANCE trial, have demonstrated a strong correlation between poor glycemic control and exacerbation of vascular complications and progression of DN (Fioretto et al., 2006; The ADVANCE Collaborative Group, 2008). Blood pressure control is also thought to be effective in reducing hyperfiltration during early DN and involves the use of classical blood pressure medications such as angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARB), and RAAS inhibitors (American Diabetes Association, 2016; James et al., 2014). However, these two strategies have contraindications in populations such as the elderly, whom are vulnerable to hypoglycemia and hypotension (Peron et al., 2015). Overtreatment with antihyperglycemic medications resulting in hypoglycemia is a prevalent issue, and may confer excessive mortality, as demonstrated by the ACCORD trial of type 2 diabetics (Ismail-Beigi et al., 2014).

Treatment of dyslipidemia is also indicated, as it is also an independent risk factor for both cardiovascular events as well as progression of DN (Rossing, Hougaard, & Parving, 2002). Studies have indicated FFAs, triglycerides, high-density lipoprotein, and cholesterol as risk factors associated with the progression of DN (Sacks et al., 2014). Statins such as simvastatin and pravastatin have been shown to experimentally reduce inflammation, AGE-mediated oxidative stress, and tubular apoptosis in addition to slowing the decline of GFR (Colhoun et al., 2009; Ishibashi et al., 2012). Because statins also reduce albuminuria while lowering risk of cardiovascular events, they are indicated in diabetics with DN, irrespective of the presence of dyslipidemia (Abe et al., 2011).

1.3 Drug Disposition

As a growing population of diabetics require more medicines to treat an increasing number of complications, the practice of drug dose adjustment according to individual needs becomes increasingly important. To this end, a thorough understanding of drug disposition—how a drug is handled within the body—is required. These principles include the absorption, distribution, metabolism, and excretion (ADME) of a drug. The following section provides an overview of ADME principles, while highlighting aspects relevant to the present study.

1.3.1 Drug Absorption, Metabolism, and Excretion

Most drugs must enter the circulation to exert pharmacological effects, but there are many barriers to entry. Most anti-diabetic drugs are taken orally, requiring them to be absorbed via the gut, which results in incomplete and variable absorption. There are several reasons for this: firstly, the drug may be inactivated by gut microbiota, digestive enzymes, or acidity in the stomach (Ritter, Lewis, Mant, & Ferro, 2008). Secondly, drug efflux transporters expressed on the apical membrane of intestinal enterocytes (the primary site of absorption) such as intestinal P-gp, MRP, and BCRP may efflux any absorbed drug from enterocytes to the gut lumen (Estudante, Morais, Soveral, & Benet, 2013; Ritter et al., 2008). Finally, enzymes in the intestinal wall metabolize drugs prior to entry (Ritter et al., 2008). After the drug passes through the gut, it does not reach systemic circulation without first entering the liver via the hepatic portal vein (Ritter et al., 2008). Drugs are further

metabolized in the liver prior to entering the systemic circulation. Enzyme-mediated metabolism of drugs in the intestine and liver is collectively known as “first-pass metabolism”. Together, microbial metabolism in the gut, intestinal efflux, and first-pass metabolism yield a decreased bioavailability of orally administered drugs (Ritter et al., 2008). This results in a variable amount of drug reaching the systemic circulation, which depends on factors such as expression of certain drug transporters and enzymes and composition of the gut microbiome (Ritter et al., 2008). Studies have shown that changes in expression of drug-metabolizing enzymes and drug transporters can result in clinically significant DDIs and ADEs (May & Schindler, 2016).

The liver is the most important site of drug metabolism. Enzyme-mediated metabolism of drugs is catalyzed by hepatic Phase I and Phase II drug-metabolizing enzymes (Ritter et al., 2008). Phase I drug metabolism consists primarily CYP drug-metabolizing enzymes which oxidize, reduce, and hydrolyze drugs (Meyer, 1996; Ritter et al., 2008). Phase II drug metabolism involves a range of different enzymes which conjugate functional groups onto drugs: UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), N-acetyltransferases (NAT), glutathione-S-transferases (GST), and methyltransferases (MT) (Meyer, 1996). The addition of these functional groups usually inactivates xenobiotics and increases their water solubility, allowing them to be more easily excreted by the kidney (Meyer, 1996). Drugs may be subjected to Phase I, Phase II, or both types of metabolism before being eliminated from the body (Meyer, 1996).

Following metabolism to more water soluble metabolites, drugs may then be eliminated by the kidneys via filtration and secretion (U. A. Meyer, 1996; Ritter et al., 2008). How well a drug is renally excreted depends on its lipid solubility; lipid-soluble molecules are virtually completely reabsorbed since they may freely diffuse through the tubular cells back into systemic circulation (Ritter et al., 2008). The proximal tubules contain OCTs and OATs on the basolateral membrane, which both secrete drugs in their metabolized and non-metabolized forms into the tubular filtrate in coordination with apically expressed MATEs (Estudante et al., 2013; Ritter et al., 2008; Yin & Wang, 2016). Other important apical secretory transporters include the efflux pumps MRPs and P-gp (Yin & Wang, 2016). Renal transporters can have profound effects on drug interactions: for instance, the anti-

histamine cimetidine may inhibit OCT- and MATE-mediated secretion of metformin, leading to significantly increased plasma concentration of substrate drugs (Somogyi, Stockley, Keal, Rolan, & Bochner, 1987; Wang, Yin, Tomlinson, & Chow, 2008).

1.3.2 Cytochrome P450 Drug Metabolizing Enzymes

CYP enzymes are central to Phase I drug metabolism and are involved in the biotransformation of over 75% of all drugs (Guengerich, 2008). CYP enzymes are organized into families and subfamilies based on similarity in homology and structure, usually beginning with an Arabic number followed by alphanumeric digits for a specific isoform—for instance, CYP3A4 indicates cytochrome P450 family 3 subfamily A member 4 (Guengerich, 2008; Phillips & Shephard, 2006). They may also be categorized by substrate, with CYP1A, CYP2B, CYP2C, CYP2E, and CYP3A being the subfamilies principally responsible for xenobiotic and drug metabolism, while other subfamilies metabolize various endogenous sterols, fatty acids, vitamins, and other compounds (Guengerich, 2008). Together, CYP3A4 and CYP2C9 are responsible for the metabolism of over 50% of the top 200 clinically prescribed drugs (Zanger & Schwab, 2013). The corresponding mouse orthologues of these two enzymes are *Cyp3a11* and *Cyp2c29*, and thereby commonly the focus of drug metabolism studies in mouse models (Lam, Jiang, Zhang, Zhang, & Smith, 2010; Pilote & Patoine, 2014; Shi et al., 2016). The primary function of CYP enzymes is to increase the excretion of lipophilic compounds by metabolizing them to more water soluble metabolites (McDonnell & Dang, 2018). CYPs are found in virtually all biological entities, including eukaryotes, bacteria, and viruses (Lamb et al., 2009; Zanger & Schwab, 2013). In humans, they are expressed in most tissues of the body but are most abundant in the liver (Meyer, 1996).

Mammalian microsomal CYP is a hemoprotein which usually performs oxidation and depends on a coupled flavoprotein electron carrier called NADPH cytochrome P450 reductase (Meyer, 1996). This complex multi-enzymatic system functions as an electron transport chain and can be found tethered to the smooth ER (Meyer, 1996). To catalyze an oxidative reaction, CYP enzymes require the presence of molecular oxygen, a substrate, and a source of electrons (Meunier, de Visser, & Shaik, 2004). The principal reaction is the insertion of one oxygen atom from molecular oxygen into an enzyme substrate,

followed by the reduction of the remaining oxygen atom into water using two electrons provided by NADPH (Meunier et al., 2004). To achieve this, CYP enzymes contain a heme group, which facilitates binding of molecular oxygen and conversion to water, while nearby substrates in substrate-binding sites may be converted into a variety of metabolites depending on the enzyme and substrate properties (e.g. hydroxylation) (Meunier et al., 2004). Thus, CYP enzymes are commonly denoted as heme-dependent monooxygenases. Not all CYP-mediated reactions are fully coupled; that is, the number of electrons and oxygen atoms consumed may deviate from their normal amounts, resulting in the generation of $H_2O_2^-$ and O_2^-, which may subsequently be converted into free radicals and exert damaging effects on the cell (Narasimhulu, 2010). Recent research has also investigated allosteric regulatory mechanisms and functional cooperativity of substrate binding specifically in CYP3A4. The binding of multiple substrate molecules to a single CYP3A4 substrate-binding pocket, or the presence of an effector (such as Mg^{2+}), may subsequently alter parameters such as degree of heme hydration by altering conformational state of the enzyme (Davydov, Baas, Sligar, & Halpert, 2009). Despite initial presumptions that CYP enzyme activity is not allosterically regulated, the scientific consensus has shifted in recent years, with most acknowledging the involvement of conformational changes of CYP3A4 as a result of allosteric regulation (Davydov & Halpert, 2008; Isin & Guengerich, 2006; Tsalkova, Davydova, Halpert, & Davydov, 2007).

CYP enzyme expression is modulated by the activity of nuclear receptors, mainly located in the cells of key drug-metabolizing tissues including the liver and small intestine (Fukuen et al., 2002; Urquhart, Tirona, & Kim, 2007). Figure 1.2 summarizes the regulatory process of nuclear receptor mediated CYP transcription. Nuclear receptors may be activated by a ligand binding to its ligand-binding domain (LBD), or modulate expression independently of ligand activation (Lehmann et al., 1998; Urquhart et al., 2007). Thus, they greatly influence the basal and inducible expression of CYP enzymes and are mechanistically involved in the interindividual variability of drug metabolism. While inactive, nuclear receptors are complexed with corepressors (Urquhart et al., 2007). When ligand activation occurs, corepressors dissociate, and nuclear receptors migrate to the nucleus, where they commonly heterodimerize with the retinoid X receptor (RXR) (Urquhart et al., 2007). These heterodimer complexes then bind to promoter and enhancer regions of target genes

to modulate their expression (Urquhart et al., 2007). Nuclear receptors pregnane X receptor (PXR), constitutive androstane receptor (CAR), and hepatocyte nuclear factor 4 alpha (HNF-4α) are the primary modulators of CYP3A4 enzyme expression (Goodwin, Hodgson, Costa, & Robertson, 2002; Lehmann et al., 1998; Tirona et al., 2003). PXR has a broad substrate specificity and is responsible for basal and induced expression of CYP3A4 (Jones et al., 2000). It is a primary binding site for many drug ligands, as many drug interactions involving changes in CYP3A4 expression are due to PXR activation (Lehmann et al., 1998). In addition, changes in PXR expression due to genetic splice variants, as well as single nucleotide polymorphisms (SNP) have been correlated with variations of CYP3A4 expression (Fukuen et al., 2002; Hustert et al., 2001; Zhang et al., 2001). CAR binds to similar ligands as PXR, and regulates CYP3A4 expression, indicating possible PXR-CAR cross-talk (Goodwin et al., 2002). HNF-4α is an important co-activator of PXR-CAR activity, since it binds immediately upstream of PXR-CAR enhancer sites and is required for PXR-CAR-mediated activation of CYP3A4 (Matsumura et al., 2004; Tirona et al., 2003). It is likely that these nuclear receptors are also implicated in the expression of CYP2C9 (Chen, Ferguson, Negishi, & Goldstein, 2004; Ferguson, Chen, Lecluyse, Negishi, & Goldstein, 2005).

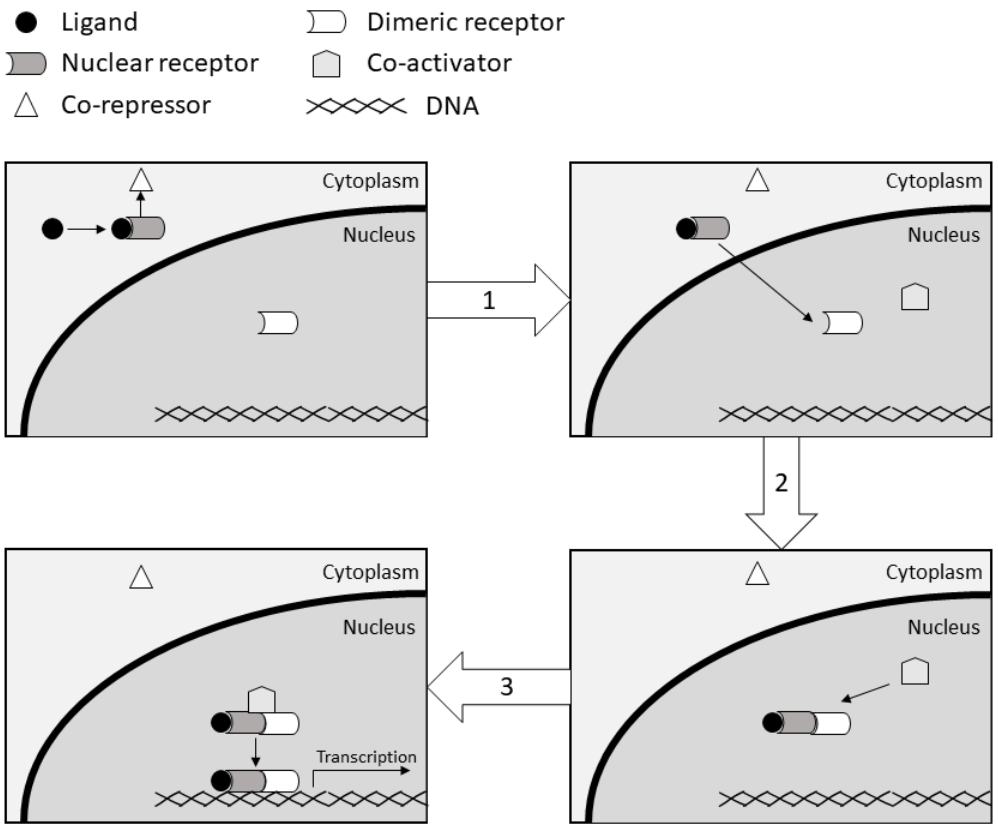

Figure 1.2. Schematic depicting the steps of nuclear receptor mediated activation of gene transcription. 1) Ligand enters the cytoplasm and binds to the nuclear receptor ligand-binding domain, causing dissociation of co-repressors. 2) The ligand-receptor complex diffuses into the nucleus, where it forms a homodimeric or heterodimeric receptor complex. 3) Co-activators are recruited to the homodimeric or heterodimeric receptor complex. The dimeric complex then binds to DNA and causes transcription of target genes.

Variations in the expression of CYP enzymes are known to exist among patients, causing variation in metabolism of drugs (McDonnell & Dang, 2018). Patients are commonly classified as poor metabolizers, intermediate metabolizers, or ultrarapid metabolizers based on their metabolic phenotype (Johansson & Ingelman-Sundberg, 2011). The sources of variation in CYP enzyme expression include, but are not limited to: genetic polymorphism, epigenetic modifications, sex, age, and disease states (Edginton & Willmann, 2008; Gandhi, Aweeka, Greenblatt, & Blaschke, 2004; Ingelman-Sundberg & Gomez, 2010; Meyer & Zanger, 1997). Loss-of-function polymorphisms involve changes in splicing and expression such as SNPs and insertions and/or deletions, while gain-of-function variations involve an increase in copy number variants (Johansson & Ingelman-Sundberg, 2008; Sadee et al., 2011). Substrate specificity and enzyme inducibility is not often affected by copy number variants (Zanger & Schwab, 2013). A study found CYP3A genes and its transcription factors are increased in human hepatoma (HepG2) cell lines following treatment with 5-aza-2'deoxycytidine, an inhibitor of DNA methylation, indicating that CYP3A expression may be affected by epigenetic modification to some degree (Dannenberg & Edenberg, 2006). A study utilizing a rat model of CKD also demonstrated that rat CYP3A2 and CYP2C11 are downregulated due to decreased histone acetylation, providing further evidence for epigenetic effects on CYP expression (Velenosi, Feere, Sohi, Hardy, & Urquhart, 2014). Sex also affects gene expression, with females having higher expression in up to 75% of CYP genes, including CYP3A4 (Zhang et al., 2011). This has been confirmed in both humans and rodents and is thought to be due to sex differences in growth hormone secretion (Waxman & Holloway, 2009). CYP3A4 was shown to have 2-fold increased protein expression in human female livers compared with male livers, and this difference is also present in mRNA (Lamba, Panetta, Strom, & Schuetz, 2010; Wolbold et al., 2003; Yang et al., 2010). While neonates have impaired drug metabolism due to lack of CYP3A4, advanced age does not seem to directly affect gene expression of CYPs *per se* but is associated with inhibition of enzymes due to polypharmacy as well as decreased renal function (Cotreau, Von Moltke, & Greenblatt, 2005; Kinirons & O'Mahony, 2004; Koukouritaki et al., 2004; Stevens, 2006). Disease states have a significant effect on CYP expression, with the majority of CYP enzymes being downregulated due to the presence of inflammatory cytokines such as IL-1β, TNF-

α, and IL-6 (Aitken, Richardson, & Morgan, 2006; Slaviero, Clarke, & Rivory, 2003). One exception is CYP2E1, which is commonly upregulated in diseases with increased retention of fatty acids and triglycerides. For instance, alcoholic and non-alcoholic fatty liver disease (NAFLD) is associated with diabetes, obesity, and metabolic syndrome, and these patients display an upregulation of CYP2E1 which may be contributing to lipid peroxidation and generation of free radicals (Buechler & Weiss, 2011). In addition, patients with liver disease, including hepatitis or cirrhosis, experience decreased activity of CYPs with the decrease in the number of functioning hepatocytes (Edginton & Willmann, 2008).

1.4 Drug Disposition in Diabetes and CKD

1.4.1 Experimental Rodent Models

In recent decades, there has been an increase in animal models to experimentally study diabetes(Heinz-Taheny, Harlan, Qi, & Heuer, 2018). However, early models had many limitations with regards to fidelity, with many missing key histological and morphological features of DN (Heinz-Taheny et al., 2018). To circumvent this, the Animal Models of Diabetic Complications Consortium (AMDCC) published criteria for using animal models of DN, which include the following: (1) progressive renal decline with hyperglycemia, (2) albuminuria, (3) histological changes, (4) GBM thickening, (5) mesangial matrix expansion, (6) interstitial fibrosis, and (7) arteriolar hyalinosis (Diabetic Complications Consortium, 2003). It is worth noting that no current model meets all the requirements, and that they should be considered goals rather than strict criteria (Alpers & Hudkins, 2011).

The STZ-induced diabetic mouse model is one of the most commonly used experimental models of type 1 diabetes due to the selective autoimmune destruction of pancreatic β-cells (Tesch & Allen, 2007). Streptozotocin is a chemotherapeutic GLUT2 substrate and is transported into pancreatic β-cells, where it causes DNA alkylation (Delaney et al., 1995; Elsner, Guldbakke, Tiedge, Munday, & Lenzen, 2000; Szkudelski, 2001). At the same time, reactive oxygen species (ROS) are formed and oxidative functions of the mitochondria are reduced (Nukatsuka, Sakurai, Yoshimura, Nishida, & Kawada, 1988). The subsequent depletion of ATP causes the generation of additional superoxide anions and ROS by xanthine oxidase, leading to formation of hydrogen peroxide and free radicals

which are directly toxic to the cells (Nukatsuka, Yoshimura, Nishida, & Kawada, 1990). This was further confirmed when pre-treatment of β cells with xanthine oxidase inhibitors prevented STZ effects (Nukatsuka et al., 1990). The use of STZ produces mild pancreatic toxicity which partly relies on secondary autoimmune destruction, imitating type 1 diabetes (Tesch & Allen, 2007). Researchers may choose to use low-dose, medium-dose, and high-dose STZ injections, but repeated low-dose injections incur the least losses due to accidental death and have the highest success rate in producing hyperglycemia (Tesch & Allen, 2007). Thus, the AMDCC includes the low-dose STZ protocol as the standard procedure for this model (Diabetic Complications Consortium, 2003). Following these guidelines yields a >90% success rate in achieving sufficient diabetes to model DN (Diabetic Complications Consortium, 2003; Tesch & Allen, 2007).

Another popular model is the *db/db* mouse model, the most widely used animal model of type 2 diabetic complications (Tesch & Lim, 2011). The *db* annotates a point mutation on the gene which encodes for leptin receptor (LEPR) (H. Chen et al., 1996). A $LEPR^{db/db}$ mutation results in a defective receptor for leptin, which results in overeating, obesity, insulin resistance, and diabetes (Lee et al., 1996; Tesch & Lim, 2011). These mice eventually present with albuminuria, renal dysfunction, and histological lesions within 24 weeks after developing hyperglycemia (Chow, Nkolic-Paterson, Ozols, Atkins, & Tesch, 2005; Chua et al., 2010). This model is genetically versatile, with many modified *db/db* mice being developed within the past decade such as $ICAM1^{-/-}$ and $Ccl2^{-/-}$ *db/db* mice (Chow et al., 2007, 2005). This versatility allows for knockout and overexpression as well as short-term intervention studies in a reliable mouse model.

The humanized CYP3A4/hPXR/hCAR mouse model is bred on a C57BL/6 background and incorporates a targeted genetic replacement of mouse *Cyp3a* isoforms *Cyp3a11*, *Cyp3a16*, *Cyp3a25*, *Cyp3a41*, *Cyp3a44*, *Cyp3a57*, and *Cyp3a59* with corresponding human orthologs CYP3A4 and CYP3A7. There are also targeted replacements of mouse *Pxr* and *Car* with their corresponding human orthologs. The general structures of nuclear receptors PXR and CAR are well-conserved between rodents and humans, but the LBD structure has significantly diverged (Kliewer, Goodwin, & Willson, 2002). Although receptors bind to similar ligands between species, the affinities for each ligand are different

(Moore et al., 2000). For instance, rifampicin induces CYP3A4 expression in humans via PXR binding, but has no effects on mouse *Cyp3a* and *Pxr* (Xie et al., 2000). On the other hand, pregnenolone-16α-carbonitrile (PCN) induces murine *Pxr* activity and *Cyp3a* transcription but has no effects on human PXR (Xie et al., 2000). To overcome these differences, this humanized mouse model is commonly used to better predict changes in human CYP3A enzyme expression with the goal of overcoming species-specific variations in drug-metabolizing enzyme expression (Cheng, Ma, & Gonzalez, 2011; Cheung & Gonzalez, 2008; Hasegawa et al., 2011). In addition to having human CYP3A4, these mice allow for the potentially synergistic cross-talk between PXR and CAR in CYP regulation, which provides a distinct advantage over earlier models which only contained one or two of human CYP3A4, PXR, and CAR (Scheer et al., 2008).

1.4.2 Altered Drug Disposition in Diabetes

CYP3A4 is the most important Phase I drug-metabolizing enzyme in the human liver, accounting for over 60% of hepatic CYP content and metabolizing 40% of the top 200 clinically prescribed drugs (Guengerich, 1999; Zanger & Schwab, 2013). Unfortunately, CYP3A4 expression may vary as much as 40-fold in individuals, hindering accurate and reproducible discernment of expression changes in observational studies (Guengerich, 1999). This highlights the importance of well-controlled experimental studies or clinical trials in humans. Previous literature about the effect of diabetes on CYP3A expression and function is contradictory. This may be due to differences in age, sex, disease state, and treatment (Wang, Shankar, Ronis, & Mehendale, 2007). In a recent study, investigators used livers from deceased diabetic donors to examine the effect of diabetes on CYP3A4 expression and activity (Dostalek, Court, Yan, & Akhlaghi, 2011). By isolating human liver microsomes from tissue and incubating them with CYP3A4 probe substrates such as midazolam and testosterone, they found a significant 95% decrease in the maximal rate of reaction (V_{Max}) of testosterone metabolism by CYP3A4 activity (Dostalek et al., 2011). Further Western blot and quantitative real-time PCR (qPCR) analysis revealed 43.5% decrease in protein and no difference in mRNA levels of CYP3A4 in hepatic tissue (Dostalek et al., 2011). However, a diabetic animal study reported different results. In this study, investigators characterized hepatic gene expression of several CYP enzymes,

nuclear receptors, and transporters in 10- and 25-week old male *db/db* mice and observed no disease-associated alterations in hepatic *Cyp3a11* expression or activity (Lam et al., 2010). A more recent study in models of type 1 and type 2 diabetes further complicated these findings. This study also utilized male STZ-injected mice as well as *db/db* mice as mouse models of type 1 and type 2 diabetes, respectively (Patoine et al., 2014). They evaluated microsomal Cyp3a expression and activity using similar techniques, including qPCR, Western blot, and metabolism of testosterone (Patoine et al., 2014). Contrary to previous results, this study revealed significant increases of 330% and 37% in hepatic Cyp3a mRNA and protein expression in type 1 diabetic mice, as well as significant increases of 195% and 60% in hepatic Cyp3a mRNA and protein expression in type 2 mice (Patoine et al., 2014). Type 1 and type 2 diabetic mice also displayed significant increases of 219% and 242% in the formation of testosterone metabolite 6β-hydroxytestosterone, respectively (Patoine et al., 2014). These studies, although contradictory, indicate the possible alteration of CYP3A expression, which may contribute to clinically significant diabetes-related ADEs and DDIs (May & Schindler, 2016).

1.4.3 Altered Drug Disposition in CKD

Although it is well-known that drug disposition changes in CKD due to reduced function of renal elimination pathways (i.e. filtration and secretion), changes in non-renal clearance pathways are less intuitive. A large proportion of drugs undergo metabolism prior to excretion, and drug metabolizing enzymes such as CYPs play a key role in non-renal drug elimination (Velenosi & Urquhart, 2014). Animal models exhibit decreased hepatic expression of CYP enzymes and altered drug metabolism in both severe and moderate CKD (Nolin, Naud, Leblond, & Pichette, 2008; Velenosi, Fu, Luo, Wang, & Urquhart, 2012). In one study, a rat model of severe CKD was generated by surgical 2/3 nephrectomy plus ligation of renal arteries to eliminate the perfusion of remaining kidney. Subsequent analysis of rat hepatic *CYP3A2* enzyme expression and activity using qPCR, Western blot, and testosterone metabolism revealed decreases of 99.6%, 91%, and 68% in relative mRNA levels, protein expression, and V_{Max} of the formation of testosterone metabolites, respectively (Velenosi et al., 2012). Rat hepatic *CYP2C11* also experienced decreases of 95%, 68%, and 82% in its respective measurements (Velenosi et al., 2012). These findings

were corroborated in a mouse model of surgically generated severe CKD by 3/4 nephrectomy. In this study, similar methods were used, including: the isolation of hepatic tissue and subsequent analysis by qPCR, Western blot, and generation of formaldehyde from the CYP3A4-mediated *N*-demethylation of erythromycin. Mice with severe CKD experienced 37%, 37%, and 25% reductions in relative Cyp3a11 mRNA levels and protein expression, as well as erythromycin metabolism by Cyp3a11, respectively (Dani et al., 2010). In addition, a review summarized the effects of CKD on hepatic transporters, causing differential uptake and efflux of certain drug substrates (Nolin et al., 2008). Of note, hepatic P-gp mediating biliary excretion experienced mRNA, protein, and activity increases of 20%, 40%, and 45% in hepatic tissue of CKD rats induced by 5/6 nephrectomy (Naud et al., 2007). The combination of altered drug metabolizing enzyme and transporter expression creates a challenge of appropriate dosing in CKD patients, as they often have unpredictable drug pharmacokinetics (Nolin et al., 2008). For instance, the antidiabetic meglitinide drug repaglinide is transported into hepatocytes by OATP1B1, metabolized by CYP3A4 and CYP2C9, and excreted into bile by hepatic P-gp. In patients with advanced DN, repaglinide exhibits a four-fold increased area under-the-curve (AUC) compared to subjects with normal renal function (Marbury et al., 2000).

1.4.4 Regulation of Drug Metabolizing Enzymes in CKD

Several mechanisms have been proposed to explain the altered CYP-mediated drug metabolism in CKD. In CKD, there is the accumulation of metabolic waste products which are normally cleared by the kidney, and this is known as uremia. Early studies have examined the effect of uremia on CYP expression and showed that mRNA and protein levels were decreased by more than 35% in rat hepatocytes treated with uremic serum (Guévin, Michaud, Naud, Leblond, & Pichette, 2002). Michaud *et al.* showed that this was ameliorated when rat hepatocytes were treated with post-dialysis serum and proposed that uremic toxins may be involved in CYP regulation (Michaud et al., 2008). Uremic toxins may originate from endogenous metabolic pathways, such as the breakdown of lipids, or from environmental exposure, such as aristolochic acid or mercurial conjugates (Mutsaers et al., 2013; Nigam et al., 2015). However, a large group of uremic toxins are mainly derived from digested foods in the gut (Jansen, Jankowski, Gajjala, Wetzels, & Masereeuw,

2017). The gut microbiome contains bacterial proteolytic enzymes that metabolize dietary proteins into uremic toxin precursors (Ramezani & Raj, 2014). Precursor molecules are then transported to the liver through the hepatic portal vein, where they may be further metabolized into uremic toxins before entering the systemic circulation (Meijers & Evenepoel, 2011). For example, dietary *L*-tryptophan is converted into indole by bacterial *L*-tryptophanase in the gut, and then further metabolized into indoxyl by CYP2E1 and subsequently indoxyl sulfate by SULT in the liver (Sasaki-Imamura, Yano, & Yoshida, 2010). Although studies on the effect of uremic toxins on hepatic drug metabolism are limited, a previous *in vitro* study has reported that incubation of hepatocytes with uremic toxins including *p*-cresol, indoxyl sulfate, and hippuric acid resulted in a 50% reduction of CYP1A2, CYP2E1, and CYP3A4 activity (Barnes, Rowland, Polasek, & Miners, 2014). In mouse and rat models of CKD, uremic toxins may reduce hepatic and kidney transporter uptake (OATPs, OATs, OCTs) by competitive inhibition or reducing expression, also resulting in decreased hepatic metabolism and proximal secretion of drugs (Sun, Frassetto, & Benet, 2006). On the other hand, hepatic and kidney efflux transporters (such as MRPs and P-gp) often show an increase in expression (Sun et al., 2006). Thus, uremic toxins may be one causative factor of altered drug disposition in uremia and CKD by altering CYP and transporter expression and activity. However, the exact mechanism by which uremic toxins cause changes in drug disposition in CKD are unknown and require further study. Michaud *et al*. also showed that NF-κB inhibition could ameliorate the effect of uremic serum on CYP expression, implicating the effect of parathyroid hormone (PTH) and inflammatory cytokines involved in these pathways (Michaud et al., 2008). In support of this hypothesis, their earlier study showed that depletion of PTH using anti-PTH antibodies in uremic serum ameliorated the downregulation of major hepatic CYPs in rat hepatocytes (Michaud et al., 2006). They also showed that parathyroidectomized rats with CKD also did not have decreased hepatic CYP levels (Michaud et al., 2006). The role of inflammatory cytokines on downregulating CYP enzymes has been previously well-characterized (Morgan et al., 2008). Direct competitive inhibition of CYP enzymes has also been proposed by several studies (Elston, Bayliss, & Park, 1993; Nolin et al., 2008). This is due to the observation that changes in clearance of CYP substrates in uremia are acute, as uremic hepatocytes treated with post-dialysis serum see a restoration of CYP expression (Michaud et al., 2008;

Nolin et al., 2006). Additionally, in one prospective cohort study, Nolin et al. observed that patients which underwent hemodialysis had higher ^{14}C excretion rates evaluated by an erythromycin breath test, and that the post-dialysis improvement in CYP3A4-mediated erythromycin metabolism was acute (Nolin et al., 2006). This further supports the notion that uremia acutely affects drug metabolism in CKD. However, other studies have observed alterations in *in vitro* hepatic uptake of erythromycin due to changes in transporters, indicating that erythromycin breath test results may not solely be an indicator of improved CYP3A4 activity (Sun, Huang, Frassetto, & Benet, 2004). Nonetheless, this study indicates that dialyzable uremic toxins may be responsible for acute changes in non-renal clearance of CYP substrates in CKD. Downstream of these mechanisms, CYP downregulation in CKD may be due to decreased nuclear receptor binding and histone acetylation, as demonstrated using chromatin immunoprecipitation (ChIP) in a surgically-induced CKD rat model (Velenosi, Feere, Sohi, Hardy, & Urquhart, 2014). In preliminary CKD studies using intestinal adsorbent AST-120, there is effective removal of uremic toxin precursors such as indole, a precursor of indoxyl sulfate. However, recovery of CYP3A and CYP2C function was not observed, indicating that uremic toxins may not be the sole regulators of CYP function and expression in CKD (Velenosi, 2015). There is a need for additional studies to elucidate the complex mechanisms behind drug metabolism changes observed in CKD.

1.5 Rationale, Objectives, and Hypothesis

1.5.1 Rationale

As previously mentioned, the prevalence of diabetes is increasing. Over 40% of these patients develop DN, which puts them at risk of developing CKD and progressing to ESRD. To manage hyperglycemia, comorbidities, kidney disease, and other diabetes-related complications, these patients often experience polypharmacy, putting them at risk for DDIs and ADEs. In addition to this, patients with DN may be at risk of pharmacokinetic changes because CYP enzymes are downregulated in CKD. For instance, common antidiabetic drugs such as thiazolidinediones, sulfonylureas, and meglitinides are mostly metabolized by CYP3A4, CYP2C9, and CYP2C8, and display unpredictable pharmacokinetic changes in patients with altered CYP expression. This issue, and its

prevalence in modern patient populations, incurs an extremely large economic cost and healthcare burden. Outcomes for patients in ESRD are poor due to a lack of a cure, with main treatments being dialysis and kidney transplantation, leading to a large population of patients requiring revised treatment regimens and personalized drug dosing.

Pharmacokinetic changes in late-stage CKD and ESRD are relatively well-characterized, and researchers are beginning to examine CYP expression over a range of CKD phenotypes. A recent study compared changes in CYP expression in rat models of moderate CKD and late CKD and demonstrated that CYP3A2 and CYP2C11 mRNA, protein, and activity are reduced by 88%, 75%, 66% and 77%, 41%, 67% for each enzyme, respectively, by only moderate CKD (Velenosi et al., 2012). In addition, a previous study characterized the expression of CYPs over a timeline of CKD progression in a diet-induced rat model of CKD, which resulted in decreases of 83%, 63%, and 360% in CYP3A2 mRNA, protein, and activity, respectively, by day 14 after induction of CKD by adenine diet (out of a 42-day progression timeline). At the same time point in CKD injury stage, CYP2C11 mRNA, protein, and activity were also reduced by 84%, 52%, and 300%, respectively (Hartjes, 2017). However, the functional expression of CYPs in early DN have not been elucidated. The majority of DN patients have early CKD as opposed to moderate or late CKD due to the relatively asymptomatic period during which hyperglycemia induces early glomerular changes. The lack of reliable early diagnostic biomarkers, as well as the large proportion of diabetics who are undiagnosed, allows for the development of early DN before pharmacological intervention. It is extremely important to characterize changes in drug metabolism in this population of patients, due to the disproportionately large number of diabetics with this phenotype, as well as polypharmacy that these patients must undergo to manage diabetic complications and comorbidities.

1.5.2 Objectives and Hypothesis

The overall aim of this project is to characterize the effects of diabetes on drug metabolizing enzymes. To this end, the focus is the function and expression of hepatic CYP drug-metabolizing enzymes, since the liver contains the highest amount of CYP enzymes compared with other tissues. In addition, CYP enzymes are responsible for the metabolism of over 75% of all drugs, while CYP3A4 and CYP2C9 together metabolize over 50% of

the top 200 clinically marketed drugs. This project focuses on CYP3A4 and CYP2C9 and their orthologous mouse enzymes, Cyp3a11 and Cyp2c29. The two specific aims of this project are 1) to examine the effect of DN on functional expression of Cyp3a11 and Cyp2c29 in an STZ-induced mouse model, and 2) to examine the effect of diabetes on the expression and function of CYP3A4 in an STZ-induced humanized CYP3A4/PXR/CAR mouse model. It is hypothesized that mice with diabetes will have decreased expression and function of hepatic CYP3A and CYP2C enzymes. Since moderate and severe CKD display pronounced downregulation of CYP3A and CYP2C enzymes, it is predicted that downregulation of these enzymes will occur in diabetes.

Chapter 2

2 Materials & Methods

2.1 Animal Study Design

Eleven male C57BL/6 mice, aged 7-8 weeks, were acquired from Charles River Laboratories (Wilmington, MA). Thirty humanized TgCYP3A4/hPXR/hCAR mice, aged 5 weeks, were acquired from Taconic Biosciences, Inc. (Rensselaer, NY). All mice were housed in clean, temperature-controlled environments with a 12-hour day/night cycle. Mice were randomly assigned into treatment (n=6 for male mice; n=15 for female mice) and control (n=5 for male mice; n=15 for female mice) groups and acclimated for at least 72 hours prior to the start of the experiment. The female TgCYP3A4/hPXR/hCAR mice were split into two separate cohorts (n=18 and n=12, with equal numbers of experimental and control mice in each cohort), to make animal husbandry easier. Each cohort received the exact same treatments as follows. To induce diabetes, animals were fasted for 6 hours before administration of 5 consecutive daily injections of STZ prepared in 0.1 M sodium citrate buffer (pH=4.5) at a dose of 50 mg/kg body weight by intraperitoneal injection, in accordance with AMDCC guidelines (Diabetic Complications Consortium, 2003). Control animals were also fasted for 6 hours and injected with equal volumes of vehicle solution. All injections were administered to animals in the same order between 2–5 P.M. in the afternoon. Mice were weighed three times a week and water and food were provided *ad libitum.* Blood glucose was measured in duplicate weekly via tail vein puncture using a Contour® Next One glucometer with corresponding glucose strips, using a diabetic blood glucose cut-off value of >13.9 mmol/L. If any mice did not reach this threshold, they were given up to five additional STZ injections. Mice that did not reach threshold values were sacrificed and excluded from the study. Hyperglycemia was monitored weekly and maintained for 16 weeks for the male C57BL/6 mice to induce DN, whereas female humanized TgCYP3A4/hPXR/hCAR mice were hyperglycemic for at least 2 weeks and not exceeding 4 weeks prior to sacrifice. Mice were sacrificed by ketamine anaesthesia followed by intracardiac puncture, and heart, pancreas, liver, kidney, and blood were collected and stored at -80 °C. All animal protocols were performed in accordance to

Canadian Council on Animal Care (CCAC) guidelines and approved by the Western University Animal Care Committee (ACC).

2.2 Glucose Tolerance Tests

Twenty-four hours prior to sacrifice, all mice were subjected to a 2-hour glucose tolerance test (GTT) to evaluate insulin sensitivity and glucose handling ability. Mice were fasted and weighed 6 hours prior to intraperitoneal injection of 30% dextrose in sterile saline solution at a dose of 2 g/kg body weight as recommended by a methodological review (Andrikopoulos, Blair, Deluca, Fam, & Proietto, 2008). Blood glucose values were measured via tail vein puncture using glucometry at 0, 15, 30, 45, 60, 90, and 120 minutes post-injection.

2.3 Disease Markers

2.3.1 Plasma Creatinine

To measure the degree of kidney disease, two markers were assessed: plasma creatinine and UACR. Previous studies from the AMDCC highlight the advantages of using a UPLC-MS method over the conventional Jaffé alkaline picrate method for the measurement of creatinine (Dunn, Qi, Bottinger, Breyer, & Sharma, 2004). Sample preparations for plasma creatinine are as follows: to isolate plasma, blood samples (collected in heparinized tubes) were centrifuged at 2,400*g* for 10 minutes and the resulting plasma transferred into new tubes. To precipitate proteins, 150 µL of ice-cold acetonitrile (ACN) with creatinine-d3 as internal standard at a concentration of 50 µM was added to 50 µL of plasma, vortexed, and incubated for 20 minutes at -20°C. Supernatant was isolated by centrifugation at 14,000*g* for 10 minutes and diluted 1:5 using ultrapure water. Prepared samples were transferred to vials and analyzed using an ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS; see Section 2.9.1). A standard curve of creatinine using concentrations of 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, and 0 µM was run to facilitate absolute quantification.

2.3.2 Urinary Albumin-to-Creatinine Ratio

UACR measurement involved measurement of albumin via enzyme-linked immunosorbent assay (ELISA) and measurement of creatinine in urine by UPLC-MS. The Abcam Mouse Urine Albumin ELISA Kit was used to determine urinary albumin (Cambridge, UK). All sample preparation and measurement protocols were performed according to the manufacturer's instructions. Appropriate concentration and dilution ranges were previously validated by the same protocol. Briefly, isolated urine was centrifuged at 800*g* and the supernatant diluted 800-fold in manufacturer-provided diluent. Human albumin standards were prepared at the following concentrations: 200, 50, 12.5, 3.13, 0.78, and 0 ng/mL in provided diluent buffer. Fifty µL of sample or standard were pipetted into each microplate well previously coated with an albumin-specific antibody and incubated for 2 hours. Wells were then washed with 200 µL of wash buffer five times and 50 µL of biotinylated albumin-specific antibody was added to each well. Following a 1-hour incubation and another wash step, 50 µL of streptavidin-horseradish peroxidase (HRP) conjugate antibody was added to each well and incubated for 30 minutes. After washing, 50 µL of chromogen substrate was added and wells were monitored for colorimetric changes or until 15 minutes had passed, when 50 µL of stop solution was added. Plates were immediately read at 450 nm on a SpectraMax® M3 spectrophotometer with a wavelength correction of 570 nm. Absorbance values were interpolated according to known concentrations of the human albumin standard curve fitted with a hyperbolic trendline.

To measure urinary creatinine, 20 µL of undiluted urine was treated with 60 µL of ice-cold ACN containing 50 µM creatinine-d3 as internal standard. Tubes were centrifuged at 14,000*g* for 10 minutes to pellet precipitate and supernatant was isolated in new tubes, diluted 100-fold with 90:10 ACN:water, and transferred to UPLC vials. The following concentrations of creatinine standards were included as a standard curve: 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, and 0 µM. Samples were analyzed using UPLC-MS as described in Section 2.9.1.

2.4 RNA Extraction and Real-Time PCR

2.4.1 RNA Extraction

Following liver isolation, RNA was extracted using TRIzol reagent (Thermo Fischer Scientific; Waltham, MA) according to manufacturer's protocol: Approximately 30–50 mg of liver tissue was sectioned and added to microtubes containing 200 µL of TRIzol reagent. Tissues were homogenized and 800 µL of TRIzol were added. Following a 15-minute incubation at room temperature, 200 µL of chloroform was added, mixed by vigorous shaking, and tubes were isolated at room temperature 2–3 minutes before being centrifuged at 12,000*g* for 15 minutes. The supernatant containing RNA was isolated and transferred to new tubes and precipitated with the addition of 500 µL isopropanol. The tubes were incubated for 10 minutes at room temperature and then centrifuged at 12,000*g* for 10 minutes. The supernatant was discarded, and RNA pellets were washed with 75% ethanol before being centrifuged at 12,000*g* for 5 minutes a final time. Once dry, the RNA was dissolved in ultrapure water. Concentrations and purity of RNA for each sample was quantified by dispensing 1 µL of RNA on a Nanodrop 2000. Following this, cDNA was synthesized from 1 µg of RNA using reverse-transcription PCR by the addition of Quanta® qScript cDNA Supermix (Quanta BioSciences; Gaithersburg, MD). The resulting cDNA was frozen and stored at -20 °C.

2.4.2 Real-Time PCR

In preparation for qPCR, synthesized cDNA was diluted 20-, 40-, and 100-fold. Primer pairs for each gene were either constructed using the National Center for Biotechnology Information (NCBI) Primer Basic Local Alignment Search Tool (Primer-BLAST) or obtained from similar studies and are listed in Table 2.1. Primer pairs were validated by cycle threshold (Ct) and melt curve analysis, and linearity was measured between average Ct and log (dilution factor) for fixed dilutions of cDNA. Once primer pairs were validated, relative mRNA for each gene was measured using qPCR as follows: Five µL of 40-fold diluted cDNA (all samples previously synthesized from 1 µg of RNA) was mixed with 0.6 µL of primer mix, 0.4 µL of ultrapure water, and 6 µL of PerfeCTa SYBR Green FastMix (Quanta BioSciences; Gaithersburg, MD). Wells were plated in triplicate for each sample

and run on a Bio-Rad CFX384 Real-Time System (Hercules, CA, USA). For all runs, Ct values were normalized to those of β-actin and relative mRNA levels was quantified using the double-delta Ct method (ΔΔCt) (Livak & Schmittgen, 2001).

Table 2.1. Mouse and human gene names, followed by NCBI accession number, primer sequences (5'–3'), and estimated product size for mouse CYP enzymes and transporters, as well as human CYP3A4, PXR, and CAR. All primers were previously validated by Ct and melt curve analysis and measured to be linear within working pooled cDNA dilutions.

Gene Name	Accession Number	Sequence (5'–3')	Product Size
mCyp3a11	NM_007818.3	FW: CTCAAGGAGATGTTCCCTGTCA RV: GGCTTGCCTTTCTTTGCCTTCT	83
mCyp2c29	NM_007815.3	FW: CATCGACCTCCTCCCCACTA RV: ACTGTAGTTCCCTTGGGGATG	87
mCyp2e1	NM_021282.2	FW: TTTCTGCAGGAAAGCGCGTG RV: TTGTAACAGGGCTGAGGTCG	138
mSlco1a1	NM_013797.5	FW: ACATGGTTCTTCTGAGGTGTG RV: GTCTGGAGAGTGGATGTCGC	314
mSlco1b2	NM_020495.1	FW: GCCTGAGTTCAGGACACCAA RV: TACCCTATGCCTTCCACCGA	100
mAbcc3	NM_001363187.1	FW: GCCCCAGACGGACTTTATCAT RV: CATTTTGCAAGGCTGCTTCAT	139
mSlc10a1	NM_001177561.1	FW: TTGCGCCATAGGGATCTTCC RV: ATCATGCCTGCCTTGAGGAC	72
CYP3A4	NM_017460.5	FW: AGCAAAGAGCAACACAGAGC RV: GTTCCATATAGATAGAGGAGCAC	145
NR1I3	NM_001077482.2	FW: ACAGTGGGGTTCCAGGTAGA RV: AGGGAGCAGGAGAGAAGAGG	128
NR1I2	NM_003889.3	FW: CCAGGACATACACCCCTTTG RV: CTACCTGTGATGCCGAACAA	60
mActb	NM_007393.5	FW: CGTAAAGACCTCTATGCCAA RV: TAAAACGCAGCTCAGTAACA	287

2.5 Microsome Isolation & Protein Quantification

2.5.1 Microsome Isolation

Hepatic microsomes were isolated from liver tissue by differential centrifugation according to the following protocol: For each sample, approximately 3–4 g of liver was transferred to ultracentrifuge tubes, and homogenized in buffer (1.15% KCl + 1 mM ethylenediaminetetraacetic acid (EDTA)) using an IKA T10 ULTRA-TURRAX homogenizer (Sigma-Aldrich; St. Louis, MO). Tubes were then centrifuged at 9,000*g* at 4 °C in an Optoma L-90K ultracentrifuge (Beckman-Coulter; Fullerton, CA) for 20 minutes. The supernatant was collected and centrifuged again at 105,000*g* for 1 hour. The supernatant was then discarded, and pellets were collected and homogenized in 200 µL of 0.1 M potassium phosphate + 20% glycerol (pH = 7.4). Separate aliquots were made for Western blotting and enzymatic activity (see Sections 2.6 & 2.7) and stored at -80 °C until use.

2.5.2 Protein Quantification

Protein content in microsomal aliquots was determined using Pierce BCA Protein Assay Kit (Thermo Fischer Scientific) according to the 96-well microplate version of manufacturer's protocol. Bovine serum albumin (BSA) standards were prepared at the following concentrations: 1000, 500, 250, 125, and 0 µg/mL, and working reagent (WR) was prepared using a 50:1 ratio of Reagent A: Reagent B. Samples and WR were plated in duplicate using a sample: WR ratio of 1:8 and incubated at 37 °C for 30 minutes or until optimal colorimetric development. Following incubation, plate absorbance was read immediately at a wavelength of 562 nm on a SpectraMax® M5 spectrophotometer with blank correction. Mean protein concentration for each sample was calculated by interpolating absorbance values according to standard curve values. Following this, additional aliquots of microsomes were diluted in buffer to 5 mg/mL and 0.8 mg/mL and stored at -80 °C for use in Western blotting and enzymatic assays (see Sections 2.6 & 2.7).

2.6 Western Blot

2.6.1 Gel Electrophoresis & Antibody Blotting

To determine relative protein expression of hepatic CYPs in microsomal fractions, proteins were electrophoresed and blotted prior to imaging. Gel electrophoresis was previously optimized for 15-well gels made with 10% resolving and 4% stacking buffers, made according to the following recipes: resolving, 4.1 mL deionized water, 3.3 mL 30% acrylamide/bis-acrylamide, 2.5 mL 1.5 M Tris-HCl (pH 8.8), and 0.1 mL 10% w/v sodium dodecyl sulfate (SDS); stacking, 6.1 mL deionized water, 1.3 mL 30% acrylamide/bis-acrylamide, 2.5 mL 1.5 M Tris-HCl (pH 6.8), and 0.1 mL 10% w/v SDS. To polymerize gels, 80 µL of ammonium persulfate (APS) and 8 µL of tetramethylethylenediamine (TEMED) were added to the resolving gel and 80 µL APS and 10 µL of TEMED were added to the stacking gel. Samples were prepared by adding sample buffer, β-mercaptoethanol, water and microsomes to achieve a loading volume of 7 µL containing 4 µg of protein for Cyp3a11 assays, and 15 µL containing 4 µg of protein for CYP3A4 assays. Samples were incubated at 80 °C for 15 minutes prior to loading. Following loading, gels were run in running buffer (Bio-Rad) at 60 V for 25 minutes followed by 120 V for 60 minutes. Proteins were then transferred to nitrocellulose membranes in transfer buffer (Bio-Rad) at 100 V for 120 minutes. Blots were washed using phosphate buffered saline + 0.1% Tween-20 (PBS-T) three times and blocked overnight using 0.6% BSA and 5% milk powder. Blots were then probed for 1.5 hours using primary antibodies, washed in PBS-T, and probed for 1 hour with secondary HRP-conjugated antibodies. The following antibody formulations were used to detect Cyp3a11: 1:8000 rabbit anti-rat CYP3A2 in PBS-T + 0.6% BSA, followed by 1:10000 goat anti-rabbit IgG conjugated to HRP in PBS-T + 0.6% BSA. To detect CYP3A4, 1:5000 rabbit anti-human CYP3A4 in PBS-T, followed by 1:8000 mouse anti-rabbit IgG conjugated to HRP in PBS-T. All protein readings were normalized to β-actin, detected using 1:10000 anti-mouse IgG-HRP conjugated to mouse anti-β-actin in PBS-T with a 1.5-hour incubation.

2.6.2 Imaging & Densitometry

Imaging was performed on a VersaDoc Imaging System (Bio-Rad) using Quantity-One v4.6.3 software (Bio-Rad). Approximately 1 mL of Luminata™ Forte HRP Substrate (Millipore; Billerica, MA) was added to blots for 1 minute, dried, and sealed using thin plastic film. Images were taken with 10-, 35-, and 60-second exposures. Densitometry analysis was performed in Quantity-One v4.6.3 software using in-suite volume analysis tools and normalizing average pixel intensity per square millimeter per band to β-actin.

2.7 Enzymatic Activity

In all enzymatic assays, 6-β-hydroxytestosterone formation was measured, as this was previously shown to be a relatively specific metabolite formed by CYP3A4 metabolism in humans and Cyp3a11 metabolism in mice (Chovan, Ring, Yu, & Baldino, 2007; Löfgren, Hagbjörk, Ekman, Fransson-Steen, & Terelius, 2004). Standard concentrations for 6-β-hydroxytestosterone used in all assays were: 50, 25, 12.5, 6.25, 3.12, 0.78, 0.39, 0.19, and 0 µM. Prior to experimentation, linearity tests were done for the formation of 6-β-hydroxytestosterone from testosterone. All assays were completed in the linear range for both reaction time and protein concentration. In performing the reaction, 0.8 mg/mL isolated hepatic microsomes and reaction buffer (50 mM potassium phosphate + 2 mM $MgCl_2$) were incubated with 1 µL of testosterone (Steraloids Inc.; Newport, RI) in 96-well V-bottom plates to achieve working concentrations of testosterone: 400, 200, 75, 25, and 12.5 µM with a final well volume of 75 µL. All microsomes were plated in duplicate at a final concentration of 0.1 mg/mL and incubated for 10 minutes at 37 °C. To initiate metabolism, 1 mM NADPH (Sigma-Aldrich) was added, and plates were incubated at 37 °C for 5 minutes. To stop the reaction, wells were immediately precipitated with 225 µL ice-cold ACN containing 80 ng/mL flurazepam (Cerilliant, Round Rock, TX). Plates were centrifuged at 4,000 rpm for 10 minutes to pellet microsomal proteins and supernatant was transferred to a new plate with a 5-fold dilution using ultrapure water. Samples were analyzed via UPLC-MS according to methods described in Section 2.9.2.

2.8 Hepatic Uremic Toxins

To determine the abundance of hepatic uremic toxins, approximately 100 mg of each mouse liver was precipitated using a 1:3 dilution and homogenized in ACN containing 2.5 µM chlorpropamide internal standard using an IKA T10 ULTRA-TURRAX homogenizer (Sigma-Aldrich). Precipitated proteins were pelleted by centrifuging at 14,000*g* for 10 minutes, supernatant extracted and diluted 5-fold in new tubes. Samples were loaded into vials and analyzed via UPLC-MS (see Section 2.9.3).

2.9 Chromatography & Mass Spectrometry

2.9.1 Creatinine

Urine and plasma sample preparations were injected using an ACQUITY UPLC I-Class autosampler (Waters; Milford, MA) at an injection volume of 2 µL with a flow rate of 0.45 mL/min. Samples were maintained at 4 °C and column temperature was maintained at 45 °C. Injected sample was eluted on a hydrophilic interaction liquid chromatography (HILIC) column, a Waters ACQUITY UPLC BEH Amide column (1.7 µM particle size, 2.1 mm × 100 mm). Solvents water + 0.1% formic acid (A) and ACN + 0.1% formic acid (B) were used as mobile phase solutions in the following ratios during elution: 0.00–0.99 min., 10% A, 90% B; 1.00–1.50 min., 40% A, 60% B; 1.51–2.50 min., 10% A, 90% B. Mass spectrometry was performed on a Xevo^{TM} G2-S Quadrupole Time-of-flight (QTof) MS (Waters), in positive electrospray ionization (ESI) and sensitivity mode, with the following source tune parameters: source temperature, 150 °C; capillary and sampling cone voltage, 2 and 40 kV; desolvation temperature, 500 °C; desolvation and cone gas flows, 50 and 1000 L/hr. Mass was acquired in a continuum data format over the 50–1200 *m/z* range, with a scan time of 0.1 s over a period of 2.5 min. For mass correction, 500 ng/mL leucine-enkephalin was used as a lock mass (556.2771 Da) and was sampled in 10 s intervals and averaged over 3 scans. Sample method and data collection was compiled in MassLynx v4.1 software (Waters). Sample quantification and peak integration was performed in TargetLynx v4.1 (Waters).

2.9.2 Testosterone Metabolites

Supernatant from reaction plates were injected using an autosampler as described above (Waters) at an injection volume of 5 µL with a flow rate of 0.50 mL/min. Samples were maintained at room temperature and column temperature was maintained at 40 °C. Injected sample was eluted on a Phenomenex® Kinetex Phenyl-Hexyl column (1.7 µM particle size, 2.1 mm × 50 mm; Torrance, CA). Solvents water + 0.1% formic acid (A) and ACN + 0.1% formic acid (B) were used as mobile phase solutions in the following elution ratios: 0.00–1.99 min., 75% A, 25% B; 2.00–2.49 min., 65% A, 35% B; 2.50–3.50 min., 20% A, 80% B; 3.51–4.50 min., 75% A; 25% B. Mass spectrometry was performed on a Xevo G2-S QTof MS in positive ESI and resolution mode, with the following source tune parameters: Source temperature, 150 °C; capillary and sampling cone voltage, 0.5 and 40 kV; desolvation temperature, 600 °C; desolvation and cone gas flows, 50 and 1200 L/hr. Mass was acquired in a continuum data format over the 50–1200 *m/z* range, with a scan time of 0.1 s over a period of 3.0 min. For mass correction, 500 ng/mL leucine-enkephalin was used as a lock mass (556.2771 Da) and was sampled in 10 s intervals and averaged over 3 scans. Sample method and data collection was compiled in MassLynx v4.1 software (Waters). Sample quantification and peak integration was performed in TargetLynx v4.1 (Waters).

2.9.3 Uremic Toxins

Hepatic homogenate sample preparations were injected using an autosampler (Waters) at an injection volume of 2 µL with a flow rate of 0.45 mL/min. Samples were maintained at 4 °C and column temperature was maintained at 45 °C. Injected sample was eluted on a reverse-phase liquid chromatography (RPLC) column, a Waters ACQUITY RPLC HSS-T3 column (1.8 µM particle size, 2.1 mm × 100 mm). Solvents water + 0.1% formic acid (A) and ACN + 0.1% formic acid (B) were used as mobile phase solutions in the following ratios during elution: 0.00–1.99 min., 99% A, 1% B; 2.00–2.50 min., 40% A, 60% B; 2.51–3.50 min., 20% A, 80% B; 3.51–4.50 min., 99% A, 1% B. Mass spectrometry was performed on the Xevo G2-S QTof (Waters), in negative ESI and sensitivity mode, with the same source tune parameters as described in Section 2.9.1. Mass was acquired in a centroid data format over the 50–1200 *m/z* range, with a scan time of 0.05 s over a period

of 3.5 min. For mass correction, 500 ng/mL leucine-enkephalin was used as a lock mass (554.2615 Da) and was sampled in 10 s intervals and averaged over 3 scans. Sample method and data collection was compiled in MassLynx v4.1 software (Waters). Sample quantification and peak integration was performed in TargetLynx v4.1 (Waters).

2.10 Statistical Analysis

For GTT AUC, plasma creatinine, UACR, mRNA levels & protein expression, enzyme metabolism V_{max} & K_m, and hepatic uremic toxin comparisons between STZ and control mice, normality of data was first evaluated using the D'Agostino-Pearson omnibus normality test. For normally distributed data, Student's unpaired parametric *t*-test was used, while non-normally distributed data was analyzed using the Mann-Whitney *U* test. Where applicable, data was interpolated from linear standard curves apart from urinary albumin, which was interpolated from a hyperbolic fit. Michaelis-Menten curves were fitted to metabolite formation graphs for enzymatic activity assays. Differences in mouse body weight, weekly blood glucose, and GTT blood glucose between treatment groups was compared using repeated measures two-way analysis of variance (ANOVA), and Sidak's multiple comparisons test was used to compare individual means. All statistical analysis and graphing were done in GraphPad Prism 6.01 (GraphPad Software Inc.; La Jolla, CA) and all data are presented as means ± standard error of the mean (SEM), with a *p*-value of less than 0.05 considered significant.

Chapter 3

3 Results

3.1 Body Weight and Blood Glucose

For male C57BL/6 mice, body weights of the STZ-treated group were significantly decreased compared to control mice from weeks 13–16 post-injection ($p<0.05$; Figure 3.1A). As previously mentioned in Section 2.1, female TgCYP3A4/hPXR/hCAR mice were split into two separate cohorts, which received the same treatments. Henceforth, body weights and blood glucose for each cohort are presented separately. For the first cohort of female mice, body weights of the STZ-treated group were significantly decreased compared with controls 1 and 3 weeks before sacrifice ($p<0.05$; Figure 3.2A). For the second cohort, body weights of STZ-treated mice were not significantly different from controls (Figure 3.2B).

For male C57BL/6 mice, blood glucose of STZ-treated mice was significantly elevated compared with controls for 15 weeks prior to sacrifice ($p<0.05$; Figure 3.1B). For the female TgCYP3A4/hPXR/hCAR mice, blood glucose of the STZ-treated group was significantly higher compared to controls for 3 and 4 weeks prior to sacrifice for each of the first and second cohorts, respectively ($p<0.05$; Figure 3.3).

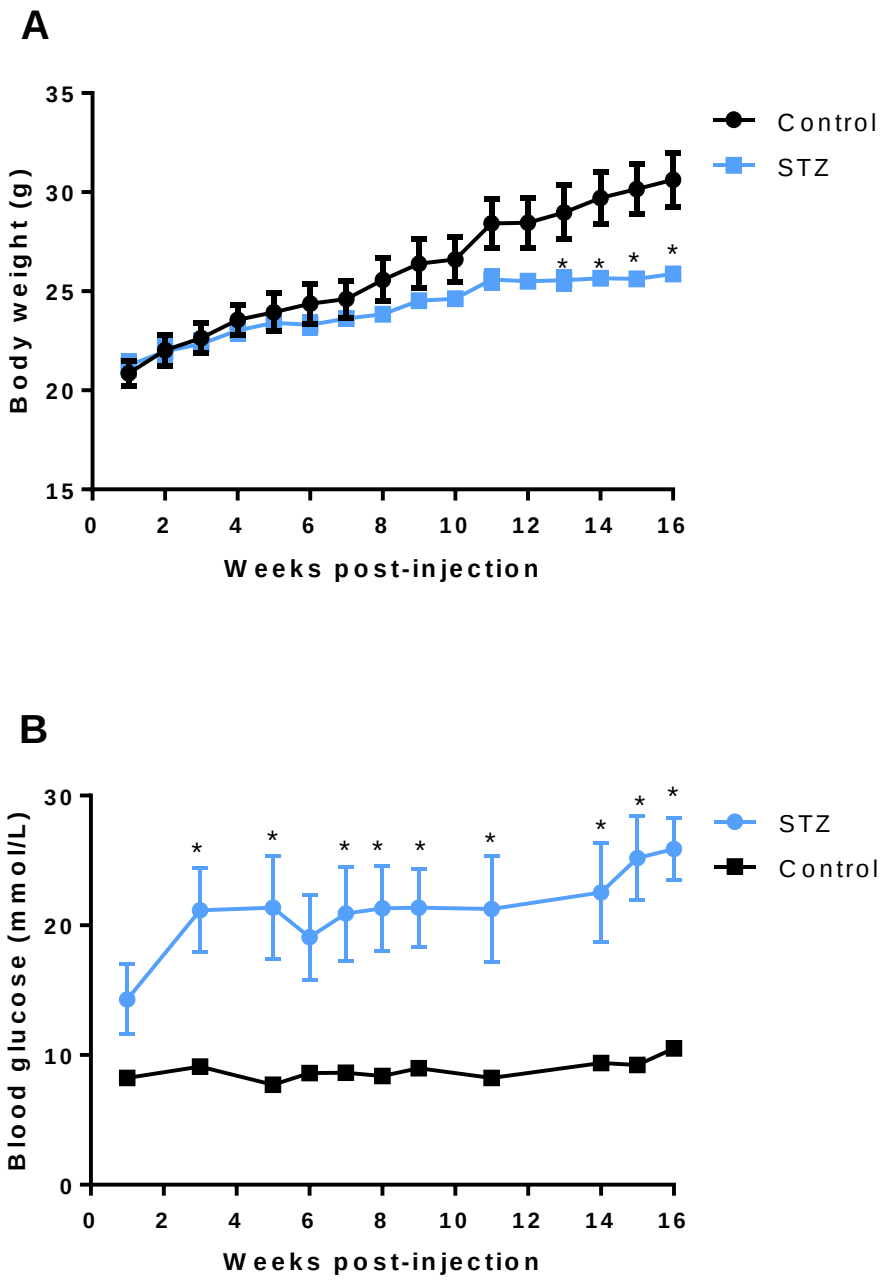

Figure 3.1. Weekly post-injection **(A)** mean body weights (g) and **(B)** mean blood glucose (mmol/L) of male C57BL/6 mice treated with STZ (*n*=6) or vehicle (*n*=5). Values are presented as means ± SEM. STZ-treated mice had a significantly decreased body weight, as well as increased blood glucose, compared with vehicle-treated mice (Repeated Measures Two-way ANOVA and Sidak's Multiple Comparisons Tests, $p<0.05$).

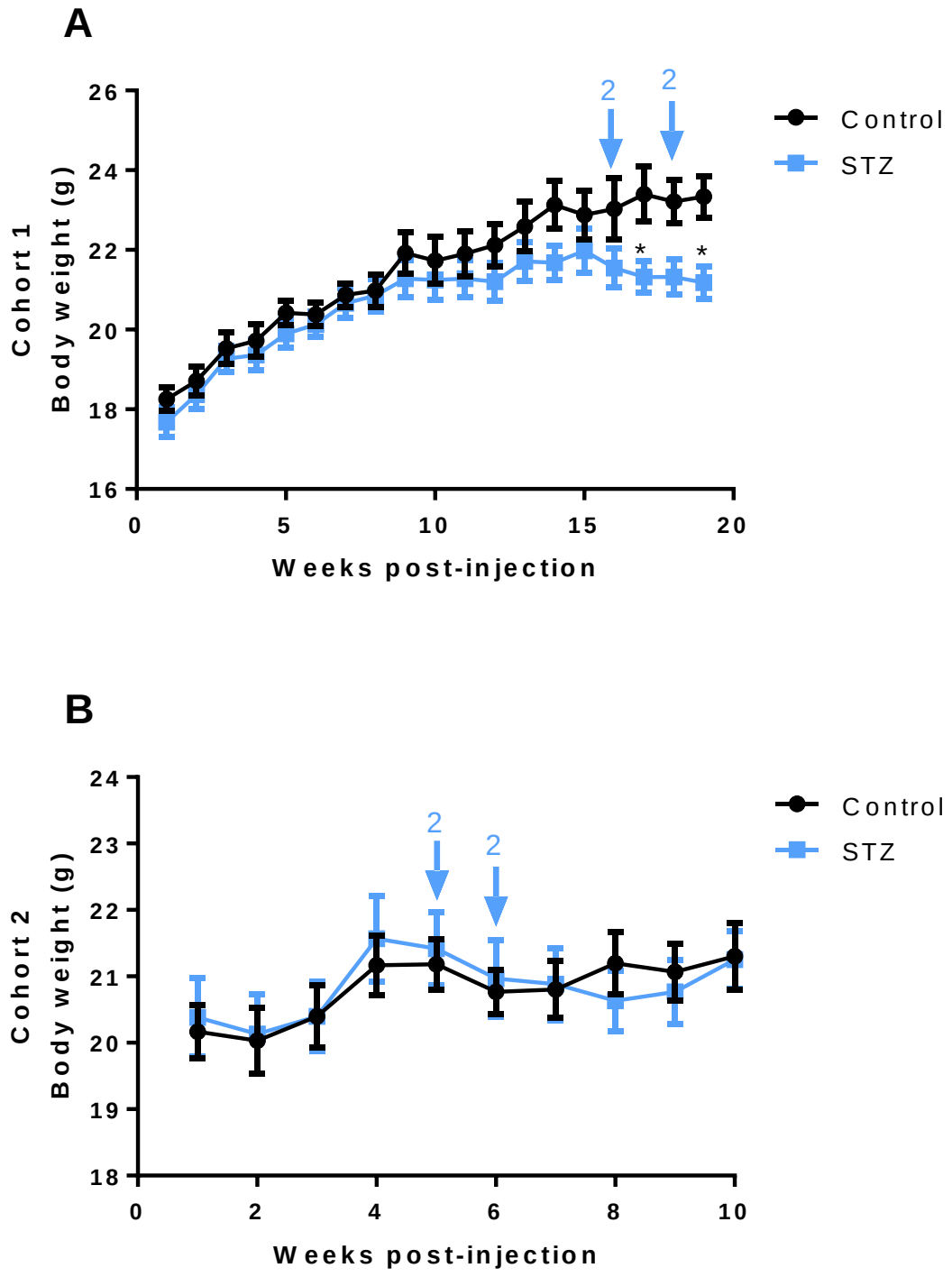

Figure 3.2. Mean body weights (g) of **(A)** female humanized TgCYP3A4/hPXR/hCAR mice treated with STZ (*n*=9) or with vehicle (*n*=9). **(B)** female humanized TgCYP3A4/hPXR/hCAR mice treated with STZ (*n*=6) or with vehicle (*n*=6). Values are presented as means ± SEM. Arrows indicate administration of extra STZ injections.

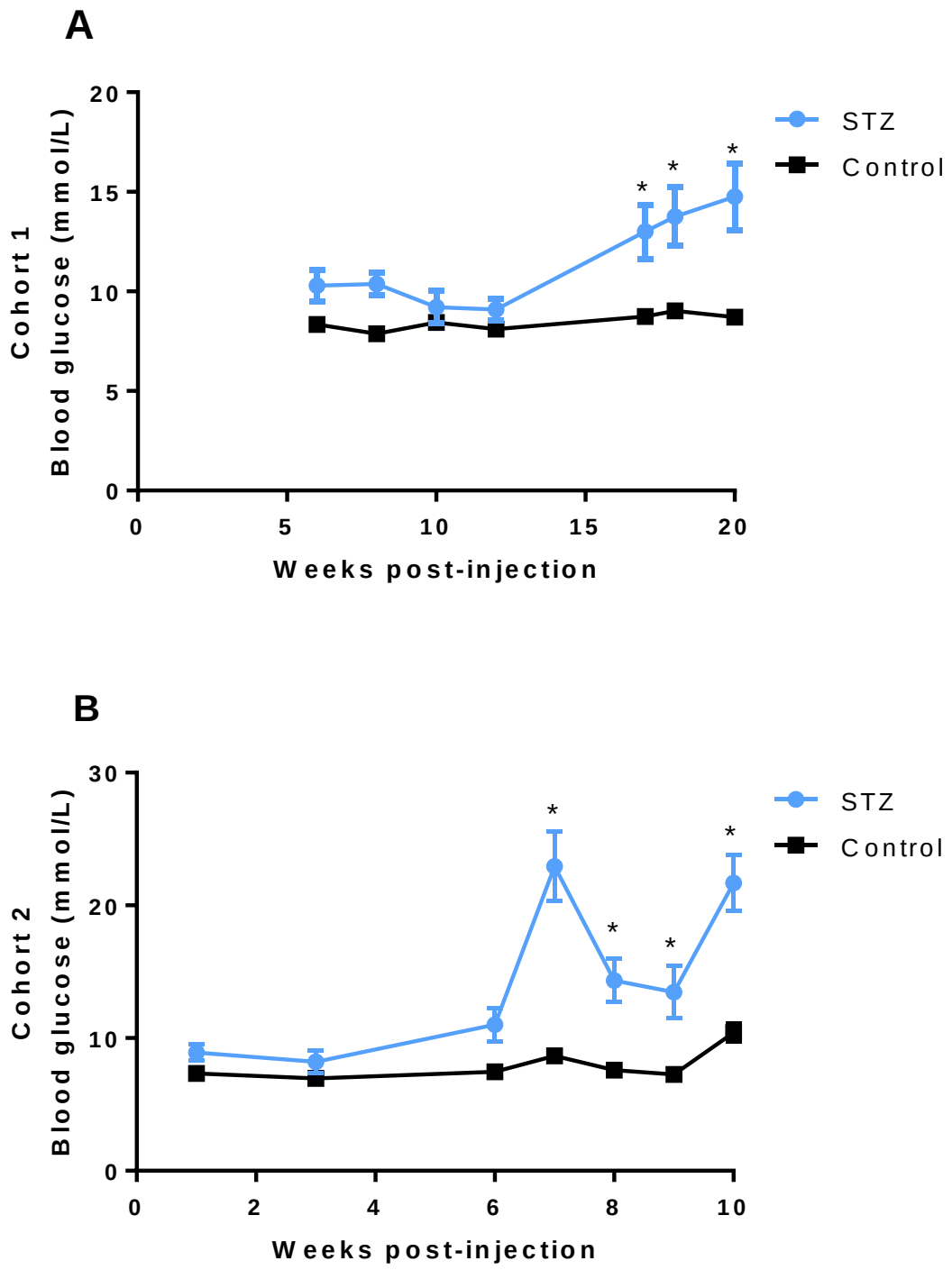

Figure 3.3. Mean blood glucose (mmol/L): **(A)** female humanized TgCYP3A4/hPXR/hCAR mice treated with STZ (*n*=9) or with vehicle (*n*=9). **(B)** female humanized TgCYP3A4/hPXR/hCAR mice treated with STZ (*n*=6) or with vehicle (*n*=6). Values are presented as means ± SEM.

3.2 Glucose Tolerance Tests

Figure 3.4 depicts blood glucose values for a 120-minute GTT performed on STZ-treated male C57BL/6 mice (n=6) and controls (n=5). STZ-treated male mice had significantly higher mean blood glucose values compared to controls at all time points (p<0.05; Figure 3.4).

Both cohorts of female TgCYP3A4/hPXR/hCAR STZ-treated mice had significantly higher mean blood glucose values compared to controls at all time points (p<0.05; Figure 3.5A & C), as well as AUC values compared to controls (p<0.05; Figure 3.5B & D). STZ-treated mice had a mean AUC increase of 4.5- and 4.7-fold compared to controls for the first and second cohorts, respectively (Figure 3.5B & D).

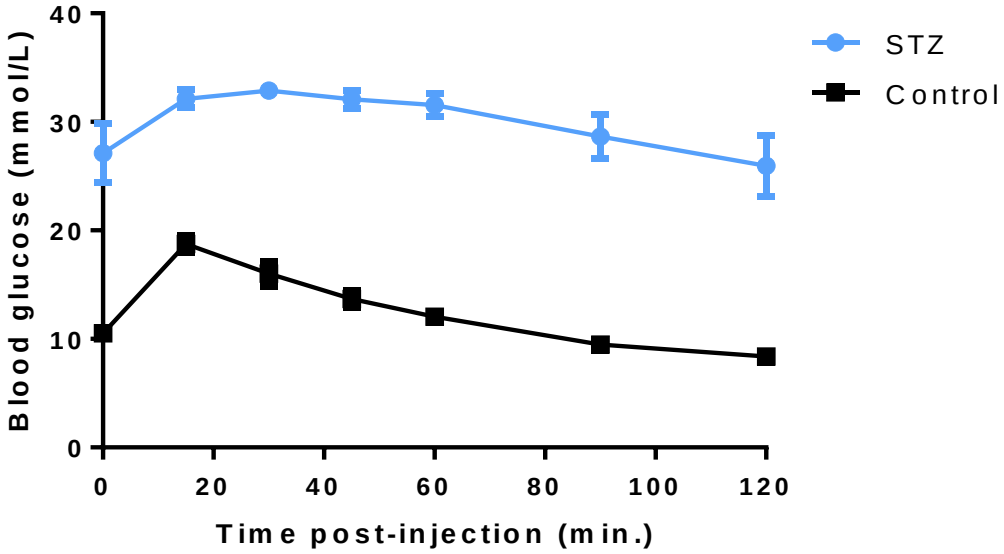

Figure 3.4. Mean blood glucose (mmol/L) of STZ-treated male C57BL/6 mice (*n*=6) and vehicle-treated mice (*n*=5) in a glucose tolerance test. Mice were administered 30% dextrose at a dose of 2 g/kg. Results presented as means ± SEM. STZ-treated mice had significantly increased mean blood glucose across all time points compared with vehicle-treated mice (Repeated Measures Two-way ANOVA and Sidak's Multiple Comparisons, $P<0.05$).

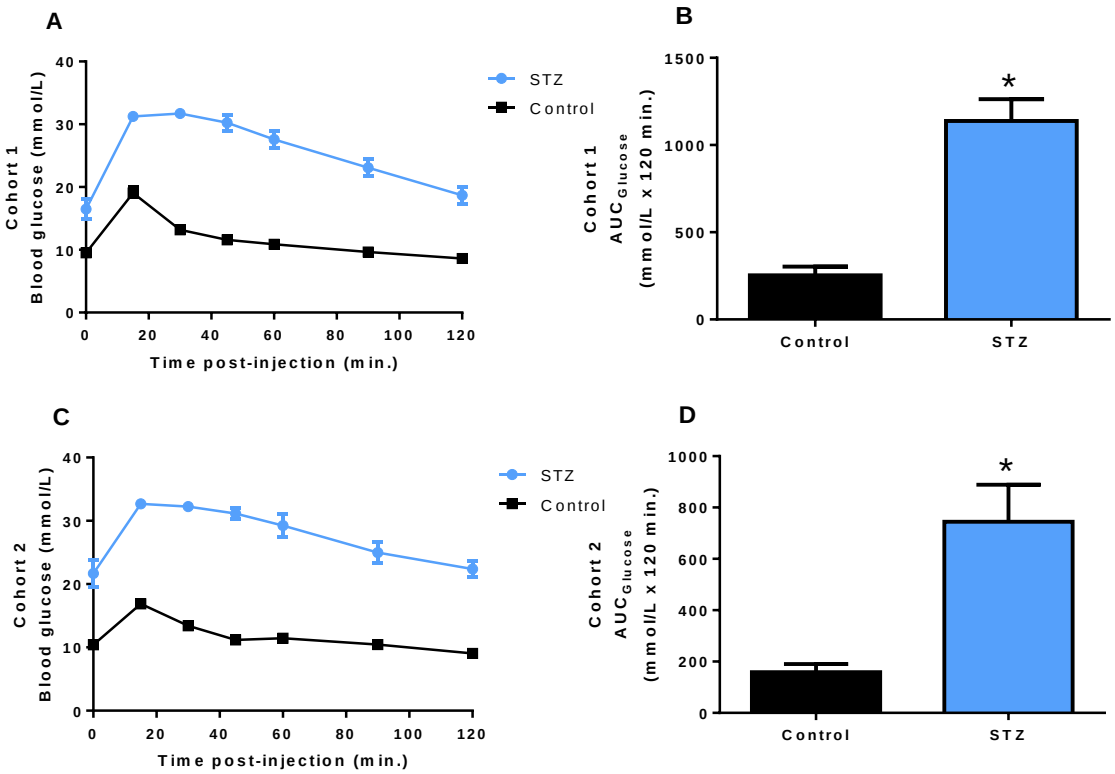

Figure 3.5. Mean blood glucose (mmol/L) of female humanized TgCYP3A4/hPXR/hCAR mice over 120 min. in a glucose tolerance test for **(A)** STZ-treated (*n*=9) and vehicle-treated (*n*=9) mice of one group and **(C)** STZ-treated (*n*=6) and vehicle-treated (*n*=6) mice of another group. Mean area under-the-curve (AUC; mmol/L x 120 min.) of **(B)** female humanized Tg CYP3A4/hPXR/hCAR STZ-treated mice (*n*=9) and vehicle-treated mice (*n*=9) for one group and **(D)** STZ-treated mice (*n*=6) and vehicle-treated mice (*n*=6) of another group. Mice were administered 30% dextrose at a dose of 2 g/kg. Results presented as means ± SEM. STZ-treated mice had significantly increased mean blood glucose and AUC across all time points compared with vehicle-treated mice (Repeated Measures Two-way ANOVA and Sidak's Multiple Comparisons, $p<0.05$).

3.3 Disease Markers

Male C57BL/6 STZ-treated mice had a mean plasma creatinine of 11.73 µM, which was not significantly different from controls, which had a mean of 11.21 µM (Figure 3.6A). Female TgCYP3A4/hPXR/hCAR STZ-treated mice had a mean plasma creatinine of 14.47 µM, which was not significantly different from the control mean of 13.83 µM (Figure 3.7A).

Mean UACR for male C57BL/6 STZ-treated mice displayed an a non-significant increase of 225.3 µg/mg compared to the control average of 116.3 µg/mg (p=0.13; Figure 3.6B;). Female TgCYP3A4/hPXR/hCAR STZ-treated mice had a mean UACR of 58.7 µg/mg, which was not significantly different from the control average of 49.8 µg/mg (Figure 3.7B).

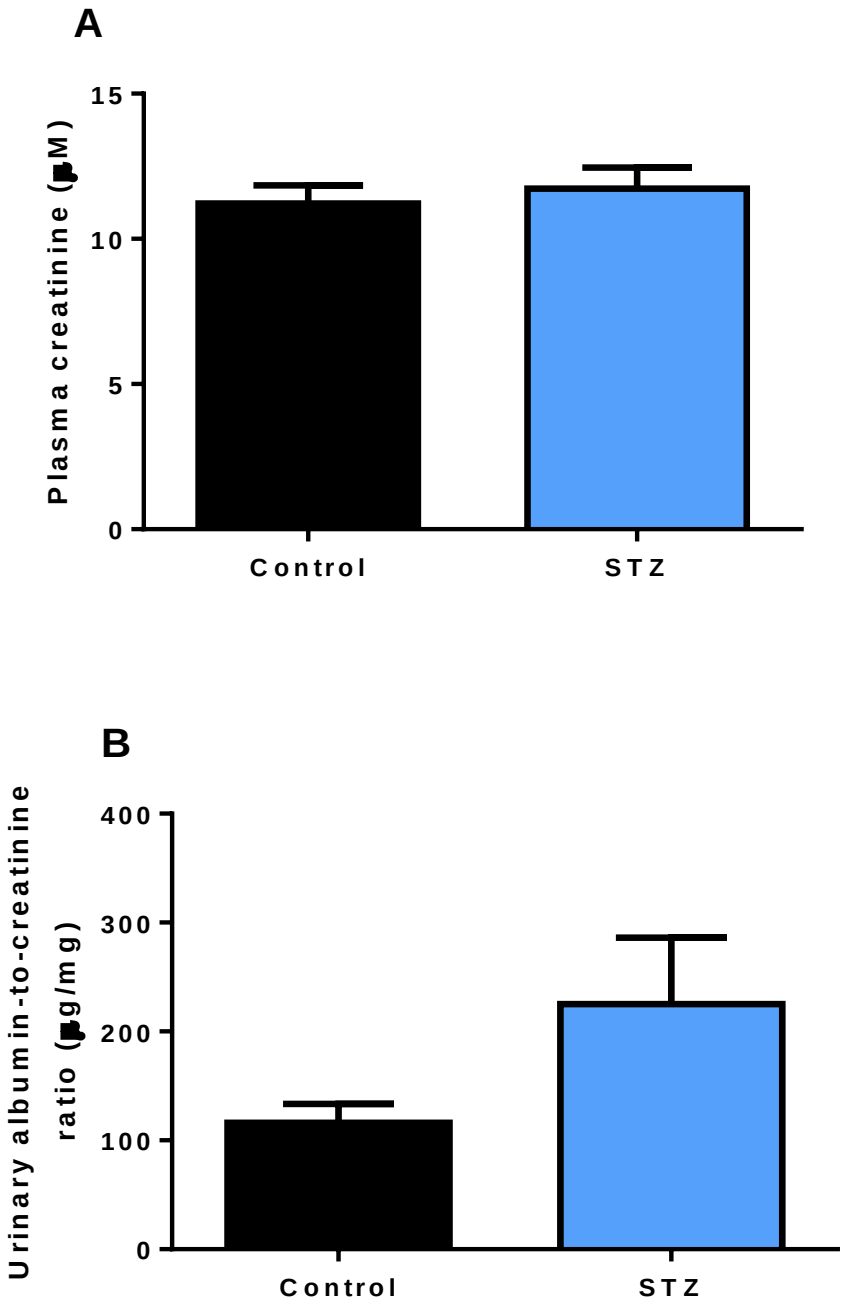

Figure 3.6. (A) Mean plasma creatinine (μM) and **(B)** urinary albumin-to-creatinine ratio (μg/mg) from male C57BL/6 mice treated with STZ (*n*=6) or vehicle (*n*=5). Results presented as mean ± SEM.

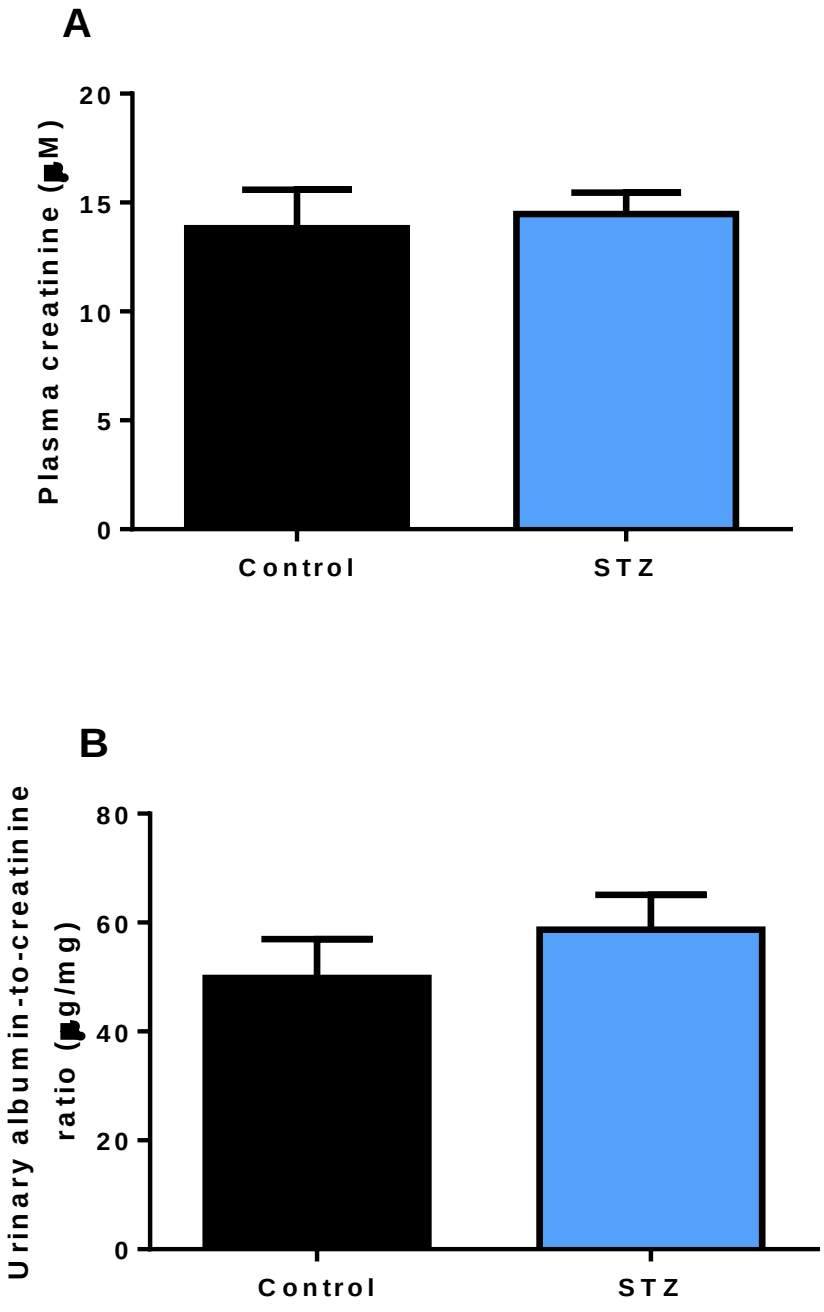

Figure 3.7. (A) Mean plasma creatinine (mmol/L) and **(B)** mean urinary albumin-to-creatinine ratio (μg/mg) in a pooled cohort of female TgCYP3A4/hPXR/hCAR mice treated with STZ (*n*=13) or with vehicle (*n*=8). Values are presented as means ± SEM.

3.4 Expression of Hepatic CYPs, Transporters, and Nuclear Receptors

Male C57BL/6 STZ-treated mice showed non-significant decreases of 13, 23, and 11% for hepatic *Cyp2c29, Cyp2e1,* and *Cyp3a11* mean relative mRNA levels compared with controls, respectively (Figure 3.8). Male STZ-treated mice showed no significant differences in hepatic mRNA levels of transporters *Oatp1a1, Oatp1b2, Mrp3,* and *Ntcp* compared to controls (Figure 3.9). Female TgCYP3A4/hPXR/hCAR STZ-treated mice showed a significant 41% decrease in mean relative hepatic *Cyp2e1* mRNA levels compared with control ($p<0.05$; Figure 3.10); however, CYP3A4 expression was not significantly different from control (Figure 3.10). Female STZ-treated mice exhibited a significant 42% decrease in hCAR levels compared with controls ($p<0.05$; Figure 3.9), as well as a non-significant decerase of 19% in hPXR levels compared with controls ($p=0.06$; Figure 3.10).

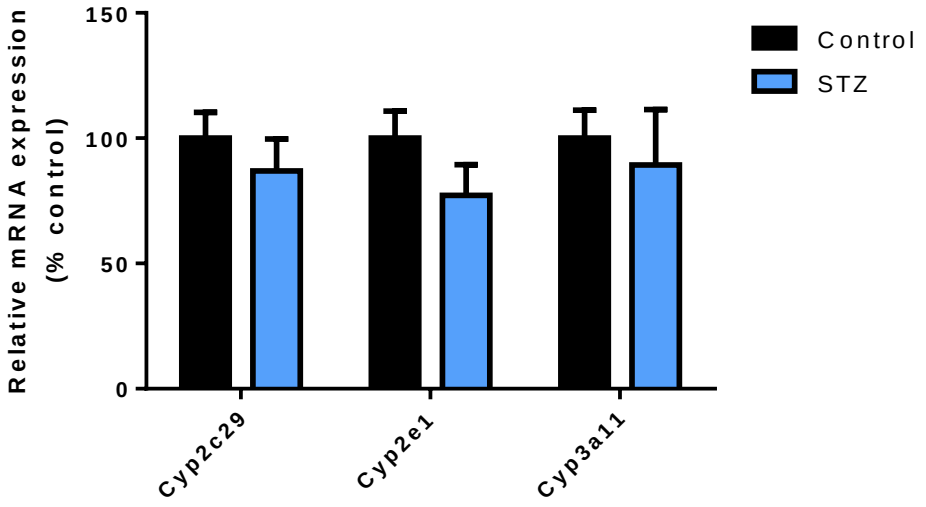

Figure 3.8. Mean relative hepatic CYP mRNA levels of male C57BL/6 mice treated with STZ (*n*=13) or vehicle (*n*=13). Experiment was performed in triplicate and mRNA levels were normalized to β-actin levels. Results are presented as mean ± SEM.

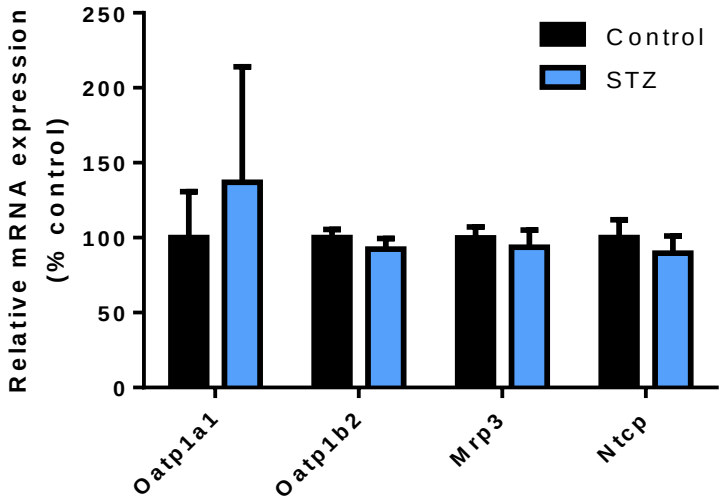

Figure 3.9. Mean relative hepatic transporter mRNA levels of male C57BL/6 mice treated with STZ (*n*=13) or vehicle (*n*=13). Experiment was performed in triplicate and mRNA levels were normalized to β-actin levels. Results are presented as mean ± SEM.

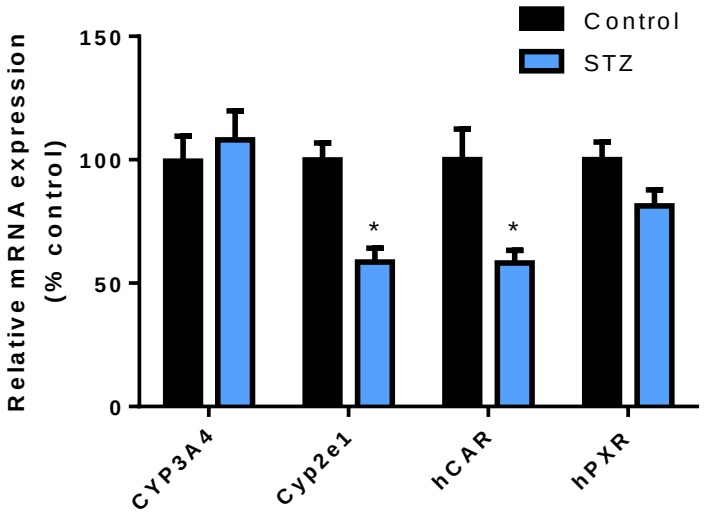

Figure 3.10. Mean relative hepatic mRNA levels of CYP enzymes and transcription factors in a pooled cohort female TgCYP3A4/hPXR/hCAR mice treated with STZ (*n*=15) or vehicle (*n*=15). Experiment was performed in triplicate and mRNA levels were normalized to β-actin levels. Results are presented as mean ± SEM.

3.5 CYP Protein Expression

Relative protein expression of Cyp3a11 showed a non-significant decrease of 27% in STZ-treated male C57BL/6 mice compared with controls (Figure 3.11). On the other hand, relative protein expression of CYP3A4 showed non-significant increase of 70% in STZ-treated female TgCYP3A4/hPXR/hCAR mice compared with control mice (p=0.07; Figure 3.12).

3.6 Enzymatic Metabolism of Testosterone

The formation of 6-β-hydroxytestosterone from testosterone was used as an indicator of CYP3A metabolic activity, as detailed previously (Chovan et al., 2007; Löfgren et al., 2004). Table 3.1 lists the average maximal rate of reaction, V_{max}, as well as the Michaelis constant (concentration of substrate at which half-maximal reaction rate is achieved), K_M, for STZ-treated (n=6) and control (n=5) male C57BL/6 mouse liver microsomes. The average V_{max} and K_M were 13 625 pmol/min/mg and 79.6 µM testosterone, respectively, for STZ-treated male C57BL/6 mouse microsomes (Table 3.1). This was not significantly different from the average V_{max} and K_M of controls, which were 12 736 pmol/min/mg and 69.6 µM, respectively (Table 3.1). A Michaelis-Menten graph of metabolite formation shows characteristically similar curves between microsomes from STZ-treated mouse livers and controls (Figure 3.13).

Table 3.2 lists the average V_{max} and K_M for liver microsomes from STZ-treated and control female TgCYP3A4/hPXR/hCAR mice. The average V_{max} for STZ-treated mouse liver microsomes was 24 587 pmol/min/mg protein, which was significantly higher than the control average of 16 262 pmol/min/mg protein (p<0.05; Table 3.2). However, the average K_M for STZ-treated mouse liver microsomes was not significantly different from control at 49.07 vs 46.18 µM, respectively (Table 3.2). A Michaelis-Menten graph of metabolite formation shows increased 6-β-hydroxytestosterone formation by STZ-treated mouse liver microsomes compared to controls (Figure 3.14).

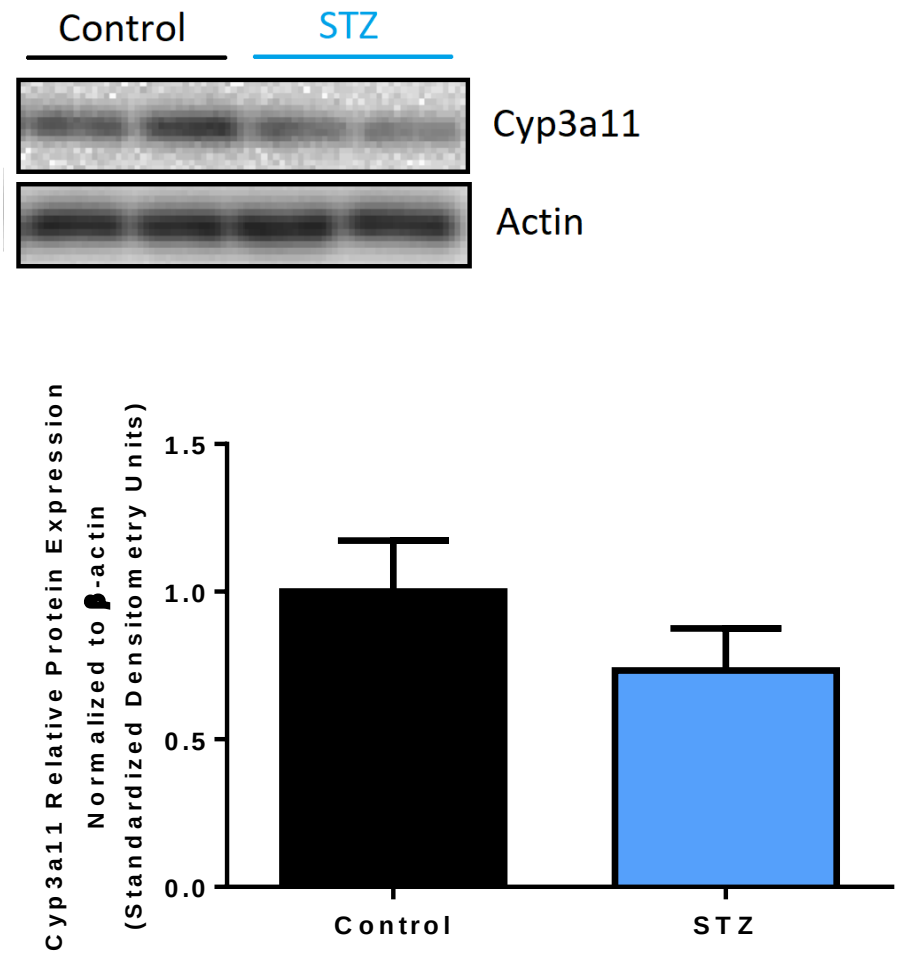

Figure 3.11. Mean relative hepatic Cyp3a11 protein expression for male C57BL/6 mice treated with STZ (*n*=13) or vehicle (*n*=13). Bands were analyzed via densitometry using Quantity One 4.6.9 (Bio-Rad Laboratories Ltd) and normalized to β-actin. Results presented as mean ± SEM.

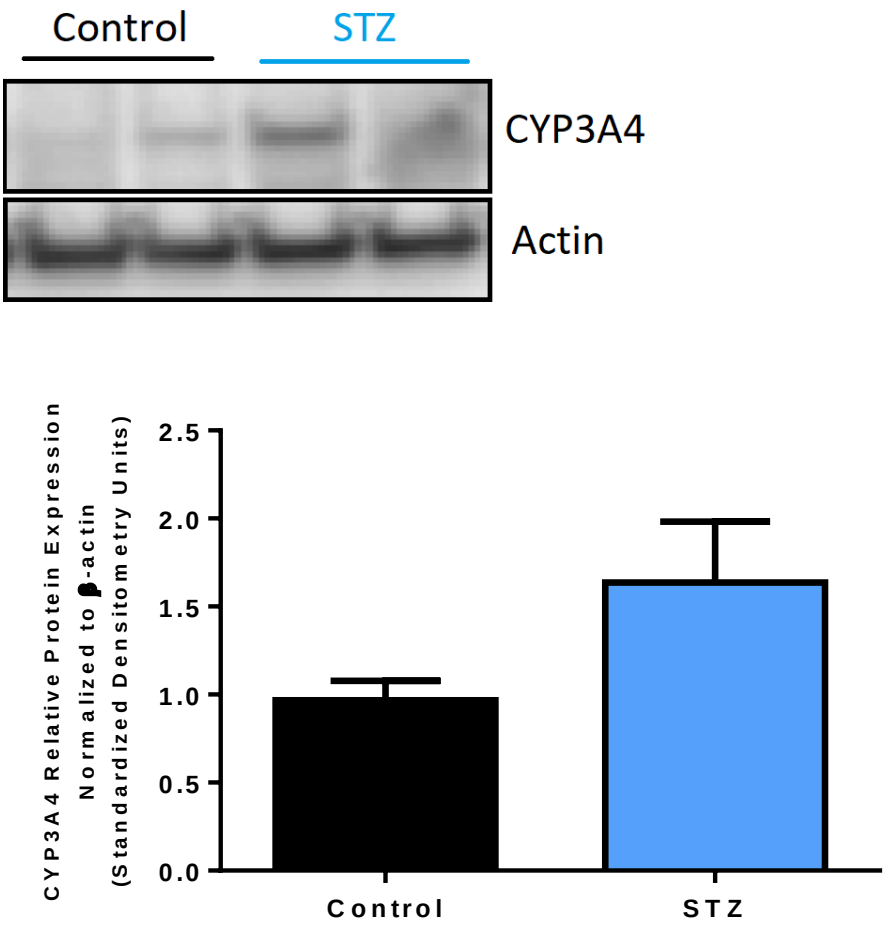

Figure 3.12. Mean relative hepatic CYP3A4 protein expression in a pooled cohort of female humanized TgCYP3A4/hPXR/hCAR mice treated with STZ (*n*=15) or vehicle (*n*=15). Bands were analyzed via densitometry using Quantity One 4.6.9 (Bio-Rad Laboratories Ltd) and normalized to β-actin. Results presented as mean ± SEM.

Table 3.1. V_{max} and K_m values for metabolism of testosterone to 6-β-hydroxytestosterone (pmol/min/mg protein) by STZ-treated (*n*=6) or vehicle-treated (*n*=5) male C57BL/6 mouse liver microsomes after incubation with 1 mM NADPH in a 5-minute reaction. Experiment was performed in duplicate and results presented as means ± SEM.

	Control	STZ	*P*-value
V_{Max} (pmol/min/mg protein)	12 736	13 625	0.8386
K_M (μM)	69.6	79.6	0.6628

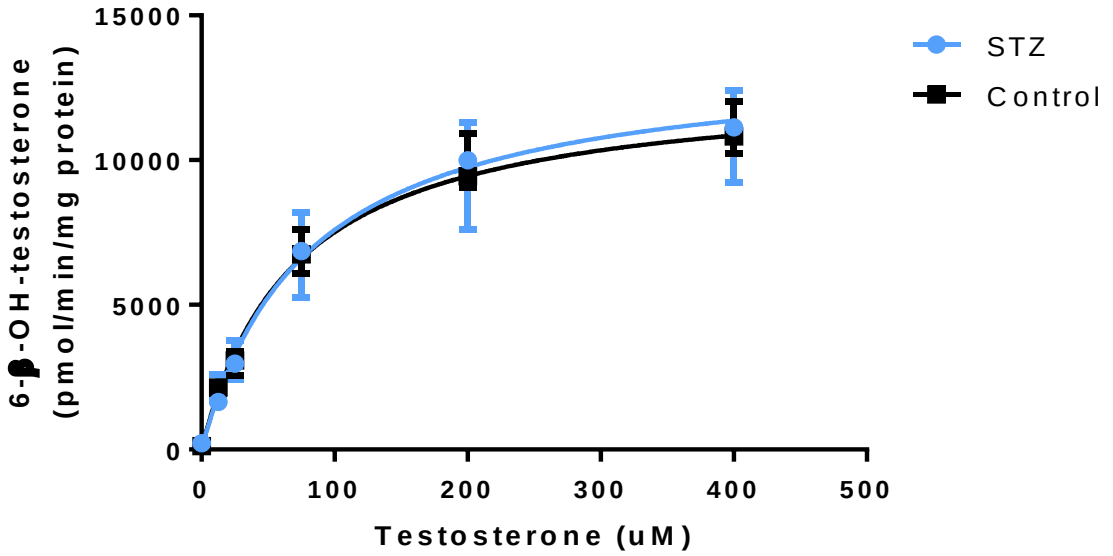

Figure 3.13. Michaelis-Menten fit of the metabolism of testosterone to 6-β-hydroxytestosterone (pmol/min/mg protein) by male C57BL/6 mice. Mice were treated with STZ (*n*=6) or vehicle (*n*=5) and isolated liver microsomes were incubated with 1 mM NADPH in a 5-minute reaction. Experiment was performed in duplicate and results presented as mean ± SEM.

Table 3.2. V_{max} and K_m values for metabolism of testosterone to 6-β-hydroxytestosterone (pmol/min/mg protein) by STZ-treated (*n*=15) or vehicle-treated (*n*=15) female TgCYP3A4/hPXR/hCAR mouse liver microsomes after incubation with 1 mM NADPH in a 5-minute reaction. Experiment was performed in duplicate and results presented as means ± SEM.

	Control	STZ	*P*-value
V_{Max} (pmol/min/mg protein)	16 262	24 587	0.0394*
K_M (μM)	46.2	49.1	0.3626

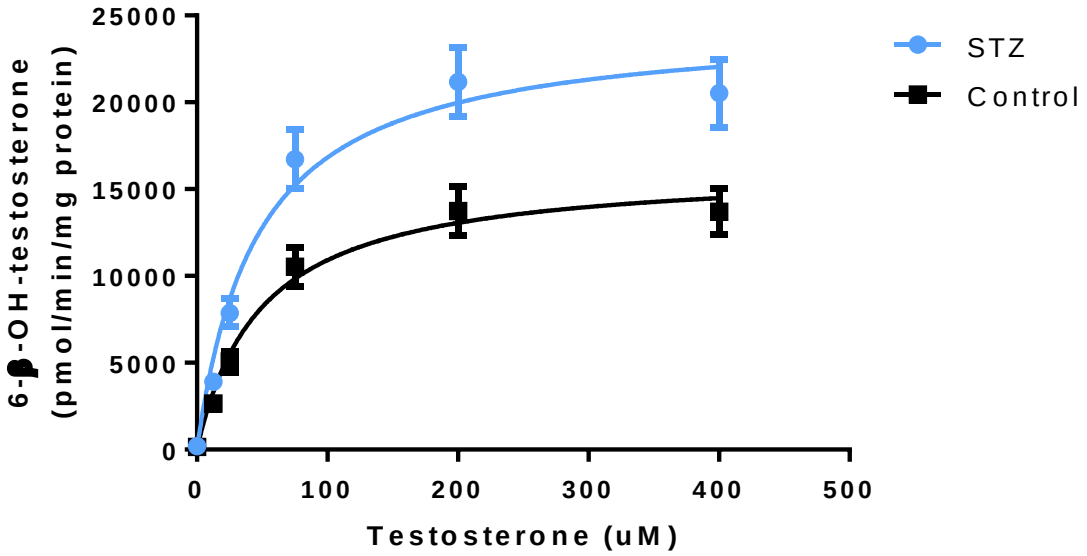

Figure 3.14. Michaelis-Menten fit of the metabolism of testosterone to 6-β-hydroxytestosterone (pmol/min/mg protein) by a pooled cohort of female TgCYP3A4/hPXR/hCAR mice. Mice were treated with STZ (*n*=15) or vehicle (*n*=15) and isolated liver microsomes were incubated with 1 mM NADPH in a 5-minute reaction. Experiment was performed in duplicate and results presented as mean ± SEM.

3.7 Hepatic Uremic Toxins

Five uremic toxins were evaluated in livers of female TgCYP3A4/hPXR/hCAR mice: hippuric acid, indoxyl sulfate, *p*-cresol sulfate, and *p*-cresol glucuronide. Of these five, only hippuric acid, indoxyl sulfate, and phenyl sulfate were detected. Average concentrations for hepatic uremic toxins were 2.41, 2.50, and 3.75 µM for hippuric acid, indoxyl sulfate, and phenyl sulfate, respectively (Figure 3.15). These were not significantly different from control averages of 2.09, 2.20, and 3.80 µM, respectively (Figure 3.15).

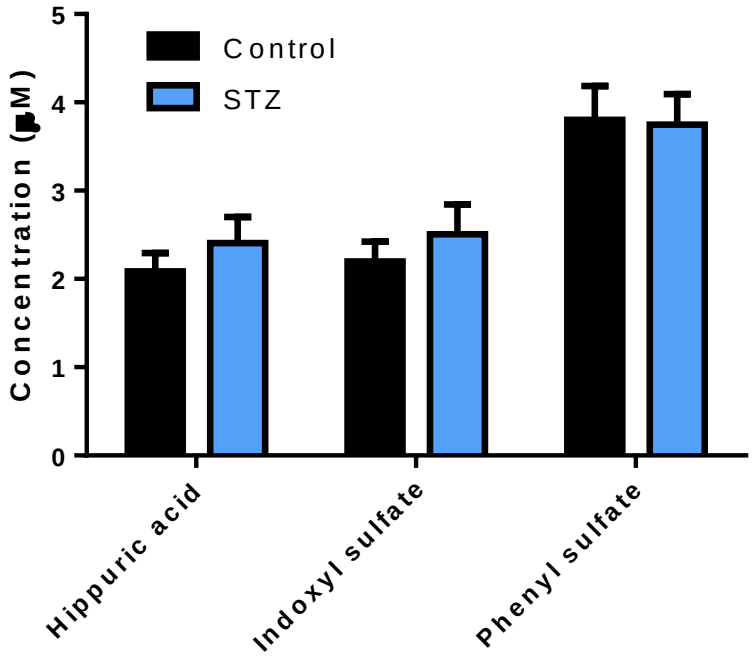

Figure 3.15. Concentrations of uremic toxins hippuric acid, indoxyl sulfate, and phenyl sulfate in liver homogenate from a pooled cohort of female TgCYP3A4/hPXR/hCAR mice treated with STZ (*n*=15) and with vehicle (*n*=15). Results presented as means ± SEM.

Chapter 4

4 Discussion

4.1 Results Summary

4.1.1 Model Validation

Male C57BL/6 mice treated with STZ showed significantly decreased gains in average body weight compared to vehicle-treated controls for 4 weeks prior to sacrifice. Body weight increases were not unexpected as mice were relatively young (7-8 weeks of age) at start of the study as recommended by methodological guidelines (Tesch & Allen, 2007). The significant decrease in body weight is likely a result of prolonged severe type 1 diabetes, inefficient glucose uptake into tissues, and initiation of catabolic processes, which are standard characteristics of this model (Imagawa et al., 1999; Kharroubi & Darwish, 2015; Yu et al., 2000). The first cohort of STZ-treated humanized female mice also exhibited decreased body weight compared to controls, indicating the presence of severe diabetes and the possibility of impaired glucose utilization. However, the second cohort of STZ-treated humanized female mice displayed no differences in body weight compared with controls despite pronounced hyperglycemia. This finding, however, does not preclude the presence of diabetes, as STZ-induced diabetic mouse models may also exhibit no differences in body weight compared with control animals (Diabetic Complications Consortium, 2003). Both female STZ-treated cohorts were given 2 × 5 total 50 mg/kg daily consecutive intraperitoneal STZ injections due to initial resistance to hyperglycemia (see Section 4.2). However, the second cohort received both injections within 6 weeks, whereas the first cohort received second set of injections up to 19 weeks after the first (due to protocol limitations). Thus, it is possible that the first cohort of STZ-treated female mice may have had more time to develop β-cell damage due to autoimmune secondary insults despite relatively low average blood glucose values, resulting in a more severe phenotype of diabetes upon additional STZ treatment.

Blood glucose values were significantly elevated in STZ-treated male C57BL/6 mice for 15 weeks post-injection, indicating the presence of hyperglycemia and diabetes. Mean blood glucose fluctuated from 20–24 mmol/L during this time, although there was

significant variation between subjects (19.3–33.3 mmol/L). Despite this variation, all subjects were above the 13.9 mmol/L cut-off range, and none were excluded from the study. Female STZ-treated TgCYP3A4/hPXR/hCAR mice exhibited a greater resistance to hyperglycemia, necessitating 5 additional STZ injections (totaling 10) for both cohorts. In addition to this, weekly blood glucose monitoring showed highly dynamic fluctuations in female mice, with some subjects experiencing a ±10 mmol/L change in blood glucose between weekly measurements. Intra-group variability was also high, with blood glucose ranges from 13.0–27.0 mmol/L and 16.4–30.9 mmol/L for the first and second cohorts, respectively. A previous study has implicated the role of estradiol and estrogen receptors in the protection of pancreatic β-cells from oxidative injury (Le May et al., 2006). In this study, *in vivo* disruption of aromatase and estrogen receptor alpha (ERα) resulted in increased vulnerability to oxidative-stress induced β-cell apoptosis upon treatment with STZ, which is rescued upon treatment with estradiol (Le May et al., 2006). This was demonstrated in both male and female mice. The authors also showed that non-diabetogenic doses of STZ has only moderate effects in wild-type mice of both sexes, while it provoked a severe diabetic phenotype in ERα-knockout mice (Le May et al., 2006). Thus, it is possible that the increased amount of estrogen in female mice may have allowed increased resistance to developing hyperglycemia to the same degree as male mice, necessitating extra STZ injections.

In addition to mean blood glucose values, GTTs were used to evaluate glucose handling differences between STZ-treated and vehicle-treated mice. Both male C57BL/6 and humanized female TgCYP3A4/hPXR/hCAR STZ-treated mice showed significantly elevated blood glucose values across all time points of a 120-minute GTT compared to controls, indicating severe hyperglycemia. In addition to this, both cohorts of female STZ-treated mice had significantly elevated baseline-corrected AUC of blood glucose values, indicating significantly impaired glucose uptake. This is further characterized by the blood glucose-time graphs, in which control mice had a steeper decline in blood glucose values after administration of glucose, while STZ-treated mice experienced a longer plateau of elevated blood glucose values. Conventionally, increased AUC and decreased glucose clearance over time indicate impaired glucose uptake, and in this case, is likely due to the autoimmune destruction of pancreatic β-cells (Andrikopoulos et al., 2008).

UACR is commonly used as a measure of kidney damage due to loss of filtration barrier integrity, allowing the excretion of albumin in urine (Tuttle et al., 2014). Urinary albumin is normalized to creatinine to account for differences in urine concentration and subject hydration (Eknoyan et al., 2003). The non-significant increase in UACR of STZ-treated male C57BL/6 mice compared to controls indicates the possibility for mild damage to the glomerular filtration barrier due to kidney damage. In addition, unchanged plasma creatinine levels between STZ-treated and control male mice show that kidney damage to the point of GFR decline is not present, as plasma creatinine is expected to increase during moderate and late stage CKD because of GFR decline and an accumulation of unfiltered creatinine (Mussap et al., 2002). Thus, male STZ-treated mice showing a slight non-significant increase in UACR with no changes in plasma creatinine may be entering the early stages of DN, which characteristically include albuminuria and hyperfiltration (Gross et al., 2005). However, whether STZ-treated male mice have glomerular hyperfiltration cannot be inferred from unchanged plasma creatinine levels. On the other hand, because female STZ-treated TgCYP3A4/hPXR/hCAR mice showed no differences in plasma creatinine and UACR compared with controls, it is unlikely that these mice experienced kidney abnormalities due to hyperglycemia.

4.1.2 CYP & Transporter Expression in Diabetes & DN

Male STZ-treated C57BL/6 mice exhibited non-significant decreasing trends in *Cyp3a11*, *Cyp2c29*, and *Cyp2e1* relative mRNA levels as well as *Cyp3a11* protein expression, which does not support the initial hypothesis that mice with early DN will have decreased expression and function of hepatic CYP3A and CYP2C enzymes. It can be concluded from the results that mice with STZ-induced diabetes leading to early DN do not experience any significant changes in expression of these CYP enzymes.

CYP2E1 mRNA expression has historically been shown to be increased in humans with both types of DM, while increases in protein expression is characteristic of primarily type 2 DM patients (Wang et al., 2003). This contrasts the decrease of *Cyp2e1* mRNA levels in the current study, but studies in human subjects are often vastly different from experimental animal models. Diabetic humans regularly take drugs which alter the expression and activity of insulin, or may take insulin directly, which has been shown to dramatically alter

the expression of CYP2E1 *in vivo* (Woodcroft, Hafner, & Novak, 2002). On the other hand, diabetic animal models are not normally administered insulin. While some diabetic animal drug metabolism studies are short and do not typically exceed 1 week after induction of diabetes, mice in the current study were diabetic for at least 15 weeks for male C57BL/6 mice and 3 weeks for female TgCYP3A4/hPXR/hCAR mice. The extended period of diabetes could lead to the production of ketone bodies, of which acetoacetate has been shown to upregulate CYP2E1 protein expression but downregulate mRNA expression in cultured rat hepatocytes (Abdelmegeed, Carruthers, Woodcroft, Kim, & Novak, 2005). In the current study, decreases in average body weight of diabetic male mice could also indicate fat breakdown in part leading to the production of ketones (Laffel, 1999). One critical point is that the authors did not examine the effects of 3-hydroxybutyrate, an equally prominent ketone in diabetes, on CYP2E1 expression (Garber, Menzel, Boden, & Owen, 1974). The nature of *in vivo* CYP2E1 alteration in long-term diabetic experimental animal models remains unknown. While CYP enzymes are generally reduced in mouse models of CKD, *Cyp2e1* typically shows no differences in protein and mRNA levels (Dani et al., 2010).

Numerous studies and reviews have previously shown that non-renal drug metabolism is impaired in CKD and involves the downregulation of Phase I and Phase II drug-metabolizing enzymes (Dani et al., 2010; Feere, Velenosi, & Urquhart, 2015; Guévin et al., 2002; Naud, Nolin, Leblond, & Pichette, 2012; Nolin, 2008; Velenosi et al., 2012). It is also known that the degree of downregulation of CYPs corresponds to the severity of CKD phenotype (Hartjes, 2017; Velenosi et al., 2012). The possible mechanisms by which this occurs are still relatively unknown. Early experiments showed that incubation of rat hepatocytes with uremic serum from CKD patients resulted in decreased mRNA and protein expression of many CYP families, including CYP3A and CYP2C (Guévin et al., 2002). Mediators in the uremic serum, such as indoxyl sulfate and *p*-cresol, are likely implicated in CYP3A and CYP2C downregulation, as shown in studies which examined their effects on drug transport and metabolism (Guévin et al., 2002; Reyes & Benet, 2011; Tsujimoto et al., 2010). Indoxyl sulfate is known to activate NF-κB, a commonly upregulated marker of inflammation and disease which has been shown to inhibit transcriptional activity of CYPs through protein-protein interactions with cofactors such as

RXR (Gu et al., 2006). However, uremic toxins are not the only source of NF-κB activation, and NF-κB is not the only regulator of CYP expression. Downregulation of drug-metabolizing enzymes have been demonstrated in many inflammation studies which implicate the actions of IL-1, IL-6, and TNF-α (Morgan et al., 2008). Although DN is primarily an inflammatory disease, it is possible that the severity of inflammation in early DN does not reach significant levels to produce significant CYP expression changes in tissues outside of the kidney. In addition, PKC-mediated phosphorylation of CYP enzymes, particularly CYP3A, mediates their ubiquitin-dependent degradation, and PKC has been shown to be upregulated in DN (Anderson, McGill, & Tuttle, 2007). These factors could explain the slight non-significant downregulation of *Cyp3a11* and *Cyp2c29* mRNA levels, as well as a slight non-significant decrease of Cyp3a11 protein expression in the liver of male STZ-treated C57BL/6 mice.

Humanized TgCYP3A4/hPXR/hCAR female STZ-treated mice exhibited no changes in CYP3A4 mRNA level but a significant decrease in hCAR and a non-significant decrease in hPXR mRNA levels. In addition to this, hepatic CYP3A4 protein expression showed a non-significant increase in STZ-treated mice. This represents a possible disconnect between transcription and activity of nuclear receptors, as hCAR mRNA is decreased but there are no significant changes in transcription of CYP3A4. This is also possible for CYP3A4, as mRNA level does not show any changes, but protein expression exhibits a non-significant increase. The disconnect between transcription and activity of nuclear receptors could be explained by regulation mechanisms of PXR and CAR. Protein-protein interactions with cofactors, as well as post-translational modifications such as acetylation and phosphorylation, have been implicated in PXR and CAR activity (Li & Wang, 2010; Oladimeji, Cui, Zhang, & Taosheng, 2016). Some of these interactions are not encompassed by the humanized mouse model: For instance, protein kinase A has been shown to synergistically interact with PXR to induce CYP3A expression in mouse hepatocytes, whereas it represses CYP3A expression in rat and human hepatocytes (Lichti-Kaiser, Xu, & Staudinger, 2009). Unfortunately, it is unknown what effect could be produced by interaction of mouse PKA with human PXR. In addition, it is unknown how these proteins are altered under diabetic conditions. Also, experimental drug metabolism studies in diabetes have reported conflicting results for the expression of CYP3A4 and its

orthologues. Studies in diabetic humans have shown significantly reduced hepatic CYP3A4 mRNA level and protein expression, while studies in type 2 diabetic rats have shown upregulated hepatic CYP3A2 (Dostalek et al., 2011; Oh et al., 2012). However, there are no studies which examine the expression of CYP3A4 in a humanized streptozotocin-induced diabetic mouse model. The STZ-induced model of diabetes also has a greater resemblance to type 1 DM. From these results, it may be possible that type 1 diabetics experience no significant changes in protein expression of CYP3A4 and PXR, but decreased CAR mRNA level.

In mice, *Oatp1a1, Oatp1b2,* and *Ntcp* are responsible for hepatic basolateral uptake of xenobiotics, while *Mrp3* mediates basolateral efflux. The entry of xenobiotics into hepatocytes, mediated by OATPs and NTCP, and inhibited by MRPs, is a major rate-limiting factor of non-renal drug clearance and drug metabolism (Shitara & Sugiyama, 2002; Sun et al., 2006; Yamazaki, Akiyama, Nishigaki, & Sugiyama, 1996). Thus, any changes in expression of these proteins may potentially exacerbate altered drug pharmacokinetics in CKD. However, mRNA levels of hepatic uptake and efflux transporters, including *Oatp1a1, Oatp1b2, Ntcp,* and *Mrp3* were unchanged in STZ-treated male C57BL/6 mice compared with controls. Therefore, altered drug pharmacokinetics in mouse models of early DN are unlikely to be affected by hepatic basolateral uptake and/or efflux transporters.

4.1.3 CYP Function in Diabetes & DN

Male C57BL/6 STZ-treated mouse liver microsomes showed no differences in the rate of conversion of testosterone into 6-β-hydroxytestosterone compared to control mice as represented by similar V_{Max} values. This implies that there are no substantial changes in *Cyp3a11* maximal metabolic activity in early DN, which does not support the initial prediction of decreased *Cyp3a11* metabolic activity. In addition to this, K_M values were not significantly different, indicating that the substrate affinity of *Cyp3a11* does not significantly change in early DN. This also precludes net effects of possible regulators on *Cyp3a11* activity and affinity, which may have previously been responsible for changes in *Cyp3a11* mRNA level and protein expression. Studies have shown that microsomal CYP activity is downregulated by uremic toxins such as indoxyl sulfate and *p*-cresol (Tsujimoto

et al., 2010). In this study, uremic toxin levels were unchanged, implying that the accumulation of uremic toxins in diabetes is not substantial, and that CYP activity is likely unaffected as a result.

Female humanized STZ-treated TgCYP3A4/hPXR/hCAR mice displayed an increase in V_{Max} of 6-β-hydroxytestosterone formation from testosterone, but no differences in K_M, indicating that the metabolic activity of CYP3A4 in diabetes is increased with no changes in substrate affinity. This is consistent with the non-significant increase in CYP3A4 protein expression. Hepatic levels of hippuric acid, indoxyl sulfate, and phenyl sulfate showed no significant differences between treatment groups; thus, it is unlikely that these toxins are implicated in the upregulated CYP3A4 metabolic activity of STZ-treated mice. Increased CYP3A4 activity in these mice, despite no changes in expression, leaves few explanations: Changes in post-translational modifications may alter total protein content and activity. For instance, CYP3A4 ubiquitin-dependent degradation is initiated by phosphorylation of several amino acid residues mediated by PKC (Wang et al., 2009). Also, PKA has previously been implicated, by interaction with nuclear receptors, in the synergistic up-regulation of CYP3A4 transcription in mouse hepatocytes (Lichti-Kaiser et al., 2009). An increase in CYP3A4 expression and function despite decreases in hCAR mRNA levels could be explained by a simultaneous increase of PKA-mediated phosphorylation of nuclear receptors, as well as a decrease in PKC-mediated ubiquitination of CYP3A4. However, PKC is increased in DN, and it is likely that activation occurs starting in early diabetes; therefore, this pathway is not likely to be responsible for CYP3A4 protein stability under these conditions (Anderson et al., 2007).

4.2 Limitations

It is important to recognize the limits of interpretation and consider the methodological contexts under which results from this study were obtained. The following section lists some examples of these limitations and, where applicable, provides alternatives for amelioration in future studies.

4.2.1 Methodological Limitations

The STZ-induced model of rodent diabetes is widely used for its ease of use, cost effectiveness, and relatively straightforward generation of hyperglycemia. Since other diabetic mouse models often involve costly genetic manipulations (such as the *db/db* mouse) or strict experimental conditions (such as high-fat diets), the STZ-induced mouse model of diabetes is frequently used (Deeds et al., 2011a). As of September 2018, a general keyword search on NCBI PubMed using "streptozotocin diabetes" yields over 54,000 results. However, the model, as well as the results obtained from experimentation, contain limitations. Firstly, STZ treatment is a form of chemically induced diabetes which relies on secondary autoimmune insulitis following an initial pancreatic insult (M Nukatsuka et al., 1988; Tesch & Allen, 2007). This is more representative of autoimmune destruction of pancreatic β-cells observed in type 1 DM, which comprises a relatively small proportion of diabetics overall (Dean, 2012; International Diabetes Federation, 2017). In addition, STZ is transported into cells by GLUT2, which is found most abundantly on pancreatic β-cells but also found in the liver and kidney (Imaeda, Kaneko, Aoki, Kondo, & Nagase, 2002; Szkudelski, 2001). Should any cellular damage occur to hepatocytes, this may cause unintended decreases of hepatic CYP and transporter expression and activity. In addition to this, any significant damage to kidney tubular cells may confound the effects of hyperglycemia on kidney damage. Measurement of liver biomarkers, such as serum aspartate aminotransferase (AST) as well as alanine aminotransferase (ALT), should be performed to ensure there are no general liver alterations which may be induced by off-target STZ effects. For instance, a recent study found significantly elevated serum AST and ALT in STZ-induced diabetic rats compared with control rats (Alkan & Celik, 2018). However, it remains difficult to determine whether serum AST and ALT elevations are due to STZ-induced hepatic injury or hyperglycemia. In addition to molecular biomarkers, abnormalities may be detected by pancreas, liver, and kidney histology. Ideally, an STZ-induced mouse model of diabetes should produce pancreatic islet cell destruction with no histological changes in liver or kidney.

In addition to potential off-target effects, STZ has been shown to induce varying degrees of hyperglycemia between individuals (Deeds et al., 2011a). Despite being widely used in

scientific literature, many investigators experience an average subject drop-out rate of 10% in the absence of extra STZ injections (Tesch & Allen, 2007). Some investigators have reported that similar STZ doses in age, sex, and strain-matched rodents have proven to be inert to some and fatal to others, and this may be exacerbated in animals provided from different suppliers (Deeds et al., 2011b). This effect was observed in the current study, with highly variable blood glucose values in both male and female STZ-injected mice. In addition, female mice were highly resistant to STZ effects, and 5 extra injections were necessary to induce diabetes in these animals. Since a fixed number of STZ injections were approved for each mouse, protocol approval for extra injections resulted in variable time periods between initial and extra injections. Therefore, although all mice were diabetic by the end of the study term, the degree of pancreatic damage may be altered between STZ-injected cohorts of female mice. This further highlights the importance of pancreatic histology to ensure similar degrees of pancreatic damage has occurred within STZ-injected mice. The resistance to hyperglycemia by female mice may be due to the protective effect of estrogen on pancreatic β-cells, as detailed in Section 4.1.1 (Le May et al., 2006).

The primary method of assessing diabetes in the current study was spot blood glucometry using glucose strips and meter intended for humans (Contour Next One). Glucometers read glucose from a 5–20 µL drop of blood, assuming a 50% hematocrit, by measuring the oxidation of glucose and subsequent generation of an electric current. To this end, conventional glucose strips are coated with glucose dehydrogenase (Kang, Kim, Jeong, Choi, & Park, 2016). However, the Contour Next One glucometer is not designed to measure extremely high levels of blood glucose, and some subjects from the male C57BL/6 STZ-injected group had blood glucose values above the 33.3 mmol/L detection limit of the glucometer. This problem becomes especially important when male C57BL/6 mice are administered GTTs, leading to blunted AUC peaks and inaccurate results from statistical tests of conventional baseline-normalized AUC between STZ-treated and control-treated groups. A possible solution would be to use veterinary glucometers, such as the AlphaTrak® or CERA-PET® glucometers (Abbott Laboratories; Abbott Park, IL; Ceragem Medisys; Seoul, Korea), which have linear ranges between 1.1–41.7 and 0.6–50.0 mmol/L, respectively (Kang et al., 2016). These glucometers are shown to be more accurate in animals than human-intended glucometers when compared to laboratory glucose

hexokinase tests (Kang et al., 2016). However, the accuracy of these glucometers has not been tested specifically in mice. As additional measures of diabetes, food and water intake should be closely monitored along with body weight. This is important because diabetics often experience polyphagia and polydipsia, altering their nutrition and hydration levels (American Diabetes Association, 2010). By keeping track of these factors, we may potentially eliminate them as confounding variables, as concentration of urine as well as hepatic CYP expression are affected by hydration and fasting, respectively.

The estimation of kidney function using plasma creatinine measurements was previously shown to be accurate when using UPLC-MS/MS methods of detection but not the conventional alkaline picrate (Jaffé reaction) method (Dunn et al., 2004). Despite the relative robustness of plasma creatinine as an indicator kidney filtration, this is still an estimation of GFR, and not a true measure of GFR. Current clinical eGFR formulas, such as CKD-EPI, Cockcroft-Gault, and MDRD account for this; however, plasma creatinine alone does not (Munikrishnappa, 2009). In addition to this, eGFR cannot be reliably measured in mice since these formulas are calibrated for humans and thus account for adjustments in human differences. Fortunately, for rodents, there exist more accurate methods of measurement which allow investigators to determine real GFR. One such method involves the use of fluorescein isothiocyanate (FITC)-inulin, which overcomes many disadvantages of plasma creatinine (Dunn et al., 2004; Qi et al., 2004). Firstly, up to 40% of creatinine excretion is due to tubular secretion, a value which is especially higher in rodents, and is greatly increased during renal injury (Darling & Morris, 1991; Eisner et al., 2011; Levey, Perrone, & Madias, 1988; Vallon et al., 2012). Secondly, creatinine levels in the blood can be influenced by age, sex, diet, and muscle mass (Munikrishnappa, 2009). As age increases, excretion of creatinine decreases, leading to an underestimation of GFR. Males tend to have higher plasma creatinine levels than females, and individuals with a greater muscle mass have markedly increased plasma creatinine levels (Munikrishnappa, 2009). Thirdly, there is a high range of calibration bias across laboratories which measure creatinine (Munikrishnappa, 2009). Some may choose to use HPLC, which entails validation tests, or the alkaline-picrate method, which overestimates creatinine values due to the high number of interfering chromogens (Dunn et al., 2004; Meyer, Meyer, Gray, & Irwin, 1985). Using FITC-inulin rectifies these issues: Firstly, inulin is freely filtered and

not secreted, reabsorbed, or metabolized to any extent (Chasis, Ranges, Goldring, & Smith, 1938). Secondly, it is an exogenously administered substance, which affords it reliability due to the elimination of bias by endogenous production or breakdown. Finally, it can be detected by measuring fluorescence using standardized spectrophotometric equipment, such as the Nanodrop 3300, which reduces inter-assay variability (Rieg, 2013). The use of FITC-inulin would therefore yield an accurate and true value of GFR. Investigators have already developed a high-throughput method for the use of FITC-inulin in mice (Rieg, 2013).

4.2.2 Species Differences

To circumvent differences in species, a humanized TgCYP3A4/hPXR/hCAR female mouse model of diabetes was utilized in the current study. For instance, despite PXR and CYP3A enzymes having a wide substrate selectivity in both mice and humans, these proteins are differentially regulated in each species (Stanley, Horsburgh, Ross, Scheer, & Roland Wolf, 2006; Xie et al., 2000). In addition to this, mice have eight *Cyp3a* gene isoforms whereas humans have three, and it is unknown to what extent additional mouse *Cyp3a* isoforms may alter the metabolism of probe drugs such as testosterone (Nelson et al., 2004). However, the humanized TgCYP3A4/hPXR/hCAR mouse model is not a perfect predictor of human regulation of CYP3A4 in diabetes. Firstly, expression of the human knock-in genes is influenced by the mouse promoter and enhancer regions. Other factors responsible for altering CYP expression include hormonal regulation, epigenetics, and nutrition intake, among others (Dannenberg & Edenberg, 2006; Jaffe, Turgeon, Lown, Demott-Friberg, & Watkins, 2002; Lahuna et al., 2000; Shi et al., 2018). For instance, growth hormone is a regulator of CYP3A4 expression (Waxman & Holloway, 2009). Early studies have proposed that despite relatively similar amino acid sequences between growth hormone in mice and humans, species-specific binding to these hormones is due to the different hindering effects from the microenvironment (Nicoll, Tarpey, Mayer, & Russell, 1986). In addition to this, humans have different dietary and xenobiotic composition from the average laboratory rodent, which may contribute to differences of CYP3A expression (Shi et al., 2018). There are also many mouse genes involved in drug disposition, such as hepatic transporters and Phase II enzymes, which may alter pharmacokinetics

independently of human CYP3A4 in these mice. In short, humanized mice provide a better representation of human drug metabolism by the specific humanized gene(s); however, our understanding of whole-system *in vivo* drug disposition is still poorly understood. In addition, care should be taken to prevent drawing inappropriate predictions of drug disposition from experimental models, despite the degree of genetic modification.

In addition to biological differences, environmental factors which affect experimental mice are also very different from human patients. Mice live in experimentally controlled conditions with little environmental influence throughout experimental duration. This is relatively short (weeks to months) compared to the progression of human DN, which may develop over decades (Heinz-Taheny et al., 2018). During this time, disease may be influenced by comorbidities such as a high-fat diet, blood pressure, pregnancy, ADEs, and polypharmacy, among many others (Lim, 2014). In addition to comorbidities, diet is also an important factor in CYP regulation. A recent study showed that meat-based diets caused a reduction in hepatic protein levels of CYP enzymes, GST, UGT, and SULT compared to casein- and soy-based diets in rats (Shi et al., 2018). While humans may consume more meat in their daily lives, casein and soy are the primary sources of protein for experimental rodents. Environmental differences between humans and rodents are impossible to replicate in experimental conditions and may be significant causes and/or results of changes in drug disposition. These environmental factors may also contribute to contradictory results of CYP expression studies in humans.

In summary, the greatest limitation of species-specific differences in drug-metabolizing enzyme expression is that clinical conditions, biological differences, and environmental variables cannot be fully replicated. It may simply be that the ideal experimental population comprises the patients themselves. To this end, large-scale clinical studies may be more effective. Observational studies should be performed with the goal of producing reasonable grounds for experimental intervention. This, however, does not undermine the importance of animal experimentation. It is difficult to measure CYP expression in live human patients as the extraction of liver samples is invasive and unethical. Thus, rodent studies are essential for the elucidation of CYP regulation mechanisms in pathological conditions. Simply put, there are many future steps we must take before we can make concrete

conclusions regarding human CYP regulation in clinical conditions, unless observations are made directly *in vivo*.

4.3 Future Studies

The current study evaluated the expression of CYP enzymes under specific experimental conditions. As highlighted in this thesis, diabetic patients may be extremely diverse in terms of genetics, physiology, disease types, comorbidities, and treatment therapies. Interested investigators should fully characterize changes in drug disposition by addressing these differences, with the goal of furthering our knowledge of personalized medicine and reducing ADE risk for patients. To this end, there are several avenues of research.

Firstly, most diabetic patients have type 2 DM, characterized by obesity, overeating, and insulin resistance (American Diabetes Association, 2010). The model utilized in this study entails a chemically-induced pancreatic β-cell destruction followed by secondary autoimmune insulitis, which is more representative of type 1 DM (Devendra et al., 2004). To examine whether drug metabolism by CYP enzymes is altered in a type 2 DM model, one may utilize a type 2 DM model such as the *ob/ob* or *db/db* mouse. Another mouse model is the TTRhRen mouse, which contains a transgenic insertion of human renin cDNA under the control of the transthyretin promoter (Thibodeau et al., 2014). This mouse is used as a model of angiotensin-dependent hypertension and has been used in addition to STZ injections to generate a hypertensive-diabetic model of DN (Thibodeau et al., 2014).

Secondly, this study has only characterized expression and functional changes in CYP enzymes. However, Phase II drug metabolizing enzymes, such as SULT, UGT, NAT, GST, and MT, may substantially contribute to drug metabolism, and greatly increase the excretion of drugs (Jancova, Anzenbacher, & Anzenbacherova, 2010). Phase II enzymes have received less attention from experimental studies, in part because drug interactions involving these enzymes are relatively rare (Jancova et al., 2010). Despite this, changes in Phase II enzymes can alter levels of drugs above or below the therapeutic window; in addition, they may also cause the formation of toxic metabolites (Jancova et al., 2010; Meyer, 1996). Transporters, sometimes referred to as Phase III enzymes, also alter the levels of drug in the body, and have been implicated in many clinically significant DDIs

(Shitara & Sugiyama, 2002). Transporters control the metabolism of substrates by allowing their uptake into metabolically active tissues such as liver (Shitara & Sugiyama, 2002; Yamazaki et al., 1996). In addition to this, kidney transporters facilitate renal secretion and reabsorption of drugs (Lote, 2013).

Thirdly, additional experiments should aim to elucidate mechanistic causes of CYP regulation. Diet studies using animal protein, casein, and soy protein have shown that animal protein consumption causes reductions in CYP2D and CYP2C, GST, UGT, and SULT compared to casein and soy diets in rat liver (Shi et al., 2018). In addition to this, meat-based protein diets caused an increase in some inflammatory factors, including MAP-1, T-kininogen 2, and galectins, while decreasing others such as ICAM-1 and C-reactive protein (Shi et al., 2018). Inflammatory factors are known to alter CYP expression to a large extent (Gu et al., 2006; Morgan et al., 2008). While diet studies are relatively straightforward, *in vivo* studies of diabetes and DN are time-consuming and costly. Investigators may consider the use of *in vitro* hepatocytes, hepatic microsomes, and renal epithelial cells for preliminary experimentation when examining novel mechanisms of CYP regulation. The current study showed decreased hCAR while CYP3A4 mRNA levels did not change, and protein expression was increased. One may use ChIP to elucidate whether the recruitment of nuclear receptors to the CYP3A4 promoter region may be decreased when hepatocytes are treated with hyperglycemia (Velenosi et al., 2014). ChIP can be performed on hepatocytes subjected to different experimental conditions; namely, hepatocytes may be incubated with growth hormone, PTH, inflammatory cytokines, or insulin, all of which have previously been shown to alter hepatic CYP expression (Gu et al., 2006; Lahuna et al., 2000; Michaud et al., 2006; Morgan et al., 2008; Woodcroft et al., 2002). In addition, *in vitro* experimentation allows investigators to better examine the effects of two or more of these conditions together, while excluding the others. Additional aspects of the model, such as whether there is a significant reabsorption of STZ in kidney proximal tubular cells, may be elucidated.

Proposed studies thus far have focused on investigating mechanistic causes of drug disposition changes, identifying changes in all systems related to drug disposition, and conducting experiments in more representative models. Finally, it may be necessary to

assess whether the amelioration of diabetes and DN may also ameliorate changes to these parameters. In patients there may be a feedback loop between pharmacological treatment of diabetes and DN and changes in drug disposition. For instance, insulin is a common pharmacological treatment for diabetics. Although insulin has been shown to alter hepatic CYP expression, it remains unclear whether the reduction of hyperglycemia will normalize altered drug disposition in diabetes (Woodcroft et al., 2002). For early DN, the sodium glucose linked transporters (SGLT) have been implicated as a contributor to hyperfiltration (Vallon, Blantz, & Thomson, 2003). Investigators proposed that glomerular hyperfiltration in early DN is caused by an increase in proximal tubule reabsorption (Bank & Aynedjian, 1990; Vallon et al., 2003). This is caused by increases in mRNA levels of SGLT proteins, as well as increased sodium reabsorption secondary to glucose reabsorption (Vestri et al., 2001). In addition, kidney growth during early DN increases net proximal reabsorption (Thomson et al., 2001). The reabsorbed sodium causes increase in blood pressure as well as an increase in tubulo-glomerular feedback, which cause increases in GFR (Vallon et al., 2003). SGLT inhibitors, such as empagliflozin, have increased glucosuria and reduced glomerular hypertrophy, inflammation, mesangial matrix expansion, and albuminuria in early DN (Gembardt et al., 2014). However, the effect of SGLT inhibitors on drug disposition in mice and humans has not been investigated. It is imperative that the effects of diabetic treatments, such as insulin and SGLT inhibitors, on drug disposition be elucidated since many clinical diabetic patients commonly receive these treatments. The primary goal is the reduction of clinically significant DDIs associated with pharmaceutical treatment, especially when administered in conditions of high polypharmacy.

4.4 Relevance & Conclusions

Drug disposition in DN and CKD is one of the major areas of pharmacology on the forefront of modern research due to the high global economic cost of diabetes (Dasgupta, 2014; Zhou et al., 2017). Results from this study show that CYP-mediated drug metabolism may potentially be slightly altered in diabetes, but not early DN, and highlights the importance of personalized treatment strategies. Specifically, mRNA levels of major CYP enzymes remain unchanged in early DN, and functional activity of CYP3A4 in humans may be significantly increased in diabetes. However, interpretation of study results is

subject to methodological limitations such as model representation and species-specific differences in biology and environment. Future studies should leverage diversity in experimental models of DN and elucidate changes in additional aspects of drug disposition such as Phase II and transporter proteins. In addition, mechanistic causes for changes in these enzymes have yet to be properly characterized. Finally, pharmacological interventions in these patients and their effects on drug disposition should be evaluated to measure whether drug disposition changes may be ameliorated with disease. Understanding changes in drug disposition is the first step to reducing clinically significant ADEs involving accumulation of drugs and metabolites outside of therapeutic ranges as well as DDIs involving polypharmacy, both of which are highly common in a rapidly increasing population of diabetics (Peron et al., 2015).

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Appendices

Appendix A: Animal use approval

Western

September 10, 2009

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

Dear Dr. Urquhart:

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

has been approved by the University Council on Animal Care. This approval is valid from **September 10, 2009** to **September 30, 2010.** The protocol number for this project is **#2009-058.**

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

ANIMALS APPROVED FOR 4 Years

SPECIES & SECT D.5.1 GROUP ID#	STRAIN &/or OTHER SPECIES DETAIL For Rodents, Also Provide Vendor Stock #	AGE or WEIGHT & SEX	4-YEAR TOTAL ANIMAL NUMBER
Rat ID# 1-36	Sprague-Dawley	200-300g, male	780
As Above ID# 37	Wistar	200-300g, male	120

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

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

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, • London, Ontario • CANADA – N6A 5C1
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Curriculum Vitae

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Presentations:

Cheng Fang, James Nugent, Emily Hartjes, Thomas Velenosi, Dylan Burger, Brad Urquhart. Expression of hepatic cytochrome P450 enzymes in mouse models of diabetic nephropathy. London Health Research Day, London, Ontario, Canada. Mar 28, 2017. (Poster)

Cheng Fang. Expression of hepatic drug metabolizing enzymes in a mouse model of diabetic nephropathy. Pre-ASN Meeting, London, Ontario, Canada. Oct 12, 2017. (Oral)

Cheng Fang, Yong Lim, Dylan Burger, Chet Holterman, Chris Kennedy, Jean-Francois Thibodeau, Brad Urquhart. Expression of hepatic cytochrome P450 enzymes in mouse models of diabetic nephropathy. American Society of Nephrology Kidney Week, New Orleans, Louisiana, United States. Oct 31–Nov 5, 2017. (Poster)

Cheng Fang, Yong Lim, Dylan Burger, Chet Holterman, Chris Kennedy, Jean-Francois Thibodeau, Brad Urquhart. Expression of hepatic cytochrome P450 drug-metabolizing enzymes in a mouse model of diabetic nephropathy. Physiology and Pharmacology Research Day, London, Ontario, Canada. Nov 7, 2017. (Poster)

Cheng Fang, Yong Lim, Dylan Burger, Chet Holterman, Chris Kennedy, Jean-Francois Thibodeau, Brad Urquhart. Expression of hepatic cytochrome-P450 drug-metabolizing enzymes in diabetes and diabetic nephropathy. London Health Research Day, London, Ontario, Canada. May 10, 2018. (Poster)

Jay Fang. Expression of hepatic cytochrome P450 drug-metabolizing enzymes in diabetes and diabetic nephropathy. Canadian Society of Pharmacology and Therapeutics Joint Annual Meeting, Toronto, Ontario, Canada. May 1–4, 2018. (Oral)

Jay Fang, James Lim, Nicholas Tonial, Dylan Burger, Chet Holterman, Chris Kennedy, Jean-Francois Thibodeau, Brad Urquhart. Expression of hepatic cytochrome P450 drug-metabolizing enzymes in diabetes and diabetic nephropathy. Canadian Society of Pharmacology and Therapeutics Joint Annual Meeting, Toronto, Ontario, Canada. May 1–4, 2018. (Poster)