CHARACTERIZATION OF THE EFFECT OF CHRONIC KIDNEY DISEASE ON HEPATIC REDUCTION: IN-VITRO AND IN-VIVO STUDIES OF WARFARIN

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CHARACTERIZATION OF THE EFFECT OF CHRONIC KIDNEY DISEASE ON HEPATIC DRUG REDUCTION: IN-VITRO AND IN-VIVO STUDIES OF WARFARIN

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Chronic kidney disease (CKD) affects the disposition of drugs by altering their renal excretion. Well-established evidence suggests that CKD also impacts the nonrenal clearance of drugs by modulating drug metabolism and transport. The overall objective of this dissertation was to investigate the effect of CKD on hepatic reduction, an important Phase I drug metabolism pathway. In order to achieve our goals, a novel analytical method was developed and validated to measure warfarin and its alcohol metabolites.

In vitro studies utilizing rat hepatic cytosol and microsomes showed significant decreases in the formation of *RS/SR*-warfarin alcohol in the CKD group compared to control, suggesting decreased reductase activity. Significantly decreased mRNA and protein expression (>30%) of selective reductase enzymes was also observed in rats with CKD. *In vitro* studies of human liver cytosol and microsomes collected from patients with end stage renal disease (ESRD) revealed a trend toward decreased formation of *RS/SR*-warfarin alcohol. A significant decrease (65%) in protein expression of carbonyl reductase 1 was observed in livers from ESRD compared to control livers. Together, the *in vitro* findings implicate transcriptionally or translationally mediated changes in reductase enzyme function in CKD.

In vivo studies assessing the effect of CKD on steady-state warfarin and warfarin alcohol disposition showed increased concentrations of *RR/SS*-warfarin alcohol along with decreased kidney function, indicating impaired renal elimination of the *RR/SS*-warfarin alcohol metabolite

in kidney disease patients. Additionally, increased free and total *S/R* warfarin observed in ESRD patients potentially suggests reduced CYP2C9 activity.

Collectively, our findings demonstrate that drug reduction is impacted by kidney function, and provide a mechanism for altered nonrenal clearance and disposition of drugs in CKD. The findings of these studies may be clinically relevant in improving the management of drug substrates of reductases, specifically warfarin, in patients with CKD. Given the high frequency with which these drugs are prescribed for CKD patients, dosing adjustments and frequent monitoring may be warranted.

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PREFACE

On my journey to complete my graduate study, I have met many people. These individuals have contributed to the successful completion of my Doctor of Philosophy degree. I would like to thank each and every one of them. First and foremost, I would like to express my sincere thanks and appreciation to my advisor Dr. Thomas Nolin for considering me to be his first graduate student. Truly, Dr. Nolin is the best guide throughout my entire graduate work, and in my life inside and outside the lab. His help, support, guidance, and expertise were extremely valuable to complete this work. The way he mentors and supervises his students in the lab is inspiring. Without Dr. Nolin's assistance, this work would have never been completed in such an impressive way.

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ABBREVIATIONS

11β-HSDs	11β-hydroxysteroid dehydrogenases
ABC	ATP binding cassette transporters
AF	Atrial fibrillation
AKR	Aldo-keto reductase
AUC	Area under the curve
BCRP	Breast cancer resistance protein
BMI	Body mass index
CBR	Carbonyl reductase
CKD	Chronic kidney disease
CLCr	Creatinine clearance
C_{max}	Maximum plasma concentration
C_{ss}	Steady-state plasma concentration
CMPF	3- carboxy-4-methyl-5-propy-2-furanpropanoic acid
CYP450	Cytochrome P450
DMEs	Drug metabolizing enzymes
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
FDA	Food and drug administration
f_e	Fraction eliminated unchanged in the urine
f_u	Fraction unbound
GC-MS	Gas chromatography with mass spectrometric
GFR	Glomerular filtration rate
HD	Hemodialysis
HR	Hazard ratio
INR	International normalized ratio
K_m	Affinity constant
LLOQ	Lower limit of quantification
MDR	Multi-drug resistance protein
MRP	Multi-drug resistance associated protein
MTBE	Methyl tert-butyl ether
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	N-acetyltransferase
NNK	4-methylnitrosamino-1-(3-pyridyl)-1-butanone
NOACs	New oral anticoagulants
NQO	NADPH-dependent quinone oxidoreductases
NSAIDs	Non-steroidal anti-inflammatory drugs

OAT	Organic anion transporters
OATP	Organic anion transporter polypeptide
OCT	Organic cation transporters
PGE	Prostaglandin E
P-gp	P-glycoprotein
PTH	Para-thyroid hormone
QC	Quality control
qRT-PCR	Quantitative real-time polymerase chain reaction
RSD	Relative standard deviation
SD	Standard deviation
SDR	Short-chain dehydrogenase/reductase
SEM	Standard error of the mean
SLC	Solute carrier transporters
$t_{1/2}$	Half-life
t_{max}	Time to reach maximum plasma concentration
UGTs	Uridine diphoshate (UDP)-glucuronosyltransferase
UPLC-MS/MS	Ultra-high performance liquid chromatography–tandem mass spectrometry
VKOR	Vitamin K epoxide reductase
V_{max}	Maximum velocity of enzymes

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 CHRONIC KIDNEY DISEASE (CKD)

1.1.1 Definition and Stages

Chronic kidney disease (CKD) is a general term that describes the progressive and heterogeneous disorders that are associated with functional and structural abnormalities of the kidney, and occur for greater than a period of three months. Two fundamental factors are used for defining CKD including the degree of kidney function (i.e., estimated glomerular filtration rate; eGFR<60 mL/min/1.73m^2) and the presence of kidney damage (i.e., proteinuria) (James et al., 2010). The National Kidney Foundation, a leading CKD advocacy organization, classifies CKD into five stages according to the degree of disease severity as indicated primarily by GFR, with increased severity for lower GFR values. **Table 1-1** presents the classification of CKD according to the eGFR values. The early stages of CKD are generally asymptomatic; however, the advanced stages are more aggressive and associated with uremia (Levey et al., 2003). The progressive decline in kidney function becomes ultimately irreversible leading to end-stage renal disease (ESRD) (eGFR<15 mL/min/1.73m^2), which requires renal replacement therapy including dialysis or kidney transplantation (Coresh et al., 2007).

Table 1-1 Classification of chronic kidney disease

CKD Stage	Description	eGFR (mL/min/1.73m^2)
1	Kidney damage with normal or elevated GFR	>90
2	Kidney damage with mildly decreased GFR	60-89
3	Moderate decrease of GFR	30-59
4	Severe decrease of GFR	15-29
5	Kidney failure	<15

Adapted from (Naud et al., 2012)

1.1.2 Risk Factors and Epidemiology

Kidney disease is the ninth leading cause of death in the United States. Several risk factors are associated with CKD and can be divided into non-modifiable and modifiable factors. The non-modifiable factors include age, race, and genetic susceptibility. Obesity, diabetes, and hypertension are the primary modifiable factors (Levey et al., 2005). Chronic kidney disease is considered a major public health problem that affects approximately 26 million Americans with a prevalence estimated to be 13% of the US population (Levey and Coresh, 2012; Weiner, 2009). The prevalence of CKD markedly increases with age from approximately 4% at age 20-39 years to about 47% in people older than 70 years, due in part, to the known decrease in kidney function with aging (Coresh et al., 2007; Zhang and Rothenbacher, 2008). Additionally, prevalence of CKD is increasing rapidly due to the epidemics of diabetes and hypertension, the two most common causes of CKD worldwide (Coresh et al., 2007). Diabetes, the primary etiology of kidney disease, accounts for about 40% of all new cases (Levey and Coresh, 2012).

1.1.3 Cardiovascular Complications and Comorbidities

CKD places patients at high risk of cardiovascular comorbidities, which is the leading cause of death in ESRD patients (Schiffrin et al., 2007; Thomas et al., 2008). CKD is associated with hemostatic defects predisposing patients to a hypercoagulable state that increases the risk for venous thromboembolism and stroke (Wattanakit and Cushman, 2009). CKD is also associated with the incidence of arrhythmias that raise the risk of stroke, morbidity, and mortality (Wetmore et al., 2013; Winkelmayer et al., 2011b). Atrial fibrillation (AF), the most common cardiac arrhythmia, independently increases the risk of ischemic stroke by 5-10-fold in hemodialysis (HD)

patients (Vazquez et al., 2009; Vazquez et al., 2003). The prevalence of AF among long-term HD patients is reported to be 13-27%, which is 10 to 20-fold higher than that in the general population (Reinecke et al., 2009). Other studies have also reported similar AF prevalence in non–dialysis-dependent CKD patients (Alonso et al., 2011; Ananthapanyasut et al., 2010; Hart et al., 2011). For example, the large population-based cohort study of Alonso et al. showed that the hazard ratio (HR) of AF is approximately 3-fold higher in patients with eGFR of 15-30 mL/min/1.73m^2 compared to individuals with normal kidney function (Alonso et al., 2011). The prevalence of AF in this observational study was 18%. Together, these data indicate that cardiovascular complications are common in patients with various stages of CKD. Because CKD is associated with thromboembolism, AF and stroke, anticoagulant use is essential for prevention and treatment of thromboembolic events in high-risk CKD patients.

1.2 EFFECT OF CKD ON PHARMACOKINETICS

Optimizing drug therapy is necessary in patients with kidney disease. Thus, it is important to assess how kidney disease may affect disposition of drugs used in this patient population. Kidney disease changes the exposure of drugs by altering their urinary excretion through the processes of filtration and active tubular secretion. Therefore, for drugs that are predominantly eliminated renally, it is intuitive that dosage adjustments are needed in patients with progressive deterioration of kidney function. However, a growing body of evidence demonstrates alterations in disposition of drugs that are eliminated by nonrenal clearance pathways (i.e., metabolism and hepatic transport), suggesting that these medications may require consideration when taken by patients with CKD. In

this section, we will provide an overview of the major pharmacokinetic concepts, and discuss how CKD may impact individual steps in drug absorption and disposition.

1.2.1 Overview of Pharmacokinetics

The pharmacokinetic properties of drugs are basically determined by absorption, distribution, metabolism, and excretion. Absorption is an important determinant of the bioavailability of drugs administered orally. Bioavailability reflects the rate and extent to which the parent drug appears in the systemic circulation. Hence, it determines how much drug will be available to bind to its target site in the desired tissue. After the drug reaches the blood, it circulates through the entire body. The volume of distribution is the term used to describe the degree to which the drug is distributed throughout the body. Metabolism accounts for the biochemical modification processes of drug that are principally mediated in the liver, the major drug detoxification organ in the body. The parent drug and/or its metabolite(s) are excreted from the body primarily through urine or bile. Metabolism and excretion are together known as drug elimination. Because drug metabolism is the major focus of this dissertation, it will be further discussed in this section.

Phase I metabolism primarily involves oxidation, reduction, and hydrolysis. The most prevalent phase I metabolism pathway is oxidation, which is mainly mediated by the cytochrome P450 (CYP450) enzymes (Guengerich, 2008). CYP450 enzymes are separated into different subfamilies and isoforms based on the similarity in their structure and function. For example, isoforms that possess more than 55% sequence homology are assigned to the same subfamily, as in the case of CYP3A4 and CYP3A5 which belong to the CYP3A subfamily (Lamba et al., 2002). In the liver, the most abundant CYP450 enzymes are CYP3A4 and CYP2C, representing about

30% and 20% of total liver CYPs, respectively (Pinto and Dolan, 2011). In fact, CYP3A accounts for around 50% of all CYP450-mediated drug metabolism (Rendic, 2002).

Phase I metabolism plays critical roles in the biosynthesis, transformation, and regulation of endogenous compounds such as sterols, fatty acids, prostaglandins and others, as well as numerous xenobiotics and drugs (Xu et al., 2005). This metabolic pathway usually converts parent compounds into more polar and biologically inactive metabolites that can be directly excreted from the body and/or further conjugated by phase II enzymes. However, it is not uncommon that drug-metabolizing enzymes (DMEs) transform drug substrates into partially or totally reactive or toxic metabolites (Sheweita, 2000). One important pathway of phase I metabolism is reduction, which accounts for the metabolism of a significant number of endogenous and exogenous substrates. This pathway is the focus of our work and will be discussed separately later in this chapter.

Parent drug and/or metabolites can be targeted by phase II metabolism through the processes of glucuronidation, sulfation, acetylation, methylation, and glutathione or amino acid conjugation. Glucuronidation is the most common phase II metabolic pathway. It is mediated by uridine diphoshate (UDP)-glucuronosyltransferase (UGTs). In most of the cases, phase II metabolism generates hydrophilic metabolites (except methylation and acetylation) that are readily excreted through urine or bile (Rowland et al., 2013; Tukey and Strassburg, 2000).

Oxidative and conjugative DMEs exhibit wide tissue distribution with high levels being observed in the liver, kidney, gut, lung, and brain. While drug metabolizing CYP450 and UGT enzymes are located in the microsomal fraction of the cell (membrane of the endoplasmic reticulum), reductase, acetylase, and sulfatase isoforms are primarily found in the cytosol (Sheweita, 2000).

Finally, drug transport has become increasingly important in determining pharmacokinetics. Transporters are transmembrane proteins that are directed toward the apical or luminal site of the membrane where they control substrate influx (uptake) or efflux in or out of the cells. Recently, transport proteins have been described by the terms phase 0 and phase III for the uptake and efflux transporters, respectively (Doring and Petzinger, 2014). Drug transporters belong to two main families; the ATP binding cassette (ABC) and the solute carrier (SLC) transporters. The ABC carriers mediate primarily drug efflux from cells to the lumen using ATP as the source of energy. The major subgroups of the ATP family are multi-drug resistance protein (MDR; also called P-glycoprotein (P-gp)), multi-drug resistance associated protein (MRP), and breast cancer resistance protein (BCRP) (International Transporter et al., 2010). On the other hand, the primary function of the SLC transporters is intracellular uptake of chemicals. SLC transporters consist of organic anion transporters (OAT), organic anion transporter polypeptide (OATP), and organic cation transporters (OCT) (Roth et al., 2012). Besides the liver, transporters are also expressed in extra-hepatic tissues such as gut, kidney, brain, adrenal gland and lung, where they act together to govern drug absorption, distribution, or elimination (Doring and Petzinger, 2014).

In the following sections, the effect of kidney disease on DMEs and transport proteins at the level of absorption and elimination (metabolism and excretion) will be discussed. **Tables 1-2** and **1-3** provide a comprehensive review of experimental studies that have demonstrated alterations of intestinal, hepatic, and renal metabolizing enzymes and transporters, respectively in the setting of kidney disease.

Table 1-2 Alterations of intestinal, hepatic, and renal metabolic enzymes in experimental kidney disease

	Intestine [1]			Liver [2-10]			Kidney [10, 11]		
	mRNA	**Protein**	**Activity**	**mRNA**	**Protein**	**Activity**	**mRNA**	**Protein**	**Activity**
CYP1A1	Decreased (32%)	Decreased (43%)	Decreased (25%)	-	Unchanged[2]	-	Decreased[11] (98%)	Decreased[11] (48%)	-
CYP1A2	-	-	-	Unchanged[4] Decreased[9]	Unchanged[4] Decreased[9] Increased[2]	Unchanged[3]	-	-	-
CYP2B1	-	Unchanged	-	-	Unchanged[2]	-	-	-	-
CYP2B2	-	-	-	-	Unchanged[2]	-	-	-	-
CYP2C6	-	Unchanged	-	-	Unchanged[4] Decreased[2,5] (38%)	-	-	-	-
CYP2C11	Unchanged	Unchanged	-	Decreased[4,5,6] (27-95%)	Decreased[2,3,4,5,6] (40-68%)	Decreased[3,6] (35-82%)	-	-	-
CYP2D1	-	-	-	Unchanged[5]	Unchanged[2,4,5]	-	-	-	-
CYP2E1	-	-	-	-	Unchanged[4]	-	-	-	-
CYP3A1	-	-	-	Decreased[4]	Decreased[3,4,5] (37-85%)	Decreased[8] (50%)	Unchanged[11]	Unchanged[11]	-
CYP3A2	Decreased (36%)	Decreased (71%)	Decreased (25%)	Decreased[4,5,6] (36-99%)	Decreased[2,3,4,5,6] (45-91%)	Decreased[3,4,6] (45-72%)	-	-	-
NAT1	-	-	-	Decreased[7] (25%)	Decreased[7] (33%)	-	-	-	-
NAT2	-	-	-	Decreased[7] (35%)	Decreased[7] (50%)	Decreased[7] (50%)	-	-	-
UGT1A	-	-	-	-	Unchanged[10]	Unchanged[10]	-	Unchanged[10]	Unchanged[10]
UGT2B	-	-	-	-	Unchanged[10]	Unchanged[10]	-	Unchanged[10]	Unchanged[10]

CYP: cytochrome P450; NAT: N-acetyltransferase; UGT: uridine diphoshate glucuronosyltransferase; (-) data are not available

Table references: **1**: (Leblond et al., 2002); **2**: (Uchida et al., 1995); **3**: (Leblond et al., 2000); **4**: (Leblond et al., 2001); **5**: (Guevin et al., 2002); **6**: (Velenosi et al., 2012); **7**: (Simard et al., 2008); **8**: (Rege et al., 2003); **9**: (Michaud et al., 2005); **10**: (Yu et al., 2006); **11**: (Naud et al., 2011)

Table 1-3 Alterations of intestinal, hepatic, and renal transport proteins in experimental kidney disease

	Intestine [1-3]			Liver [3-5]			Kidney [3, 5-7]		
	mRNA	**Protein**	**Activity**	**mRNA**	**Protein**	**Activity**	**mRNA**	**Protein**	**Activity**
MRP2	Unchanged[2]	Decreased[2] (60%)	Decreased[2] (25%)	Increased[4,5] (40-90%)	Increased[5] (70%) Unchanged[3,4]	-	Increased[5,7] (40-200%)	Increased[5,7] (82-124%)	-
MRP3	Unchanged[2,3]	Decreased[2] (35%) Unchanged[3]	-	Increased[3]	Increased[3]	-	Unchanged[3] Increased[7] (92%)	Unchanged[3] Increased[7] (292%)	-
MRP4	-	-	-	Unchanged[3]	-	-	Increased[7] (72%) Unchanged[3]	Increased[7] (178%)	-
NPT1	-	-	-	-	-	-	Decreased[7] (46%)	Decreased[7] (38%)	-
OAT1	-	-	-	-	-	-	Decreased[7] (78%)	Decreased[7] (48%) Unchanged[6]	-
OAT2	-	-	-	-	-	-	Decreased[7] (98%)	Decreased[7] (40%)	-
OAT3	-	-	-	-	-	-	Decreased[7] (69%)	Decreased[7] (77%) Unchanged[6]	-
OATK1/K2	-	-	-	-	-	-	Decreased[7] (90%)	Decreased[7] (82%)	-
OATP1	-	-	-	-	-	-	Decreased[7] (97%)	Decreased[7] (74%)	-
OATP2	-	Unchanged[2]	-	Unchanged[4]	Decreased[4] (40%)	-	Increased[7] (125%)	Increased[7] (144%)	-
OATP3	Unchanged[2]	Unchanged[2]	-	-	-	-	Increased[7] (179%)	Increased[7] (108%)	-
OATP4	-	-	-	-	-	-	Decreased[7] (92%)	Decreased[7] (68%)	-
OATP5	-	-	-	-	-	-	Decreased[7] (95%)	-	-

OCT-1	-	-	-	-	-	-	-	Unchanged[6]	-
OCT-2	-	-	-	-	-	-	-	Decreased[6] (67%)	Decreased[6] (82%)
P-gp	Unchanged[1, 2]	Decreased[2] (65%) Unchanged[1]	Decreased[1, 2] (30-33%)	Increased[4] (50%) Unchanged[5]	Increased[4] (25%) Unchanged[5]	Increased[4] (63%)	Decreased[7] (48%) Unchanged[5]	Decreased[7] (44%) Unchanged[5]	Decreased[7]
URAT1	-	-	-	-	-	-	Decreased[7] (75%)	Decreased[7] (38%)	-

MRP: multi-drug resistance associated protein; NPT1: sodium-dependent phosphate transport protein 1; OAT: organic anion transporter; OATP: organic anion transporter polypeptide; OCT: organic cation transporter; P-gp: P-glycoprotein; URAT: urate transporter; (-) data are not available

Data were obtained from the following references: **1**: (Veau et al., 2001); **2**: (Naud et al., 2007); **3**: (Gai et al., 2014); **4**: (Naud et al., 2008); **5**: (Laouari et al., 2001); **6**: (Ji et al., 2002); **7**: (Naud et al., 2011).

1.2.2 Effect of CKD on Absorption

The 5/6th nephrectomy model has been widely used to evaluate the impact of CKD on pharmacokinetics. The model represents a progressive impairment of kidney function. In this 2-stage model, a two-third nephrectomy of the left kidney is followed by a complete right nephrectomy seven days later. The targeted organ (i.e., liver, kidney, intestine) is harvested 6-7 weeks after the surgery to assess the effect of kidney disease on enzyme or transporter function and expression (Leblond et al., 2000; Naud et al., 2011). Alterations in the metabolic enzymes located in enterocytes and transporters lining the apical membrane and endothelial cells will impact the amount of drug reaching the systemic circulation (bioavailability), and thus its pharmacological action (Momper et al., 2010). Previous studies using the 5/6th nephrectomy model have shown reduced activity and expression of intestinal CYP3A (by up to 25% and 71%, respectively) and CYP1A (by up to 25% and 43%, respectively) in rats. However, protein levels of CYP2B1, CYP2C6, and CYP2C11, and mRNA level of CYP2C11 were not affected (Leblond et al., 2002).

In addition to DMEs, intestinal transporters were also shown to be differentially affected by CKD. One study showed a decrease in the activity of P-gp, but not intestinal mRNA expression in rats with CKD induced by 5/6th nephrectomy (Veau et al., 2001). Decreased P-gp activity has been recently confirmed by Naud and colleagues who showed that the activity of the efflux transporters P-gp and MRP2 as well as their intestinal protein expression were reduced in rats with kidney disease (Naud et al., 2007). However, this decrease in protein expression of P-gp and MRP is not parallel with their mRNA levels as they remained unaffected in nephrectomized rats (Naud et al., 2007). Together, this suggests the contribution of translational modifications and/or

degradation to reduced protein levels and therefore the activity of transporters in CKD rats (Naud et al., 2007).

Decreased activity of DMEs and drug efflux by P-gp in the intestine may together explain increased bioavailability of tacrolimus in rats with CKD (Okabe et al., 2000) and increased maximum plasma concentration of fexofenadine observed in CKD patients (Nolin et al., 2009). However, these findings contradict other observations documented in clinical pharmacokinetic studies of drugs in ESRD. For instance, the pharmacokinetics of oral midazolam and the intestinal bioavailability of erythromycin were not affected by ESRD, suggesting that intestinal CYP3A is not significantly altered in kidney disease (Nolin et al., 2009; Sun et al., 2010). Together, these conflicting results, especially for erythromycin, indicate the complex interplay between DMEs and transporters. Additionally, this may also suggest that other factors such as changes in drug first-pass metabolism could contribute to altering drug bioavailability in CKD.

1.2.3 Effect of CKD on Distribution

One important factor that influences drug distribution is the extent to which the drug binds to plasma or tissue proteins. Changes in plasma protein binding of high clearance drugs may affect the unbound fraction of in plasma, which could alter drug pharmacological action and clinical response. Thus, evaluation of the effect of disease state on drug plasma protein binding is important. Protein binding of many acidic drugs is reduced in CKD, which may result in increased volume of distribution. Decreased plasma protein binding has been correlated with plasma levels of urea and creatinine (Reidenberg, 1977; Sjoholm et al., 1976; Vanholder et al., 1988). For instance, binding of diazepam, salicylic acid, phenytoin, theophylline, and warfarin is considerably impaired in uremic serum (Reidenberg, 1977; Sjoholm et al., 1976; Vanholder et al., 1988).

Decreased plasma protein binding in uremia can be explained by reduced affinity of drugs to albumin, due to 1) conformational changes resulting in abnormal albumin or 2) the presence of chemical inhibitors in uremic serum (i.e., uremic toxins) that displace drugs from their binding sites (Sjoholm et al., 1976; Vanholder et al., 1993; Vanholder et al., 1988).

1.2.4 Effect of CKD on Hepatic Elimination

The liver is the major organ contributing to drug biotransformation in the body. Various experimental studies have investigated the effect of CKD on hepatic drug metabolism and transport. The metabolic activity of CYP2C11 and CYP3A2 measured using aminopyrine and erythromycin as probe substrates, respectively, was reduced up to 35% in rats with severe kidney disease (Leblond et al., 2000). This was also observed recently in rats with moderate CKD (induced by one-third removal of the left kidney and complete nephrectomy of the right kidney) suggesting that earlier stages of kidney impairment may also affect nonrenal drug clearance (Velenosi et al., 2012). Besides activity, protein expression of CYP2C11 and CYP3A has been shown to be consistently reduced in moderate and severe experimental kidney disease (Leblond et al., 2001; Leblond et al., 2000; Rege et al., 2003; Velenosi et al., 2012). This decrease was secondary to reduced mRNA expression documented by downregulation of CYP2C11, CYP3A1, and CYP3A2 genes. However, inconsistent results were obtained with respect to the expression of CYP1A2 and CYP2C6 (Leblond et al., 2001; Michaud et al., 2005; Rege et al., 2003; Uchida et al., 1995). Protein and mRNA levels of CYP2D and CYP2E1 remained unaffected (Leblond et al., 2001; Rege et al., 2003; Uchida et al., 1995). Generally, the experimental animal models of CKD demonstrate the presence of differential effect of kidney impairment on CYP450 functional

expression. However, the expression and activity of CYP2C11 and CYP3A2 were consistently decreased in rats with CKD.

In order to test whether the alterations in the functional expression of CYP450 observed in animal models can be translated to humans, *in vivo* studies have been conducted using phenotypic probe substrates that are metabolized by specific CYP450 isoforms. Previous studies assessed the activity of CYP3A, the most abundant isoform in the liver, in patients with ESRD, utilizing the erythromycin breath test (Dowling et al., 2003). Patients with ESRD had 28% lower baseline erythromycin breath test demonstrating reduced CYP3A activity (Dowling et al., 2003). However, this finding may not be exclusively due to CYP3A activity since erythromycin is also a substrate for drug transporters that contribute to its elimination. A follow-up study aimed to identify whether the decrease in nonrenal clearance of erythromycin is as a result of reduced hepatic and/or gut metabolism (Sun et al., 2010), found that hepatic clearance of erythromycin is decreased in ESRD patients by 31% relative to controls receiving IV infusion of erythromycin. With oral administration, erythromycin bioavailability was increased by 36%, and that was attributed to change in liver, but not gut metabolism mediated by CYP3A (Sun et al., 2010). This is in contrast to the findings of Nolin et al., who showed that the pharmacokinetics of midazolam, a probe substrate for CYP3A, are not different between controls and ESRD patients receiving conventional HD therapy (Nolin et al., 2009). The contradictory observations may be explained by the effect of CKD on transport proteins, given that erythromycin is a substrate for the uptake transporter OATP and efflux transporter P-gp (Frassetto et al., 2007). CYP2C9 is another critical isoform that accounts for about 20% of drug metabolism (Miners and Birkett, 1998). The activity of CYP2C9 was investigated in ESRD patients using warfarin as a substrate. A 50% increase of warfarin *S/R*

ratio has been observed in ESRD patients compared with control subjects, suggesting reduced CYP2C9 activity in patients with kidney disease (Dreisbach et al., 2003).

Phase II conjugation is a crucial pathway for detoxification and excretion of several drugs from the body. The conjugation pathways have not been widely investigated in kidney disease, despite the fact that, a number of studies have shown decreases in liver metabolism of drugs that are metabolized by phase II pathways in kidney disease. Drugs that are primarily metabolized by acetylation such as procainamide and isoniazid were found to be accumulated in CKD (du Souich and Erill, 1978; Kim et al., 1993). Indeed, the effect of CKD on acetylation was clearly supported by around 30% decrease in protein and mRNA expression of N-acetyltransferase (NAT) enzymes, and a 50% reduction in NAT2 activity in rat with CKD relative to controls (Simard et al., 2008). Moreover, the down-regulation in protein (33%) and mRNA (23%) expression of NAT2 has been also shown in a murine model of CKD (Dani et al., 2009). Additionally, clinical studies have shown that the metabolic clearance of drugs that are extensively eliminated by glucuronidation such as metoclopromide, zidovudine, and morphine is significantly reduced in CKD patients (Lehmann et al., 1985; Osborne et al., 1993; Singlas et al., 1989). However, Yu and coworkers have shown negligible effect of CKD on the function and expression of liver UGTs in nephrectomized rats (Yu et al., 2006). These findings further demonstrate the disparity of the effect of CKD on drug metabolism.

Hepatic drug transport is also important in drug disposition since many drugs are substrates for both CYP450 enzymes and transport proteins. As discussed with intestinal transporters, those located in the liver are also differentially affected by kidney disease. Hepatic P-gp activity was shown to be increased in rats with CKD secondary to increased protein and mRNA expression (Naud et al., 2008). This in contrast to what is already known about intestinal P-gp (Naud et al.,

2007). On the other hand, protein level of OATP2 was decreased, and MRP2 remained unaffected (Naud et al., 2008). This effect on protein was not associated with the change in gene expression since mRNA levels of OATP2 and MRP2 were either unaffected or increased, respectively (Naud et al., 2008). A recent study also demonstrated that protein and mRNA expression of MRP3 and OST-α was up-regulated in rats with CKD (Gai et al., 2014). Together, these data may suggest that CKD decreases the functional expression of uptake transporters involved in drug absorption, and increases the functional expression of efflux transporters contributing to drug elimination. This may eventually impact drug disposition by altering its bioavailability through the intestine and the extent to which it is transported and be available to metabolizing enzymes located in the liver. For example, decreased efflux activity of P-gp in the intestine, and decrease uptake activity of OATP in the liver would increase drug bioavailability and exposure.

Fexofenadine is a non-specific probe substrate of drug transporters that has been used to assess the effect of kidney disease on drug transporters in humans. Fexofenadine oral clearance is decreased in ESRD, suggesting either down-regulation of P-gp efflux transport in the intestine and/or decreased hepatic uptake by OATPs (Nolin et al., 2009). This observation has been recently confirmed by the results of a study that evaluated transporter activity in patients with glomerulonephritis using fexofenadine as a substrate, and documented a 40% reduction in fexofenadine oral clearance, which could be translated to decreased transporter activity (Joy et al., 2014).

1.2.5 Effect of CKD on Renal Excretion

Urinary excretion of drugs and/or their metabolites is primarily mediated through the processes of filtration and active tubular secretion. These routes are highly affected by kidney impairment,

resulting in alterations in pharmacokinetics. Thus, dosing adjustments are applied to patients with CKD for drugs that are principally eliminated unchanged in the urine. DMEs and transporters are also expressed in the kidney and can contribute to elimination of drugs and their intracellular accumulation within the kidney. Therefore, several studies have been conducted to investigate whether CKD impacts DMEs and transporters located in the kidney (Lalande et al., 2014; Naud et al., 2012). The results demonstrate a differential impact of kidney disease on transporters. For example, while expression of OATs, OATP1, OCT2, and P-gp was reduced, levels of MRPs, OATP2, and OATP3 were up-regulated in nephrectomized rats (Ji et al., 2002; Laouari et al., 2001; Naud et al., 2011). These alterations in kidney transporters have resulted in increased intra-renal accumulation of [^{14}C]benzylpenicillin (substrate for OATs and MRPs) and [^{3}H]digoxin (substrate for P-gp) in the kidney of uremic rats (Naud et al., 2011). The latter study also showed decreased expression of CYP1A in CKD rats (Naud et al., 2011). Together, these data demonstrate that decreased expression of transporters in CKD may reduce renal excretion mediated by active tubular secretion, and thereby increase drug plasma exposure. Because renal elimination through filtration and protein binding of drugs (fraction unbound) are also affected by kidney disease, the ultimate effect of impaired kidney function on renal elimination may be drug dependent.

Phase II renal UGT enzymes were also assessed in rats with kidney disease. The protein levels and activity of UGT2B and UGT1A were decreased in nephrectomized rats; however, this reduction was similar to that observed in the control pair-fed group, suggesting that food restriction but not kidney impairment contributed to decreased UGT expression and activity (Yu et al., 2006).

1.2.6 Mechanisms Contributing to Altered Drug Metabolism and Transport in CKD

Impairment of drug metabolism and transport in CKD may be explained by two major mechanisms attributed to the accumulated uremic toxins and inflammatory cytokines. These toxins may 1) directly inhibit or 2) modulate protein and mRNA expression of DMEs and transport proteins. Multiple studies have shown that the activity of DMEs is impacted by uremic serum, indicating the direct inhibition of drug metabolism by uremic toxins (Michaud et al., 2005; Simard et al., 2008). This was established by exploring N-demethylation of erythromycin, O-deethylation of 7-ethoxyresorufin, and N-acetylation of p-aminobenzoic acid as indictors of CYP3A, CYP1A, and NAT activity, respectively (Michaud et al., 2005; Simard et al., 2008). Studies have also reported that protein and mRNA expression of several DMEs is modulated when normal rat hepatocytes were incubated with serum collected from rats or patients with CKD (Guevin et al., 2002; Michaud et al., 2005; Tsujimoto et al., 2012). For instance, levels of CYP1A, CYP2C, and CYP3A were reduced at mRNA and protein levels. Whether this decrease in expression is as a result of impaired transcription and translation or changes in mRNA and protein stability is unclear, and needs further investigation. The decline in the activity of selected DMEs (i.e., CYP3A, CYP2C, NAT) can be temporarily reversed after hemodialysis, but completely resolved after renal transplantation (Kim et al., 1993; Michaud et al., 2008; Nolin et al., 2006).

Similarly, the function and expression of transport proteins located in intestine, liver, and kidney were also impacted by uremic serum (Naud et al., 2011; Naud et al., 2007; Naud et al., 2008; Sun et al., 2004; Tsujimoto et al., 2012). For example, the observation of increased rhodamine-123 permeability from apical to basolateral side of Caco-2 or HK-2 cells treated with uremic serum versus control, indicates decreased P-gp activity (Naud et al., 2011; Naud et al., 2007). Moreover, increased P-gp and decreased OATP2 protein expression was observed in

hepatocytes after treatment with CKD rat serum (Naud et al., 2008). Of note, the most common toxins implicated in alterations of DMEs and transporters in CKD include indole-3-acetic acid, 3-indoxyl sulfate, *p*-cresol, hippuric acid, 3- carboxy-4-methyl-5-propy-2-furanpropanoic acid (CMPF), para-thyroid hormone (PTH), and cytokines (Barnes et al., 2014; Tsujimoto et al., 2013; Volpe et al., 2014).

1.2.7 Does CKD Affect Drug Reduction?

As we previously mentioned, reduction is an important pathway for elimination of an array of drugs commonly used in clinical practice. Unlike phase I oxidation and phase II conjugation, reduction has been studied very little in CKD. In fact, the disposition of several drugs that are extensively eliminated by reduction has been significantly impacted by the presence of kidney disease. For example, naltrexone exposure is dramatically increased in ESRD patients on hemodialysis, which could be explained in part by decreased elimination (Kambia et al., 2004). Moreover, a 30% decrease in the metabolic clearance of idarubicin was reported in patients with creatinine clearance (CLCr) of <60 mL/min (Camaggi et al., 1992). Given the high contribution of reductases to elimination of these drugs (Dayton and Inturrisi, 1976; Kang and Weiss, 2003), these findings demonstrate that, besides oxidation, phase I reduction of drugs may also be impaired in kidney disease. **Table 1.4** summarizes the effect of kidney disease on the clinical pharmacokinetics of several reductase drug substrates.

Table 1-4 Clinical pharmacokinetics of selected drug substrates of reductases in CKD patients

Drug	Kidney Function (# of Pts)	f_e (%)	CL_t	$t_{1/2}$	C_{max}	AUC	Comments
Boceprevir[1]	Normal (6)	3	178 (L/h)	1.73 (h)	1730 (ng/mL)	5760 (ng*h/mL)	No dose adjustment necessary in patients with ESRD, including those receiving dialysis
	ESRD/ HD (8)		193 (L/h)	2.2 (h)	1340 (ng/mL)	5150 (ng*h/mL)	
Bupropion[2]	Normal (17)	0.5	414 (L/h)	8.1 (h)	66.1 (ng/mL)	490 (ng*h/mL)	The authors state that despite the increases in the exposure observed in CKD patients, dosage recommendations cannot be given
eGFR 15-59 (mL/min)	Moderate-severe (10)		155 (L/h)[$]	19.4 (h)[$]	123 (ng/mL)[$]	1100 (ng*h/mL)[$]	
Dolasetron[3]	Normal (24)	<1	114.9 (mL/min/kg)	8.6 (min)	-	286.5 (ng*h/mL)	Despite increased exposure, the authors state that there is no need for dosage adjustment due to high variability observed between patients
CLCr 41-80 (mL/min)	Mild-moderate (12)		55.7 (mL/min/kg)	22 (min)[$]	-	685.9 (ng*h/mL)[$]	
CLCr 11-40 (mL/min)	Moderate-severe (12)		117.5 (mL/min/kg)	30.1 (min)[$]	-	426.0 (ng*h/mL)	
CLCr <= 10 (mL/min)	ESRD/HD (12)		66.1 (mL/min/kg)	11.1 (min)	-	594.4 (ng*h/mL)[$]	
Doxorubicin[4]	Non-HD (8)	5-12	0.894 (L/h/kg)	-	-	21 (ng* h/mL*kg)	The authors suggest to carefully monitor HD patients receiving doxorubicin therapy
	ESRD/HD (5)		0.52 (L/h/kg)[$]	-	-	36 (ng* h/mL*kg)[$]	
Idarubicin[5]	Normal (6)	<3	122.8 (L/h)	15.24 (h)	-	186.5 (ng*h/mL)	-
CLCr <60 (mL/min)	Moderate-severe (6)		83.4 (L/h)[$]	19.03 (h)	-	258.2 (ng*h/mL)[$]	
Ketanserin[6]	Normal (10)	<3	6.4 (L/h)	18 (h)	198 (ng/mL)	625 (ng*h/mL)	No dose adjustment necessary in spite of increased free fraction due to reduced plasma binding in kidney failure
CLCr <30 (mL/min)	Severe-ESRD (6)		6.33 (L/h)	29 (h)	127 (ng/mL)	632 (ng*h/mL)	
	ESRD/ HD (6)		5.94 (L/h)	28 (h)	60.7 (ng/mL)	637 (ng*h/mL)	
Ketoprofen[7]	Normal (7)	4	0.0766 (L/h/kg)	2.03 (h)	3.29 (mg/L)	11.2 (mg*h/L)	The authors suggest decrease the dose or the frequency in patients with severe CKD
CLCr 15-60 (mL/min)	Moderate-severe (5)		0.0443 (L/h/kg)[$]	3.17 (h)[$]	3.68 (mg/L)	16.7 (mg*h/L)	
Naltrexone[8]	Normal (6)	<2	278.4 (L/h)	8.8 (h)	9-44 (ng/mL)	-	The authors concluded that the clinician could determine whether dosage adjustments are necessary in HD patients after careful monitoring
	ESRD/HD (7)		118 (L/h)[$]	1.48 (h)	255 (ng/mL)[$]	739.46 (ng*h/mL)	

Oxcarbazepine[9]	Normal (6)	<1	-	-	1.23 (µmole/L)	4.2 (µmole*h/L)	The authors suggest reducing the dose by 50% in moderate CKD patients. However, dosage adjustments in severe CKD cannot be proposed from this study
CLCr 30-80 (mL/min)	Mild (6)		-	-	1.67 (µmole/L)	8.3 (µmole*h/L)	
CLCr 10-29 (mL/min)	Moderate (7)		-	-	1.45 (µmole/L)	9.2 (µmole*h/L) $	
CLCr <10 (mL/min)	Severe (7)		-	-	1.82 (µmole/L)	7.9 (µmole*h/L)	
Pentoxifylline[10]	Normal (9)	<1	-	-	393 (ng/mL)	2008 (ng*hr/mL)	The authors state that although pentoxifylline kinetics were not affected by CKD, dosage adjustment and close monitoring are recommended due to active metabolites accumulation
CLCr 30-80 (mL/min)	Moderate (6)		-	-	187 (ng/mL)	1020 (ng*hr/mL)	
CLCr <30 (mL/min)	Severe (10)		-	-	152 (ng/mL)	829 (ng*hr/mL)	

AUC: area under the plasma concentration–time curve; CKD: chronic kidney disease; CLCr: creatinine clearance; Clt: total drug clearance; C_{max}: peak plasma concentration; eGFR: estimated glomerular filtration rate; ESRD: end-stage renal disease; f_e: percentage of drugs excreted unchanged in the urine; HD: hemodialysis; $t_{1/2}$: half-life; (-): not available

$ indicates statistically significant difference from the control group

Data were obtained from the following references: **1**: (Treitel et al., 2012); **2**: (Turpeinen et al., 2007); **3**: (Dimmitt et al., 1998); **4**: (Yoshida et al., 1994); **5**: (Camaggi et al., 1992); **6**: (Barendregt et al., 1990); **7**: (Stafanger et al., 1981); **8**: (Kambia et al., 2004); **9**: (Rouan et al., 1994); **10**: (Paap et al., 1996).

1.3 PHASE I HEPATIC REDUCTION

Compounds bearing a carbonyl group such as aldehydes and ketones are susceptible to reduction generating products with a hydroxyl group. The metabolites are more hydrophilic and can be conjugated by phase II enzymes or excreted unchanged without any further modification (Oppermann, 2007). Metabolism by reduction is considered the major elimination pathway for a variety of endogenous compounds and xenobiotics such as drugs, as the carbonyl moiety is quite common in these substrates. **Table 1-5** provides a list of drugs that are metabolized through reduction pathway. Reduction can be either a deactivation and detoxification process producing inactive metabolites that are eliminated from the body, or can sometime produce toxic metabolites as is case with doxorubicin (Hoffmann and Maser, 2007). Additionally, reduction can act as an activation method for pro-drugs such as nabumetone and tibolone, producing pharmacologically active metabolites (Malatkova and Wsol, 2014).

Our understanding of drug reduction pathway is limited, in part, due to relatively little research that has been conducted in this area. In fact, reduction has been poorly investigated in the literature compared to that of CYP450-mediated oxidation, which usually receives much more attention. However, recent development and advances in molecular biology has improved the opportunity for the researchers to study reductase enzymes and their important roles in endogenous and exogenous biotransformation (Malatkova and Wsol, 2014).

Table 1-5 List of drugs that are substrates for reductase enzymes

Drug	Class	Drug	Class
L-691,121*	Antiarrhythmic	Haloperidol*[$]	Antipsychotic
Ketotifen*	Antiasthmatic	Timiperone*	Antipsychotic
Benfluron*	Anticancer	Boceprevir*	Antiviral
Daunorubicin	Anticancer	S-1360	Antiviral
Dimefluron	Anticancer	Levobunolol	β-Blocker
Doxorubicin	Anticancer	Metyrapone	Diagnostic
Epirubicin	Anticancer	Ethacrynic Acid[$]	Diuretic
Idarubicin*	Anticancer	Pentoxifylline[$]	Hemorheological
Iododoxorubicin	Anticancer	Oxisuran	Immunosuppressive
Mitomycin C	Anticancer	Eperisone	Muscle Relaxant
Oracin*	Anticancer	HY-770	Muscle Relaxant
Wortmannin*	Anticancer	Tolperisone	Muscle Relaxant
Warfarin[$]	Anticoagulant	CS-670	NSAID
Nafimidone	Anticonvulsant	Flobufen*	NSAID
Bupropion[$]	Antideprassant	Ketoprofen	NSAID
E/Z-10-oxonortriptyline	Antideprassant	Loxoprofen	NSAID
Acetohexamide*	Antidiabetic	Nabumetone*	NSAID
Dolasetron*	Antiemetic	Methylnaltrexone	Opiate antagonist
Oxcarbazepine*	Antiepileptic	Naloxone	Opiate antagonist
Fenofibrate	Antihyperlipidemia	Naltrexone*	Opiate antagonist
Triadimefon	Antifungal	Glucocorticoids[$]	Steroid
Befunolol*	Antihypertensive	Norethynodrel*	Steroid
Ketanserin*	Antihypertensive	Tibolone*	Steroid
TA-510	Anti-inflammatoy	Naftazone	Vasoprotectant
Bromperidol*	Antipsychotic	Menadione	Vitamin (K3)

NSAID: non-steroidal anti-inflammatory drug

*Drugs have reduction as a major metabolic pathway

[$]Drugs that are commonly used in kidney disease patients

Several reductase enzymes have been implicated in the elimination of reactive carbonyl ligands. Similar to other DMEs, reductases are separated into different families, groups, and individual isoforms based on the homology in their structure and amino acid sequence (**Figure 1-1**). In general, reductase enzymes are grouped into carbonyl reducing enzymes and quinone reductases. Carbonyl reducing enzymes belong to two fundamentally distinct enzyme families, namely the short-chain dehydrogenase/reductase (SDR) and the aldo-keto reductase (AKR) that are further separated into different subgroups. On the other hand, carbonyl reduction by quinone reductases is principally mediated by the NADPH-dependent quinone reductases (NQO) (Forrest and Gonzalez, 2000; Oppermann, 2007). In the following sections, a detailed description of reductase subgroups and specific isoforms will be presented.

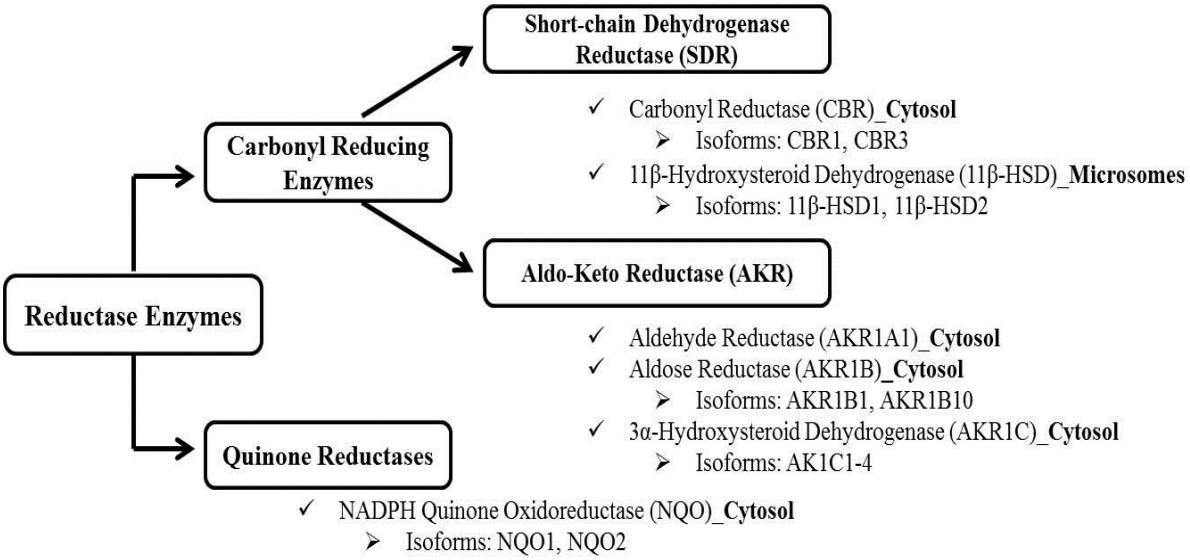

Figure 1-1 Major human reductase isoforms and their sub-cellular location

1.3.1 Carbonyl Reducing Enzymes

1.3.1.1 Short-chain dehydrogenase/reductase (SDR)

The SDR superfamily of reductase enzymes is mainly located in the cytosol and mediates reduction primarily by the carbonyl reductases (CBRs). Further, the 11β-hydroxysteroid dehydrogenases (11β-HSDs), which are the main microsomal reductases, belong to the SDR superfamily (Hoffmann and Maser, 2007). Other enzymes of the SDR family that may contribute to carbonyl reduction are dicarbonyl/xylulose reductase (DCXR) and dehydrogenase/ reductase SDR family located in the cytosol (Matsunaga et al., 2006), and retinol dehydrogenase and 17β-hydroxysteroid dehydrogenases located in the microsomes (Skarydova and Wsol, 2012).

Carbonyl Reductases (CBRs)

CBR isoforms: The major subgroup of the SDR superfamily that mediates cytosolic reduction of carbonyl-containing compounds is carbonyl reductase. CBRs are monomeric proteins that are mainly located in the cytosolic fraction of the cell and have a molecular weight of about 30 KDa (Malatkova et al., 2010). CBRs are NADPH-dependent reductases, indicating that they prefer nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor in the carbonyl reduction of xenobiotics. Three CBR isoforms have been identified in humans which are CBR1, CBR3, and CBR4 (Malatkova et al., 2010). While CBR1 and CBR3 are located in the cytosol (Persson et al., 2009), CBR4 is expressed in the mitochondria (Endo et al., 2008). CBR enzymes show wide tissue distribution. For instance, CBR1 is highly expressed in the liver and kidney with lower amounts found in the placenta and the nervous system, however, CBR3 shows high levels in the pancreas, prostate, and ovary (Malatkova et al., 2010). The liver expression of CBR1 is about 10-fold higher than CBR3 (Bains et al., 2010). The mitochondrial CBR4 is almost

exclusively expressed in the liver, but at levels lower than CBR1. It is worth mentioning that another isoform of CBR called CBR2 is identified in rodents and does not have any homolog in humans. CBR1 and CBR3 are the two best studied isoforms and are also called SDR21C1 and SDR21C2, respectively, according to the SDR nomenclature classification system (Malatkova et al., 2010). In fact, these two isoforms share 70% in the identity, which could explain why they show overlapping substrate specificity (Miura et al., 2008).

Endogenous substrates of CBR: CBRs play an important role in the metabolism and biotransformation of many biological compounds such as prostaglandins, steroids, lipids, and aldehydes. For example, the reduction of the 9-keto group of prostaglandins E1 and E2 (PGE1 and PGE2) to PGF2α is mediated by CBR1 (Schieber et al., 1992). Moreover, isatin, an indole derivative that is involved in regulation of blood pressure, stress, and sedation is found to be a substrate for both CBR1 and CBR3 (Usami et al., 2001). These data suggest that CBRs regulate the biological activity and biotransformation of many endogenous ligands.

Exogenous substrates of CBR: Several xenobiotic compounds act as substrates for CBRs. These could be aldehydes, ketones or quinones in origin. Phenanthrenequinone, the product of poly aromatic hydrocarbons, can be toxic to the body through DNA alkylation or generation of reactive oxygen species. Reduction of this quinone to the hydroquinone form by CBRs is a detoxification process, because the resulting metabolite is readily conjugated and eliminated from the body, thus, this process protects the body from the harmful effect of quinones (Jarabak and Harvey, 1993). The carcinogenic tobacco product, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK), is primarily eliminated through reduction. It is a common substrate for various reductase enzymes, of which, CBRs play a fundamental role and showed the highest catalytic efficiency in its metabolism (Atalla et al., 2000; Skarydova et al., 2012).

CBRs are also implicated in the metabolism of different group of drugs. The list of substrates includes mainly anticancers (i.e., benfluron, daunorubicin, doxorubicin, idarubicin, oracin), non-steroidal anti-inflammatory drugs (NSAIDS) (loxoprofen and nabumetone), antipsychotics (brompepridol, haloperidol, trimiperone), and others such as bupropion, dolasetron, metyrapone and pentoxifylline (Barski et al., 2008; Malatkova and Wsol, 2014). Daunorubicin and doxorubicin are the major CBR substrates that have been extensively studied (Lal et al., 2010; Skarka et al., 2011). Metabolism of these anti-cancer agents at C13 position generates the alcoholic forms that have much lower cytotoxic activity compared to the parent drug. However, these alcohol metabolites are believed to be responsible for cardiotoxicity, the major dose limiting toxicity of anthracyclines (Ax et al., 2000; Forrest et al., 2000). While CBR1 shows 10-fold higher activity toward daunorubicin reduction; CBR3 prefers doxorubicin as a substrate (Bains et al., 2010; Kassner et al., 2008). Generally speaking, CBR3 endogenous and exogenous substrates are very limited, and it usually shows lower affinity (high K_m) and maximum enzyme capacity (low V_{max}) compared with CBR1. Collectively, the findings of several studies imply that CBR1 is the major carbonyl reductase of the SDR family involved in drug reduction (Pilka et al., 2009).

11β-Hydroxysteroid Dehydrogenases (11β-HSDs)

11β-hydroxysteroid dehydrogenases are the major SDR members that catalyze carbonyl reduction in the microsomes. Two microsomal HSD isoforms have been described to date, 11β-HSD1 (SDR26C1) and 11β-HSD2 (SDR9C3), which vary in their biological activity and tissue distribution (Skarydova and Wsol, 2012). Of these, the NADPH-dependent 11β-HSD1 reductase is the most well-investigated and characterized isoform involved in the metabolism of xenobiotics. While 11β-HSD1 is predominantly expressed in the liver and adipose tissues, 11β-HSD2, the

NAD-dependent dehydrogenase, is abundant in the mineralocorticoid target tissues like kidney, colon, and to a lower extent in placenta (Masuzaki and Flier, 2003; Odermatt and Kratschmar, 2012).

11β-HSDs substrates: Glucocorticoides are important in the regulation of various biological processes. Besides their metabolic function, they are also critical modulators of inflammation and immune response. The inter-conversion between endogenous cortisone and cortisol is mediated through 11β-HSDs. Cortisone is the inactive form that has a carbonyl moiety at C11 position. Reduction at this position is mediated by 11β-HSD1 producing the biologically active alcohol form (i.e., cortisol). Conversely, the reverse reaction of cortisol deactivation to cortisone is catalyzed by 11β-HSD2 (Draper and Stewart, 2005). Thus, regulation of the availability and physiological functions of corticosteroids is governed by the 11β-HSDs activity. In addition, recent studies suggested that treatment with synthetic therapeutic corticosteroids might be impacted by the functional expression of 11β-HSDs (Diederich et al., 2002; Gathercole et al., 2013).

The 11β-HSD1 plays a crucial role in the metabolism of xenobiotics. The contribution of 11β-HSD2 to drug detoxification is poorly studied so far, and it is suggested that it mainly contributes to endogenous glucocorticoids biotransformation (Skarydova and Wsol, 2012). Several drugs are substrates for the 11β-HSD1 including acetohexamide, benfluron, bupropion, daunorubicin, eperisone, ketoprofen, metyrapone, oracin, and prednisone (Malatkova and Wsol, 2014). Of these, bupropion and eperisone are primarily metabolized by 11β-HSD1 (Meyer et al., 2013; Yoo et al., 2011). Metabolism by 11β-HSD1 can eliminate the biological activity of drugs as in case of benfluron, or producing toxic metabolites as with daunorubicin (Barski et al., 2008; Malatkova and Wsol, 2014). Additionally, active metabolites can be generated by the metabolic

reduction of drugs. For instance, bupropion is metabolized by oxidation mediated by CYP2B6 which generates hydroxybupropion, as well as by reduction mediated by reductases, with the latter being the major pathway and occurs in the cytosol and microsomes (Skarydova et al., 2014). Metabolism by reduction produces the diastereoisomers erythro- and threohydrobupropion that possess about 20% activity of the parent drug bupropion (Molnari and Myers, 2012). A recent study reported that bupropion metabolism is catalyzed primarily in the microsomes, producing threohydrobupropion as a predominant metabolite followed by hydroxybupropion and erythrobupropion (Meyer et al., 2013). Moreover, pro-drugs can also be activated by reduction. For example, prednisone is a pro-drug that is metabolized by 11β-HSD1 to prednisolone, which is the biologically active form (Diederich et al., 2002). Taken together, 11β-HSD1 is the major microsomal reductase enzyme located in the liver and implicated in phase I drug reduction. In addition to drugs, 11β-HSD1 also contributes to the detoxification of other xenobiotics such as the tobacco toxin (NNK) and quinones, and thus protecting the body from harm (Maser et al., 2006).

1.3.1.2 Aldo-keto reductase (AKR)

The second superfamily of the carbonyl reducing enzymes is the AKR. This group of reductases is further separated into multiple subfamilies including AKR1, AKR6, and AKR7 (Barski et al., 2008). AKR1 is the largest subfamily and is divided into AKR1A, 1B, and 1C. AKR1A is called aldehyde reductase because it mainly reduces aldehyde-containing substrates into their respective alcohols, though it is not aldehyde specific. In fact, a broad spectrum of endogenous and xenobiotic carbonyls are reduced by AKR1A including succinic semialdehyde, 4-carboxybenzaldehyde, 4-hydroxynonenal, haloperidol, metyrapone, loxoprofen, and daunorubicin (Oppermann, 2007). AKR1A1 is abundantly expressed in kidney, liver, central nervous system (CNS), and intestine (O'Connor et al., 1999).

Two major isoforms of aldose reductase (AKR1B) have been identified, AKR1B1 and AKR1B10. AKR1B1 is well known for its activity toward glucose reduction to sorbitol (Gabbay, 2004). This reaction is responsible for microvascular complications associated with diabetes. In fact, several AKR1B1 inhibitors are under development to prevent tissue injury associated with diabetes (Comer and Ciulla, 2005). AKR1B1 is ubiquitously expressed and contributes to biotransformation of several endogenous substrates such as corticosteroids and lipid peroxidase products; however, its role in drug reduction has not clear (Barski et al., 2008; Jin and Penning, 2007). On the other hand, AKRB10 is mainly expressed in the liver, colon, and small intestine, and it contributes to the metabolism of dolasetrone, nabumetone, and oracin (Martin et al., 2006).

The primary AKR group that mediates xenobiotic phase I reduction is AKR1C. In humans, four isoforms have been characterized, AKR1C1, AKR1C2, AKR1C3, and AKR1C4, that share about 85% homology (Jez et al., 1997). The isoform, AKR1C1, is highly expressed in the liver and other tissues. AKR1C2 and AKR1C3 are predominantly expressed in prostate and mammary gland, and to a lower extent in the liver. AKR1C4 is exclusively found in the liver (Jin and Penning, 2007). The major endogenous substrates for AKR1C are steroids, bile acids, and prostaglandins. Because of their ability to transform the dihydrodiols of aromatic hydrocarbons, these reductases are also called dihydrodiol dehydrogenases (Barski et al., 2008).

AKR1C is the most important reductase that contributes to drug reduction. It shows wide and overlapping drug substrate specificity. AKR1C metabolizes drugs from different pharmacological classes such as anticancers, NSAIDS, antipsychotics, opiate antagonists and others (Matsunaga et al., 2006). AKR1C1 and AKR1C4 seem to be the most important, due in part to their high expression in the liver, the major detoxification organ in the body (Oppermann, 2007). The degree of involvement of these reductases to individual drug reduction is variable. For

example, the NSAID drug nabumetone was shown to be metabolized by all four AKR1C isoforms; however, the catalytic activity was the highest for AKR1C4, and the lowest for AKR1C2 (intrinsic clearance = 1.94 vs. 0.28 mL/mg/min, respectively) (Skarydova et al., 2013).

1.3.2 Quinone Reductases

Several enzyme systems have been reported to metabolize quinone-containing compounds such as those of the SDR or AKR families and thioredoxin reductase, however, the NADPH-dependent quinone reductase type 1 (NQO1, DT diaphorase) is the most extensively investigated (Siegel et al., 2012). NQO1 is a cytosolic reductase that protects cells from oxidative stress, and catalyzes the two-electron reduction of quinones to hydroquinones forms (Dinkova-Kostova and Talalay, 2000). NQO1 is expressed at high levels in the epithelial tissues and adipocytes, nevertheless, low levels are found in the liver (Siegel and Ross, 2000). The anticancer quinone, mitomycin C, has been shown to be activated by NQO1. Mitomycin C is commonly used in solid tumors including pancreas, lung, and breast cancer. Reduction of mitomycin C by NQO1 results in production of the pharmacologically active leucomitomycin C (MMC hydroquinone) that cross-linked to the DNA and initiates cancer cell death (Danson et al., 2004; Siegel et al., 1990). Besides quinones, NQO1 also catalyzes the metabolism of azo and nitroaromatic compounds. Of note, NQO2 is another quinone reductase that has been reported to mediate quinone reduction, without utilizing NAPDH as a cofactor. Its role in drug reduction is unknown (Oppermann, 2007).

1.4 WARFARIN

1.4.1 Warfarin Pharmacotherapy

Warfarin is an oral vitamin K antagonist that has been the gold standard anticoagulant for the treatment and prevention of thromboembolic disorders since its introduction more than 50 years ago. It acts by inhibiting vitamin K epoxide reductase (VKOR), the enzyme that is involved in hepatic activation of vitamin k-dependent clotting factors II, VII, IX, and X and proteins C and S (Hirsh et al., 2001). Warfarin is administered clinically as a racemic mixture consisting of both *R*- and *S*- enantiomers, with the latter being 3-5 times more potent in inhibiting VKOR enzyme (Breckenridge et al., 1974).

Warfarin is a very widely used drug in the clinical practice. It is prescribed to approximately 2 million Americans each year, and currently there are around 50 million people in the US under warfarin therapy (Kim et al., 2009). The widespread use of warfarin might be attributed, in part, to its established efficacy for stroke prevention in the general medical population, and the rapid reversal of its action by vitamin K in case of over-anticoagulation. The effectiveness of warfarin is supported by a previous meta-analysis, which showed that warfarin use in AF patients is associated with 65% reduction in the relative risk of stroke compared to placebo or untreated patients (Hart et al., 2007). The analyses also showed that warfarin is about 40% more effective than antiplatelet therapy for stroke prevention (Hart et al., 2007). Despite its extensive use, treatment with warfarin is challenging and complicated due to its narrow therapeutic window, high likelihood for food and drug interactions, and large inter-individual variability (Hirsh et al., 2003). The bleeding rate in AF patients who are under long-term warfarin therapy varies from 0.4 to 17% per year (Fang et al., 2011). Additionally, most patients under warfarin

treatment spend about one-third of the time outside the INR target range (Baker et al., 2009; Jones et al., 2005). This demonstrates the challenge of optimizing warfarin therapy. Thus, frequent monitoring of the international normalized ratio (INR) and individualized warfarin dosing are necessary to achieve safe and effective clinical outcomes.

Individualizing warfarin therapy requires consideration of patient demographic factors, comorbid conditions such as liver disease, and variations in the genes encoding VKOR and CYP2C9 (White, 2010). Various dosing algorithms have been developed for estimation of therapeutic warfarin dose based on integration of patient's clinical and pharmacogenetic data (Gage et al., 2004; Klein et al., 2009; Lenzini et al., 2010). Kidney function has been shown to be an important determinant of *S*-warfarin metabolic clearance (Gong et al., 2011), as well as a powerful and independent covariate for predicting warfarin-associated bleeding risk (Fang et al., 2011; Gage et al., 2006; Pisters et al., 2010). Recently, a failure in pharmacogenetic-based algorithms for estimation of warfarin dosing has been observed (Schwartz et al., 2011). The combined genetic and clinical factors explained approximately 62% of warfarin dosing variability, however, the model overestimated the dose for 15 out of 16 patients requiring <2 mg/day warfarin dose. Based on patients' data, the authors suggested that kidney disease is a potential variable that may be associated with lower dosing requirements in elderly patients (Schwartz et al., 2011).

1.4.2 Warfarin Use in CKD

Warfarin is commonly used among CKD patients, similar to that observed in the general medical population (Reinecke et al., 2009). Approximately 25% of ESRD patients use warfarin, primarily to prevent vascular access thrombosis during dialysis (Elliott et al., 2007; Miller et al., 2006) and/or to prevent thromboembolic complications in patients with AF (Hart et al., 2011; Yang et al., 2010).

Warfarin use is also prevalent in patients with mild/moderate CKD (Hart et al., 2011). Kidney disease patients are treated with warfarin despite the sparse evidence about its anticoagulant safety and efficacy in the CKD population. In fact, warfarin management in CKD is derived from studies that have evaluated the effectiveness of warfarin in other medical patients. Patients with advanced CKD are usually excluded from the clinical trials of warfarin therapy (Bansal, 2014). Additionally, there has been no randomized controlled study that directly assessed the safety and efficacy of warfarin for stroke prevention in HD patients (Winkelmayer and Turakhia, 2014). The lack of evidence based use of warfarin, primarily in HD patients, likely contributes to the high incidence of poor response in patients with CKD.

1.4.3 Warfarin Dosing Requirements in CKD

As previously mentioned the variability in warfarin dosing is partially accounted for by the differences in the clinical and genetic background of patients, which could eventually influence warfarin pharmacokinetics and pharmacodynamics. Recent studies have shown that dosing requirements are also affected by the presence of kidney disease. In a prospective cohort study of 578 patients who were followed for up to two years, Limdi and colleagues showed that patients with severe CKD (eGFR of <30 mL/min/1.73m^2) required 20% lower warfarin dosages, had higher proportion of INRs outside the therapeutic range, and were at increased over-anticoagulation (HR = 1.49) and major bleeding risks (HR = 2.4) compared with patients who had mild or no CKD. The findings remained significant even after adjustment for patient's clinical and genetic variables (Limdi et al., 2009). The same group further reported that patients with moderate CKD (eGFR of 30-59 mL/min/1.73m^2) require approximately 10% warfarin dose reduction compared to patients with mild or no CKD (Limdi et al., 2010). Recent retrospective studies have confirmed these

findings, demonstrating dose reductions in CKD/ ESRD of up to 24% compared to control (Kleinow et al., 2011; Sakaan et al., 2014). One of these investigations has also documented that CKD patients are at high risk of decreased anticoagulation stability, thus they are required to visit anticoagulation clinics more frequently for warfarin dose manipulation (Kleinow et al., 2011). As indicated, warfarin dosing requirements are varied between CKD patients and other patients with normal kidney function; therefore, better understanding of factors that affect warfarin dosing in CKD may identify the appropriate and optimum warfarin therapeutic dosage for kidney disease patients, which would improve its clinical safety and efficacy. The recent findings suggest that the current approaches for warfarin management in CKD patients, which are reflections of those applied for the general medical patients, may be not suitable and they should be modified so they can account for the level of kidney function.

1.4.4 Warfarin Outcomes in CKD

Because AF patients with CKD experience an increased risk of bleeding and stroke, researchers questioned whether treating CKD patients with warfarin will be beneficial in preventing thromboembolic complications and will minimize the risk. Thus, several observational studies have investigated the safety profile and effectiveness of warfarin in CKD patients, demonstrating an association between increased risk of poor outcomes, such as hemorrhage, stroke, and mortality, and impaired kidney function (Chan et al., 2009a). The bleeding rate associated with warfarin anticoagulation in HD patients is 2-fold higher than those receiving no warfarin treatment (Elliott et al., 2007). A retrospective study of 255 HD patients reported that the risk of major bleeding increases with warfarin and/or aspirin use up to four or five times (Holden et al., 2008). This finding has also been supported recently in a study of 1626 HD patients, which showed a 44%

increased risk of bleeding in warfarin-treated patients (Shah et al., 2014). In addition to bleeding complications, there is an evidence of increased risk of mortality in ESRD patients (Chan et al., 2009a). The recent epidemiological retrospective study of 41,425 incident HD patients showed that warfarin, clopidogrel, and aspirin were commonly prescribed among long-term HD patients. However, their use was significantly associated with increased risk for mortality. Warfarin hazard ratio (1.27) was higher than that for clopidogrel (1.24) and aspirin (1.06) (Chan et al., 2009a). In a follow-up study of 1,671 incident HD patients with preexisting atrial fibrillation, the same group also documented increased risk for new stroke in warfarin users but not with either users of clopidogrel or aspirin (Chan et al., 2009b). These strokes were more likely to be hemorrhagic in nature (Winkelmayer et al., 2011a). In both studies, however, the authors suggested that the confounding by indication cannot be ruled out.

The above findings are in conflict with other reports that showed improved outcomes for CKD patients treated with warfarin. The most modern observational, prospective, multi-center cohort study of 24,317 patients (5292 warfarin users) demonstrated a lower risk of the composite outcome of death and ischemic stroke, with no increase in bleeding risk with warfarin treatment across all kidney disease levels (Carrero et al., 2014). Other elegant well-matched observational studies have also elucidated that warfarin use in CKD patients is associated either with decreased or in-different risk of mortality, stroke and bleeding in CKD patients, primarily those under hemodialysis (Hart et al., 2011; Lai et al., 2009; Olesen et al., 2012; Winkelmayer et al., 2011a). It is therefore obvious that further investigations are warranted to explore the possible reasons behind the anticoagulant instability of warfarin in patients with CKD.

1.4.5 Warfarin versus the New Oral Anticoagulants

Several new oral anticoagulants (NOACs) have been recently approved for prevention and treatment of thromboembolism and as stroke prophylaxis in patients with AF. While dabigatran works as a direct thrombin inhibitor, rivaroxaban and apixaban act by inhibiting clotting factor X activation (Shameem and Ansell, 2013). Unlike warfarin, the NOACs have limited drug and food interactions, shorter half-lives, and do not require frequent lab monitoring of INR. However, these drugs are more expensive, and lack a specific antidote that would be essential to reverse their anticoagulation effect in case of toxicity (Reinecke et al., 2013). Most importantly, the NOACs are excreted unchanged in the urine in considerable amounts (25-80%), which indicates that dosage adjustment is necessary in patients with varying degrees of kidney impairment, especially those with severe CKD (Harder, 2012).

These novel oral anticoagulants have been investigated for their efficacy and safety as compared to warfarin; however, most often patients with CrCl < 30 mL/min were usually excluded from these clinical trials. Dabigatran at 150 mg twice daily was more effective than warfarin in stroke prevention in AF patients, however, similar efficacy was also observed with dabigatran 110 mg twice daily. The risk of major bleeding was similar and lower at 150 mg and 110 mg dabigatran, respectively, as compared with warfarin (Connolly et al., 2009). These findings remained consistent even after categorizing patients according to their kidney function (Hijazi et al., 2014). The randomized double-blinded control trial (ROCKET AF) investigated effectiveness and safety of rivaroxaban versus warfarin in AF patients, demonstrated the non-inferiority of rivaroxaban to warfarin in stroke prevention. Also, the risk of major bleeding was not different between the two groups (Patel et al., 2011). In the ARISTOTLE clinical trial, apixaban showed higher efficacy than warfarin in stroke prevention and reducing mortality with lower rates of major bleeding,

irrespective of the degree of kidney disease (Granger et al., 2011; Hohnloser et al., 2012). A recent systematic review and meta-analysis compared the use of new oral anticoagulants and vitamin K antagonists in CKD patients and reported no significant difference between the two groups in terms of efficacy outcomes of stroke and embolism, as well as the risk of major bleeding (Harel et al., 2014).

The aforementioned findings raise many concerns and challenges for the value of warfarin use in CKD, leading many people to doubt and question the risk versus benefit of using warfarin in CKD, particularly in dialysis patients (Bennett, 2006; Finazzi and Mingardi, 2009; Sood et al., 2009; Yang et al., 2010). With this controversy, definitive conclusions can only be obtained after conducting large randomized controlled trials (Granger et al., 2011). Until more data are available, clear understanding of mechanisms that lead to altered warfarin dose-response in CKD would be helpful in generating new approaches that would have the potential to improve warfarin management and pharmacotherapy especially in the CKD population. Because the NOAC agents have not been investigated across all levels of kidney function, definitive conclusion about switching from warfarin to NOAC cannot be drawn at this time.

1.4.6 Warfarin Metabolism

1.4.6.1 Phase I warfarin oxidation

Warfarin is a highly metabolized drug that undergoes phase I and phase II metabolism in the liver. Phase I metabolism of warfarin is separated into oxidation and reduction reactions (**Figure 1-2**). Warfarin oxidation, which is mediated by CYP450 enzymes, shows stereoselective and regioselective features (Kaminsky and Zhang, 1997). The more potent isomer, *S*-warfarin, is more rapidly cleared by hepatic oxidation reactions, almost exclusively by CYP2C9 (~90%) producing

S-7-OH as a major hydroxylated metabolite (Kaminsky and Zhang, 1997). On the other hand, several CYP enzymes (CYPs 1A2, 2C8, 2C19, and 3A4) are involved in the metabolism of *R*-warfarin into its hydroxy-metabolites (Kaminsky and Zhang, 1997; Wadelius et al., 2007). CYP1A2 is the principal enzyme catalyzing *R*-6-hydroxylation (Zhang et al., 1995). CYP2C19 has an affinity toward production of *R*-8-OH and *R*-6-OH, favoring the former metabolite (Kaminsky et al., 1993; Wienkers et al., 1996). CYP3A4 has a 5-fold higher affinity toward *R*-warfarin compared with *S*-warfarin to generate exclusively *R*-10-OH metabolite (Ngui et al., 2001; Rettie et al., 1992). Because *S*-warfarin is almost exclusively metabolized by CYP2C9, the ratio between the two enantiomers (*S/R* warfarin) is currently used as phenotypic index of CYP2C9 activity (Dreisbach et al., 2003). Warfarin oxidation results in loss of anticoagulant activity. As such, the inactive warfarin hydroxy-metabolites can be either excreted unchanged in the urine, or further metabolized by phase II conjugation. Warfarin oxidation has received more attention to date due to its significant contribution to warfarin metabolism and overall elimination.

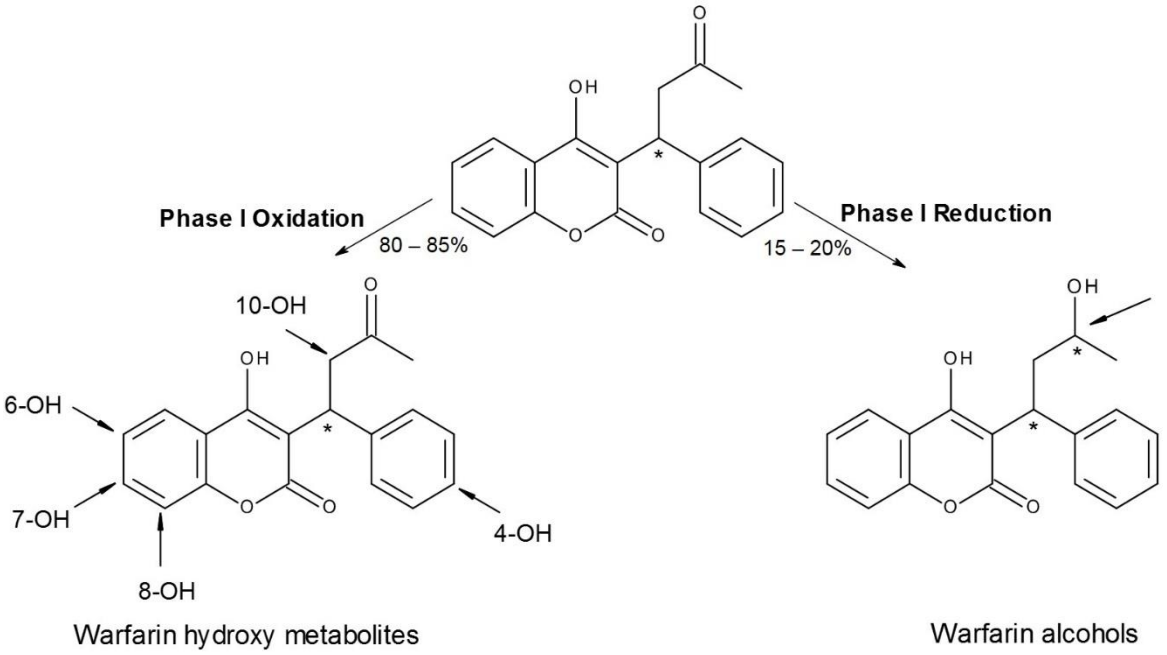

Figure 1-2 Phase I warfarin oxidation and reduction pathways

1.4.6.2 Phase I warfarin reduction

Reduction is the second major pathway of phase I warfarin biotransformation that accounts for approximately 15-20% of total warfarin metabolism (Lewis et al., 1974). The acetonyl side chain reduction of warfarin by hepatic reductases generates warfarin alcohols. Warfarin reduction results in creation of another chiral center on warfarin structure, thus, four stereoisomers of warfarin alcohols are produced (*RS*, *RR*, *SS*, and *SR*). These alcohols are separated into two pairs (mirror images): Alcohol 1; the *RS*/*SR* diastereoisomer, and Alcohol 2; the *RR*/*SS* diastereoisomer (Chan et al., 1972).

Previous reports have shown that the NADPH-dependent reductases contribute to warfarin reduction. It is also known that warfarin reduction is predominantly catalyzed in the cytosol (Moreland and Hewick, 1975). *In vitro* studies showed that, after incubation of liver cytosolic

fractions with either warfarin or individual warfarin enantiomers, *R*-warfarin was reduced into primarily *RS* alcohol (77%), and *S*-warfarin was reduced to primarily *SS* alcohol (57%) (Moreland and Hewick, 1975). This indicates that the alcohol products of warfarin reduction are mainly in *S*-configuration. The findings also demonstrate that the rate of *R*-warfarin reduction is higher than *S*-warfarin, and human reductases show 4-fold more activity toward warfarin reduction than rats (Moreland and Hewick, 1975). In order to assess the ability of microsomal reductases to metabolize warfarin, a study was conducted in several species using cytosolic and microsomal fractions of liver tissue (Hermans and Thijssen, 1989). The authors reported that the cytosolic reductases play a predominant role in warfarin reduction. Nonetheless, the microsomal enzymes have undetectable (in rats) or generally low (in humans) activity toward warfarin reduction; human microsomes metabolized the *R*-enantiomer to primarily the alcohol 1 (*RS*) metabolite (Hermans and Thijssen, 1989). In an effort to understand the contribution of cytosolic reductases to warfarin metabolism, a study was performed utilizing two purified rabbit liver cytosolic fractions and various reductase enzymes inhibitors. Warfarin reduction was inhibited by menadione, furosemide, indomethacin, and prostaglandin E2 in both fractions, suggesting that warfarin reduction is more likely to be catalyzed by several isoforms of the carbonyl reductase enzymes (Hermans and Thijssen, 1992; Hermans and Thijssen, 1993). To summarize, warfarin reduction shows substrate and product stereoselectivity. While *R*-warfarin is preferred as a substrate, the favored product is the *S*-configuration. Additionally, warfarin reduction primarily occurs in the cytosol. Nevertheless, the specific reductase isoforms that are involved in warfarin metabolism have not been characterized to date.

Unlike warfarin hydroxy-metabolites, warfarin alcohols are excreted unchanged in the urine (Lewis et al., 1974). Moreover, the steady-state plasma levels of warfarin alcohol in patients

with long term anticoagulation therapy are comparable to that of 7-OH-warfarin, the most abundant warfarin hydroxy-metabolite, or even to the parent warfarin enantiomers, with the *RS* isomer being the most common warfarin alcohol in plasma (Chan et al., 1994; McAleer et al., 1992). Most importantly, compared to hydroxy-metabolites, warfarin alcohols exert anti-coagulant activity. The oral administration of individual warfarin alcohols to healthy volunteers resulted in decreased plasma levels of vitamin-k dependent clotting factors with subsequent prolongation in prothrombin time. The anticoagulant response was equivalent for all four alcohols; however, it was more sustained after administration of *RS* alcohol, which could be attributed partially to its long half-life (34 h for *RS* versus 13 h for *RR*, *RS*, and *SS*) (Chan et al., 1972; Lewis and Trager, 1971; Lewis et al., 1973). In addition, the recent *in vitro* findings have demonstrated that warfarin alcohols are 6-fold less potent than warfarin in their ability to inhibit VKOR enzyme (IC_{50} 12.5 vs. 2.2 µM) (Gebauer, 2007). Since warfarin alcohols possess anticoagulant activity and achieve considerably higher plasma concentrations, alteration of their disposition may contribute to the variability in warfarin response and dosing requirements in CKD patients.

1.4.6.3 Phase II warfarin conjugation

The hydroxy products of warfarin phase I metabolism can be further stereo- and regioselectively conjugated primarily by UGT isoforms, producing glucuronidated hydroxy-metabolites that are excreted principally in the urine (Jones et al., 2010). Several UGTs are involved in warfarin glucuronidation. Using human liver microsomes and recombinant human UGTs, Zielinska and coworkers illustrated that five UGTs were shown to be active toward warfarin hydroxy-metabolites conjugation, including UGTs 1A1, 1A3, 1A8, 1A9, and 1A10 (Zielinska et al., 2008). The role of the extrahepatic UGT1A8 and UGT1A10 was significant in clearance of warfarin metabolites. The 6-OH metabolite was primarily metabolized by the extrahepatic UGT1A10.

Different UGTs recognized 7-OH and 8-OH metabolites with the highest activities related to UGT1A1 and UGT1A10, respectively. Lastly, UGT1A10 showed a little activity toward 4'-OH, nevertheless, 10-OH as well as warfarin were not recognized by any of the screened enzymes (Zielinska et al., 2008). These data suggest that UGT1A1 and UGT1A10 are the two principal UGT isoforms that catalyze warfarin conjugation. Additionally, the combined functional expression of hepatic and extrahepatic (i.e., stomach and intestines) UGTs derives the capacity and efficacy of this pathway toward warfarin elimination. Recently, the study of Bratton and colleagues has demonstrated the stereoselective effect of UGTs in metabolizing warfarin hydroxy-metabolites. For instance, UGT1A8 and UGT1A9 selectively recognized *S*-8-OH metabolite, with no activity toward *R*-8-OH enantiomer (Bratton et al., 2012). Collectively, the complementary role of conjugative enzymes toward warfarin metabolism may open new avenues to better understand their contribution to warfarin disposition and response.

1.5 SUMMARY, RESEARCH HYPOTHESIS AND OBJECTIVES

CKD is among the major public health problems that affect people worldwide. The prevalence of CKD is increasing due to the growing global epidemics of diabetes and hypertension. Deterioration of kidney function alters pharmacokinetics as a result of impaired filtration and/or active secretion processes, and may place patients at high risk of drug toxicity. Thus, dosing adjustments are important for drugs that are cleared unchanged via the kidneys. In addition to the effect of kidney disease on renal clearance of drugs, elimination mediated by drug metabolizing enzymes and transport proteins is also impacted. This suggests that drugs that are eliminated nonrenally may need dosing adjustments when prescribed to CKD patients. As described earlier, numerous studies have demonstrated alterations of drug metabolism and transport in kidney disease. This was evaluated from *in vitro* studies utilizing sub-cellular fractions or cell culture, to *in vivo* studies using phenotypic probe substrates. However, these studies were limited to phase I drug oxidation, phase II conjugation, and transport.

Reduction is another critical drug metabolism pathway that accounts for the metabolism of a significant number of drugs and endogenous compounds. Alterations of drug metabolism catalyzed by reductase enzymes may influence drug disposition and its pharmacological response. In fact, several clinical studies outlined earlier demonstrated changes in exposure and metabolic clearance of drugs metabolized primarily by reduction. However, to date, limited information exists about whether kidney disease alters the activity and expression of reductase enzymes. Thus, the major goal of this dissertation work was to systematically evaluate the effect of kidney disease on hepatic reductase functional expression. This body of work will improve our understanding of the effect of kidney disease on drug disposition, nonrenal clearance in particular. It may also help improve drug management in patients with impaired kidney function.

The primary hypothesis guiding this work is that kidney disease decreases the functional expression of reductase enzymes located in the liver, resulting in altered drug disposition. In order to perform these assessments, we employed warfarin as a pharmacological probe substrate. As discussed earlier, warfarin is metabolized by reduction to produce distinct warfarin alcohol metabolites, and their formation rate may be used as a surrogate of the activity of reductase enzymes. In addition, utilizing warfarin as a substrate could explain mechanisms of altered warfarin dose-response in the setting of kidney disease.

To test the central research hypothesis, it was first necessary to develop a novel analytical assay capable of quantifying warfarin alcohol metabolites. This was achieved by utilizing advanced and modern techniques and instruments as presented in chapter 2. The second objective was to investigate the effect of experimental kidney disease on hepatic reductase function and expression. This was completed *in vitro* using subcellular fractions of livers collected from control rats and those subjected to nephrectomy as described in chapter 3. Next, we predicted that human reductase isoforms will be impacted by kidney disease. Thus, the third objective was to explore the effect of ESRD on the activity and expression of human hepatic reductases. This work was accomplished *in vitro* using liver tissue collected from patients with and without ESRD as detailed in chapter 4. The last objective was to evaluate the steady-state pharmacokinetics of warfarin in patients with impaired kidney function. This was achieved by collecting blood samples from patients with varying degrees of kidney function, and conducting analytical work, pharmacokinetic and data analyses as presented in chapter 5. The last study may allow a potential translation of pre-clinical findings into humans. Finally, the conclusions, limitations, and the recommended future directions of this thesis work are discussed in chapter 6.

2.0 DETERMINATION OF WARFARIN ALCOHOL METABOLITES USING ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY: METHOD DEVELOPMENT AND VALIDATION

[**Alshogran OY**, Ocque AJ, Zhao J, Day BW, Leblond FA, Pichette V, Nolin TD. Determination of warfarin alcohols by ultra-high performance liquid chromatography-tandem mass spectrometry: application to *in vitro* enzyme kinetic studies. *J Chromatogr B Analyt Technol Biomed Life Sci* **944**: 63-68, 2014].

2.1 ABSTRACT

A sensitive, accurate, and reproducible ultra-high performance liquid chromatography–tandem mass spectrometry method was developed and validated for determination of warfarin and its alcohol metabolites (*RS/SR*- and *RR/SS*-warfarin alcohol) in 10 mM Tris-HCl incubation buffer (pH 7.4). Sample preparation involved acidification with 4% formic acid, followed by liquid-liquid extraction using methyl tert-butyl ether. Chromatographic separation was achieved using a Hypersil Gold C18 (2.1 × 100 mm, 1.9 µm) analytical column with gradient elution of solvent A (water containing 0.01% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). The flow rate was 0.4 mL/min and the total run time was 5 min. Detection of analytes was performed using heated electrospray ionization (negative mode) and selected reaction monitoring. Excellent linearity was observed for all analytes over the standard curve concentration ranges of 100-10,000 ng/mL for warfarin, and 0.5-250 ng/mL for warfarin alcohols. The intra- and inter-day accuracy and precision for analytes were within ±10.0%. Excellent recovery and negligible matrix effects were observed. The method is sensitive, accurate and reproducible, and was successfully applied to *in vitro* enzyme kinetic studies of warfarin in rat liver cytosol.

2.2 INTRODUCTION

Warfarin is administered clinically as a racemic mixture of both *R*- and *S*-enantiomers, and is highly metabolized, exhibiting regioselective and stereoselective metabolism (Jones et al., 2010; Kaminsky and Zhang, 1997). It undergoes phase I oxidation mediated mainly by CYP450 enzymes, CYP2C9 in particular, producing hydroxy metabolites that can be further metabolized by phase II conjugation (Jones et al., 2010; Kaminsky and Zhang, 1997). The effect of various factors (e.g., disease, inflammation, genetic polymorphisms) on CYP2C9 function (Zanger and Schwab, 2013) and their subsequent impact on warfarin dose requirements has been extensively studied (Cavallari et al., 2010; Jorgensen et al., 2009), and this information is now used to optimize warfarin therapy (Klein et al., 2009; Lenzini et al., 2010).

As discussed previously in chapter 1, reduction is an important metabolic pathway for warfarin elimination. Warfarin undergoes reduction by hepatic reductases, which generate warfarin alcohols of two diastereoisomers (alcohol 1 and alcohol 2) (Chan et al., 1972; Lewis et al., 1974). Warfarin reduction is catalyzed predominantly in the cytosol, producing warfarin alcohol 1 as the major metabolite (Hermans and Thijssen, 1989; Moreland and Hewick, 1975). Although reduction accounts for up to 20% of warfarin metabolism (Lewis et al., 1974) and produces pharmacologically active alcohol metabolites (Lewis et al., 1973), the effect of altered reductase function on the disposition of warfarin and its alcohol metabolites has not been studied to date. In order to do so, a robust and validated analytical method for the quantitative determination of each analyte is required.

Several analytical assays have been developed for measuring the enantiomers of hydroxywarfarin metabolites in biological samples including HPLC with UV (Miura et al., 2011), combination of UV/fluorescence and circular dichroism (Takahashi et al., 1997) or MS detection

(Jones et al., 2011; Uno et al., 2007; Zuo et al., 2010), capillary zone electrophoresis with UV detection (Zhou et al., 2003), and micellar electrokinetic chromatography with MS detection (Wang et al., 2013). However, few methods have been reported for the determination of warfarin alcohol metabolites. These include conventional HPLC methods with UV or fluorescence detection (Banfield and Rowland, 1984; Fasco et al., 1979; Fasco et al., 1977; Wong and Davis, 1989), and gas chromatography with mass spectrometric (GC-MS) detection (Duffield et al., 1979). In general, HPLC with UV or fluorescence detection methods are not as sensitive and selective as MS techniques and require larger sample volume to achieve high sensitivity. The reported GC-MS method is complex, and requires extensive sample preparation including a derivatization step (Duffield et al., 1979). In addition, each of the reported methods include minimal or no validation parameters for detection and quantitation of warfarin alcohols.

Recently, LC-MS techniques are considered as the gold standard for quantification of pharmaceuticals in different biological matrices, in part, due to the inherent selectivity and sensitivity. To date, use of contemporary LC-MS for the measurement of warfarin alcohols has not been reported. Thus, the goal of this work was to develop and validate an efficient, simple, robust, and rapid high throughput ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) assay for determination of warfarin and its alcohol metabolites.

The method was validated according to the current U.S. Food and Drug Administration (FDA) guidelines for bioanalytical method validation [http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM368107.pdf]. The method was utilized to examine the effect of CKD on the activity of hepatic reductases using warfarin as a probe as we will discuss in the following chapters of this thesis.

2.3 METHODS

2.3.1 Chemical and Reagents

Warfarin ($C_{19}H_{16}O_4$), *d5*-7-hydroxywarfarin ($C_{19}H_{11}D_5O_5$, used as internal standard for warfarin alcohols), formic acid, NADPH, magnesium chloride, and tris(hydroxymethyl)aminomethane (Trizma base) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Warfarin alcohols ($C_{19}H_{18}O_4$) were synthesized as previously described with the assistance of Dr. Billy Day laboratory at the University of Pittsburgh (Chan et al., 1972; Trager et al., 1970). Racemic *d5*-warfarin ($C_{19}H_{11}D_5O_4$, used as internal standard for warfarin) was purchased from Toronto Research Chemical (North York, Ontario, Canada). Hydrochloric acid, methyl tert-butyl ether (MTBE), methanol, OPTIMA LC-MS grade water, and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). All chemicals were LC-MS grade or of the highest purity available. Nitrogen gas (ultra-pure, >99.9%) was produced by a Parker Balston nitrogen generator (Haverhill, MA, USA). Argon gas (ultra-pure, >99.9%) was provided by Valley (Wheeling, WV, USA).

2.3.2 Equipment and LC-MS/MS Conditions

Liquid chromatography was performed with an Accela series UHPLC system (Thermo Scientific, San Jose, CA, USA) including an autosampler and ultra-high performance binary pump. Chromatographic separation of the samples was achieved with a Thermo Hypersil Gold C18 (2.1 × 100 mm, 1.9 µm) analytical column, connected to 0.22 µm frit filter. The flow rate was 400 µL/min and composed of solvent A (water containing 0.01% formic acid), and solvent B

(acetonitrile containing 0.1% formic acid). The gradient consisted of 60% solvent A and 40% B for 0-2.0 min, followed by 35% A and 65% B from 2.0-3.0 min. The column was then re-equilibrated to initial conditions for 2 min. The total run time was 5 min. The autosampler was maintained at 10ºC and the column temperature was held at 40ºC.

Mass spectrometric detection was performed on a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific San Jose, CA, USA) equipped with a heated electrospray ionization source (HESI). Analytes were detected in negative ionization mode using selected reaction monitoring. The spray voltage was set to 3000V and the vaporizer temperature was set to 360ºC. The sheath gas and auxiliary gas were set to 65 and 55 (arbitrary units), respectively. The ion transfer tube was set to 350ºC. Collision gas (argon) pressure was set at 1.5 mTorr. Scan width was set to 0.01 *m/z*, scan time was set to 0.5 seconds, and full width at half maximum was set to 0.7 *m/z* for quadrupole one (Q1) and three (Q3). The ion transition and collision energy of the analytes are presented in **Table 2.1**. Signal output was captured and processed with Xcaliber software v2.2 (Thermo Scientific, San Jose, CA, USA).

Table 2-1 MS conditions of the analytes and internal standards in the negative electrospray ionization mode

Analyte	Precursor→Product (*m/z*)	Collision Energy (V)
Warfarin	307.1 → 161.1	17
d5-warfarin	312.2 → 161.2	22
Warfarin alcohols	309.1 → 250.3	26
d5-7-hydroxywarfarin	328.1 → 177.1	22

2.3.3 Preparation of Calibration Standard and Quality Control Samples

Each analyte (warfarin and warfarin alcohols) was dissolved in methanol to obtain a 1.0 mg/mL stock solution. These stock solutions were mixed with methanol to prepare intermediate working solutions that were spiked into blank 10 mM Tris-HCl incubation buffer (pH 7.4) to create calibration standards at concentrations of 100, 200, 1000, 2000, 5000, and 10000 ng/mL for warfarin, and 0.5, 5.0, 25, 50, 125, and 250 ng/mL for warfarin alcohols. Three quality control samples (LQC, MQC, and HQC) containing both analytes were made by spiking a separate 1.0 mg/mL stock solution into blank Tris-HCl solution at concentrations of 300, 4000, and 8000 ng/mL for warfarin, and 1.5, 100, and 200 ng/mL for warfarin alcohols. Preliminary experiments showed that recoveries of analytes were not affected by the presence of microsomal or cytosolic protein and cofactors. Therefore, standards and QCs were prepared in the incubation buffer alone. All stock solutions, standards and QC samples were stored at -80ºC to simulate the storage conditions of study samples.

2.3.4 Sample Preparation

An internal standard working solution containing 5.0 µg/mL *d5*-warfarin and 1.2 µg/mL *d5*-7-hydroxywarfarin was prepared in methanol. A 100 µL aliquot of sample (incubation sample, calibration standards, or quality controls) was spiked with 20 µL internal standard solution, and then acidified by adding 250 µL 4% formic acid in water. Sample extraction was carried out using liquid-liquid extraction procedure by adding 2 mL MTBE and placing on a shaker with continuous mixing for 20 min at room temperature. The upper organic layer was removed and evaporated to dryness for 10 min under a stream of nitrogen at 37ºC. The dried residue was reconstituted with

150 µL of acetonitrile-water (25:75, v/v). Samples were loaded into autosampler vials and 20 µL was injected into the LC-MS/MS system.

2.3.5 Assay Validation

2.3.5.1 Calibration and linearity

Calibration curves were constructed using six concentrations of warfarin and warfarin alcohols. Each calibration level was run in duplicate for three days, except for the lower limit of quantification (LLOQ), which was run in triplicate. For each curve, the absolute peak-area ratios of the analyte to the internal standard were calculated and plotted against the nominal analyte concentration. Calibration curves were generated by equal weighting quadratic log-log regression analysis.

2.3.5.2 Accuracy and precision

Accuracy and precision were determined by the analysis of QC samples spiked at three concentrations (LQC, MQC, and HQC). Six replicate QC samples at each concentration were analyzed daily for two days, followed by analysis of twelve replicate QC samples at each concentration on the third day, for a total of n = 24 QC samples at each QC level. Intra-day accuracy and precision were determined from the twelve replicates on day 3, and inter-day accuracy and precision were calculated from all 24 QC samples. The calculated mean concentration relative to the nominal concentration was used to express accuracy (% bias) as follows: %bias = (calculated concentration – nominal concentration)/ nominal concentration×100. The relative standard deviation (% RSD) was calculated from the QC values and used to estimate the precision as follows: %RSD = (standard deviation/mean) ×100. The results of each run were

accepted when the accuracy and precision are falling within ±15% deviation from the nominal concentrations.

A dilution analysis was performed on samples spiked to twice the concentration of the highest standard, then diluted 1:4, 1:20, or 1:100 with blank Tris-HCl buffer before analysis. Each dilution level was processed in triplicate and back calculated against the standard curves.

2.3.5.3 Recovery, process efficiency and matrix effect

Extraction recovery of warfarin alcohols was determined by comparing the absolute response (peak areas) of the analytes spiked in blank Tris-HCl buffer before and after the extraction. Process efficiency was evaluated by comparing the response of analytes spiked in the buffer before extraction with that of neat solutions spiked in mobile phase, which was defined as 100% recovery. Matrix effect was assessed by comparing the response of the analytes spiked in the buffer after extraction with that of neat solutions spiked in mobile phase, representing 100% (no matrix effect). All experiments were conducted at three QC levels in four replicates. Samples were analyzed using standard curves generated from warfarin alcohol standards as described previously.

2.3.5.4 Stability

Stability was evaluated at low and high QC levels in triplicate. Bench-top stability was tested by analyzing samples that were left out on the bench top at room temperature for four hours before analysis. Autosampler stability was determined for processed samples stored at 10ºC, which were re-injected and analyzed against a new standard curve up to 24 hours post-processing. Freeze-thaw stability was assessed for samples that were subjected to three freeze-thaw cycles at 24 hour intervals from -80°C to room temperature prior to analysis. The results of all tested samples were compared with the recovery from samples that were freshly prepared and defined as 100% control.

In all cases, solutions were considered to be stable as long as the measured concentration deviated from control by less than 15%.

2.3.6 Application of the Method

The analytical method was applied to the quantification of alcohol metabolites generated after *in vitro* warfarin reduction. Briefly, liver cytosol was isolated from rat liver tissue by a standard differential centrifugation procedure (van der Hoeven and Coon, 1974). Cytosolic protein (0.5 mg/mL) was incubated with warfarin (20-2000 µM) and 5 mM $MgCl_2$ in 10 mM Tris-HCl buffer (pH 7.4). Reactions were started by the addition of 1 mM NADPH and conducted for 30 min at 37ºC. Incubation conditions were optimized with respect to time and protein concentration. Following incubation, warfarin alcohols were measured as described above. A Michaelis-Menten model was used to fit the formation of alcohol metabolites, and total reductase enzyme activity was estimated (GraphPad Software Inc., San Diego, CA).

2.4 RESULTS

2.4.1 Chromatographic Separation

Representative chromatograms of analytes at the LLOQ and in a cytosolic reaction mixture are shown in **Figure 2.1.** The analytes were identified based on retention time and mass spectra of the injections of individual analytical standards. The retention times were approximately 2.23, 2.61 and 2.86 min for warfarin alcohol 2, warfarin alcohol 1 and warfarin, respectively. The peaks of interest were well separated and free from evidence of ion suppression or enhancement.

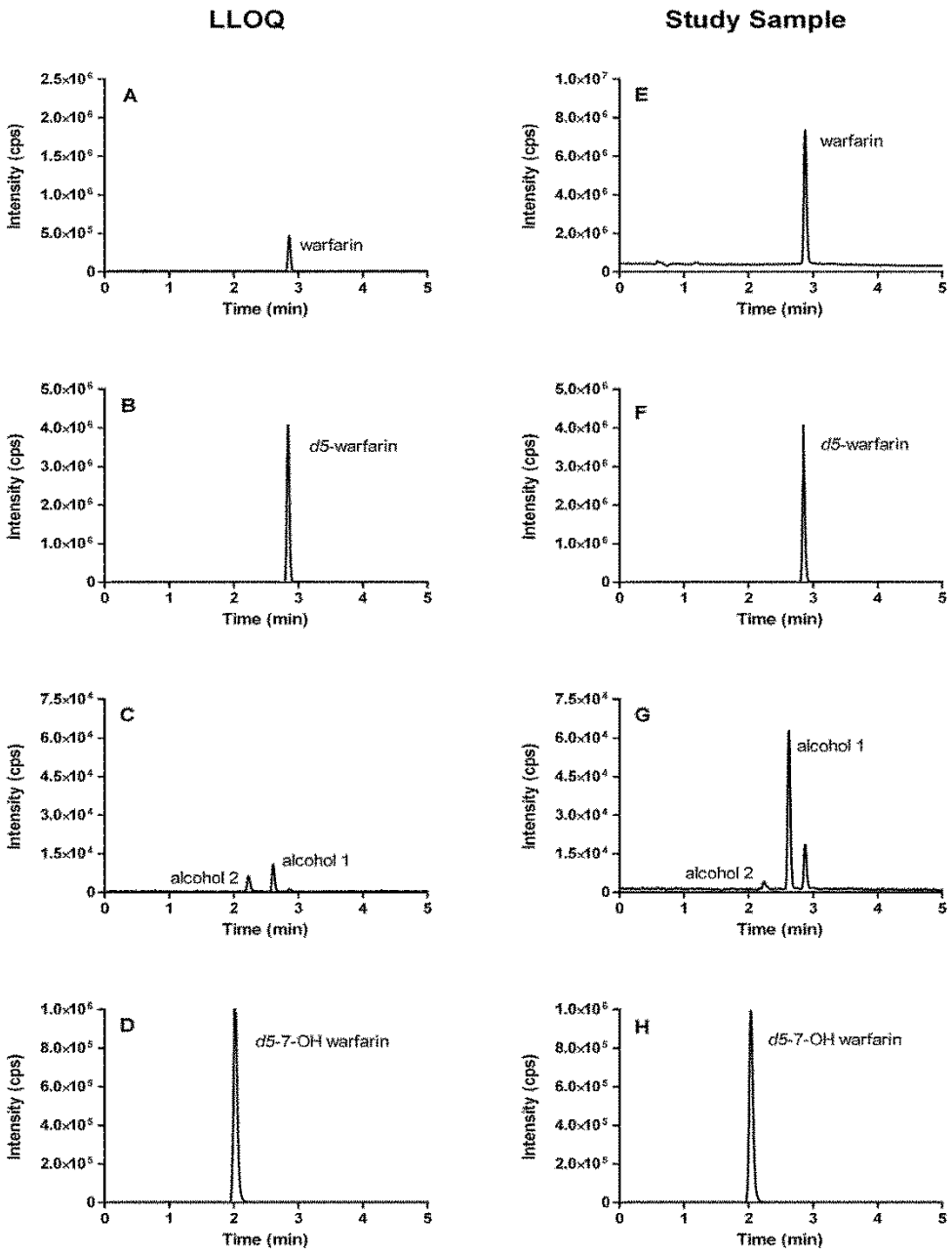

Figure 2-1 Representative chromatograms of incubation buffer at LLOQ and a cytosolic incubation sample

(A) warfarin: 100 ng/mL; (B, F) internal standard *d5*-warfarin; (C) warfarin alcohols: 0.5 ng/mL; (D, H) internal standard *d5*-7-hydroxywarfarin; (E) warfarin: 7,941 ng/mL; (G) warfarin alcohol 2 & alcohol 1: 0.85, 7.97 ng/mL, respectively.

2.4.2 Assay Validation

2.4.2.1 Calibration and linearity

Calibration curves were obtained over concentration ranges of 100-10,000 ng/mL for warfarin, and 0.5-250 ng/mL for warfarin alcohols, with a correlation coefficient (r^2) greater than 0.996 for all curves. The LLOQ for each calibration curve demonstrated acceptable accuracy and precision (RSD and bias were within ±11% and ±5%, respectively, **Table 2.2**), and signal-to-noise was greater than 10:1. The intra- and inter-day accuracy and precision were within ±12% for all calibration standards (**Table 2.2**).

Table 2-2 Intra-and inter-day accuracy (%Bias) and precision (%RSD) for calibration standards

	Nominal Conc	Intra-day[a]		Inter-day[b]	
Analyte	(ng/mL)	% Bias	% RSD	% Bias	% RSD
Warfarin					
	100 (LLOQ)	-2.4	4.7	-5.4	7.8
	200	6.2	0.3	2.8	3.9
	1000	-4.2	2.0	-3.2	3.4
	2000	4.7	2.6	6.4	3.7
	5,000	-5.8	1.8	-7.1	1.5
	10,000	3.4	1.8	3.5	1.5
Warfarin alcohol 1					
	0.5 (LLOQ)	1.1	10.7	1.2	6.5
	5	-1.7	1.3	-3.6	4.9
	25	-8.2	8.6	-1.3	6.9
	50	11.7	2.3	5.9	7.4
	125	3.6	4.1	1.8	4.2
	250	-5.0	0.9	-3.1	3.9
Warfarin alcohol 2					
	0.5 (LLOQ)	0.3	10.8	0.4	8.8
	5	2.1	3.1	0.4	2.9
	25	-7.3	6.3	-2.8	5.5
	50	5.4	1.6	2.0	5.4
	125	3.2	3.4	2.7	3.6
	250	-2.5	2.1	-1.9	2.4

[a] 3 replicates for LLOQ, 2 replicates for other standards

[b] 9 replicates for LLOQ; 6 replicates for other standards

2.4.2.2 Accuracy and precision

The intra-day and inter-day accuracy (% bias) and precision (% RSD) were determined at warfarin QC concentrations of 300, 4000, and 8000 ng/mL, and alcohols concentrations of 1.5, 100, and 200 ng/mL over three validation runs. Assay bias ranged from -10.0 to 8.8%, while the RSD ranged from 4.0 to 8.2% (**Table 2.3**). In all cases, bias and RSD values were within ±10% for all analytes indicating an accurate and a reproducible assay. Acceptable accuracy and precision were also observed during the dilution analysis, as bias and RSD were within ±14.4%, and 4.8% in all samples, respectively.

Table 2-3 Intra-and inter-day accuracy (%Bias) and precision (%RSD) for quality controls

Analyte	Level	Nominal Conc (ng/mL)	Intra-day[a]		Inter-day[b]	
			% Bias	% RSD	% Bias	% RSD
Warfarin						
	LQC	300	-10	6.9	-8.9	5.7
	MQC	4,000	-4.4	5.6	-4.7	6.3
	HQC	8,000	0.79	5.8	-1.1	6.5
Warfarin alcohol 1						
	LQC	1.5	-4.4	7.0	-2.9	8.2
	MQC	100	5.2	6.7	5.8	6.3
	HQC	200	2.2	5.0	0.53	5.1
Warfarin alcohol 2						
	LQC	1.5	5.7	5.7	3.9	6.6
	MQC	100	8.8	4.8	7.7	5.0
	HQC	200	7.2	4.0	3.5	5.7

[a] 12 replicates for LQC, MQC, HQC

[b] 24 replicates for LQC, MQC, HQC

2.4.2.3 Recovery, process efficiency and matrix effect

The extraction recoveries and process efficiency of analytes, which were determined at three QC levels, ranged from 73.2% to 90.2%, and 76.3% to 93.6% respectively (**Table 2.4**). Overall, the recoveries and process efficiency of analytes were consistent and reproducible. The matrix effect was negligible, with measured concentrations deviating by -2.7% to 11% from neat samples (**Table 2.4**).

Table 2-4 Extraction recovery, process efficiency and matrix effect of analytes in incubation buffer

Analyte	Nominal Conc (ng/mL)	Extraction Recovery (%, mean)	Process Efficiency (%, mean)	Matrix Effect (%, mean)
Warfarin				
	300	79.2	78.3	98.8
	4,000	88.4	92.0	104
	8,000	90.2	93.6	104
Warfarin alcohol 1				
	1.5	73.2	76.3	104
	100	79.8	81.8	102
	200	81.0	82.4	102
Warfarin alcohol 2				
	1.5	77.5	92.0	111
	100	80.5	79.3	98.5
	200	80.7	78.5	97.3

n=4 for each QC level

2.4.2.4 Stability

Bench-top, autosampler, and freeze-thaw stability were assessed for analytes in the buffer at LQC and HQC levels. Samples were found to be stable for 4 hours at room temperature (bench-top), and 24 hours post analysis at 10ºC in the refrigerated autosampler. Additionally, three freeze-thaw cycles had no effect on the stability of analytes in the buffer. The mean measured concentrations of analytes in the buffer ranged from 95% to 112% of freshly analyzed samples, indicating adequate stability under all conditions tested (**Table 2.5**).

Table 2-5 Stability for quality controls (LQC & HQC) of warfarin and its alcohol metabolites

Analyte	Target (ng/mL)	Stability*					
		Bench-top (4 hrs)		Autosampler (24 hrs)		Three Freeze/Thaw cycles (24 hrs)	
		% of Target	%RSD	% of Target	%RSD	% of Target	%RSD
Warfarin							
	300	106	1.8	103	8.6	99.6	2.5
	8,000	111	0.45	95.1	2.3	111	0.85
Warfarin alcohol 1							
	1.5	112	3.5	103	5.9	111	7.4
	200	103	3.6	101	0.82	95.9	1.1
Warfarin alcohol 2							
	1.5	102	3.0	111	7.9	106	9.2
	200	104	2.7	101	0.86	101	2.5

*Data are presented as means of n=3 for LQC and HQC levels

2.4.3 Assay Application

The current method was successfully applied to the *in vitro* assessment of warfarin reduction. Michaelis-Menten plots of warfarin alcohols generated after incubation of rat cytosol with various concentrations of warfarin are shown in **Figure 2.2**. The metabolic activities (V_{max}) for cytosolic reductases producing alcohol 1 and alcohol 2 were 33.2 and 9.9 pmol/mg protein/min, respectively. The affinity constant values (K_m) were 434.8 and 1,583 µM for alcohol 1 and alcohol 2, respectively. These results imply that the current method is well suited and reliable for enzyme kinetic studies of warfarin requiring high-throughput quantitative determination of warfarin alcohol metabolites, as also shown in chapters 3 and 4 using subcellular fractions isolated from rat liver tissue and human liver tissue, respectively.

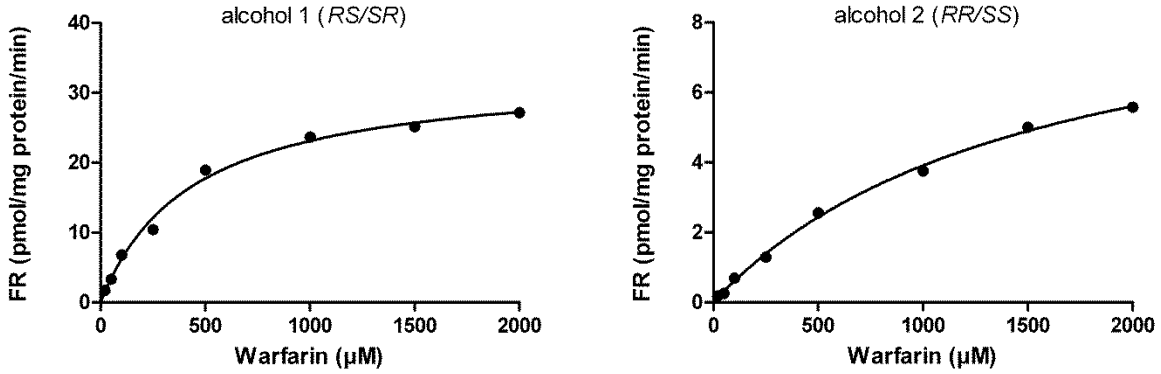

Figure 2-2 Michaelis-Menten plots for formation of warfarin alcohols in rat liver cytosol

Incubation was conducted using 0.5 mg/mL rat liver cytosolic protein in the presence of 1 mM NADPH, 5 mM $MgCl_2$, and various concentrations of warfarin (20-2000 µM) for 30 min at 37ºC. The y-axis represents the formation rate of warfarin alcohol metabolites. Controls omitting warfarin or NADPH showed negligible formation of warfarin alcohol metabolites.

2.5 DISCUSSION

In order to support studies exploring warfarin reduction, we aimed to develop and validate a simple UHPLC-MS/MS method for determination of warfarin and warfarin alcohol metabolites. In here, we present a robust method to measure warfarin and its alcohol metabolites in cellular fraction incubates. This is the first validated UHPLC-MS/MS method reporting quantification of warfarin alcohol metabolites. Furthermore, we have successfully applied the processing conditions and extraction procedure of the current method for the measurement of warfarin alcohols in incubates of rat cytosolic cellular fractions. Collectively, these results suggest that our assay is valid and reproducible, and can be used successfully to quantify warfarin alcohols after *in vitro* enzyme kinetic studies.

The assay was developed in the negative ionization mode because of the enhanced sensitivity. The MS conditions were chosen to achieve optimum selectivity, specificity and sensitivity for the analytes. The liquid chromatography separation of the analytes was performed with gradient elution of the mobile phase. The selected percentages of formic acids in the mobile phase provided the optimum clear separation of the analytes with sharp peaks and short run time. In this assay, we used deuterated warfarin as an internal standard for measuring warfarin. However, a deuterated warfarin alcohol is not commercially available. Thus, we used deuterated 7-hydroxy-warfarin as internal standard for measuring warfarin alcohols. The selected internal standards were eluted close to the analytes (warfarin and warfarin alcohols) and facilitated a short run time of 5 min.

The assay has been validated according to the FDA guidelines. The intra and inter-day accuracy and precision of analytes were within ±10% suggesting the validity and reproducibility of our method. In addition, the assay shows excellent (more than 73%) and consistent extraction

recovery of the analytes from the incubation buffer. We have not noticed any matrix effect of the Tris-HCl incubation buffer on the recovery of the analytes as there was less than 11% difference in the anayltes response. This was also confirmed by the absence of any ion suppression or enhancement by post-column infusion experiment.

To simulate the real life situations, the calibration standards and QCs were stored at -80°C, and the QC samples were exposed to various conditions to test for their stability. Our samples were found to be stable under the three explored conditions that may be subjected to prior to analysis. These include bench-top, autosampler, and freeze-thaw stability.

The presented method was successfully applied to the measurement of warfarin alcohol metabolites generated during *in vitro* enzyme kinetic studies with rat liver tissue (chapter 3) and human liver tissue (chapter 4).. In rat tissue, the metabolic formation of warfarin alcohol 1 after cytosolic incubation with warfarin was about three-fold higher than alcohol 2. Also, the affinity constant (K_m) for alcohol 1 was 3.6 fold lower than that of alcohol 2 suggesting higher affinity toward alcohol 1 formation in the cytosol. This is in agreement with previous reports, which showed that warfarin alcohol 1 is the major reduced warfarin metabolite that is produced in the cytosol (Hermans and Thijssen, 1989; Moreland and Hewick, 1975).

The previous reported methods for quantification of warfarin alcohols in different matrices have some limitations. These include inadequate sensitivity (LLOQ 10-40 ng/mL), extensive pre-analysis work, large sample volume (250-1500 µL), long run time (7-60 min), and complexity (Banfield and Rowland, 1984; Duffield et al., 1979; Fasco et al., 1979; Fasco et al., 1977; Wong and Davis, 1989). Our modern and unique method has several advantages over the previously published non-LC/MS assays including: 1) simple and rapid sample processing based on liquid-liquid extraction with no derivatization step; 2) a small sample volume requirement of 100 µL; 3)

short run time of 5 min; 4) high sensitivity with 0.5 ng/mL lower limit of quantification; 5) validation according to the FDA guidelines.

In conclusion, a simple, rapid, accurate, and precise UHPLC-MS/MS method for the determination of warfarin and warfarin alcohols has been developed and validated. The short run-time and high sensitivity and specificity associated with LC-MS/MS renders this method valuable for studies relating to warfarin reduction.

3.0 EFFECT OF EXPERIMENTAL KIDNEY DISEASE ON THE FUNCTIONAL EXPRESSION OF HEPATIC REDUCTASES

[**Alshogran OY**, Naud J, Ocque AJ, Leblond FA, Pichette V, Nolin TD. Effect of experimental kidney disease on the functional expression of hepatic reductases. *Drug Metab Dispos* **43**: 100-106, 2015].

3.1 ABSTRACT

CKD can affect the nonrenal clearance of drugs by modulating the functional expression of drug metabolizing enzymes and transporters. The impact of CKD on phase I oxidative and phase II conjugative metabolism has been extensively studied. However, the impact of CKD on hepatic drug reduction, an important phase I drug metabolism pathway, has not been investigated. We aimed to assess the effect of experimental CKD on hepatic reduction, using warfarin as a probe substrate. Cytosolic and microsomal fractions were isolated from liver tissue harvested from 5/6th-nephrectomized and control rats (n=10 per group). The enzyme kinetics for warfarin reduction were evaluated in both fractions, and formation of warfarin alcohols was used as an indicator of hepatic reductase activity. Selective inhibitors were employed to identify hepatic reductases involved in warfarin reduction. mRNA and protein expression of reductases were quantified using qRT-PCR and Western blotting, respectively. Formation of *RS/SR*-warfarin alcohol was decreased by 39% (P<0.001) and 43% (P<0.01) in cytosol and microsomes, respectively in livers from CKD rats compared to control rats. However, *RR/SS*-warfarin alcohol formation was unchanged in the cytosol and microsomes. mRNA expression of cytosolic CBR1 and AKR1C3, and microsomal 11β-HSD1 was significantly reduced by 34%, 93% and 35% (P<0.05), respectively in livers from CKD compared with control rats. Additionally, protein expression of the corresponding reductases was significantly down-regulated by >40% (P<0.05) in CKD rats versus controls. Collectively, these results suggest that the functional expression of hepatic reductases is selectively decreased in kidney disease. These findings support the hypothesis of altered nonrenal clearance, exposure, and response of certain drugs in CKD patients.

3.2 INTRODUCTION

Chronic kidney disease is a major public health problem that affects approximately 26 million Americans (Weiner, 2009). Kidney disease is described as the general term of the progressive and heterogeneous disorders that are associated with functional and structural abnormalities of the kidney (Levey and Coresh, 2012). Patients with CKD commonly exhibit unpredictable changes in drug disposition and response, largely due to decreased renal clearance of drugs excreted unchanged by the kidney, as well as altered nonrenal drug clearance (Nolin et al., 2008). As we previously detailed in chapter 1 numerous experimental and clinical studies have documented remarkable decreases in the functional expression of drug metabolizing enzymes in CKD (Lalande et al., 2014; Naud et al., 2012), including particularly phase I oxidation mediated by CYP450 enzymes (Yeung et al., 2014), and phase II conjugation via acetylation (Simard et al., 2008), while phase II glucuronidation is not affected (Yu et al., 2006). The effect of CKD on other drug metabolic pathways is less clear.

As described earlier, carbonyl reduction represents an important phase I drug metabolism pathway (Malatkova and Wsol, 2014). Carbonyl reductases can be classified into quinone reductases and carbonyl reducing enzymes (Oppermann, 2007). Reduction by quinone reductases is mediated primarily by NQO1 (Oppermann, 2007; Siegel et al., 2012). The carbonyl reducing enzymes are further separated into two major superfamilies; SDR that includes primarily cytosolic CBR and microsomal 11β-HSD isoforms (Gathercole et al., 2013; Skarydova and Wsol, 2012), and AKR that mainly comprises the AKR1C subgroup (Malatkova et al., 2010; Matsunaga et al., 2006). Collectively, reductase enzymes play key roles in the biotransformation of diverse endogenous compounds such as bile acids, glucocorticoids, prostaglandins, and numerous drugs (e.g., bromperidol, bupropion, doxorubicin, haloperidol, ketoprofen, naltrexone, and warfarin)

(Malatkova and Wsol, 2014; Oppermann, 2007; Skarydova et al., 2014). Although the pharmacokinetics of several drugs that are reductase substrates, including idarubicin (Camaggi et al., 1992) and bupropion (Turpeinen et al., 2007) are altered in patients with CKD, the effect of impaired kidney function on hepatic reductase enzymes has not been assessed to date.

This study aimed to investigate the effect of severe CKD on the expression and function of several hepatic reductases in rats. This was accomplished utilizing warfarin as a probe substrate based on the premise that it undergoes reduction with formation of distinct alcohol metabolites, namely *RS*/*SR*-warfarin alcohol (alcohol 1), and *RR*/*SS*-warfarin alcohol (alcohol 2) (Alshogran et al., 2014b; Chan et al., 1972; Lewis et al., 1974). Moreover, recent evidence indicates that warfarin dosing requirements and response are altered in patients with impaired kidney function (Chan et al., 2009b; Granger and Chertow, 2014; Limdi et al., 2009; Shah et al., 2014), suggesting that warfarin disposition may also be altered in CKD. Our approach includes conducting enzyme kinetic studies to determine the activity of hepatic reductases in rat liver cytosol and microsomes. The mRNA and protein expression of selective hepatic reductases that mediate warfarin metabolism was also assessed.

3.3 METHODS

3.3.1 Chemical Reagents

Warfarin, NADPH, magnesium chloride, tris(hydroxymethyl)aminomethane (Trizma base), quercetin, flufenamic acid, indomethacin, dicoumarol, and 18β-glycyrrhetinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Warfarin alcohols were synthesized as previously described (Chan et al., 1972; Trager et al., 1970). Organic solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Primers used for PCR quantification were purchased from Eurofins MWG Operon (Huntsville, AL, USA). Unless otherwise specified, all other chemicals and reagents were purchased from Sigma-Aldrich. All chemicals were LC-MS grade or of the highest purity available.

3.3.2 Experimental Model

Male Sprague-Dawley rats (Charles River, Saint-Charles, PQ, Canada) that weighed 200-300 g were housed in the Research Center animal care facility and maintained on a standard rat chow and water *ad libitum* on a 12-h light/dark cycle. Rats were allowed to acclimatize for at least three days before conducting any experimental procedure. All experiments were conducted according to the Canadian Council on Animal Care guidelines for care and use of laboratory animals.

Studies were performed in two experimental groups of rats: control (n=10) and CKD (n=10). Control rats were pair-fed the same amount of rat chow that was ingested by CKD rats on the previous day. Severe CKD was induced by a standard two-stage 5/6th nephrectomy model as previously described (Leblond et al., 2000). This model represents an extreme and progressive

stage of uremia. Briefly, rats in the CKD group underwent a two-third nephrectomy of the left kidney that was followed by a complete right nephrectomy seven days later. Rats in the control group were subjected to two sham laparotomies. At day 41 of the surgery urine was collected over a period of 24 hour to determine creatinine clearance. Forty two days after the initial surgery, rats were scarified by decapitation. Liver tissue was harvested and stored at -80^{o}C. Blood was collected for the measurement of serum creatinine and urea concentrations to determine the extent of kidney disease.

3.3.3 Isolation of Hepatic Cytosolic and Microsomal Fractions

Liver cytosol and microsomes were isolated by a standard differential centrifugation as described previously with slight modifications (Hermans and Thijssen, 1989). In brief, frozen tissue was homogenized in ice-cold buffer (50 mM Tris-HCl buffer, 150 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol and 20% glycerol, pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride as a protease inhibitor and 0.113 mM butylated hydroxytoluene as an anti-oxidant. The homogenate was centrifuged at 20,000*g* for 30 min at 4°C. The supernatant was then centrifuged at 140,000*g* for 60 min at 4°C. The resulting 140,000*g* soluble supernatant was collected as a cytosol, and the microsomal pellet was washed twice and resuspended in 0.02 M Tris-HCl (pH 7.4) containing 0.25 M sucrose. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. Cytosolic and microsomal proteins were stored at -80^{o}C until analysis.

3.3.4 *In Vitro* Warfarin Reduction Using Cytosolic and Microsomal Fractions

The metabolic activity of rat hepatic reductases that catalyze warfarin reduction was assessed using cytosolic and microsomal cellular fractions of control and CKD liver tissue. Incubations were conducted using 0.5 mg/mL of either cytosolic or microsomal protein in 10 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ and various concentrations of warfarin (20, 50, 100, 250, 500, 1000, 1500, and 2000 µM). Reactions were started by the addition of 1 mM NADPH and conducted for 30 min at 37ºC. The final reaction volume was 500 µL. Substrates were added in acetone with a final concentration consistently maintained at 1%. Controls omitting substrate/NADPH were included in each incubation. Conditions were optimized during preliminary experiments to achieve linear formation of warfarin alcohol metabolites with respect to incubation time and protein concentration. Incubations were quenched by adding 500 µL ice-cold acetonitrile. Samples were briefly vortex-mixed, placed on ice for 15 min and centrifuged at 14,000 rpm for 10 min to pellet the protein. Metabolites were extracted using methyl tert-butyl ether, evaporated to dryness under nitrogen gas, and reconstituted with acetonitrile-water (25:75, v/v) prior to the injection onto the LC-MS system. Warfarin alcohol metabolites were quantified by UPLC-MS/MS as we have described in chapter 2 (Alshogran et al., 2014b). The intra-day accuracy and precision of analytes ranged from 90 to 108.8% and from 4 to 11%, respectively. The inter-day accuracy and precision ranged from 91 to 107.7% and from 5 to 8.2%, respectively (Alshogran et al., 2014b).

3.3.5 Identification of Rat Enzymes Involved in Warfarin Reduction

To identify rat hepatic reductases that are involved in the carbonyl reduction of warfarin, pooled liver cytosol or microsomes of control rats were incubated with warfarin (1 mM) and a chemical inhibitor under the optimized conditions described in the previous section. **Figure 3-1** depicts the classes of reductase enzymes and the corresponding chemical inhibitors utilized in this study. The inhibitors and concentrations (10 and 100 μM) used, and their selectivity for reductase enzymes were based on the previous reports, as follows: quercetin for cytosolic CBRs (Hermans and Thijssen, 1992; Tong et al., 2010); flufenamic acid for cytosolic AKRs (Atalla et al., 2000; Molnari and Myers, 2012; Rosemond et al., 2004); indomethacin for both cytosolic CBRs and AKRs (Tong et al., 2010; Usami et al., 2001); dicoumarol for cytosolic NQO1 (Tong et al., 2010); and 18β-glycyrrhetinic acid for microsomal 11β-HSD1 (Breyer-Pfaff et al., 2004; Molnari and Myers, 2012). The concentrations of the inhibitors utilized were based on the IC_{50} or K_i values reported in citations following each inhibitor. The concentration of the substrate (warfarin = 1 mM) was based on the K_m value estimated from the activity studies in the previous sections. All inhibitors were dissolved in a mixture of dimethylsulfoxide-methanol (50:50, v/v) except for dicoumarol, which was prepared in 1% sodium hydroxide in water. Organic solvents were consistently maintained at a final concentration of 0.5% in all incubations. Control reactions were performed with the vehicles in the absence of the chemical inhibitors. All experiments were conducted in triplicate.

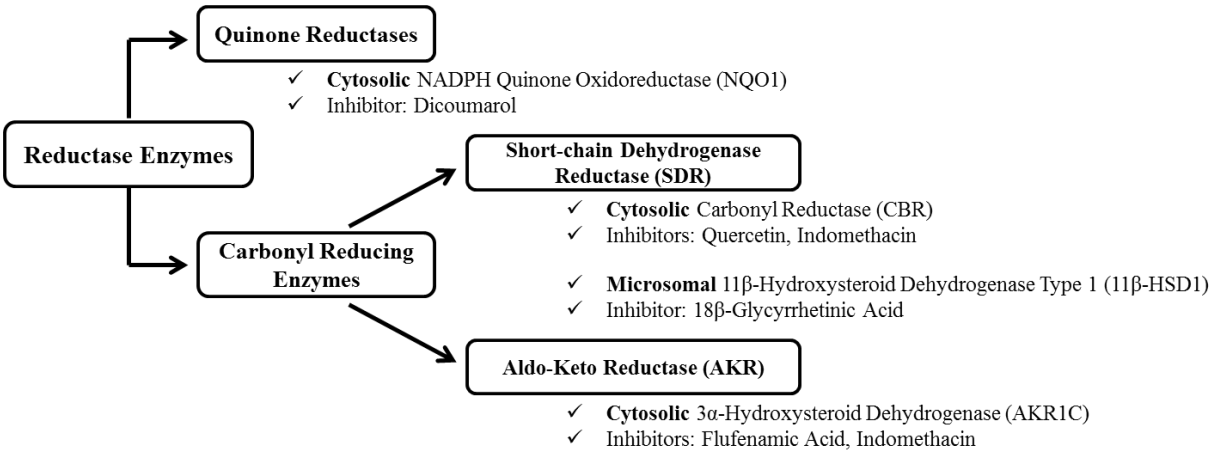

Figure 3-1 Schematic representation of the hepatic reductase enzymes and inhibitors investigated in the current study

Cytosolic reductases can be classified into quinone reductases (NQO) and carbonyl reducing enzymes. Carbonyl reducing enzymes comprises two major superfamilies; the short-chain dehydrogenase reductase (SDR) and the aldo-keto reductase (AKR) enzymes. The main groups of these super-families implicated in ketone reduction of drugs are cytosolic carbonyl reductases (CBRs) and cytosolic 3α-hydroxysteroid dehydrogenases (AKR1C), respectively. 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) is the principal SDR enzyme catalyzes drug reduction in the microsomes. The selective inhibitors utilized in this study are: dicoumarol for NQO1, quercetin and flufenamic acid for CBR and AKR1C respectively. Indomethacin is a non-specific inhibitor for both CBR and AKR1C. 18β-glycyrrhetinic acid is selective for 11β-HSD1 in the microsomes.

3.3.6 RNA Isolation and Quantitative Real-Time PCR Analysis

Total RNA was extracted from liver tissue using TRIzol reagent (Invitrogen, San Diego, CA). RNA concentration and purity were measured by spectrophotometry at 260 nm. One microgram of total RNA was used to prepare the cDNA by reverse transcription using SuperScript III reverse transcriptase (Invitrogen, San Diego, CA, USA) in a 20-µL reaction volume. Expression of rat CBR1, CBR3, AKR1C3, AKR1C14, NQO1, 11β-HSD1, CYP1A2, CYP2D1, CYP2E1 and GAPDH genes was quantified by qRT-PCR performed with the ABI Prism 7300 system (Applied Biosystems, Foster City, CA, USA) using the SYBR Green master mix (Roche, Indianapolis, IN, USA) and specific primers (**Table 3-1**). Hepatic reductase mRNA levels were normalized to GAPDH and expressed relative to the controls using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Table 3-1 Nucleotide sequence for the primers used in qRT-PCR for rat enzymes

mRNA	Left Primer (5'-3')	Right Primer (5'-3')	Predicted Product Size (base pair)
CBR1	TCCACATTCAAGCAGAGGTG	CACTCTGCCTTGGGGTTTTA	98
CBR3	ACCACATGGTAGAGTGGTGA	AGTGTGTCACATCGGAACCT	99
AKR1C3	CCTGTGTGCAACCAGGTAGA	CCATAGGCAACCAGAACGAT	95
AKR1C14	TTCCTGTACTGGGGTTTGGA	CAGAGTCAAAATGGCGGAAT	108
NQO1	GCTTGACACTACGATCCGC	CACAGCAGCCTCCTTCATG	117
11β-HSD1	AGCATTGCCATCATCTCCTC	GTGGAAAAGAACCCATCCAG	98
CYP1A2	GGCAGGTCAACCATGATGAG	CTTGTCGATGGCCGTGTT	92
CYP2D1	CTGCAGGTGGACCTCAGTAA	ATGGGCTTCCAACCCTTC	95
CYP2E1	GGGACATTCCTGTGTTCCAG	GTCTCGGAGAATGCTTAGGG	104
GAPDH	TGCCACTCAGAAGACTGTGG	GGATGCAGGGATGATGTTCT	85

3.3.7 Western Blot Analysis

The protein expression of cytosolic CBR1 and AKR1C18 [also known as 20 alpha-hydroxysteroid dehydrogenase (20α-HSD)], and microsomal 11β-HSD1 was determined using Western blotting. Samples (50 µg total protein) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Transferred membranes were blocked for 1 hr at room temperature using 5% (w/v) non-fat powdered milk dissolved in PBS. Membranes were then incubated overnight at 4°C with specific primary antibodies diluted in 0.5% non-fat powdered milk dissolved in PBS (1:1000 rabbit polyclonal anti-CBR1, Abcam (Cambridge, MA); 1:2000 rabbit polyclonal anti-20α-HSD, Kera Fast (Boston, MA); 1:200 rabbit anti-11β-HSD, CAYMAN chemicals (Ann Arbor, MI); or 1:1000 mouse monoclonal anti-β-actin, GeneTex (Irvine, CA). After washing four times with PBS-Tween (0.1% Tween-20 in PBS), membranes were incubated for 1 hr at room temperature with HRP-conjugated secondary antibodies diluted in 0.5% milk (1:4000 goat anti-rabbit, Sigma; or 1:1000 goat anti-mouse, Sigma. The membranes were then washed with PBS-Tween, and the immune complexes were revealed by chemiluminescence Lumilight (Roche, Indianapolis, IN) detection system on the Fujifilm intelligent dark box equipped with the LAS-4000 camera (Fujifilm, Piscataway, NJ). Band intensity was quantified by densitometry using Multi Gauge Software (Fujifilm) and normalized to β-actin (*This work was conducted in collaboration with Dr. François Leblond and Dr. Vincent Pichette, at the University of Montreal, Canada*).

3.3.8 Data and Statistical Analysis

The formation rate of alcohol metabolites was calculated from their measured concentrations. A non-linear regression Michaelis-Menten kinetic model was used to fit the formation of alcohol metabolites, and the maximum velocity of enzymes (V_{max}) and the affinity constant (K_m) were estimated and compared between groups using GraphPad Prism (Version 5; GraphPad Software Inc., San Diego, CA). Formation rate of alcohol metabolites in the inhibition studies was expressed as percent of control incubations, and statistical differences in formation of alcohols in the presence or absence of inhibitors were determined using student's *t*-test. Reductases mRNA and protein expression of CKD rats were expressed relative to that of controls, and statistical differences between the two groups were assessed using student's *t*-test. *P* value of <0.05 was considered significant for all comparisons. The results are expressed as mean ± SEM.

3.4 RESULTS

3.4.1 Characteristics of Control and CKD Rats

Table 3-2 presents biochemical parameters and weight of the two studied groups of rats. Successful induction of severe kidney disease was confirmed by measuring the serum concentrations of creatinine and urea, the markers of kidney function. The levels of serum creatinine were significantly elevated in CKD group compared to controls. Likewise, the serum levels of urea were significantly higher in CKD rats versus controls. Body weight was not different between the two groups of rats.

Table 3-2 Characteristics of control pair-fed and CKD rats

	Control Pair-Fed (n=10)	**CKD (n=10)**	***P*-value**
Body Weight (g)	386.0 ± 38.4	333.9 ± 75.0	NS
Serum creatinine (μM)	40.4 ± 8.7	203.4 ± 83.9	<0.001
Creatinine clearance (μL/100 g of body weight/min)	359.7 ± 79.3	68.8 ± 41.3	<0.001
Serum urea (mM)	3.6 ± 1.1	38.3 ± 26.1	<0.001

NS: non-significant

3.4.2 *In Vitro* Cytosolic and Microsomal Warfarin Reduction

To determine the impact of experimental kidney disease on hepatic reductase activity, we assessed warfarin reduction *in vitro* using rat liver cytosol and microsomes. Both cellular fractions were shown to generate warfarin alcohol metabolites, and the enzyme kinetics for alcohols were determined in this study.

In the cytosol, V_{max} for formation of warfarin alcohol 1 was significantly decreased by 39% in livers from CKD compared with control rats (23.3 ± 1.29 versus 38.0 ± 1.97 pmol/mg protein/min, *P*<0.001; **Figure 3-2A**). V_{max} for formation of warfarin alcohol 2 was not different between the two group of rats (8.74 ± 0.47 versus 8.43 ± 0.61 pmol/mg protein/min, *P*=NS), and virtually superimposable Michaelis-Menten curves were observed (**Figure 3-2B**).

In microsomes, V_{max} of microsomal reductases producing warfarin alcohol 1 was significantly decreased by 43% in livers from CKD rats compared to control rats (5.49 ± 0.45 versus 9.57 ± 1.25 pmol/mg protein/min, *P*<0.01; **Figure 3-2C**). The results for the metabolic

production of warfarin alcohol 2 in CKD group were unable to fit the Michalis-Menten model. However, the formation of alcohol 2 at the maximum substrate concentration (2000 µM warfarin) was markedly reduced by 87% in livers from CKD rats compared with controls (**Figure 3-2D**). The estimated Mechalis-Menten kinetic parameters (V_{max} & K_m) of cytosolic and microsomal studies are presented in **Table 3-3**.

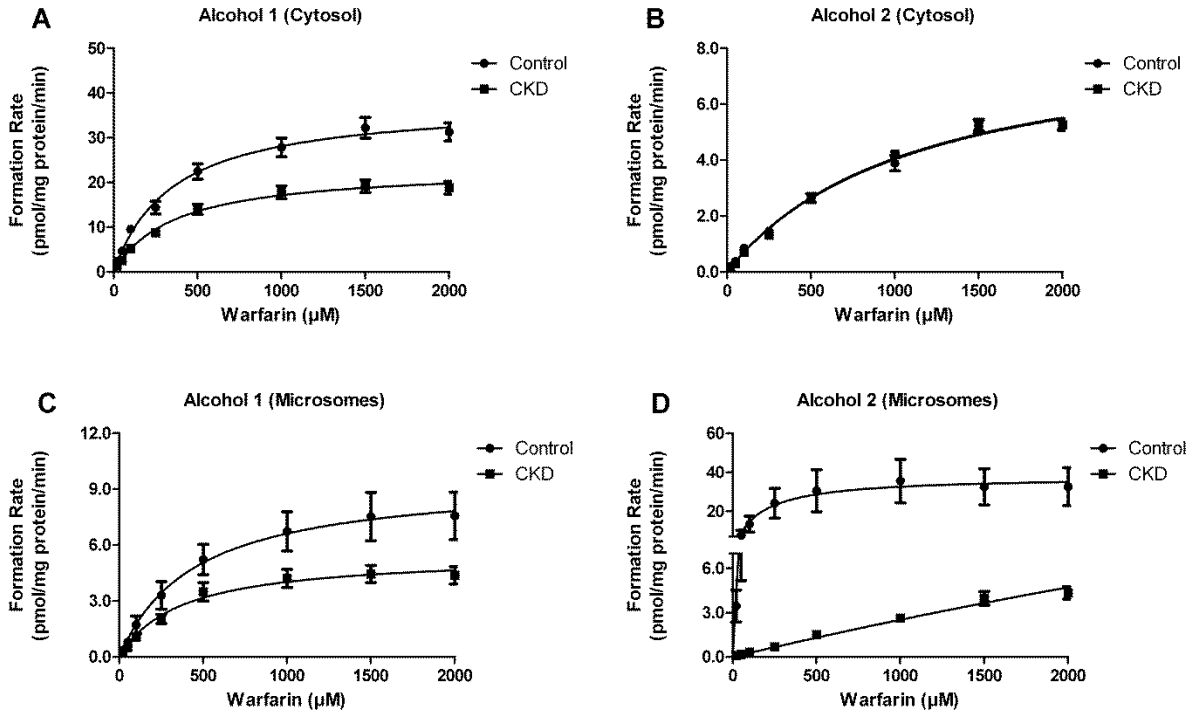

Figure 3-2 Effect of CKD on kinetic formation of warfarin alcohols in cytosol and microsomes Michaelis-Menten plots for the formation of warfarin alcohol 1 (A) and alcohol 2 (B) in rat liver cytosol, and alcohol 1 (C) and alcohol 2 (D) in rat liver microsomes of both control and CKD rats. Each point presents the mean ± SEM of 10 rats in each group.

Table 3-3 Michaelis-Menten kinetic parameters for warfarin reduction in control and CKD rat liver cytosol and microsomes

	Alcohol 1 (*RS/SR*)			**Alcohol 2 (*RR/SS*)**		
	K_m (μM)	V_{max} (pmol/mg protein/min)	Cl_{int} (μL/mg protein/min)	K_m (μM)	V_{max} (pmol/mg protein/min)	Cl_{int} (μL/mg protein/min)
Cytosol						
Control	352.0 ± 59.6	38.0 ± 1.97	0.117 ± 0.016	1091 ± 167.4	8.43 ± 0.609	0.008 ± 0.0008
CKD	359.3 ± 64.1	23.3 ± 1.29***	0.065 ± 0.007**	1149 ± 127.1	8.74 ± 0.466	0.008 ± 0.0004
Microsomes						
Control	446.9±174.7	9.57 ± 1.25	0.021 ± 0.005	158.0 ± 105.6	37.7 ± 6.17	0.243 ± 0.08
CKD	365.6 ± 95.8	5.49 ± 0.45**	0.014 ± 0.002	No fit	No fit	No fit

Data are presented as mean ± SEM of 10 rats in each group. Cl_{int} represents the intrinsic clearance (the enzymatic efficiency: V_{max}/K_m)
** $P < 0.01$ compared with control.
*** $P < 0.001$ compared with control.

3.4.3 Identification of Reductase Enzymes Catalyzing Warfarin Reduction

Selective inhibitors were employed to identify reductase enzymes involved in warfarin reduction. The relative formation of warfarin alcohol 1 and alcohol 2 in the presence of inhibitors as normalized to control incubations is depicted in **Figures 3-3A and 3-3B,** respectively. Cytosolic reduction of warfarin to its alcohols was inhibited by quercetin (inhibitor of CBR), flufenamic acid (inhibitor of AKR1C), indomethacin (inhibitor of CBR and AKR1C), and dicoumarol (inhibitor of NQO1) in a concentration-dependent manner. The magnitude of inhibition to alcohol 1 and alcohol 2 at 10 μM inhibitor concentration ranged from 7-26% and 9-28%, respectively. The extent of inhibition at 100 μM was markedly increased and ranged from 33-81% and 32-70% for formation of alcohol 1 and alcohol 2, respectively. All inhibitions in the cytosol were statistically significant except for the formation of alcohol 1 in presence of 10 μM indomethacin, and alcohol 2 in presence of 10 μM of both flufenamic acid and dicoumarol. Quercetin, an inhibitor of CBR, exhibited the most potent inhibition. Microsomal reduction of warfarin to alcohol 1 and alcohol 2 was significantly inhibited by 15% and 44% respectively at 100 μM 18β-glycyrrhetinic acid (inhibitor of 11β-HSD1).

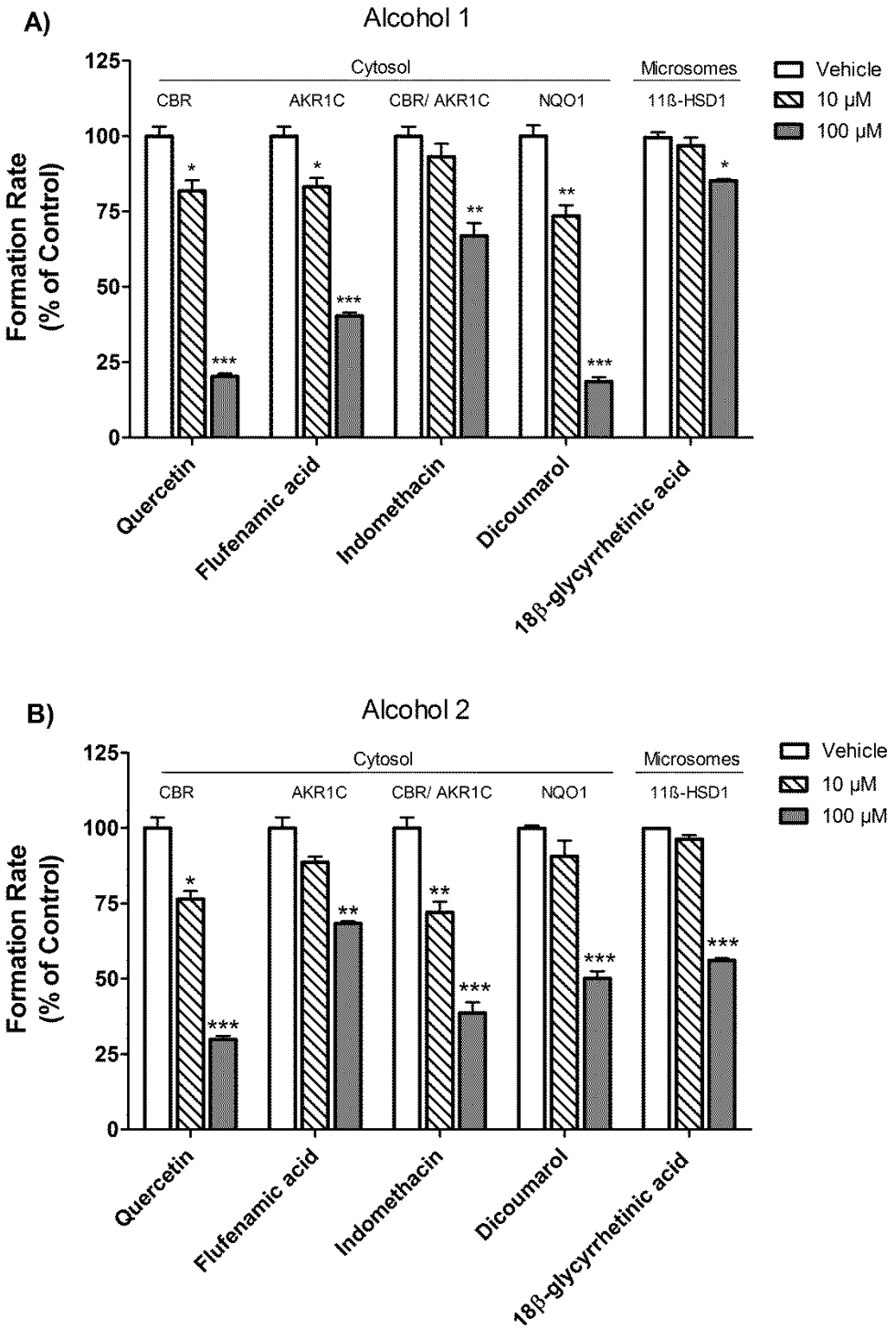

Figure 3-3 The effect of reductase inhibitors on warfarin reduction in rats

Formation of warfarin alcohol 1 (A) and alcohol 2 (B) was investigated in the presence of carbonyl reducing enzyme inhibitors in pooled rat liver cytosol (quercetin, flufenamic acid, dicoumarol, and indomethacin) and microsomes (18β-glycyrrhetinic acid). The corresponding reductase enzymes being targeted by the inhibitors are also included. Incubations were conducted at two different inhibitor concentrations (10 and 100 μM). Results are presented as mean ± SEM of triplicate incubations. Statistical differences are as follows: * (P<0.05); ** (P<0.01), and *** (P<0.001) compared with control incubations which were arbitrarily defined as 100%.

3.4.4 mRNA and Protein Expression of Hepatic Reductase Isoforms

To determine whether the decrease in the metabolic activity of hepatic reductases in kidney disease was secondary to reduced mRNA expression, mRNA levels encoding cytosolic and microsomal reductase isoforms were measured by qRT-PCR (**Figure 3-4**). Although the results show that kidney disease has differential effects on the expression of hepatic reductases, many of the investigated reductases were down-regulated. The mRNA expression of cytosolic CBR1 was significantly decreased by 34% ($P<0.001$) in CKD rats compared with controls, but cytosolic CBR3 was not changed. While the mRNA expression of cytosolic AKR1C3 significantly decreased by 93% ($P<0.001$), the expression of AKR1C14 is significantly increased by 53% in CKD compared to control rats ($P<0.05$). Cytosolic NQO1 expression remained unchanged. Lastly, mRNA expression of the microsomal 11β-HSD1 isoform was significantly reduced by 35% ($P<0.05$) in CKD rats compared with controls. In order to further confirm the validity of our reductase mRNA expression findings in CKD rats, we measured the expression of selective CYP450 isoforms that are not known to be affected by kidney disease as additional controls. Consistent with previous findings (Leblond et al., 2001; Michaud et al., 2005; Rege et al., 2003), we found that mRNA levels of CYP1A2, CYP2D1, and CYP2E1 were not statistically different between CKD and control rats.

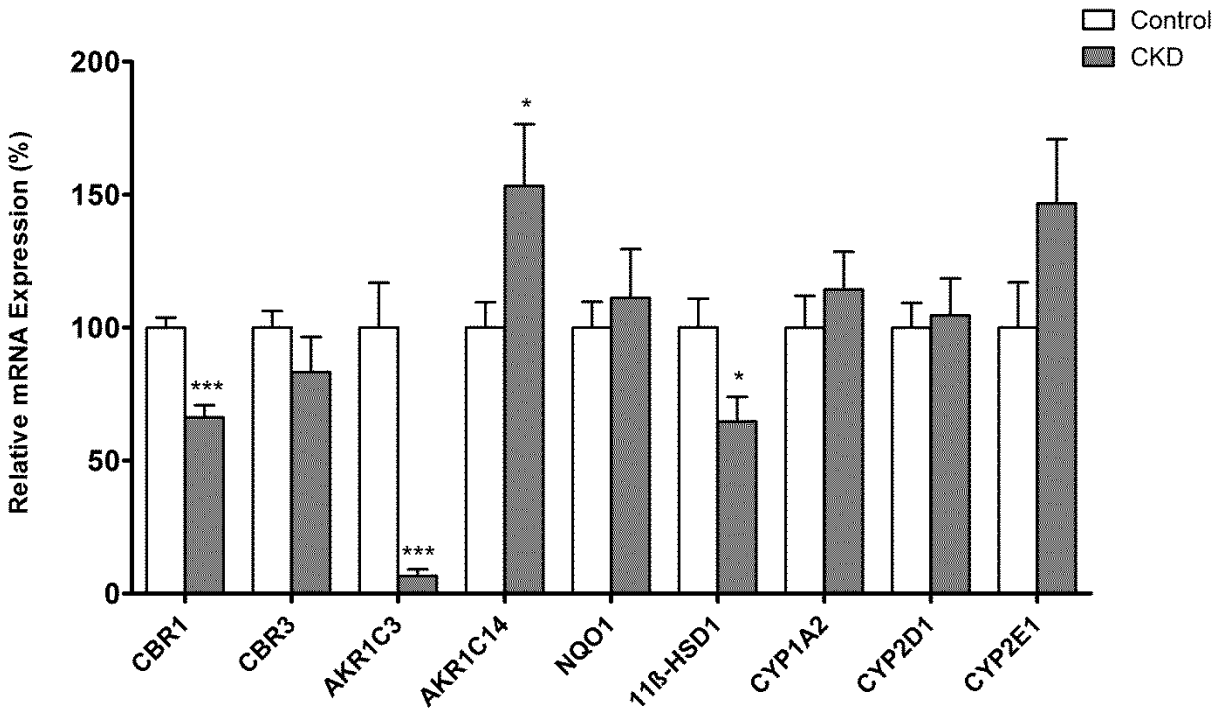

Figure 3-4 Effect of CKD on mRNA expression of rat hepatic reductases

The mRNA expression of cytosolic (CBR1, CBR3, AKR1C3, AKR1C14, and NQO1) and microsomal (11β-HSD1) rat hepatic reductases, as well as CYP1A2, CYP2D1, and CYP2E1 in control and CKD rats. The mRNA expression of reductase isoforms was normalized to that of GAPDH. The results of CKD group were normalized to the control which was arbitrarily defined as 100%. Experiments were conducted in duplicates, and results are presented as mean ± SEM of 10 rats in each group. * (P<0.05) and *** (P<0.001) compared with control.

Figure 3-5 depicts the results of the analysis of protein expression of hepatic reductases in CKD and control rats. Consistent with the findings of mRNA expression, we also found that protein expression of CBR1, AKR1C18 (encoded by AKR1C3), and 11β-HSD1 was decreased by 43%, 76%, and 70% respectively in CKD rats versus controls (P<0.05).

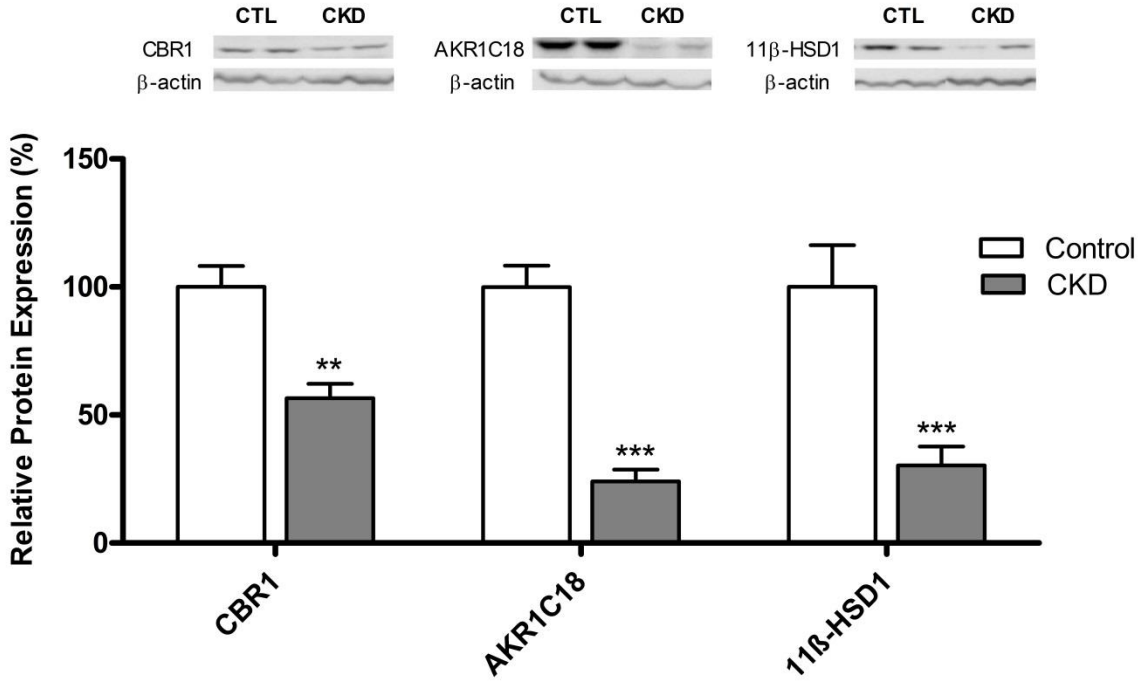

Figure 3-5 Effect of CKD on protein expression of rat hepatic reductases

Protein expression of cytosolic (CBR1 and AKR1C18) and microsomal (11β-HSD1) reductases in control and CKD rat livers. The densitometry units of reductases protein expression was normalized to that of β-actin. The standardized densitometry units of control rats were arbitrarily defined as 100%. The upper panels represent blots of two control and two CKD rats. Results are presented as mean ± SEM of 10 rats in each group. ** (P<0.01) and *** (P<0.001) compared with control.

3.5 DISCUSSION

The present study investigated the impact of experimental kidney disease on hepatic reduction using warfarin as a probe substrate. The enzyme kinetics of warfarin in both cytosolic and microsomal fractions of rat liver tissue were assessed. In addition, the effect of kidney disease on mRNA and protein expression of selective hepatic reductases was evaluated. This study demonstrates that the metabolic activities of selective hepatic reductases catalyzing warfarin reduction are decreased in CKD secondary to down-regulation of mRNA and protein expression of the enzymes (cytosolic CBR1 and AKR1C3, and microsomal 11β-HSD1). These findings suggest that CKD can alter the nonrenal clearance of drugs mediated by hepatic reduction.

Carbonyl reducing enzymes catalyze the reduction of a diverse range of endogenous and exogenous xenobiotics. This includes numerous drugs representing several classes including anticoagulants, antidiabetics, antihypertensives, anticancers, opiate antagonists, NSAIDs, antihistamines, diuretics, and many others (Malatkova and Wsol, 2014; Rosemond and Walsh, 2004). Several pharmacokinetic studies have shown that the disposition of drugs that are primarily metabolized by reduction is altered in patients with kidney disease. For instance, bupropion exposure is dramatically increased by 126% in patients with impaired kidney function, suggesting reduced metabolic clearance (Turpeinen et al., 2007). Moreover, a 30% decrease in the metabolic clearance of idarubicin was reported in patients with creatinine clearance of <60 mL/min (Camaggi et al., 1992). This suggests that phase I reduction of drug may be affected by kidney disease.

In the present study we evaluated the impact of experimental kidney disease on the metabolic activity of cytosolic and microsomal reductases. Our results indicate that warfarin reduction is stereo-selectively decreased in both cytosolic and microsomal liver fractions of CKD rats. The metabolic activity (represented by V_{max}) of cytosolic reductases generating warfarin

alcohol 1 was decreased by 39%, and formation of alcohol 2 was not affected. This suggests that there are multiple reductase enzymes implicated in warfarin reduction in the cytosol that are differentially affected by kidney disease. Dramatic decreases in the metabolic activity of microsomal reductases generating alcohol 1 and alcohol 2 was also observed. However, a definitive conclusion regarding the total activity of microsomal reductases generating alcohol 2 cannot be drawn due to unsuccessful fit of the model to the CKD data. This can be explained, in part, by the linear formation of alcohol 2 observed in the microsomes over the range of the substrate concentrations used. On the other hand, no change on the Michaelis Menten constant (K_m) of warfarin for formation of warfarin alcohols was observed in both fractions. Our results are in agreement with the previous findings showing that alcohol 1 is the major warfarin reductive metabolite in the cytosol (Hermans and Thijssen, 1989; Moreland and Hewick, 1975). Overall, our results suggest that kidney disease may impact hepatic reductases similar to other phase I and phase II metabolic pathways.

In order to identify the carbonyl reductase enzymes that play a role in warfarin reduction, we utilized selective chemical inhibitors for cytosolic and microsomal reductases. Alcohol formation in the cytosol was inhibited up to 81% by quercetin, flufenamic acid, and dicoumarol suggesting the contribution of CBRs, AKRs, and NQO1 respectively, to warfarin reduction. In fact, the potent inhibition of warfarin reduction observed by quercetin, demonstrates the significant contribution of CBRs to warfarin reduction. We also showed that indomethacin, a non-selective inhibitor for both cytosolic CBRs and AKRs (Atalla et al., 2000; Rosemond and Walsh, 2004), significantly inhibited the formation of alcohols by up to 60%. This provides further evidence to the involvement of the two families in warfarin reduction. Our results are in agreement with the previous reports that showed that 4'-nitrowarfarin reduction in liver cytosol of human and rabbit

is strongly inhibited by quercetin and indomethacin (Hermans and Thijssen, 1992; Hermans and Thijssen, 1993). Our results also showed that microsomal warfarin reduction is inhibited by 18β-glycyrrhetinic acid, which indicates that 11β-HSD1 is involved in the microsomal reduction of warfarin. Together, our findings suggest that the microsomal 11β-HSD1 and cytosolic isoforms of CBR, AKR and NQO are responsible for warfarin reduction in rats, with major contribution possibly by CBR isoforms. However, further investigations using recombinant enzyme preparations are required to confirm the specific reductase isoforms involved in warfarin reduction.

Previous studies have demonstrated that the decrease in enzyme activity in CKD is partially due to the down-regulation of protein and mRNA expression of drug metabolizing enzymes (Leblond et al., 2001; Leblond et al., 2002; Simard et al., 2008; Velenosi et al., 2012). Based on the inhibition data, we measured the mRNA expression of cytosolic CBR1, CBR3, AKR1C3, AKR1C14, and NQO1, and microsomal 11β-HSD1 in livers of rats with CKD and control rats. Our study showed, for the first time, that the mRNA expression of hepatic reductases catalyzing warfarin reduction is decreased in the livers of CKD rats. In fact, we were able to detect decreased expression of several carbonyl reductases implicated in drug metabolism, in both cytosolic (CBR1 and AKR1C3) as well as microsomal fractions (11β-HSD1) from the livers. The negligible effect of CKD observed on the expression of CBR3 and NQO1, and the up-regulation observed for AKR1C14 indicates an isoform-selective effect of kidney disease on reductase expression. Our results also showed that protein levels of CBR1, AKR1C18, and 11β-HSD1 are significantly reduced in CKD rats. These findings are similar to that observed for the corresponding genes, suggesting that reduced protein levels could be explained by the decrease in mRNA expression. Collectively, our results suggest that reduced metabolic activity of hepatic reductases in CKD could be, in part, secondary to decreased mRNA and protein expression. The decrease in reductase

activity (V_{max}) and the negligible effect on warfarin affinity (K_m) to reductase enzymes observed in kidney disease rats further support that the decrease in the activity is secondary to reduced mRNA and protein expression of the enzymes. It is well acknowledged that uremic toxins accumulated in uremia may be a reason for the diminished expression of certain CYP450 enzymes (Guevin et al., 2002; Michaud et al., 2005; Simard et al., 2008). However, further studies are warranted to elucidate this with regard to reductases.

Our findings may have important clinical implications, as they may in part explain why the disposition of drugs that are metabolized through phase I reduction pathways (e.g., bupropion, idarubicin) is altered in patients with impaired kidney function. Moreover, our results may shed insight into warfarin treatment in patients with CKD, which is challenging because of the high inter-individual variability observed in the warfarin dose-response relationship (Kimmel et al., 2013). Besides differences in the genetic and clinical background of patients, recent studies have shown that patients with severe CKD require lower warfarin maintenance dosages compared with those with mild or no CKD (Limdi et al., 2009). Moreover, multiple studies suggest that warfarin use in CKD patients is associated with increased risk of poor outcomes such as hemorrhage, stroke, and mortality (Chan et al., 2009a; Chan et al., 2009b; Shah et al., 2014). Although unproven, our findings of decreased warfarin reduction due to decreased metabolic activity of hepatic reductases may contribute to, and could provide a novel mechanistic explanation for, altered warfarin dose requirements and response in CKD patients.

In conclusion, in this study we have demonstrated that CKD selectively decreases the metabolic activity of cytosolic and microsomal hepatic reductases, secondary to down-regulation in mRNA and protein expression of selective hepatic reductases. **Figure 3-6** summarizes the effect of CKD on the activity and expression of reductase enzymes catalyzing warfarin reduction.

Collectively, our data suggest that kidney disease changes the nonrenal clearance of certain drugs mediated by hepatic reduction. The results of this investigation may provide a novel mechanistic explanation for reduced warfarin dose requirements and altered responses observed in CKD patients.

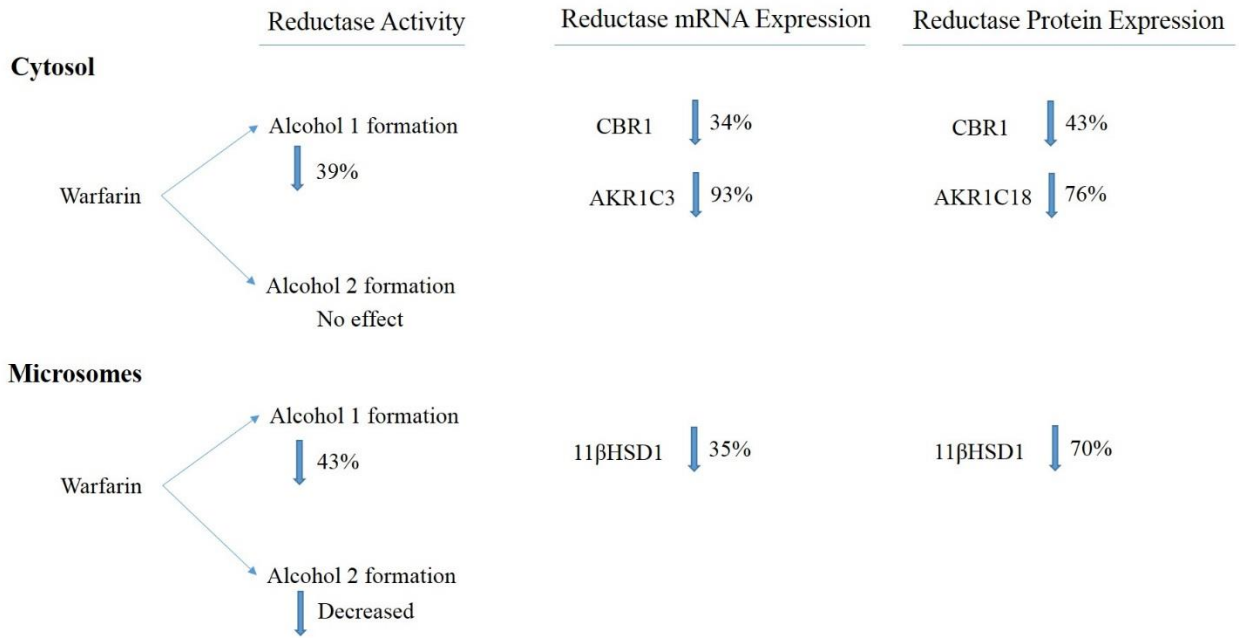

Figure 3-6 Schematic presentation of the effect of experimental kidney disease on the activity and expression of reductases catalyzing warfarin reduction in cytosol and microsomes

4.0 EFFECT OF END STAGE RENAL DISEASE ON THE FUNCTIONAL EXPRESSION OF HUMAN HEPATIC REDUCTASE DRUG METABOLIZING ENZYMES

4.1 ABSTRACT

The functional expression of hepatic drug metabolizing enzymes and transporters are altered in ESRD, leading to impaired nonrenal drug clearance. Reduction is a crucial pathway for the metabolism of an array of drugs and endogenous compounds. We aimed to assess the effect of ESRD on the functional expression of human hepatic reductases using warfarin as a probe substrate. Cytosolic and microsomal subcellular fractions were isolated from liver tissue collected from deceased ESRD (n=10) and deceased control patients (n=11). Metabolic activity, mRNA and protein expression of hepatic reductases were assessed by conducting enzyme kinetic studies, qRT-PCR, and Western blotting, respectively. No significant difference in the formation of *RS/SR*-warfarin alcohol was observed in cytosol or microsomes of ESRD livers relative to controls. Although not statistically significant, a decrease in mRNA expression of CBR1 and AKR1C1-4 of up to 67% was observed in ESRD livers compared to controls (P=NS). A significant (65%) decrease in CBR1 protein expression was observed in ESRD livers versus controls (P<0.05). Together, these results demonstrate a trend toward decreased functional expression of selective hepatic reductases in livers of ESRD patients, which may partially explain altered nonrenal drug clearance of CBR1 drug substrates in ESRD. Given the large inter-individual variability observed, future studies with larger sample size are warranted to confirm these findings.

4.2 INTRODUCTION

The previous chapter evaluated the effect of experimental CKD on the function and expression of rat hepatic reductases. We observed decreased activity of rat hepatic reductases generating warfarin alcohol 1 in cytosol and microsomes. A selective decrease in mRNA and protein expression of rat hepatic reductases was also observed. Together, this suggests that the nonrenal clearance of drugs mediated by reduction is impaired in the setting of CKD.

As we previously discussed in the introduction chapter, CKD modifies bioavailability and disposition of drugs that are nonrenally eliminated, through its effect on the function and/or expression of DMEs and transport proteins (Naud et al., 2012; Nolin et al., 2008; Yeung et al., 2014). These alterations have been demonstrated in clinical studies suggesting that human enzymes are also impacted by CKD. Based on the findings presented in the previous chapter, this study aimed to evaluate whether human hepatic reductases are also affected by kidney disease. To achieve our goals, we systematically explored the impact of ESRD on mRNA and protein expression of human hepatic reductases, as well as their metabolic activity using warfarin as a pharmacological probe substrate. This work was conducted in human liver tissue collected from recently deceased ESRD and control patients. This study is essential to further expand our findings of the effect of experimental kidney disease on drug reduction in humans. In addition, the findings of this investigation may help explain mechanisms behind altered clinical pharmacokinetics of drug substrates of hepatic reductase (e.g., doxorubicin (Yoshida et al., 1994), idarubicin (Camaggi et al., 1992), naltrexone (Kambia et al., 2004)) observed in CKD patients.

4.3 METHODS

4.3.1 Chemicals and Human Liver Samples

Warfarin, NADPH, magnesium chloride, tris(hydroxymethyl)aminomethane (Trizma base), quercetin, flufenamic acid, indomethacin, dicoumarol, and 18β-glycyrrhetinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Warfarin alcohols were synthesized as previously described (Chan et al., 1972; Trager et al., 1970). Organic solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Primers used for PCR quantification were purchased from Eurofins MWG Operon (Huntsville, AL, USA). Unless otherwise specified, all other chemicals and reagents were purchased from Sigma-Aldrich. Cadaveric human liver specimens were procured by the National Disease Research Interchange (NDRI) from 10 ESRD patients who had previously been treated with standard hemodialysis and 11 control subjects who had no evidence of CKD. Samples were maintained at -80°C until conducting the experiments.

4.3.2 Preparation of Human Liver Cytosol and Microsomes

Cytosolic and microsomal subcellular fractions of human livers were isolated by standard differential centrifugation as described previously with slight modifications (Hermans and Thijssen, 1989). Briefly, frozen liver tissue was weighed and homogenized in 50 mM Tris-HCl ice-cold buffer (pH 7.4) containing 150 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 20% glycerol in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (0.1 mM) and the anti-oxidant butylated hydroxytoluene (0.113 mM). The homogenate was centrifuged at 20,000*g* for 30 min at 4°C to yield the S9 fraction supernatant. The S9 fraction was centrifuged at 140,000*g*

for 60 min at 4°C. The resulting 140,000*g* soluble supernatant was collected as a cytosol, and the microsomal pellet was washed twice and resuspended in 0.02 M Tris-HCl (pH 7.4) containing 0.25 M sucrose. Protein concentrations were determined with a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions, using bovine serum albumin as a standard protein. Aliquots of cytosolic and microsomal proteins were stored at -80ºC until further analysis.

4.3.3 Warfarin Reduction in Human Liver Cytosol and Microsomes

The enzymatic activity of human reductases was investigated in cytosolic and microsomal fractions of control and ESRD liver tissue using warfarin as a substrate. Cytosolic or microsomal proteins (0.5 mg/mL) were incubated with various concentrations of warfarin (20, 50, 100, 250, 500, 1000, 1500, and 2000 µM) in 10 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ in a final reaction volume of 500 µL. Reactions were started by the addition of 1 mM NADPH and conducted for 30 min at 37ºC in a shaking water bath. Substrates were added in acetone with a final solvent concentration consistently maintained at 1%. Controls omitting substrate/NADPH were included in each incubation. Incubation time and protein concentration were optimized in preliminary experiments to achieve linear formation of alcohol metabolites. Metabolic reactions were terminated by the addition of 250 µL ice-cold acetonitrile. Then, samples were briefly vortex-mixed, placed on ice for 15 min and centrifuged at 14,000 rpm for 10 min to pellet the protein. The generated warfarin alcohol metabolites in the supernatant were measured using UHPLC-MS/MS as we previously described in chapter 2 (Alshogran et al., 2014b).

4.3.4 Inhibition of Warfarin Reduction in Human Liver Cytosol and Microsomes

To identify the contribution of selective human reductases to carbonyl reduction of warfarin, pooled human liver cytosol or microsomes of the control group were incubated with warfarin (1 mM) and a chemical inhibitor, under the conditions described in the previous section. We have employed the same inhibitors and concentrations described in chapter 3 which include the following: quercetin for cytosolic CBRs (Hermans and Thijssen, 1992; Tong et al., 2010); flufenamic acid for cytosolic AKRs (Atalla et al., 2000; Molnari and Myers, 2012; Rosemond et al., 2004); indomethacin for both cytosolic CBRs and AKRs (Tong et al., 2010; Usami et al., 2001); dicoumarol for cytosolic NQO1 (Tong et al., 2010); and 18β-glycyrrhetinic acid for microsomal 11β-HSD1 (Breyer-Pfaff and Nill, 2004; Molnari and Myers, 2012). Control reactions were performed with the vehicles in the absence of the chemical inhibitors.

4.3.5 RNA Isolation, cDNA Synthesis, and Quantitative Real-Time PCR Analysis

TRIzol reagent (Invitrogen, San Diego, CA) was used to extract total RNA from liver tissue. RNA concentration and purity were determined by spectrophotometry at 260 nm. One microgram of total RNA was reverse-transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen, San Diego, CA, USA). Each cDNA sample was diluted 4-fold and 2 µL of the dilution was used for qPCR analysis. The relative mRNA expression of human liver cytosolic CBR1, CBR3, AKR1C1-4, and NQO1, microsomal 11β-HSD1, and GAPDH was quantified by qRT-PCR performed using the ABI Prism 7300 system (Applied Biosystems, Foster City, CA, USA), SYBR Green master mix reagent (Roche, Indianapolis, IN, USA) and specific primers (**Table 4-1**). All samples were run in duplicate. Hepatic reductase mRNA levels were normalized to GAPDH and

expressed relative to the controls using the comparative quantitation ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen, 2001).

Table 4-1 Nucleotide sequence for the primers used in qRT-PCR of human reductases

mRNA		Primers (5'-3')	Predicted Product Size (base pair)
CBR1	Left	TTTGGTACCCGAGATGTGTG	98
	Right	TTAAGGGCTCTGACGCTCAT	
CBR3	Left	TCACCTTATGGGGTGTCCAAGTTGG	138
	Right	CCCATCCATGTCTGTCTTCACTGGT	
AKR1C1	Left	CTTGGCAAAAAAGCACAAGCGA	125
	Right	CAAACACCTGCACGTTCTGTCTGAT	
AKR1C2	Left	AACAAGCCAGGGCTCAAGTA	92
	Right	GACTTGCAGAAATCCAGCAG	
AKR1C3	Left	GCAGGCAGCTGGAGATGAT	91
	Right	CTCCGGTTGAAATACGGATG	
AKR1C4	Left	GATCCTCAACAAGCCAGGAC	95
	Right	TGCAGAAATCCAGCAGTTTG	
NQO1	Left	ACGAGCCCAGCCAATCAG	116
	Right	CAGCCTCCTTCATGGCATAG	
11β-HSD1	Left	AAGCAGAGCAATGGAAGCAT	108
	Right	GAAGAACCCATCCAAAGCAA	
GAPDH	Left	CTCAAGGGCATCCTGGGCTACA	110
	Right	TGGTCGTTGAGGGCAATGCC	

4.3.6 Western Blotting

Twenty-five microgram of total protein was loaded and electrophoresed on a 12% SDS-polyacrylamide gel. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane over 2 hr. Non-specific binding sites were blocked with 5% bovine serum albumin (BSA) in 1x Tris-buffered saline Tween (TBST) for 1 hr at room temperature. Membranes were then probed overnight at 4ºC with a specific primary antibody diluted in blocking buffer (1:1500 mouse monoclonal anti-CBR1, Santa Cruz Biotechnology; 1:2000 goat polyclonal anti-AKR1C, Santa Cruz Biotechnology; or 1:1000 mouse monoclonal anti-β actin, Sigma-Aldrich). Blots were washed three times with TBST and then incubated with secondary antibody conjugated with HRP for 1 hr at room temperature (1:1000 sheep anti-mouse, GE healthcare Life Sciences; or 1:5000 donkey anti-goat, Santa Cruz Biotechnology). Finally, blots were washed and bands were visualized using chemiluminescence ECL detection system. Band intensity was analyzed densitometrically using Image-J analysis software (National Institutes of Health, Bethesda, MD) and normalized to β-actin.

4.3.7 Data and Statistical Analysis

Enzyme kinetics for formation of warfarin alcohol metabolites were analyzed using a non-linear Michaelis-Menten regression model. The kinetic parameters including (V_{max}) and (K_m) were estimated and compared between groups using GraphPad Prism (Version 5; GraphPad Software Inc., San Diego, CA). In the inhibition studies, formation rate of alcohol metabolites was expressed as percent of control incubations, and statistical differences in alcohols formation in the presence or absence of inhibitors were determined using a student's *t*-test. mRNA and protein expression of

reductases of livers of ESRD group were expressed relative to that of controls, and statistical differences between the two groups were assessed using student's *t*-test. The threshold of significance was *P* value <0.05. All data are presented as mean ± SEM.

4.4 RESULTS

4.4.1 Human Samples and Patient Characteristics

Table 4-2 presents clinical characteristics and demographic features of patients from whom liver samples were obtained right after their death. Patients were matched with respect to age, gender, body mass index (BMI), and race. The number of comorbid conditions was unsurprisingly higher in patients with ESRD compared to that in controls. The most commonly observed comorbid conditions in our sample were hypertension, congestive heart failure, and diabetes. Cardiovascular disease was the major cause of death in our patient sample. All medications taken by patients have not been shown to impact drug reduction, except for furosemide which was taken by one individual in each group, and previous data suggest that it may act as an inhibitor for hepatic reductases (Malatkova and Wsol, 2014).

Table 4-2 Demographics and patients characteristics

Characteristic	**Control (n=11)**	**ESRD (n=10)**	***P***
Age (year; mean ± SD)	70.9 ± 9.7	74.8 ± 5.3	NS
BMI (kg/m^2; mean ± SD)	22.7 ± 4.8	25.8 ± 7.3	NS
Missing (n)	1	0	
Gender [n (%)]			
Male	7 (63.6)	7 (70%)	NS
Female	3 (27.3)	3 (30%)	
Missing	1 (9.1)	0 (0)	
Race [n (%)]			
Caucasian	11 (100)	8 (80)	NS
African	0 (0)	2 (20)	
No. of Comorbid Conditions [n (%)]*			
Low (0 or 1)	5 (45.5)	0 (0)	0.016
Medium (2 to 4)	6 (54.5)	7 (70)	
High (≥5)	0 (0)	3 (30)	

* The most commonly observed comorbid conditions in our sample were hypertension (10 patients), congestive heart failure (8), diabetes (6), chronic obstructive pulmonary disease (5), and coronary artery disease (4)

4.4.2 Effect of ESRD on Warfarin Reduction in Cytosol and Microsomes

To determine the impact of ESRD on human hepatic reductase activity, we assessed *in vitro* steady-state enzyme kinetics of warfarin reduction in a substrate concentration range of 20-2000 µM, using liver cytosol and microsomes of patients with and without ESRD. The estimated Michaelis-Menten kinetic parameters (V_{max} & K_m) for the formation of alcohol metabolites in cytosolic and microsomal fractions are presented in **Table 4-3**.

As depicted in **Figure 4-1** and **Table 4-3**, the estimated steady-state kinetic parameters (V_{max} & K_m) as well as the intrinsic clearance (Cl_{int}) for formation of warfarin alcohol metabolites in cytosol and microsomes were not significantly different between ESRD and control livers. However, a trend of 22-27% decreased V_{max} for formation of alcohol 1 and 29-41% increased warfarin K_m value was observed in ESRD livers versus controls (*P*=NS).

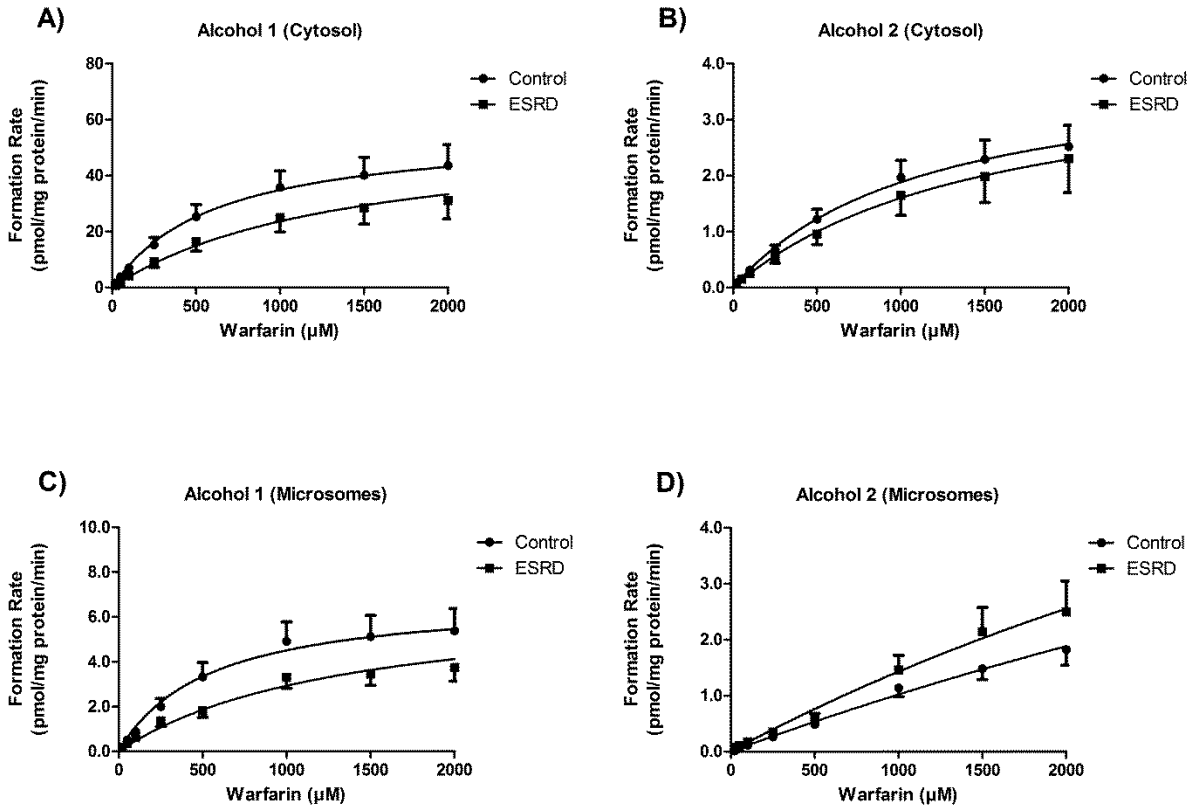

Figure 4-1 Effect of kidney disease on the metabolic activity of human hepatic reductases that catalyze warfarin reduction

Michaelis-Menten plots for the formation of warfarin (A) alcohol 1 and (B) alcohol 2 catalyzed by human liver cytosol, and (C) alcohol 1 and (D) alcohol 2 in human liver microsomes of both control and ESRD livers. Liver cytosolic or microsomal protein (0.5 mg/mL) was incubated with various concentrations of warfarin (20-2000 µM) for 30 min at 37ºC in the presence of cofactors. Each point represents the mean ± SEM of n=11 control and n=10 ESRD livers.

Table 4-3 Michaelis-Menten kinetic parameters for warfarin reduction in liver cytosol and microsomes of control and ESRD patients

	Alcohol 1 (*RS/SR*)			**Alcohol 2 (*RR/SS*)**		
	K_m (μM)	V_{max} (pmol/mg protein/min)	Cl_{int} (μL/mg protein/min)	K_m (μM)	V_{max} (pmol/mg protein/min)	Cl_{int} (μL/mg protein/min)
Cytosol						
Control	677.3 ± 273.4	58.7 ± 9.28	0.088 ± 0.015	1119 ± 465.9	3.99 ± 0.78	0.0037 ± 0.00052
ESRD	900.6 ± 486.0	45.8 ± 10.8	0.052 ± 0.012	1580 ± 1166	4.12 ± 1.65	0.0027 ± 0.00058
Microsomes						
Control	594.7 ± 259.4	7.23 ± 1.18	0.0125 ± 0.0023	No fit	No fit	No fit
ESRD	767.9 ± 300.1	5.30 ± 0.85	0.0071 ± 0.00098	No fit	No fit	No fit

Data are presented as mean ± SEM of n=11controls and n=10 ESRD
Cl_{int} represents the intrinsic clearance (the enzymatic efficiency: V_{max}/K_m)
The differences in enzyme kinetic parameters between the two groups were not statistically significant.

4.4.3 Contribution of Human Hepatic Reductases to Warfarin Reduction

The relative contribution of human reductases to warfarin reduction in pooled human liver cytosol or microsomes was identified by employing selective chemical inhibitors of reductase enzymes. The relative formation of warfarin alcohol 1 and alcohol 2 in the presence of inhibitors as normalized to control (vehicle) incubations is depicted in **Figures 4-2A and 2B,** respectively.

Our results showed that quercetin, flufenamic acid and indomethacin (inhibitors of CBRs and AKR1Cs), and dicoumarol (inhibitor of NQO1) inhibited warfarin reduction in the cytosol in a concentration-dependent manner. The inhibition of alcohol 1 and alcohol 2 formation in the cytosol at 10 μM inhibitor concentration ranged from 31-39% and 16-40%, respectively. The extent of inhibition at 100 μM was markedly increased and ranged from 73-86% and 56-81% for formation of alcohol 1 and alcohol 2, respectively. All inhibitions were statistically significant at both inhibitor concentrations (10 at 100 μM) (P<0.05). Microsomal reduction of warfarin to alcohol 1 and alcohol 2 was not inhibited by 10 or 100 μM 18β-glycyrrhetinic acid. In fact, an inhibitor concentration of 250 μM was needed to inhibit warfarin reduction in the microsomes by 43% (P<0.05).

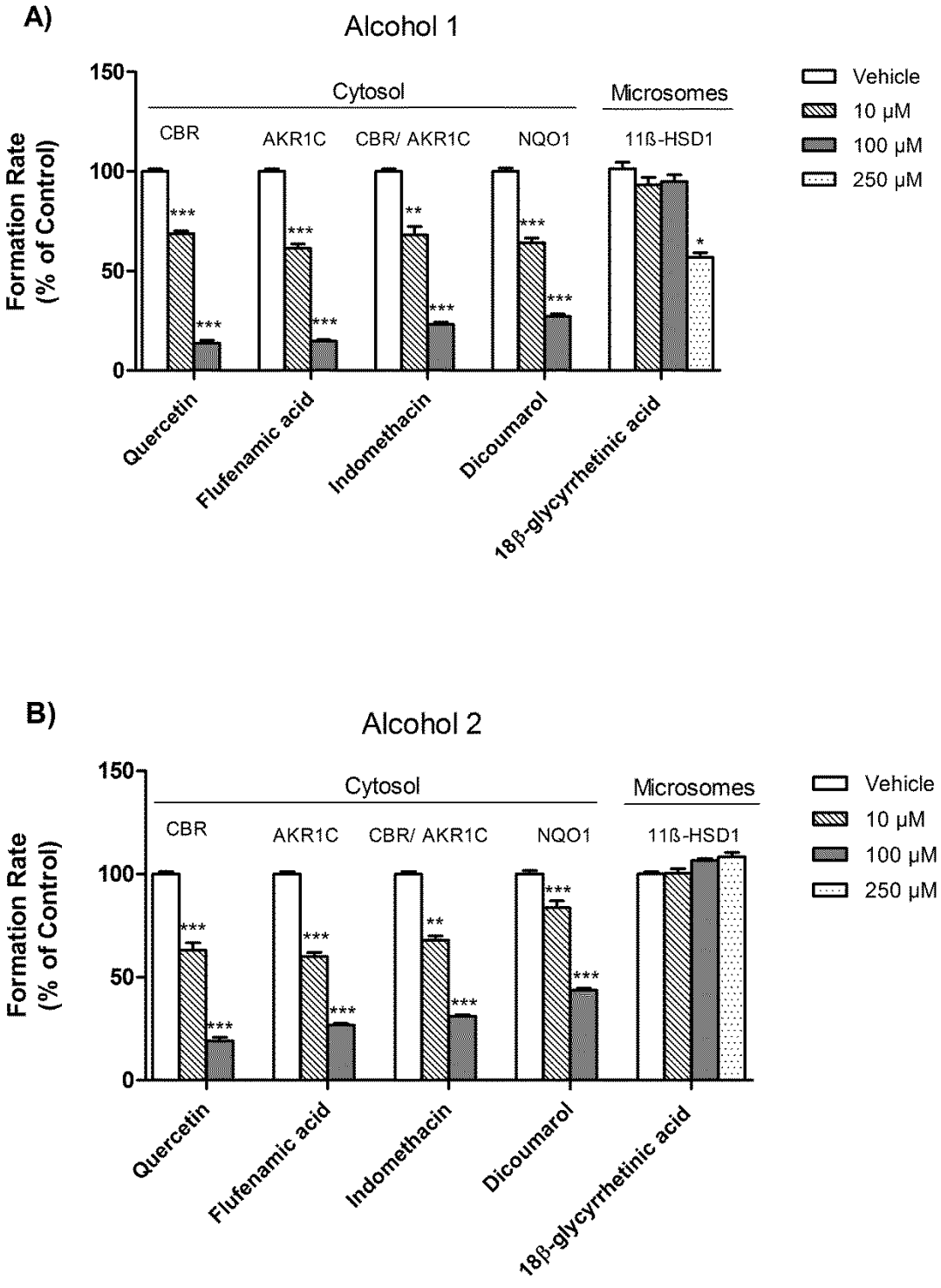

Figure 4-2 Effect of reductase inhibitors on human warfarin reduction

Formation of warfarin (A) alcohol 1 and (B) alcohol 2 was assessed in the presence of selective carbonyl reducing enzyme inhibitors in pooled human liver cytosol (quercetin, flufenamic acid, dicoumarol, and indomethacin) and microsomes (18β-glycyrrhetinic acid). The corresponding reductase enzymes being targeted by the inhibitors are also included. Incubations were conducted at 10 and 100 µM inhibitor concentration in cytosol, and 10, 100, and 250 µM in microsomes. Data are presented as mean ± SEM of triplicate incubations. Statistical differences are as follows: **P*<0.05; ***P*<0.01, and ****P*<0.001, compared with control incubations which were arbitrarily defined as 100%.

4.4.4 Effect of Kidney Disease on mRNA and Protein Expression of Human Hepatic Reductases

To determine whether the observed trend of decreased reductase metabolic activity in ESRD livers was secondary to reduced mRNA expression, we assessed mRNA levels encoding cytosolic (CBR1, CBR3, AKR1C1-4, and NQO1) and microsomal (11β-HSD1) human hepatic reductase isoforms by qRT-PCR. **Figure 4-3** displays the relative mRNA expression of hepatic reductases in livers from patients with ESRD and control subjects. Our mRNA expression data showed a similar trend, with a decline in mRNA levels of cytosolic CBR1 and AKR1C1, AKR1C2, AKR1C3, and AKR1C4 of 56%, 63%, 67%, 50% and 28% respectively, in ESRD livers versus controls. However, the observed down-regulation was not statistically significant. Conversely, significant up-regulation in mRNA expression of cytosolic CBR3 was observed in ESRD livers relative to control livers ($P<0.05$). mRNA expression of cytosolic NQO1 and microsomal 11β-HSD1 remained unaltered.

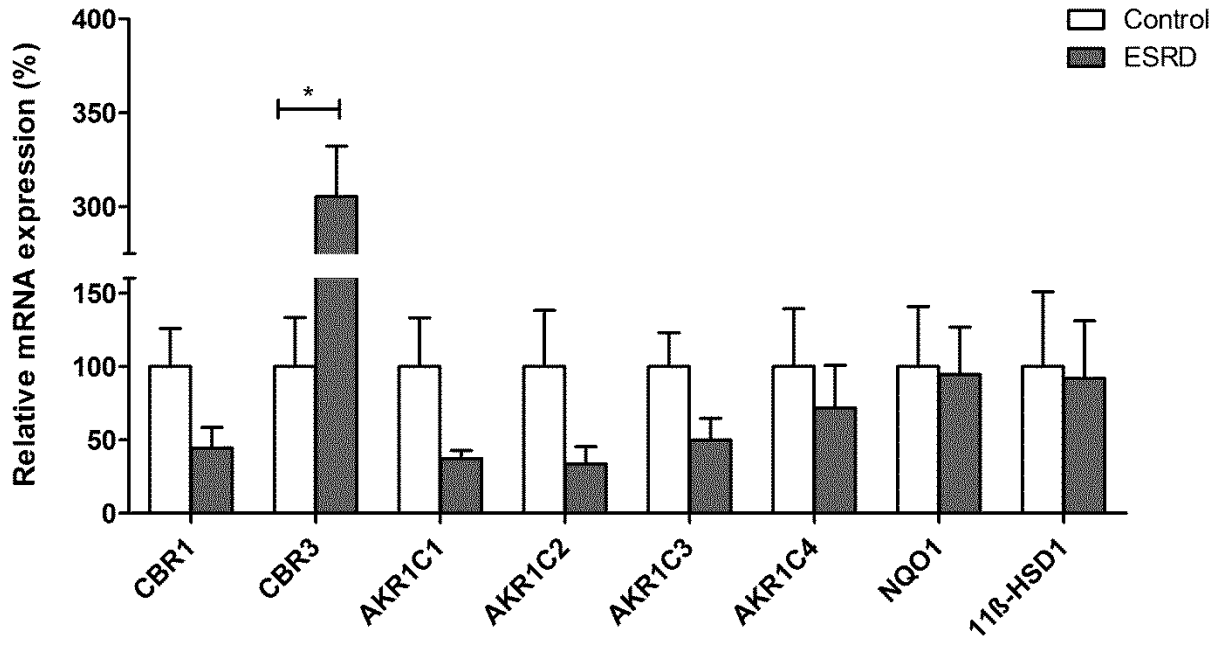

Figure 4-3 Effect of ESRD on mRNA expression of human hepatic reductases

mRNA expression of cytosolic (CBR1, CBR3, AKR1C1-4, and NQO1) and microsomal (11β-HSD1) human hepatic reductases in control and ESRD livers as determined by quantitative RT-PCR. mRNA expression of reductase isoforms was normalized to that of GAPDH. The results of ESRD group were normalized to the control which was arbitrarily defined as 100%. Experiments were conducted in duplicates, and data are presented as mean ± SEM of n=11 control and n=10 ESRD livers. * (P<0.05) compared with control.

To assess whether protein expression of CBR1 and AKR1C (AKR1C1, AKR1C2, AKR1C3, and AKR1C4 isoforms) in ESRD parallels mRNA expression, we examined CBR1 and total AKR1C protein expression using Western blotting (**Figure 4-4**). Our data showed that CBR1 protein was significantly down-regulated by 65% in ESRD livers compared to controls (P<0.05), while total AKR1C protein expression was unaffected.

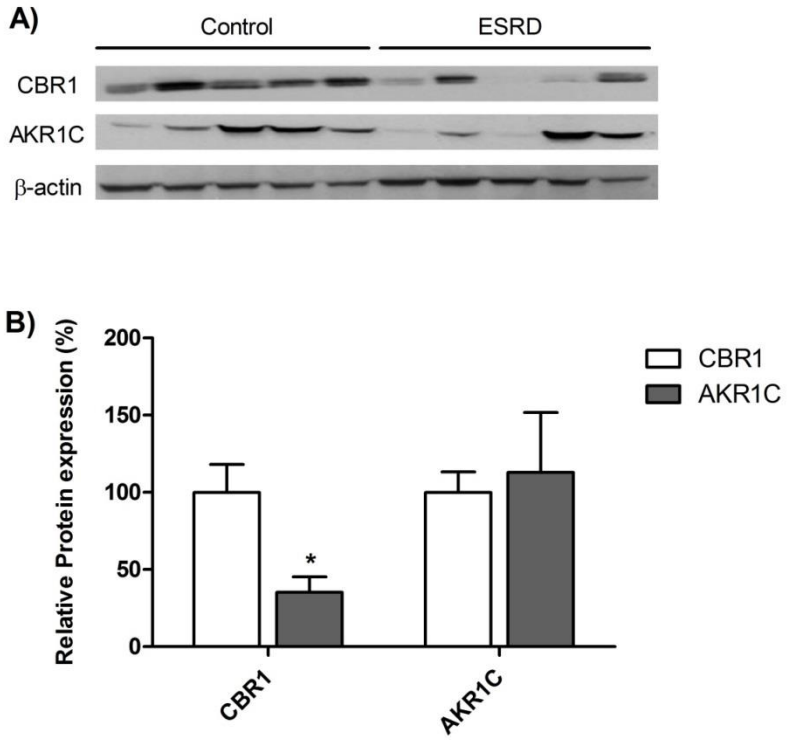

Figure 4-4 Effect of ESRD on protein expression of human hepatic reductases

Protein expression of cytosolic (CBR1 and AKR1C) human hepatic reductases in livers of patients with ESRD and control subjects examined by Western blotting. (A) Representative blots showing the expression of reductases and the internal control (β-actin) in both groups. (B) Densitometric analysis of protein expression as normalized to the β-actin. The densitometry units of ESRD group were normalized to that of control which was arbitrarily defined as 100%. Data are presented as mean ± SEM of n=11 control and n=10 ESRD livers. * (P<0.05) compared with control.

4.5 DISCUSSION

This study evaluated the effect of ESRD on the metabolic activity and expression of reductase enzymes in the cytosolic and microsomal liver fractions from humans with ESRD and control subjects. A significant downregulation in CBR1 protein expression was observed in livers from ESRD patients. The findings of this study support the previous data observed in rat model of CKD as described earlier in chapter 3. Together, these results indicate that in patients with ESRD, hepatic reduction of drugs mediated by the CBR1 isoform may be compromised.

Multiple clinical and experimental studies have established impaired drug metabolism and transport in the setting of kidney disease (Yeung et al., 2014) The results of the experimental studies also demonstrated that the change in mRNA and protein expression of drug metabolizing enzymes may mechanistically explain and at least partially contribute to the alterations in their function (Lalande et al., 2014). As reported in the previous chapter, the metabolic activity of hepatic reductases generating warfarin alcohols in cytosol and microsomes was selectively decreased in rats with experimental kidney disease, secondary to the differential downregulation of mRNA and protein expression of reductase isoforms (Alshogran et al., 2014a). The impact of kidney disease on the functional expression of human hepatic reductase is currently unknown.

The appreciation of the importance of reduction pathways in drug metabolism has been growing. Notably, numerous recent reports have strongly recommended investigating carbonyl reduction of drugs during the processes of drug discovery and development (Malatkova and Wsol, 2014). Reductases catalyze the reduction of a wide variety of drugs including anticoagulants, antidiabetics, antihypertensives, anticancers, opiate antagonists, NSAIDs, antihistamines, and diuretics (Rosemond and Walsh, 2004). As discussed in chapter 1, the disposition of many of these

drugs is shown to be altered in patients with CKD suggesting that phase I drug reduction could also be modulated by kidney disease.

In this study we first assessed the metabolic activity of reductases in subcellular fractions of liver tissue from ESRD patients and control subjects. Although the difference in alcohol 1 production did not reach statistical significance, a trend toward selectively decreased cytosolic (by 22%) and microsomal (by 27%) hepatic reductase activity in ESRD livers was observed. Notably, large interindividual variability in formation rate ranging from 51-75% coefficient of variation (CV) with alcohol 1 to 65-126% CV with alcohol 2 was observed. The greatest variability was observed in ESRD livers, which may have contributed to the non-significant findings. Post-hoc power analysis revealed that our study was underpowered to determine a significant difference in reductase activity between the two groups. Using two-sided student's t-test with alpha of 0.05, our study had only 13% power to detect approximately a 20% difference in formation of alcohol 1 in cytosol between ESRD and control livers (the calculated effect size was 0.4). Although the inter-individual variability in human patients is expected to be higher than rats, the observed trend toward decreased alcohol 1 formation in humans with ESRD is consistent, in part, with our findings observed in the experimental model of kidney disease as reported in chapter 3.

A preferential production of alcohol 1 was seen in the cytosol as indicated by its 8-fold higher metabolic formation in the cytosol compared to the microsomes. This is in agreement with previous studies that assessed *R* and *S*-warfarin reduction in the cytosolic and microsomal fractions of human liver tissue, and demonstrated a preference toward production of warfarin alcohol 1 in the cytosol (Hermans and Thijssen, 1993; Moreland and Hewick, 1975).

We further investigated whether ESRD affects mRNA and protein expression of hepatic reductases. Selective chemical inhibitors of reductases were utilized to evaluate the contribution

of different group of reductase enzymes to warfarin reduction, because the specific human reductase isoforms that catalyze warfarin reduction have not been characterized to date. Overall, the inhibition studies suggest the involvement of cytosolic NQO1, CBRs, and AKR1C, as well as microsomal 11β-HSD1 isoforms in warfarin reduction. These results agree, in part, with the previous findings of Hermans and Thijssen who demonstrated that 4'-nitrowarfarin reduction in human liver cytosol is strongly inhibited by quercetin and indomethacin, inhibitors of CBR and AKR1C reductase isoforms, respectively (Hermans and Thijssen, 1993). The diverse levels of inhibition observed with these inhibitors revealed that the relative contribution of cytosolic reductases toward warfarin reduction is different. Perhaps, the highest degree of inhibition demonstrated by quercetin may indicate that CBRs are the predominant reductases involved in warfarin reduction. In addition, the inhibition pattern observed in human cytosol and microsomes is similar to that observed in rat livers as reported in the previous chapter. However, higher concentration of 18β-glycyrrhetinic acid was needed to inhibit 11β-HSD1 isoform in human liver fractions. This may be attributed to; first) low overall contribution of microsomal reductases to warfarin reduction, second) the low concentration of inhibitor used relative to substrate concentration (10 or 100 μM vs 1000 μM respectively). Together, these results may indicate similarity between human and rat reductase isoforms.

We found that mRNA expression of hepatic reductases is differentially affected by ESRD. A non-significant decline of up to 67% in mRNA levels of CBR1 and AKR1C isoforms, and a significant upregulation of CBR3 mRNA expression was observed in ESRD livers versus controls. These findings may be partially attributed to accumulation of inflammatory cytokines that have been shown to differentially modulate the expression of several drug metabolizing enzymes and transporters as previously discussed in chapter 1 (Michaud et al., 2008; Reyes and Benet, 2011;

Tsujimoto et al., 2013; Volpe et al., 2014). In fact, the finding of increased CBR3 expression is consistent with the results of Malatkova and co-workers who elucidated that treatment of colon (HT-29) and liver (HepG2) cell lines with the inflammatory mediators TNF-α and IL-1β induced the expression of CBR3 up to 20-fold (Malatkova et al., 2012). Additionally, INF-γ has been shown to increase mRNA expression of CBR3 by 5.6-fold in human bronchial epithelial cells (Pawliczak et al., 2005).

Because CBR3 is expressed in much lower levels in human liver compared to CBR1 and AKR1C (Malatkova et al., 2010), and contributes minimally to drug metabolism *in vivo* (Pilka et al., 2009), along with limited available resources to evaluate all enzymes, we chose to subsequently evaluate the corresponding protein expression of CBR1 and total AKR1C. Our data showed a significant down-regulation in CBR1 protein in ESRD livers; however, protein level of total AKR1C was unchanged. The disproportionate decrease in CBR1 protein versus mRNA expression observed may suggest the contribution of post-translational mechanisms to its downregulation in ESRD (Greenbaum et al., 2003; Vogel and Marcotte, 2012). The observation of decreased expression of CBR1 protein in ESRD may be clinically important, because this isoform is highly expressed in the liver and contributes significantly to the biotransformation of multiple endogenous compounds (e.g., prostaglandins, steroids, lipids) and metabolism of several drugs such as doxorubicin, dolasetron and nabumetone (Kassner et al., 2008; Malatkova et al., 2010). For instance, decreased doxorubicin clearance and the corresponding increase in its exposure observed in hemodialysis patients could be partially explained by decreased expression of CBR1 protein (Yoshida et al., 1994). Taken together, these data suggest that kidney disease may selectively impair reductase protein expression. However, further well-powered investigations are required to confirm the results of the effect of ESRD on reductase mRNA expression.

In summary, CBR1 protein expression is significantly decreased in ESRD livers. Given the significant contribution of CBR1 to drug reduction, the findings could explain alterations of clinical pharmacokinetics of CBR1 drug substrates (i.e., idarubicin) in CKD patients. The findings also suggest that elimination of certain drugs that are metabolized by the CBR1 isoform could be impaired as a result of the alterations in its expression. However, due to the large inter-individual variability observed, future adequately powered studies with larger sample size are warranted to further confirm these findings. These results are consistent, in part, with our data reported in previous chapter where we established selective decrease in the expression of rat hepatic reductases due to kidney disease.

5.0 EFFECT OF CKD ON STEADY STATE WARFARIN DISPOSITION IN HUMANS: A PILOT STUDY

5.1 ABSTRACT

Warfarin metabolism is decreased in CKD, suggesting its nonrenal clearance can be altered in CKD patients. However, little is known about warfarin pharmacokinetics in patients with different levels of kidney function. This work was conducted to explore the impact of impaired kidney function on steady-state pharmacokinetics of warfarin in humans. Blood samples were collected from patients (total n=25) with varying levels of kidney function (i.e., eGFR ranging from 3-80 mL/min/1.73m^2), who were on long-term warfarin therapy. Patients were separated into 5 groups according to the eGFR values. Total and free concentrations of warfarin, warfarin enantiomers, and alcohol metabolites were measured in serum and serum ultrafiltrate, respectively, using LC-MS/MS. Warfarin clearance, *S/R* warfarin ratio, and exposure of warfarin alcohols were compared between groups using ANOVA. Regression analysis was performed to determine the impact of kidney function and other covariates on warfarin clearance. Also, the activity of warfarin alcohols against clotting factor XIa activity was assessed *in vitro*. Warfarin clearance was not different between patients with varying levels of kidney function. eGFR was a significant predictor of total *S*-warfarin clearance (P=0.045). The exposure of warfarin alcohol 2 was 6-fold higher in patients with ESRD compared to control/mild CKD patients (P<0.05). Further, warfarin *S/R* ratio was 2.5-fold higher in ESRD patients versus control/mild CKD patients (P<0.05). No differences were observed in protein binding of warfarin between patients with varying levels of CKD. Additionally, *in vitro* studies showed negligible effect of warfarin alcohols against the activity of FXIa. Collectively, our data provide evidence for increased exposure of the warfarin alcohol 2 metabolite as well as decreased CYP2C9-mediated warfarin metabolism in ESRD patients. These findings may partially explain decreased warfarin dosing requirements and increased risk of poor outcomes in CKD patients.

5.2 INTRODUCTION

Patients on warfarin anticoagulant therapy exhibit high variability in dosing requirements and response. Several dosing algorithms incorporating clinical and genetic information that account for the inter-individual variability have been developed to improve warfarin dosing. However, these attempts have only accounted for approximately 60% of the variation in warfarin dosing, stressing the importance of additional factors that should be considered (Kimmel et al., 2013; Liao et al., 2014; Stergiopoulos and Brown, 2014). Recently, kidney function has been shown to be an important determinant of *S*-warfarin metabolic clearance in a pharmacokinetic model developed based on the measured plasma concentrations of *S*-warfarin in 167 patients (Gong et al., 2011). In addition, kidney function acts as a critical covariate in clinical schemes for predicting warfarin bleeding risk (Fang et al., 2011; Gage et al., 2006; Pisters et al., 2010). Warfarin is administered clinically as a racemic mixture of *R* and *S* enantiomers, which have different pharmacokinetic profiles and pharmacological potency (Fasco and Principe, 1982; Hewick and McEwen, 1973). Warfarin is nearly completely absorbed after oral administration, highly bound to plasma albumin (99%), and has a long half-life of about 40 hours (Holford, 1986). Warfarin is eliminated almost completely by metabolism, followed by urinary excretion of the metabolites, with only a very small percentage of the dose being recovered unchanged in the urine (Jones and Miller, 2011).

Altered nonrenal clearance of drugs in kidney disease (Yeung et al., 2014) suggests that the disposition of the highly metabolized drugs, such as warfarin, may be impacted by impaired kidney function. This is supported by our work (presented in chapters 3 and 4) that demonstrates diminished warfarin reduction, along with the earlier findings of reduced activity of CYP2C9 (Dreisbach et al., 2003) in kidney disease. Alterations in warfarin exposure may be detrimental, as it may result in severe clinical consequences in cases of enhanced and reduced anticoagulant

effects. In line with this, several recent observational studies have shown decreased warfarin dose requirements and increased bleeding risk and stroke in CKD patients receiving warfarin (Winkelmayer and Turakhia, 2014), suggesting that warfarin pharmacokinetics may be altered in patients with impaired kidney function. Together, these findings may indicate that kidney function is a significant factor to be considered when predicting warfarin dose besides other known clinical (age, diet, medications interactions, and liver disease) and genetic (CYP2C9 and VKORC1 genotypes) parameters (Cavallari et al., 2010).

Limited information is available on the elimination of warfarin in patients with CKD. To date, only one study has evaluated warfarin disposition in patients with kidney disease. This study included five subjects as controls and four patients with CKD (CLCr 11-48 mL/min/1.73m^2) (Bachmann et al., 1977). After single dose warfarin administration, the results revealed shorter warfarin elimination half-life in CKD patients versus control group (mean ± SEM: 29.9 ± 5 h vs. 44.8 ± 6 h, respectively) (Bachmann et al., 1977). This study has several limitations: 1) only two groups of participants were recruited with no evidence of inclusion of patients on hemodialysis; 2) evaluated only total concentration of warfarin without assessing the free concentration; and 3) did not assess differences in disposition of warfarin enantiomers in patients with impaired kidney function. To address these gaps, we explored the effect of varying degrees of kidney function on steady-state disposition of warfarin enantiomers. The disposition of warfarin alcohol active metabolites and their activity against vitamin-k independent clotting factor (FXIa) were also assessed. We hypothesized that warfarin clearance is decreased in patients with progressive kidney disease as a result of reduced non-renal clearance.

5.3 METHODS

5.3.1 Clinical Study Design and Participants

Patients on long-term anticoagulant warfarin therapy were eligible if they exhibited stable INR values for at least four previous clinic visits. Eligible patients were stratified into 5 groups based on eGFR calculated using the CKD-EPI equation (Levey et al., 2009) as follows: normal/mild kidney function (eGFR > 60 mL/min/1.73m^2), moderate CKD (eGFR 30–60 mL/min/1.73m^2), severe CKD (eGFR 15-30 mL/min/1.73m^2), ESRD (eGFR < 15 mL/min/1.73m^2 and not requiring renal replacement therapy), and ESRD/HD (eGFR < 15 mL/min/1.73m^2 and on hemodialysis). Patients were excluded if they had a history of malabsorption or clinical/ laboratory evidence of liver disease, drink alcohol of exceeding 3 standard drinks daily, and receive concurrent treatment with drugs that strongly interact with warfarin such as amiodarone (enzyme inhibitor) and rifampicin (enzyme inducer). Patient characteristics including age, gender, BMI, race, serum albumin concentration, warfarin weekly dose, and indication for warfarin therapy were recorded. Patients were given warfarin at a time similar to that at which they usually self-administered this medication at home. After warfarin administration, a single blood sample (15 mL) was collected on the day of the clinic visit close to the mid-point of drug administration (i.e., approximately 12 hours after the last dose). Because warfarin and its metabolites are highly bound to plasma proteins (>98%) (Chan et al., 1993; Lomonaco et al., 2013), we assume that their removal by hemodialysis will be very minimal, and collecting blood samples before or after hemodialysis will not have significant effect on the measured concentrations. Blood samples were allowed to clot, and then centrifuged to collect the serum, which was stored at -80^{o}C until analysis. The study protocol was

approved by the Research and Ethics Committee of Maisonneuve-Rosemont Hospital. All subjects provided written informed consents prior to participation in the study.

5.3.2 Determination of Total and Free Warfarin and Alcohol Metabolites

Total serum concentrations of racemic warfarin and warfarin alcohol metabolites were determined using a validated LC-MS/MS analytical method as previously described in chapter 2 (Alshogran et al., 2014b). In brief, serum (100 µL) was combined with the internal standard mixture of *d5*-warfarin and *d5*-7-hydroxywarfarin, and then extracted using TMBE. Warfarin and warfarin alcohols were separated using a Thermo Hypersil Gold C18 (2.1 × 100 mm, 1.9 µm) analytical column, connected to 0.22 µm frit filter. Chromatographic separation was achieved under gradient of solvent A (water containing 0.01% formic acid), and solvent B (acetonitrile containing 0.1% formic acid) over 5 min. Specific MS/MS experimental conditions are reported in chapter 2. Calibration curves were linear ($r^2 \geq 0.994$) over a range of 50-5000 ng/mL for warfarin, 10-750 ng/mL for warfarin alcohol 1, and 1-150 ng/mL for warfarin alcohol 2. The intra- and inter-day accuracy and precision for all analytes in the analytical assay were within ±15%.

A validated chiral assay was employed to measure total concentrations of *R*- and *S*-warfarin enantiomers. Analytes were resolved using Astec Chirobiotic V column (150 mm × 2.1 mm ID, 5 µm) and an Astec Chirobiotic V guard column (10 mm × 2.1 mm ID; 5 µm), each supplied by Supelco (Bellefonte, PA, USA), with gradient elution of solvent A [10 mM ammonium acetate (pH 5.0) and acetonitrile (90:10, v/v)] and of solvent B (acetonitrile). The run time was 10 min per sample. All analytes were detected in negative ionization mode using selected reaction monitoring. The ion transitions in the chiral assay were *m/z* 307.106 →161.144 for warfarin (collision energy = 21V) and *m/z* 312.162 → 255.298 for *d5*-warfarin (collision energy = 26V). Standard curves

were linear ($r^2 \geq 0.997$) over a range of 25-2500 ng/mL for both enantiomers. The intra- and inter-day accuracy and precision for analytes were within ±10%.

For the determination of the free fraction of warfarin, warfarin enantiomers and alcohol metabolites, 500 µL of serum was ultrafiltered at 4000 rpm for 30 min at 4^{o}C using Centrifree ultrafiltration devices (Millipore, MA, USA). Serum ultrafiltrate (200 µL) was extracted as describe in chapter 2 using MTBE. Chromatographic separation and MS/MS detection were also as described earlier. Standard curves were linear ($r^2 \geq 0.992$) over the range of 1-50 ng/mL for warfarin and warfarin alcohol 1, and 0.5-25 ng/mL for warfarin enantiomers and alcohol 2 metabolite. The intra- and inter-day accuracy and precision for all analytes were within ±15%.

5.3.3 Pharmacokinetic Analyses

Based on the assumptions of steady-state conditions, full compliance, and complete oral bioavailability, one point oral clearance was calculated for warfarin and its enantiomers as follows: $CL_{total} = D/(C_{ss} \times \tau)$, in which D is the daily warfarin dose (half the racemic dose for the enantiomers), C_{ss} is the steady-state concentration of analytes, and τ is the dosing interval=24 h. The validity of the steady-state and full compliance assumptions were verified by recording the INR values of the patients in the previous four clinic visits before blood collection. The INR should be stabilized and maintained within the therapeutic range over that period of time. Because warfarin has a long elimination half-life (~50h for *R*-warfarin and 30h for *S*-warfarin) (Maddison et al., 2013), obtaining a concentration in a single blood sample collected close to the mid-point of dosing interval will be approximate to the C_{ss} (Jensen et al., 2012). The free fraction (f_u) of analytes was calculated by dividing the free unbound concentration (C_{free}) by the total concentration (C_{total}). The unbound clearance (CL_u) was calculated as follows: $CL_u = CL_{total}/f_u$ or $D/(C_{free} \times \tau)$.

5.3.4 Statistical Analyses

Mean differences in demographic variables and PK parameters were compared between CKD groups using one-way ANOVA for continuous variables and chi square test for categorical variables. Significant ANOVA was followed by a *post hoc* Bonferroni analysis. Pearson's correlation analysis was performed to express strength of relationships between variables. Multivariate linear regression analysis was used to determine the relative effect of age, gender, weight, BMI, race, eGFR, albumin concentration, and blood sampling time on outcome measures (i.e., warfarin clearance, *S/R* warfarin ratio, fraction unbound) as appropriate. Analysis was conducted using STATA software (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP) and GraphPad Prism (Version 5; GraphPad Software Inc., San Diego, CA). $P<0.05$ was considered significant for all comparisons. Results are expressed as mean ± SD.

5.3.5 Effect of Warfarin Alcohols on Clotting Factors Xa and XIa Activity In-vitro

The activity of warfarin alcohols against purified human vitamin-K independent (XIa) and dependent (Xa) clotting factors was determined *in vitro* as previously described with minor modifications (Ma et al., 2013; Wong et al., 2011; Wong et al., 2008). Reagents used in these experiments included: human FXa and FXIa from Haematologic Technologies (Essex Junction, VT, USA); chromogenic substrates S-2765 (for FXa) and S-2366 (for FXIa) from Chromogenix AB (distributed by DiaPharma Group, West Chester, OH, USA); and apixaban from Cayman Chemical (Ann Arbor, MI, USA). Working solutions of clotting factors were prepared in the assay buffer (50 mM Tris, 100 mM NaCl, 0.01% BSA, pH 7.4). Clotting factor (0.5 nM) was pre-

incubated with different concentrations of either warfarin alcohol 1 (5 nM – 32.2 μM), warfarin alcohol 2 (5 nM – 32.2 μM), warfarin (5 nM – 32.4 μM), or apixaban (0.25 nM – 2 μM) in 96-well microtiter plates for 10 min at room temperature. Control incubations included clotting factor with the vehicle only (methanol for warfarin and alcohols, DMSO for apixaban). The reaction was initiated by the addition of the substrate dissolved in water (S-2765 or S-2366) to a final concentration of 200 μM. The mixture was incubated at room temperature for 30 min. Substrate hydrolysis (which reflects clotting factor activity) was monitored by measuring the absorbance at 405 nm using a plate reader. Absorbance values were plotted against drug concentrations. Apixaban was used as a positive control for FXa and as a negative control for FXIa experiments. Similar experiments were also conducted at 0.5 nM FXIa by varying the concentrations of the S-2366 substrate (0-800 μM) and warfarin alcohols (0-32 μM). Data analysis was performed using GraphPad Prism.

5.4 RESULTS

5.4.1 Patient Characteristics

A total of 25 patients with a mean age of 73.4 ± 11 were included in the study. Participants were separated according to their kidney function as follows: normal/mild CKD (eGFR (mean ±SD) = 72 ± 6.8 mL/min/1.73m^2; n=6), moderate CKD (eGFR= 42.8 ± 5.2; n=4), severe CKD (eGFR= 19 ± 3.4, n=5), ESRD with no renal replacement therapy (eGFR= 12.6 ± 2.8; n=5), and ESRD on HD (eGFR= 4.6 ± 2; n=5). The most common indications for warfarin therapy were atrial fibrillation (52%) and thromboembolic disease (32%). The mean warfarin weekly dose (mg) was 28.6 ± 14.3 (range: 7-57.5 mg/week). The mean serum albumin level was 3.9 ± 0.35 g/dL (range: 3.1-4.6 g/dL). Patient characteristics in each CKD group were matched including age, gender, race, BMI, serum albumin, warfarin dose, and blood sampling time as listed in **Table 5-1**.

Table 5-1 Demographic and clinical characteristics of study patients

	All patients (n=25)	**Normal/Mild (n=6)**	**Moderate (n=4)**	**Severe (n=5)**	**ESRD (n=5)**	**ESRD/HD (n=5)**	***P***
eGFR (mL/min/1.73m^2)	31.4 ± 26.6	72 ± 6.8	42.8 ± 5.2	19 ± 3.4	12.6 ± 2.8	4.6 ± 2.0	<0.001
Age (year)	73.4 ± 11	65 ± 10.9	70.5 ± 12.6	79.4 ± 4.7	80.4 ± 9.9	72.6 ± 10.6	0.1051
Weight (kg)	81.5 ± 15.7	81.5 ± 8.0	82.6 ± 9.9	83.4 ± 11.4	81.2 ± 15.6	78.7 ± 30.5	0.9942
BMI (kg/m^2)	29.8 ± 4.9	29 ± 2.4	30.0 ± 3.4	30.5 ± 4.3	29.5 ± 5.3	30.4 ± 8.8	0.9891
Gender (% male)	52	50	50	60	80	20	0.4390
Race (% white)	96	100	100	100	100	80	0.3840
Serum albumin (g/dL)	3.9 ± 0.35	4.2 ± 0.32	4.1 ± 0.25	3.9 ± 0.22	4.0 ± 0.29	3.7 ± 0.48	0.1713
Warfarin dose (mg/week)	28.6 ± 14.3	39.5 ± 15.4	25.6 ± 8.3	28.6 ± 13.6	20.3 ± 8.7	28.4 ± 19	0.2702
Blood sampling time (h)	15.9 ± 3.3	16.2 ± 3.1	16.1 ± 1.0	15.4 ± 3.1	16.4 ± 5.3	15.2 ± 3.6	0.9776

5.4.2 Warfarin Clearance and Kidney Disease

No differences were found in mean values of the total and free steady-state clearance of warfarin, *R*-warfarin, and *S*-warfarin among well-matched patients with different degrees of kidney disease (**Figure 5-1**).

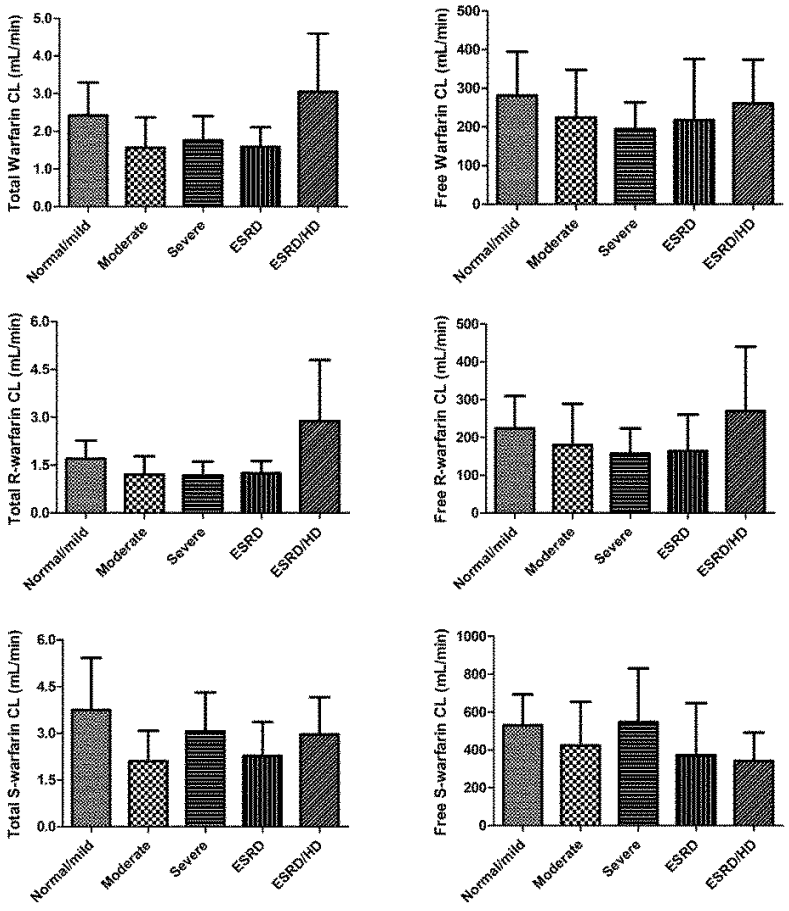

Figure 5-1 Clearance of total warfarin, free warfarin, and warfarin enantiomers in patients with varying levels of kidney disease

Clearance of total warfarin, free warfarin, and warfarin enantiomers was calculated from a single point plasma concentration close to the mid-point of warfarin administration. Data were compared using ANOVA test.

Multiple regression analysis was performed to determine the contribution of different clinical parameters to the variability in warfarin clearance (**Table 5-2**). The results indicate a significant association between total warfarin and *R*-warfarin clearance with age and serum albumin ($P<0.05$), and a significant association between free warfarin and *R*-warfarin clearance with age only ($P<0.05$) after adjustment for sex, BMI, race, blood sampling time, and eGFR. Age was associated with a significant decline in total and free warfarin clearance by 0.049 and 5.5 mL/min per year of age, respectively, and by 0.044 and 5.3 mL/min for total and free *R*-warfarin, respectively. In addition, each unit increase in serum albumin was associated with a significant decrease in total warfarin and *R*-warfarin clearance by 0.12 and 0.15 mL/min, respectively.

On the other hand; eGFR was the only significant contributor to total *S*-warfarin clearance ($P=0.045$), with a 0.025 mL/min increase in clearance for every 1 mL/min/1.73m^2 increase in eGFR. None of the covariates contributed to free *S*-warfarin clearance.

Table 5-2 Significant predictors of clearance of total and free warfarin and its enantiomers using multiple regression analysis

Outcome	**Predictor**	**Beta**	**SE**	***P***
Total Warfarin CL (mL/min)	Age	-0.049	0.017	0.01
	Serum albumin	-0.124	0.051	0.025
Total *R*-warfarin CL (mL/min)	Age	-0.044	0.018	0.024
	Serum albumin	-0.154	0.054	0.009
Total *S*-warfarin CL (mL/min)	eGFR	0.025	0.012	0.045
Free Warfarin CL (mL/min)	Age	-5.548	2.116	0.016
Free *R*-warfarin CL (mL/min)	Age	-5.257	1.999	0.015
Free *S*-warfarin CL (mL/min)	None			

SE: standard error

Age was the only significant modifier of average weekly warfarin dose for all patients. As age increases by one year, the average weekly warfarin dose decreases by 0.75 mg. In addition, significant negative relationships were observed between age and eGFR (r=-0.449, *P*=0.024) as well as age and weekly warfarin dose (r=-0.526, *P*=0.007) (**Figure 5-2**).

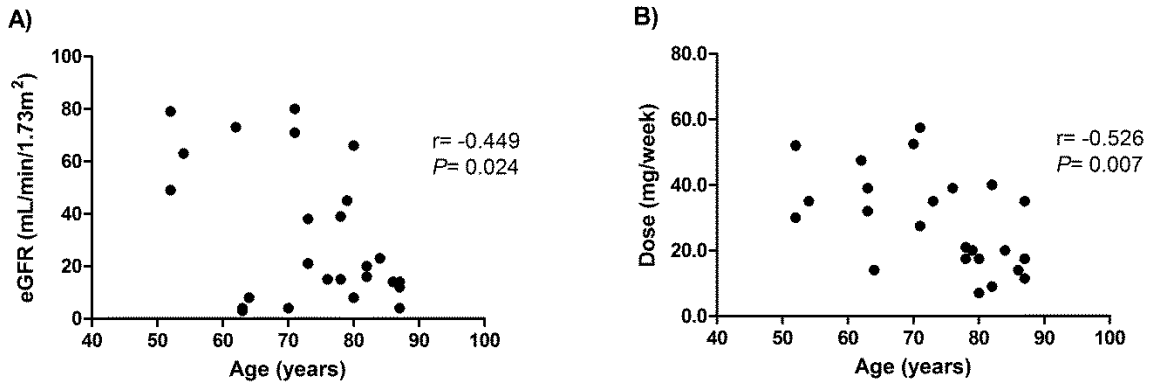

Figure 5-2 Relationship between eGFR values and weekly warfarin dose with age in study patients

5.4.3 Warfarin Alcohols Disposition and Kidney Disease

Total and free dose-normalized alcohol 2 concentrations were significantly dependent on kidney function. Total warfarin alcohol 2 exposure was 6.5-fold higher in patients with ESRD compared to those with normal/mild CKD (64.3 ± 34.6 versus 9.9 ± 3.87 ng/mg*mL; *P*=0.015) (**Figure 5-3**). Free warfarin alcohol 2 exposure was also about 6.5-fold higher in both patients with ESRD compared to those with normal/mild CKD (0.51 ± 0.29 versus 0.08 ± 0.08 ng/mg*mL; *P*=0.016), and in ESRD/HD group versus normal/mild CKD (0.49 ± 0.24 versus 0.08 ± 0.08 ng/mg*mL; *P*=0.025) (**Figure 5-3**). Similar significant trends were also observed with regard to total and free

alcohol 2 when normalized to the corresponding warfarin concentrations. Warfarin alcohol 1 exposure was not different between kidney disease patients.

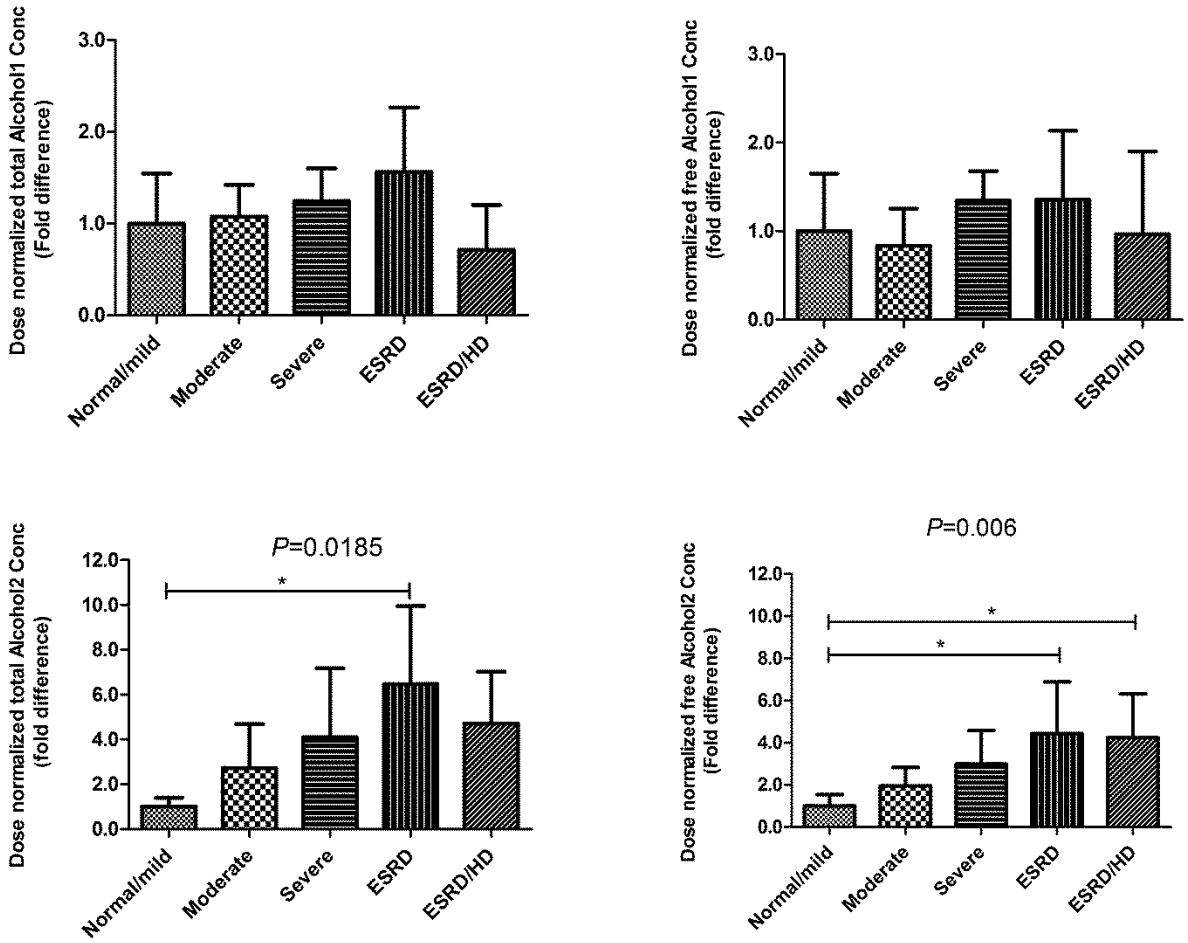

Figure 5-3 Fold difference in dose normalized warfarin alcohol concentrations in patients with varying degrees of kidney disease

P value obtained from ANOVA analysis.

* indicates statistically different from the normal/mild group.

5.4.4 *S/R* Warfarin Ratio and Kidney Disease

Mean total and free *S/R* warfarin ratio was significantly different between CKD patients (*P*=0.014 and *P*=0.018, respectively). Post hoc analysis revealed that *S/R* ratio was 2.5-fold higher in ESRD/HD patients compared to severe CKD group for total (0.93 ± 0.32 versus 0.39 ± 0.07; *P*=0.016) and free *S/R* ratios (0.76 ± 0.23 versus 0.30 ± 0.06; *P* =0.018). Total *S/R* was also 2-fold significantly higher in ESRD/HD compared to normal/mild CKD group (0.93 ± 0.32 versus 0.47 ± 0.09; *P*=0.039) (**Figure 5-4**).

eGFR was the only significant predictor for total (*P*=0.013) and free (*P*=0.043) *S/R* ratio in the regression model with a decrease in total and free *S/R* ratio by 0.006 and 0.004 receptively, with a unit increase in eGFR. Other variables included in the model (age, sex, BMI, race, serum albumin, sampling time and dose) did not have a significant contribution.

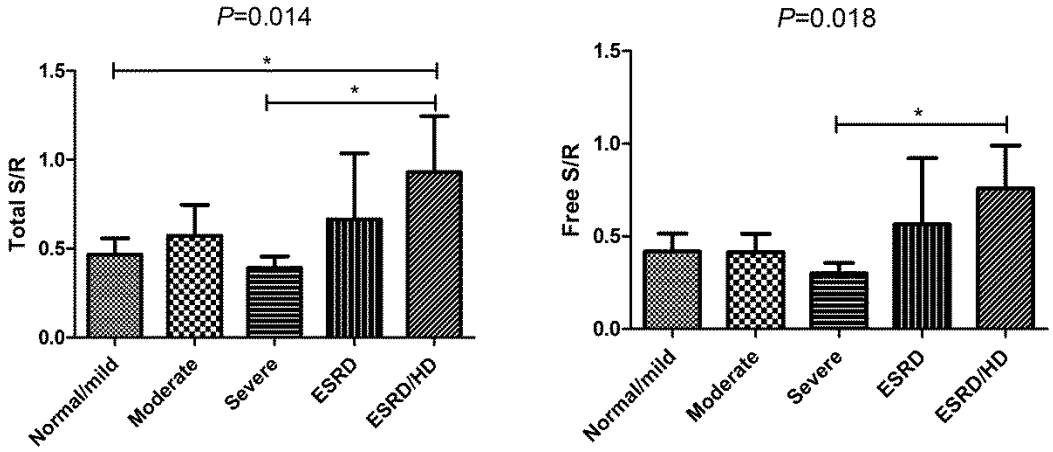

Figure 5-4 Total and free *S/R* warfarin ratio by degree of kidney disease

P value obtained from ANOVA analysis.

* indicates statistically significant.

5.4.5 Protein Binding of Warfarin and Warfarin Alcohols

The free unbound fractions of warfarin, *R*-warfarin, *S*-warfarin, alcohol 1, and alcohol 2 were not different between kidney disease patients. Considering the entire sample, the mean ± SD unbound percent was 0.92 ± 0.24 for warfarin, 0.84 ± 0.24 for *R*-warfarin, 0.70 ± 0.23 for *S*-warfarin, 0.97 ± 0.39 for alcohol 1, and 0.93 ± 0.29 for alcohol 2.

5.4.6 In-vitro Effect of Warfarin Alcohols on Human FXa and FXIa Activity

At concentrations up to 32 µM, warfarin alcohols and warfarin had negligible direct effect on clotting factors Xa and XIa activity *in vitro*. As expected, apixaban inhibited the activity of factor Xa in a concentration-dependent manner, but did not affect the activity of factor XIa. The IC50 (defined as the concentration needed to inhibit the activity by 50%) for apixaban inhibition of factor Xa was around 4 nM (**Figure 5-5** and **Figure 5-6**), which is comparable with a previous report (Jiang et al., 2009).

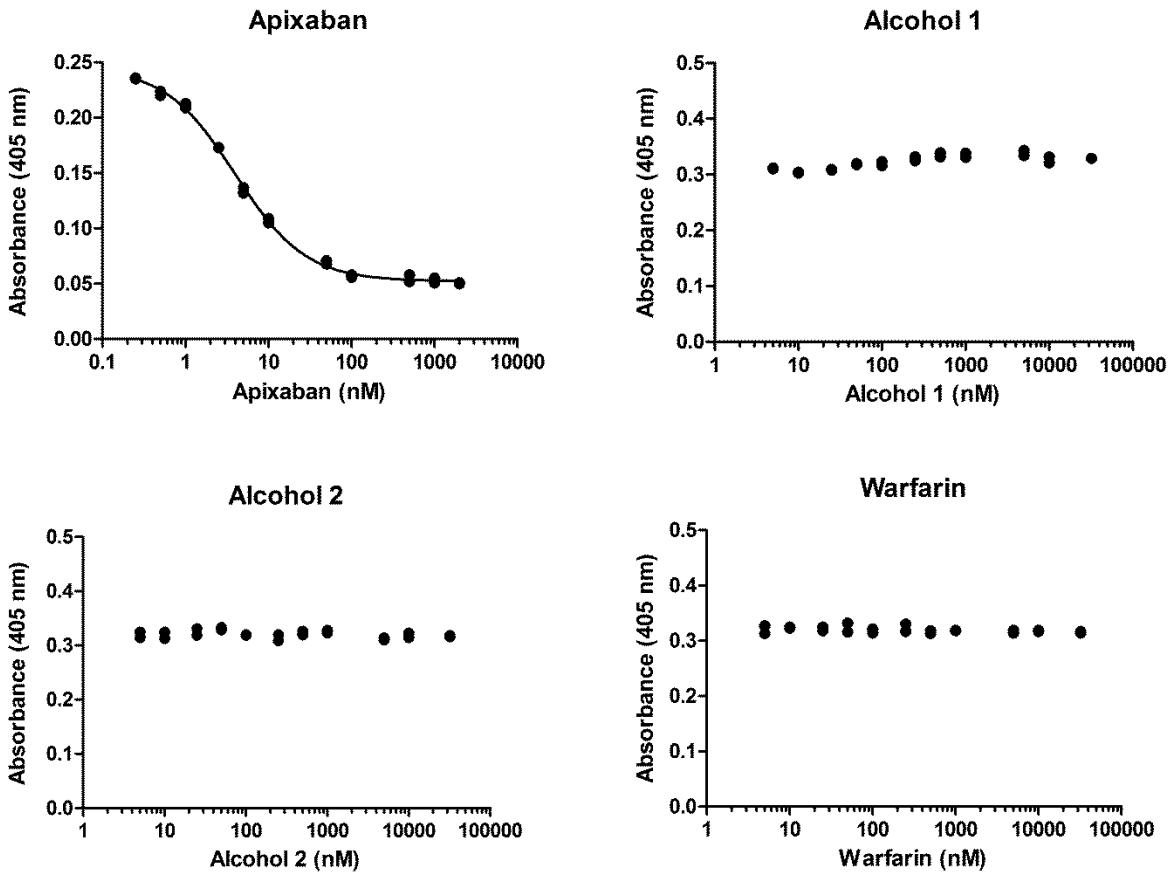

Figure 5-5 The effect of warfarin alcohol metabolites on the activity of human factor Xa

Clotting factor FXa (0.5 nM) was pre-incubated with different concentrations of either warfarin alcohol 1, warfarin alcohol 2, warfarin, or apixaban (used as a positive control). The reaction was initiated by the addition of the substrate S-2765 to a final concentration of 200 µM. Substrate hydrolysis was monitored by measuring the absorbance at 405 nm. The estimated IC_{50} of apixaban inhibition of FXa was close to 4 nM.

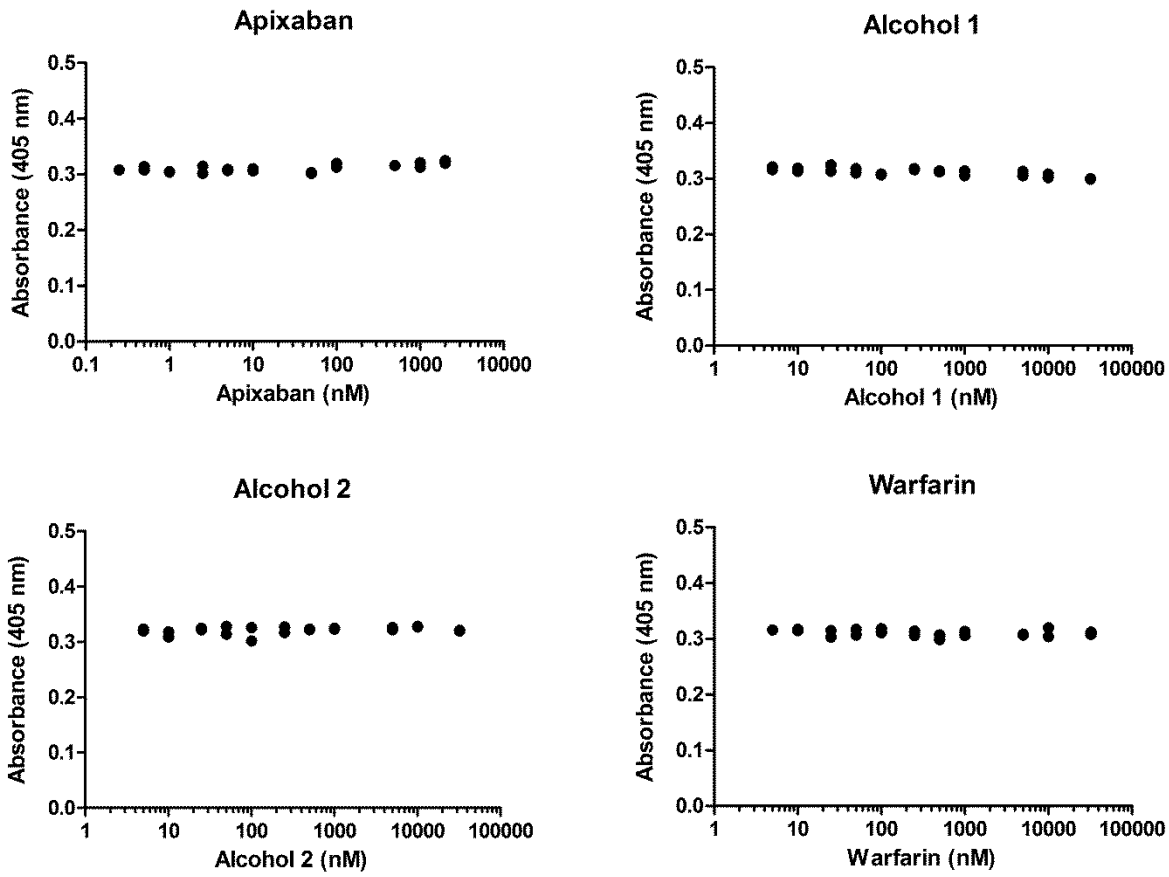

Figure 5-6 The effect of warfarin alcohol metabolites on the activity of human factor XIa

Clotting factor FXIa (0.5 nM) was pre-incubated with different concentrations of either warfarin alcohol 1, warfarin alcohol 2, warfarin, or apixaban (used as a negative control). The reaction was initiated by the addition of the substrate S-2366 to a final concentration of 200 µM. Substrate hydrolysis was monitored by measuring the absorbance at 405 nm.

Our results also indicate that warfarin alcohols did not influence FXIa activity when the clotting factor was incubated with different substrate and inhibitor (alcohols) concentrations. As depicted in **Figure 5-7**, the plots of absorbance versus S-2366 concentration were almost superimposable at different concentrations of warfarin alcohols (0, 0.5, 1, 5, 10, and 32 µM), suggesting the lack of effect.

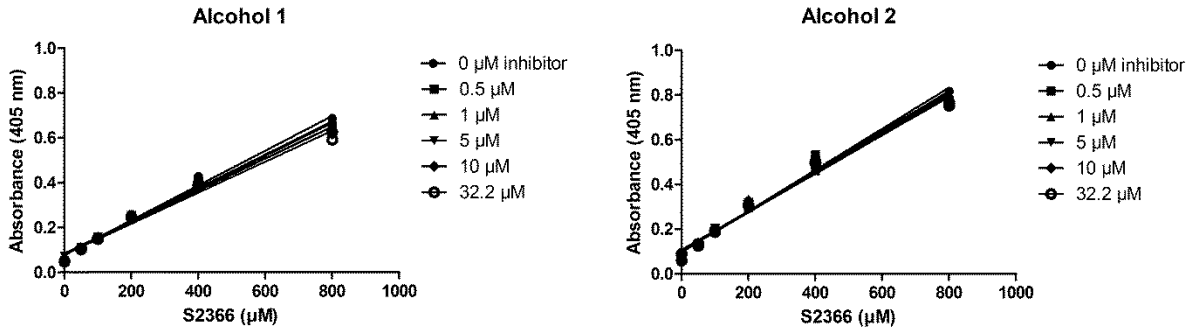

Figure 5-7 Plot of warfarin alcohols effect on human factor XIa activity at different substrate (S-2366) concentrations

Clotting factor FXIa (0.5 nM) was pre-incubated with different concentrations of either warfarin alcohol 1 or warfarin alcohol 2. The reaction was initiated by the addition of the substrate S-2366 at different concentrations ranging from 0-800 µM. Substrate hydrolysis was monitored by measuring the absorbance at 405 nm.

5.5 DISCUSSION

In this pilot study the steady-state kinetics of warfarin were evaluated in patients with varying degrees of kidney function. We have shown that kidney function is a significant predictor of total *S*-warfarin clearance. The results further reveal that the disposition of warfarin alcohol 2 and the enantiomeric warfarin (*S/R*) ratio are increased in patients with ESRD compared to those with normal/mild kidney function. Together, these data demonstrate that kidney disease may impact the nonrenal clearance of warfarin mediated by CYP2C9, and may alter the renal elimination and therefore the exposure of warfarin alcohol metabolites.

S-warfarin is primarily metabolized through CYP2C9 to produce warfarin hydroxy metabolites. Conversely, *R*-warfarin is metabolized through reduction and oxidation pathways with major contribution by hepatic reductases that generate alcohol metabolites (Kaminsky and Zhang, 1997). In this study, we found that kidney function is a significant predictor of total clearance of *S*-warfarin. This was supported by our findings of a 2.5-fold increase in total and free *S/R* warfarin in ESRD/HD patients compared to those with less severe kidney disease, which suggests reduced CYP2C9 activity in CKD. These results agree with a previous report showing that total *S/R* warfarin is increased by 50% in ESRD patients compared to controls (Dreisbach et al., 2003). However, we couldn't find any differences in *S/R* in other groups of patients who have less compromised kidney function (i.e., moderate and severe CKD). Together, these data suggest that *S*-warfarin clearance may be impacted by severity of kidney disease; on the other hand, we found that age and serum albumin concentration are significant predictors of *R*-warfarin clearance.

Our finding of decreased warfarin dose requirements with age may be explained by the known physiological reduction of liver and kidney function with age (Kinirons and O'Mahony, 2004; Meyer, 1989). These factors may contribute to the decreased total warfarin clearance in

older patients. Our data are in part consistent with the recent investigation of Jensen et al., who showed that *R*- and *S*-warfarin clearance is decreased by 0.3-0.5% per year of age (Jensen et al., 2012). Our results also showed a minor correlation between warfarin dose and kidney disease (eGFR) (r=0.389, *P*=0.056). Because age was a significant predictor of warfarin clearance and dose, this may have confounded our findings of the minor correlation between eGFR and warfarin dose, as well as the observation of eGFR being a significant predictor of total *S*-warfarin clearance. Thus, future well-matched investigations for age, or others that could assess warfarin clearance in different age groups are warranted to further clarify these observations.

Warfarin reduction contributes to about 20% of its total metabolism (Lewis et al., 1974). In addition, the alcohol metabolites produced by reduction exert anti-coagulant pharmacological activity mediated by decreased plasma levels of vitamin k-dependent clotting factors (Lewis et al., 1973). One study documented that warfarin alcohols are 6-fold less potent than warfarin in their ability to inhibit the VKOR enzyme *in vitro* (IC_{50} 12.5 vs. 2.2 µM) (Gebauer, 2007). Our study showed that the exposure of warfarin alcohol 2, but not alcohol 1, was increased with a decline in kidney function. Overall, increased exposure of warfarin alcohol 2 in CKD patients may partially contribute to the alterations in warfarin anticoagulant response and dosing requirements observed in kidney disease patients.

Previous epidemiologic studies have reported poor outcomes such as stroke and mortality with warfarin therapy in CKD patients with INR in the therapeutic range (Chan et al., 2009a; Chan et al., 2009b). This has led us to hypothesize that warfarin alcohol metabolites may be altered in these patients and may contribute to the anticoagulant effect that is mediated through vitamin k-independent clotting factors, which cannot be measured by the INR. Our *in vitro* studies, however, demonstrated negligible direct effect of warfarin alcohols on the activity of FXIa, an important

vitamin k-independent clotting factor in the coagulation cascade. Thus, further investigations are warranted to assess activity of clotting factors in CKD patients and their association with anticoagulant response.

It has been suggested that protein binding of drugs is impaired in uremia (Reidenberg, 1977). Consistent with the literature (Lomonaco et al., 2013), we found that warfarin enantiomers and warfarin alcohols are highly bound to plasma protein (99%), nevertheless, we couldn't demonstrate any alterations of protein binding of these analytes in patients with decreased kidney function. This finding contrasts with a previous report which demonstrated impaired warfarin protein binding in uremia (Sjoholm et al., 1976). This discrepancy may be attributed to the small sample size and to the technical differences in measuring warfarin albumin binding. For instance, previous investigations utilized indirect techniques to measure warfarin protein binding without quantifying the free drug concentrations (Sjoholm et al., 1976). Thus, future investigations with larger number of patients are needed to further corroborate this conclusion.

The major limitation of our study is the small sample size. Post-hoc power analysis revealed that this study is underpowered to detect a significant difference in warfarin clearance between kidney disease patients. Using F-test with alpha level of 0.05, our study had only 62% power to detect differences ranging from 27-35% in total warfarin clearance between patients with different degrees of kidney disease (the calculated effect size was 0.64). The study has other limitations such as incomplete recording of patient medical and medication history, as well as lack of CYP2C9 and VKOR1C genotyping.

In summary, this study demonstrates that kidney disease selectively alters the exposure of warfarin alcohol metabolites and also decreases the nonrenal clearance of warfarin mediated by CYP2C9. These data suggest that kidney disease does not significantly impact warfarin protein

binding. Overall, the findings may partially explain altered warfarin dosing requirements in the setting of kidney disease.

6.0 CONCLUSIONS AND FUTURE DIRECTIONS

6.1 SUMMARY AND CONCLUSIONS

The objective of this dissertation work was to characterize the effect of CKD on nonrenal drug clearance mediated by hepatic reduction. This was accomplished by 1) development and validation of a novel analytical method capable of measuring warfarin and its alcohol metabolites in various biological matrices, 2) investigating the effect of experimental kidney disease on rat hepatic reductase expression and activity utilizing warfarin as a substrate, 3) assessing human reductase expression and activity in livers of patients with or without ESRD, employing warfarin as a substrate, and 4) exploring steady-state warfarin pharmacokinetics in a pilot study of patients with different levels of kidney function. The important findings of each research chapter are summarized in the following sections.

In the first part of this research, we report the development and validation of a sensitive, rapid, and robust UHPLC-MS/MS assay for quantitating warfarin and warfarin alcohol metabolites in microsomal and cytosolic incubates. This is the first LC/MS method validated for that purpose. The method was successfully applied to measure the analytes after *in vitro* incubations of subcellular fractions with warfarin as demonstrated in chapters 2-4. In addition, the method was utilized to quantify warfarin and its alcohol metabolites in the clinical PK study as shown in chapter 5. The high sensitivity of the assay offers an advantage in identifying the unbound concentrations of warfarin and its alcohol metabolites.

In the second part of this work, the assay developed was employed to evaluate hepatic reductase activity in rats subjected to nephrectomy. The effect of experimental kidney disease on gene and protein expression of the primary rat reductases found in liver cytosol and microsomes was also investigated. This study demonstrated that the activity of hepatic reductases is selectively impaired by kidney disease. Further, we demonstrated that mRNA and protein expression of

hepatic reductases are differentially impacted by kidney disease. However, significant down-regulation was observed with many of the investigated reductases. The findings from chapter 3 suggest that the nonrenal clearance of drugs mediated by reduction pathways is impaired in kidney disease, and also implicate transcriptionally or translationally mediated changes in enzyme function.

In the third part of this research, the *in vitro* function, mRNA and protein expression of human reductases were investigated in cadaveric livers collected from ESRD patients and control subjects. This work was conducted to evaluate whether the previous findings in rats with nephrectomy can be translated to humans. A trend toward selective decreases in the activity of human reductases in livers of ESRD patients was demonstrated. In addition, a trend towards decreased mRNA expression of several reductase isoforms was also documented. These findings were not statistically significant, partially due to the large variability (%CV) observed.. We have also documented the first evidence of a significant reduction in protein expression of CBR1 in livers from ESRD patients. These findings from chapter 4 suggest that human hepatic reductases may also be altered in the setting of kidney disease. The findings may also provide a mechanistic explanation for altered drug disposition in kidney disease; especially those metabolized by reduction. This novel discovery advances our understanding of the effect of CKD on this pathway of nonrenal drug clearance.

In the final section of this dissertation, the steady-state pharmacokinetics of warfarin were assessed in patients with kidney disease. We have shown that eGFR is a significant predictor of total *S*-warfarin clearance. Also, increased *S/R* warfarin ratio was observed in ESRD patients compared to controls suggesting reduced CYP2C9 activity. We also demonstrated, for the first time, that the exposure of warfarin alcohol 2 metabolite is increased along with reduced kidney

function, suggesting reduction in its renal elimination. However, these alcohols were shown to have negligible effects on the activity of vitamin k-independent clotting factor (FXIa) as determined *in vitro*. These results presented in chapter 5 may highlight the importance of alcohol metabolites to the inter-individual variability of warfarin dosing.

6.2 CLINICAL IMPLICATIONS

The analytical assay that was developed and validated for measuring warfarin and its alcohol metabolites may be relevant for use in standard laboratories or other facilities that are equipped with modern instrumentation (i.e., mass spectrometry), and would benefit future investigations that require quantifying warfarin and metabolites in biological matrices. The method is highly sensitive and useful for measuring low concentrations as well as the unbound concentrations of warfarin and its metabolites.

Several clinical studies have shown that the pharmacokinetics of drugs eliminated nonrenally can be altered in kidney disease, resulting in changes in drug bioavailability and disposition. Our studies also demonstrate that phase I metabolic reduction is modulated in kidney disease. This finding provides a mechanism for altering drug disposition of hepatic reductase drug substrates in the setting of kidney disease. Because several drugs are primarily metabolized by the reduction pathway (e.g., haloperidol, idarubicin, boceprevir, naltrexone, nabumetone), our data suggest that these drugs may need to be carefully dosed and regularly monitored in CKD patients. Thus, our results offer potential insight to improve pharmacotherapy in CKD patients by developing better approaches that incorporate kidney function as a covariate when calculating the dose in this patient population.

There are numerous endogenous substrates for hepatic reductase enzymes such as steroids, prostaglandins, fatty acids, etc. Diminished functional expression of reductases in kidney disease may change the degree to which these substrates are biotransformed, and that may be associated with biological consequences in CKD patients. For example, decreased activity of 11β-HSD1 in kidney disease may result in reduced production of the active form cortisol, and that may modulate the inflammation and immune system response processes in the body.

Warfarin treatment in patients with kidney disease, especially those with ESRD, is challenging because of the increased risk of bleeding and stroke in this patient population. Our findings in the clinical PK study demonstrate alterations in the disposition of warfarin alcohol 2 in kidney disease patients. Increased exposure of warfarin alcohol 2, which exerts anticoagulant activity by inhibiting the VKOR enzyme, may enhance the overall pharmacological action of warfarin in patients with kidney disease. This corroborates the importance of considering kidney function in warfarin dosing and management. The current approaches for estimation of warfarin dose in CKD patients are reflections of those developed in the general medical population. In light of our findings, these approaches may be not suitable for CKD patients and have to be modified to account for changes in kidney function. Starting at lower warfarin doses may be warranted in CKD patients to avoid toxicity. However, the benefit of these new approaches should be tested in prospective randomized controlled trials.

6.3 LIMITATIONS AND FUTURE DIRECTIONS

The results presented in this dissertation provide insights and open multiple avenues for future studies relating to the effect of kidney disease on non-renal clearance of drugs. The findings act as the basis and foundation for future preclinical and clinical investigations in the setting of kidney disease. These future studies would be necessary to further mechanistically explain, validate, support, and expand upon our data. It is important to note that our studies have also some limitations that must be considered and highlighted. A list of drawbacks and proposed future directions of this work is presented below.

The inhibition studies demonstrated that isoforms of CBR and AKR as well as 11β-HSD reductases may contribute significantly to warfarin reduction. This was concluded by utilizing chemical inhibitors of reductase enzymes. However, the inhibitors used are selective for subgroups, but are not isoform specific. In addition, these inhibition studies were conducted without inclusion of positive controls (e.g., doxorubicin, naltrexone, bupropion), because the analytical assays that are required for measuring these analytes are not currently available in our laboratory. Hence, future investigations using recombinant enzyme systems of individual reductase isoforms with positive controls would be essential to confirm the specific reductases that are involved in warfarin reduction. This information is important and would be helpful in understanding the contribution of specific reductases to the overall variability in warfarin dose-response.

The number of human liver samples from patients with and without ESRD that were utilized in the study as described in chapter 4 is relatively small (n=10, n=11, respectively). The results of post-hoc power analysis revealed that our study was underpowered to detect a significant difference in reductase activity between ESRD and control livers. Further, the presence of co-

morbid conditions and the cause of death might confound our observations of the trend toward selectively decreased reductase activity and expression in livers of patients with ESRD. For instance, diabetes mellitus has been shown to alter the expression and activity of several CYP450 enzymes as well as the 11β-HSD isoform (Dostalek et al., 2011; Zhang et al., 2009). In our sample of ESRD livers, most patients had a history of diabetes and that may have confounded our findings. Variability in the region of the liver from which samples were collected may also have contributed to the variability observed between human liver samples. Therefore, future well-matched studies with larger sample size are warranted to further confirm these data.

Our studies in chapters 3 and 4 demonstrated alterations in activity, mRNA or protein expression of reductase enzymes in the setting of kidney disease. However, further investigations are required to assess mechanistically the reasons behind these alterations. For example, changes in protein stability or activity over time in cells exposed to uremia can be explored by pulse-chase analysis (Yamaguchi et al., 2009). In addition, post-translational modifications of reductase enzymes can be assessed by Western blotting using specific antibodies, or by peptide analysis using mass spectrometry (Larsen et al., 2006). Lastly, alterations in reductase enzymes mediated through nuclear transcriptional factors and epigenetic modulation in promoter region of the genes can be explored by chromatin immunoprecipitation (ChIP) techniques (Velenosi et al., 2014).

Although it is controversial, several studies have shown that genetic polymorphisms in reductase isoforms are associated with function and expression (Bains et al., 2010; Lal et al., 2010; Voon et al., 2013). In our studies of human liver tissue, genetic testing has not been performed on the liver samples. Polymorphic expression of reductases may contribute to the variability in the reductase activity observed in control and ESRD human liver samples. Thus, future investigations with larger sample size considering patients genotypes will be essential to validate our findings.

A recent study has shown that moderate CKD alters activity and expression of various CYP450 (Velenosi et al., 2012). The 5/6th nephrectomy rat model utilized in chapter 3 represents an advanced and progressive stage of uremia. In addition, the human liver tissue used in chapter 4 was collected from patients with ESRD. Thus, our assessments were limited to the effect of advanced kidney disease on hepatic reduction. Continued investigations that incorporate earlier stages of kidney disease will be necessary to test whether hepatic reductases are also impacted with moderate changes in kidney function. Measuring creatinine levels over time after the first surgery would be necessary to determine how fast creatinine concentrations and kidney function are stabilized after the nephrectomy.

Previous published reports have established that uremic toxins including cytokines and inflammatory mediators (e.g., *p*-cresol, hippuric acid, 3-indoxyl sulfate, PTH, benzyl alcohol, indole-3-acetic acid) play major roles in modulating the function and expression of drug metabolizing enzymes and transport proteins (Lalande et al., 2014; Yeung et al., 2014). Hence, future studies are required to investigate whether these toxins, which are present at high concentrations in uremic serum, can mechanistically explain our findings of decreased activity and expression of reductases in CKD. This can be tested by incubation of normal human/rat hepatocytes with different concentrations of uremic toxins or uremic serum followed by assessing the activity (using probe substrates) and expression (mRNA and protein) of reductase enzymes. Assessing the activity and expression will determine if uremia directly or indirectly affects reductase enzymes. In addition, exploring the effect of uremia on the expression of nuclear factors that mediate the transcriptional regulation of reductase genes such as Nrf2 and LXR (Chen and Zhang, 2012) will be helpful to determine whether the alterations in reductase expression in uremia is transcriptionally mediated. To simulate *in vivo* observations, hepatocyte systems are a better

approach to utilize compared to subcellular microsomal or cytosolic fractions. Applying different concentrations and individual cytokines may help identifying specific uremic toxins that have a major impact on reductive metabolism, and whether the clinically relevant concentrations have any impact on drug reduction.

Our findings in chapter 5 revealed negligible effects of warfarin alcohols on the activity of clotting factor XIa *in vitro*. Thus, future studies are required to explore other possible mechanisms for increasing the bleeding risk of warfarin in CKD patients. This may be investigated clinically or by using rat or mouse models of kidney disease and coagulopathy (Hogan et al., 2002).

The findings of decreased functional expression of hepatic reductases as well as altered warfarin alcohol exposure in CKD provide evidence that warfarin disposition may be altered in patients with CKD. This finding may support dosing optimization of warfarin in CKD patients, and consideration of kidney function as a covariate when developing new novel warfarin dosing algorithms. However, future randomized clinical trials of warfarin in patients with varying degrees of kidney disease would be necessary to definitively conclude the safety and efficacy of warfarin use in CKD patients. Our findings may also be applied to other drugs that are eliminated nonrenally primarily by reductases and are commonly used in CKD patients (e.g., bupropion, haloperidol, naltrexone). Dosing adjustments of these medications may also be necessary and would have to be validated in the setting of kidney disease.

Although it is an accepted approach (Chan et al., 1994; Jensen et al., 2012; Routledge et al., 1979), one point oral clearance as we performed in chapter 5 is not the optimal kinetic procedure for studying the effect of CKD on warfarin clearance. A study compared the clearance values based on a single plasma concentration measurement and total exposure using the area under the concentration-time curve (AUC), and demonstrated that a single point determinant of

warfarin clearance at steady-state was comparable with the AUC based clearance in only 6 of the 10 subjects included in the study. This highlights the limitation of this approach in calculating the oral clearance (McAleer et al., 1997). Therefore, collecting multiple blood samples at steady state and assessing clearance based on the AUC measurements would be a better approach to assess steady state disposition of warfarin in CKD patients. Additionally, formation clearance of alcohol metabolites could be measured in order to assess the effect of kidney disease on reductase activity. This can be conducted by collecting urine samples to measure alcohol metabolites as well as blood samples to quantify warfarin concentrations in plasma. Our study did not measure the exposure of warfarin hydroxy metabolites generated by CYP450-mediated warfarin oxidation. Because these metabolites are excreted renally and along with the evidence of decreased CYP2C9 activity in ESRD (Dreisbach et al., 2003), assessing the effect of kidney disease on the disposition of hydroxy warfarin metabolites should be considered in future investigations. It is previously reported that the role of transporters (OATP) on warfarin disposition is minimal (Frymoyer et al., 2010), thus it is unlikely that altered drug transport confounded our results.

The warfarin clinical study was conducted in a small number of patients (n=25). The post-hoc power analysis indicated that our study was underpowered to detect a significant difference in total warfarin clearance between patients with varying degrees of kidney function. The small sample size along with the high interpatient variability may have impacted our final conclusions. In addition, complete patient information was not recorded. For example, genotyping of VKOR1 and CYP2C9 was not conducted, and some of patients' comorbid conditions and medical therapy were missing. These clinical parameters may have confounded our final observations. Therefore, to overcome these issues, future large multicenter studies considering all necessary patient demographic and clinical characteristics are warranted.

BIBLIOGRAPHY

Alonso A, Lopez FL, Matsushita K, Loehr LR, Agarwal SK, Chen LY, Soliman EZ, Astor BC, and Coresh J. Chronic kidney disease is associated with the incidence of atrial fibrillation: the Atherosclerosis Risk in Communities (ARIC) study. *Circulation* **123**: 2946-2953, 2011.

Alshogran OY, Naud J, Ocque AJ, Leblond F, Pichette V, and Nolin TD. Effect of Experimental Kidney Disease on the Functional Expression of Hepatic Reductases. *Drug Metab Dispos* **43**: 100-106, 2014a.

Alshogran OY, Ocque AJ, Zhao J, Day BW, Leblond FA, Pichette V, and Nolin TD. Determination of warfarin alcohols by ultra-high performance liquid chromatography-tandem mass spectrometry: Application to in vitro enzyme kinetic studies. *J Chromatogr B Analyt Technol Biomed Life Sci* **944**: 63-68, 2014b.

Ananthapanyasut W, Napan S, Rudolph EH, Harindhanavudhi T, Ayash H, Guglielmi KE, and Lerma EV. Prevalence of atrial fibrillation and its predictors in nondialysis patients with chronic kidney disease. *Clin J Am Soc Nephrol* **5**: 173-181, 2010.

Atalla A, Breyer-Pfaff U, and Maser E. Purification and characterization of oxidoreductases-catalyzing carbonyl reduction of the tobacco-specific nitrosamine 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) in human liver cytosol. *Xenobiotica* **30**: 755-769, 2000.

Ax W, Soldan M, Koch L, and Maser E. Development of daunorubicin resistance in tumour cells by induction of carbonyl reduction. *Biochem Pharmacol* **59**: 293-300, 2000.

Bachmann K, Shapiro R, and Mackiewicz J. Warfarin elimination and responsiveness in patients with renal dysfunction. *J Clin Pharmacol* **17**: 292-299, 1977.

Bains OS, Karkling MJ, Lubieniecka JM, Grigliatti TA, Reid RE, and Riggs KW. Naturally occurring variants of human CBR3 alter anthracycline in vitro metabolism. *J Pharmacol Exp Ther* **332**: 755-763, 2010.

Baker WL, Cios DA, Sander SD, and Coleman CI. Meta-analysis to assess the quality of warfarin control in atrial fibrillation patients in the United States. *J Manag Care Pharm* **15**: 244-252, 2009.

Banfield C and Rowland M. Stereospecific fluorescence high-performance liquid chromatographic analysis of warfarin and its metabolites in plasma and urine. *J Pharm Sci* **73**: 1392-1396, 1984.

Bansal N. The Debate on Warfarin Use in Dialysis Patients With Atrial Fibrillation: More Fuel for the Fire. *Am J Kidney Dis* **64**: 677-680, 2014.

Barendregt JN, Van Peer A, Van Der Hoeven JG, Van Oene JC, and Tjandra YI. Ketanserin pharmacokinetics in patients with renal failure. *Br J Clin Pharmacol* **29**: 715-723, 1990.

Barnes KJ, Rowland A, Polasek TM, and Miners JO. Inhibition of human drug-metabolising cytochrome P450 and UDP-glucuronosyltransferase enzyme activities in vitro by uremic toxins. *Eur J Clin Pharmacol* **70**: 1097-1106, 2014.

Barski OA, Tipparaju SM, and Bhatnagar A. The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. *Drug Metab Rev* **40**: 553-624, 2008.

Bennett WM. Should dialysis patients ever receive warfarin and for what reasons? *Clin J Am Soc Nephrol* **1**: 1357-1359, 2006.

Bratton SM, Mosher CM, Khallouki F, Finel M, Court MH, Moran JH, and Radominska-Pandya A. Analysis of R- and S-hydroxywarfarin glucuronidation catalyzed by human liver microsomes and recombinant UDP-glucuronosyltransferases. *J Pharmacol Exp Ther* **340**: 46-55, 2012.

Breckenridge A, Orme M, Wesseling H, Lewis RJ, and Gibbons R. Pharmacokinetics and pharmacodynamics of the enantiomers of warfarin in man. *Clin Pharmacol Ther* **15**: 424-430, 1974.

Breyer-Pfaff U, Martin HJ, Ernst M, and Maser E. Enantioselectivity of carbonyl reduction of 4-methylnitrosamino-1-(3-pyridyl)-1-butanone by tissue fractions from human and rat and by enzymes isolated from human liver. *Drug Metab Dispos* **32**: 915-922, 2004.

Breyer-Pfaff U and Nill K. Carbonyl reduction of naltrexone and dolasetron by oxidoreductases isolated from human liver cytosol. *J Pharm Pharmacol* **56**: 1601-1606, 2004.

Camaggi CM, Strocchi E, Carisi P, Martoni A, Tononi A, Guaraldi M, Strolin-Benedetti M, Efthymiopoulos C, and Pannuti F. Idarubicin metabolism and pharmacokinetics after intravenous and oral administration in cancer patients: a crossover study. *Cancer Chemother Pharmacol* **30**: 307-316, 1992.

Carrero JJ, Evans M, Szummer K, Spaak J, Lindhagen L, Edfors R, Stenvinkel P, Jacobson SH, and Jernberg T. Warfarin, kidney dysfunction, and outcomes following acute myocardial infarction in patients with atrial fibrillation. *JAMA* **311**: 919-928, 2014.

Cavallari LH, Langaee TY, Momary KM, Shapiro NL, Nutescu EA, Coty WA, Viana MA, Patel SR, and Johnson JA. Genetic and clinical predictors of warfarin dose requirements in African Americans. *Clin Pharmacol Ther* **87**: 459-464, 2010.

Chan E, McLachlan AJ, Pegg M, MacKay AD, Cole RB, and Rowland M. Disposition of warfarin enantiomers and metabolites in patients during multiple dosing with rac-warfarin. *Br J Clin Pharmacol* **37**: 563-569, 1994.

Chan E, McLachlan AJ, and Rowland M. Warfarin metabolites: stereochemical aspects of protein binding and displacement by phenylbutazone. *Chirality* **5**: 610-615, 1993.

Chan KE, Lazarus JM, Thadhani R, and Hakim RM. Anticoagulant and antiplatelet usage associates with mortality among hemodialysis patients. *J Am Soc Nephrol* **20**: 872-881, 2009a.

Chan KE, Lazarus JM, Thadhani R, and Hakim RM. Warfarin use associates with increased risk for stroke in hemodialysis patients with atrial fibrillation. *J Am Soc Nephrol* **20**: 2223-2233, 2009b.

Chan KK, Lewis RJ, and Trager WF. Absolute configurations of the four warfarin alcohols. *J Med Chem* **15**: 1265-1270, 1972.

Chen WD and Zhang Y. Regulation of aldo-keto reductases in human diseases. *Front Pharmacol* **3**: 35, 2012.

Comer GM and Ciulla TA. Current and future pharmacological intervention for diabetic retinopathy. *Expert Opin Emerg Drugs* **10**: 441-455, 2005.

Connolly SJ, Ezekowitz MD, Yusuf S, Eikelboom J, Oldgren J, Parekh A, Pogue J, Reilly PA, Themeles E, Varrone J, Wang S, Alings M, Xavier D, Zhu J, Diaz R, Lewis BS, Darius H, Diener HC, Joyner CD, Wallentin L, Committee R-LS, and Investigators. Dabigatran versus warfarin in patients with atrial fibrillation. *N Engl J Med* **361**: 1139-1151, 2009.

Coresh J, Selvin E, Stevens LA, Manzi J, Kusek JW, Eggers P, Van Lente F, and Levey AS. Prevalence of chronic kidney disease in the United States. *JAMA* **298**: 2038-2047, 2007.

Dani M, Boisvert C, Michaud J, Naud J, Lefrancois S, Leblond FA, and Pichette V. Down-regulation of liver drug-metabolizing enzymes in a murine model of chronic renal failure. *Drug Metab Dispos* **38**: 357-360, 2009.

Danson S, Ward TH, Butler J, and Ranson M. DT-diaphorase: a target for new anticancer drugs. *Cancer Treat Rev* **30**: 437-449, 2004.

Dayton HE and Inturrisi CE. The urinary excretion profiles of naltrexone in man, monkey, rabbit, and rat. *Drug Metab Dispos* **4**: 474-478, 1976.

Diederich S, Eigendorff E, Burkhardt P, Quinkler M, Bumke-Vogt C, Rochel M, Seidelmann D, Esperling P, Oelkers W, and Bahr V. 11beta-hydroxysteroid dehydrogenase types 1 and 2: an important pharmacokinetic determinant for the activity of synthetic mineralo- and glucocorticoids. *J Clin Endocrinol Metab* **87**: 5695-5701, 2002.

Dimmitt DC, Shah AK, Arumugham T, Cramer MB, Halstenson C, Horton M, and Weir SJ. Pharmacokinetics of oral and intravenous dolasetron mesylate in patients with renal impairment. *J Clin Pharmacol* **38**: 798-806, 1998.

Dinkova-Kostova AT and Talalay P. Persuasive evidence that quinone reductase type 1 (DT diaphorase) protects cells against the toxicity of electrophiles and reactive forms of oxygen. *Free Radic Biol Med* **29**: 231-240, 2000.

Doring B and Petzinger E. Phase 0 and phase III transport in various organs: Combined concept of phases in xenobiotic transport and metabolism. *Drug Metab Rev* **46**: 261-282, 2014.

Dostalek M, Court MH, Yan B, and Akhlaghi F. Significantly reduced cytochrome P450 3A4 expression and activity in liver from humans with diabetes mellitus. *Br J Pharmacol* **163**: 937-947, 2011.

Dowling TC, Briglia AE, Fink JC, Hanes DS, Light PD, Stackiewicz L, Karyekar CS, Eddington ND, Weir MR, and Henrich WL. Characterization of hepatic cytochrome p4503A activity in patients with end-stage renal disease. *Clin Pharmacol Ther* **73**: 427-434, 2003.

Draper N and Stewart PM. 11beta-hydroxysteroid dehydrogenase and the pre-receptor regulation of corticosteroid hormone action. *J Endocrinol* **186**: 251-271, 2005.

Dreisbach AW, Japa S, Gebrekal AB, Mowry SE, Lertora JJ, Kamath BL, and Rettie AE. Cytochrome P4502C9 activity in end-stage renal disease. *Clin Pharmacol Ther* **73**: 475-477, 2003.

du Souich P and Erill S. Metabolism of procainamide in patients with chronic heart failure, chronic respiratory failure and chronic renal failure. *Eur J Clin Pharmacol* **14**: 21-27, 1978.

Duffield AM, Duffield PH, Birkett DJ, Kennedy M, and Wade DN. Plasma quantitation of warfarin and warfarin alcohol by gas chromatography chemical ionization mass spectrometry in patients on warfarin maintenance therapy. *Biomed Mass Spectrom* **6**: 208-211, 1979.

Elliott MJ, Zimmerman D, and Holden RM. Warfarin anticoagulation in hemodialysis patients: a systematic review of bleeding rates. *Am J Kidney Dis* **50**: 433-440, 2007.

Endo S, Matsunaga T, Kitade Y, Ohno S, Tajima K, El-Kabbani O, and Hara A. Human carbonyl reductase 4 is a mitochondrial NADPH-dependent quinone reductase. *Biochem Biophys Res Commun* **377**: 1326-1330, 2008.

Fang MC, Go AS, Chang Y, Borowsky LH, Pomernacki NK, Udaltsova N, and Singer DE. A new risk scheme to predict warfarin-associated hemorrhage: The ATRIA (Anticoagulation and Risk Factors in Atrial Fibrillation) Study. *J Am Coll Cardiol* **58**: 395-401, 2011.

Fasco MJ, Cashin MJ, and Kaminsky LS. A novel method for the determination of warfarin and its metabolites in plasma *Journal of Liquid Chromatography* **2**: 565-575, 1979.

Fasco MJ, Piper LJ, and Kaminsky LS. Biochemical applications of a quantitative high-pressure liquid chromatographic assay of warfarin and its metabolites. *J Chromatogr* **131**: 365-373, 1977.

Fasco MJ and Principe LM. R- and S-Warfarin inhibition of vitamin K and vitamin K 2,3-epoxide reductase activities in the rat. *J Biol Chem* **257**: 4894-4901, 1982.

Finazzi G and Mingardi G. Oral anticoagulant therapy in hemodialysis patients: do the benefits outweigh the risks? *Intern Emerg Med* **4**: 375-380, 2009.

Forrest GL and Gonzalez B. Carbonyl reductase. *Chem Biol Interact* **129**: 21-40, 2000.

Forrest GL, Gonzalez B, Tseng W, Li X, and Mann J. Human carbonyl reductase overexpression in the heart advances the development of doxorubicin-induced cardiotoxicity in transgenic mice. *Cancer Res* **60**: 5158-5164, 2000.

Frassetto LA, Poon S, Tsourounis C, Valera C, and Benet LZ. Effects of uptake and efflux transporter inhibition on erythromycin breath test results. *Clin Pharmacol Ther* **81**: 828-832, 2007.

Frymoyer A, Shugarts S, Browne M, Wu AH, Frassetto L, and Benet LZ. Effect of single-dose rifampin on the pharmacokinetics of warfarin in healthy volunteers. *Clin Pharmacol Ther* **88**: 540-547, 2010.

Gabbay KH. Aldose reductase inhibition in the treatment of diabetic neuropathy: where are we in 2004? *Curr Diab Rep* **4**: 405-408, 2004.

Gage BF, Eby C, Milligan PE, Banet GA, Duncan JR, and McLeod HL. Use of pharmacogenetics and clinical factors to predict the maintenance dose of warfarin. *Thromb Haemost* **91**: 87-94, 2004.

Gage BF, Yan Y, Milligan PE, Waterman AD, Culverhouse R, Rich MW, and Radford MJ. Clinical classification schemes for predicting hemorrhage: results from the National Registry of Atrial Fibrillation (NRAF). *Am Heart J* **151**: 713-719, 2006.

Gai Z, Chu L, Hiller C, Arsenijevic D, Penno CA, Montani JP, Odermatt A, and Kullak-Ublick GA. Effect of chronic renal failure on the hepatic, intestinal, and renal expression of bile acid transporters. *Am J Physiol Renal Physiol* **306**: F130-137, 2014.

Gathercole LL, Lavery GG, Morgan SA, Cooper MS, Sinclair AJ, Tomlinson JW, and Stewart PM. 11beta-hydroxysteroid dehydrogenase 1: translational and therapeutic aspects. *Endocr Rev* **34**: 525-555, 2013.

Gebauer M. Synthesis and structure-activity relationships of novel warfarin derivatives. *Bioorg Med Chem* **15**: 2414-2420, 2007.

Gong IY, Schwarz UI, Crown N, Dresser GK, Lazo-Langner A, Zou G, Roden DM, Stein CM, Rodger M, Wells PS, Kim RB, and Tirona RG. Clinical and genetic determinants of warfarin pharmacokinetics and pharmacodynamics during treatment initiation. *PLoS One* **6**: e27808, 2011.

Granger CB, Alexander JH, McMurray JJ, Lopes RD, Hylek EM, Hanna M, Al-Khalidi HR, Ansell J, Atar D, Avezum A, Bahit MC, Diaz R, Easton JD, Ezekowitz JA, Flaker G, Garcia D, Geraldes M, Gersh BJ, Golitsyn S, Goto S, Hermosillo AG, Hohnloser SH, Horowitz J, Mohan P, Jansky P, Lewis BS, Lopez-Sendon JL, Pais P, Parkhomenko A, Verheugt FW, Zhu J, and Wallentin L. Apixaban versus warfarin in patients with atrial fibrillation. *N Engl J Med* **365**: 981-992, 2011.

Granger CB and Chertow GM. A pint of sweat will save a gallon of blood: a call for randomized trials of anticoagulation in end-stage renal disease. *Circulation* **129**: 1190-1192, 2014.

Greenbaum D, Colangelo C, Williams K, and Gerstein M. Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol* **4**: 117, 2003.

Guengerich FP. Cytochrome p450 and chemical toxicology. *Chem Res Toxicol* **21**: 70-83, 2008.

Guevin C, Michaud J, Naud J, Leblond FA, and Pichette V. Down-regulation of hepatic cytochrome p450 in chronic renal failure: role of uremic mediators. *Br J Pharmacol* **137**: 1039-1046, 2002.

Harder S. Renal profiles of anticoagulants. *J Clin Pharmacol* **52**: 964-975, 2012.

Harel Z, Sholzberg M, Shah PS, Pavenski K, Harel S, Wald R, Bell CM, and Perl J. Comparisons between novel oral anticoagulants and vitamin K antagonists in patients with CKD. *J Am Soc Nephrol* **25**: 431-442, 2014.

Hart RG, Pearce LA, and Aguilar MI. Meta-analysis: antithrombotic therapy to prevent stroke in patients who have nonvalvular atrial fibrillation. *Ann Intern Med* **146**: 857-867, 2007.

Hart RG, Pearce LA, Asinger RW, and Herzog CA. Warfarin in atrial fibrillation patients with moderate chronic kidney disease. *Clin J Am Soc Nephrol* **6**: 2599-2604, 2011.

Hermans JJ and Thijssen HH. The in vitro ketone reduction of warfarin and analogues. Substrate stereoselectivity, product stereoselectivity and species differences. *Biochem Pharmacol* **38**: 3365-3370, 1989.

Hermans JJ and Thijssen HH. Stereoselective acetonyl side chain reduction of warfarin and analogs. Partial characterization of two cytosolic carbonyl reductases. *Drug Metab Dispos* **20**: 268-274, 1992.

Hermans JJ and Thijssen HH. Properties and stereoselectivity of carbonyl reductases involved in the ketone reduction of warfarin and analogues. *Adv Exp Med Biol* **328**: 351-360, 1993.

Hewick DS and McEwen J. Plasma half-lives, plasma metabolites and anticoagulant efficacies of the enantiomers of warfarin in man. *J Pharm Pharmacol* **25**: 458-465, 1973.

Hijazi Z, Hohnloser SH, Oldgren J, Andersson U, Connolly SJ, Eikelboom JW, Ezekowitz MD, Reilly PA, Siegbahn A, Yusuf S, and Wallentin L. Efficacy and safety of dabigatran compared with warfarin in relation to baseline renal function in patients with atrial fibrillation: a RE-LY (Randomized Evaluation of Long-term Anticoagulation Therapy) trial analysis. *Circulation* **129**: 961-970, 2014.

Hirsh J, Dalen J, Anderson DR, Poller L, Bussey H, Ansell J, and Deykin D. Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. *Chest* **119**: 8S-21S, 2001.

Hirsh J, Fuster V, Ansell J, and Halperin JL. American Heart Association/American College of Cardiology Foundation guide to warfarin therapy. *Circulation* **107**: 1692-1711, 2003.

Hoffmann F and Maser E. Carbonyl reductases and pluripotent hydroxysteroid dehydrogenases of the short-chain dehydrogenase/reductase superfamily. *Drug Metab Rev* **39**: 87-144, 2007.

Hogan KA, Weiler H, and Lord ST. Mouse models in coagulation. *Thromb Haemost* **87**: 563-574, 2002.

Hohnloser SH, Hijazi Z, Thomas L, Alexander JH, Amerena J, Hanna M, Keltai M, Lanas F, Lopes RD, Lopez-Sendon J, Granger CB, and Wallentin L. Efficacy of apixaban when compared with warfarin in relation to renal function in patients with atrial fibrillation: insights from the ARISTOTLE trial. *Eur Heart J* **33**: 2821-2830, 2012.

Holden RM, Harman GJ, Wang M, Holland D, and Day AG. Major bleeding in hemodialysis patients. *Clin J Am Soc Nephrol* **3**: 105-110, 2008.

Holford NH. Clinical pharmacokinetics and pharmacodynamics of warfarin. Understanding the dose-effect relationship. *Clin Pharmacokinet* **11**: 483-504, 1986.

International Transporter C, Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM, Hoffmaster KA, Ishikawa T, Keppler D, Kim RB, Lee CA, Niemi M, Polli JW, Sugiyama Y, Swaan PW, Ware JA, Wright SH, Yee SW, Zamek-Gliszczynski MJ, and Zhang L. Membrane transporters in drug development. *Nat Rev Drug Discov* **9**: 215-236, 2010.

James MT, Hemmelgarn BR, and Tonelli M. Early recognition and prevention of chronic kidney disease. *Lancet* **375**: 1296-1309, 2010.

Jarabak J and Harvey RG. Studies on three reductases which have polycyclic aromatic hydrocarbon quinones as substrates. *Arch Biochem Biophys* **303**: 394-401, 1993.

Jensen BP, Chin PK, Roberts RL, and Begg EJ. Influence of adult age on the total and free clearance and protein binding of (R)- and (S)-warfarin. *Br J Clin Pharmacol* **74**: 797-805, 2012.

Jez JM, Bennett MJ, Schlegel BP, Lewis M, and Penning TM. Comparative anatomy of the aldo-keto reductase superfamily. *Biochem J* **326 (Pt 3)**: 625-636, 1997.

Ji L, Masuda S, Saito H, and Inui K. Down-regulation of rat organic cation transporter rOCT2 by 5/6 nephrectomy. *Kidney Int* **62**: 514-524, 2002.

Jiang X, Crain EJ, Luettgen JM, Schumacher WA, and Wong PC. Apixaban, an oral direct factor Xa inhibitor, inhibits human clot-bound factor Xa activity in vitro. *Thromb Haemost* **101**: 780-782, 2009.

Jin Y and Penning TM. Aldo-keto reductases and bioactivation/detoxication. *Annu Rev Pharmacol Toxicol* **47**: 263-292, 2007.

Jones DR, Boysen G, and Miller GP. Novel multi-mode ultra performance liquid chromatography-tandem mass spectrometry assay for profiling enantiomeric hydroxywarfarins and warfarin in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* **879**: 1056-1062, 2011.

Jones DR and Miller GP. Assays and applications in warfarin metabolism: what we know, how we know it and what we need to know. *Expert Opin Drug Metab Toxicol* **7**: 857-874, 2011.

Jones DR, Moran JH, and Miller GP. Warfarin and UDP-glucuronosyltransferases: writing a new chapter of metabolism. *Drug Metab Rev* **42**: 55-61, 2010.

Jones M, McEwan P, Morgan CL, Peters JR, Goodfellow J, and Currie CJ. Evaluation of the pattern of treatment, level of anticoagulation control, and outcome of treatment with warfarin in patients with non-valvar atrial fibrillation: a record linkage study in a large British population. *Heart* **91**: 472-477, 2005.

Jorgensen AL, Al-Zubiedi S, Zhang JE, Keniry A, Hanson A, Hughes DA, Eker D, Stevens L, Hawkins K, Toh CH, Kamali F, Daly AK, Fitzmaurice D, Coffey A, Williamson PR, Park BK, Deloukas P, and Pirmohamed M. Genetic and environmental factors determining clinical outcomes and cost of warfarin therapy: a prospective study. *Pharmacogenet Genomics* **19**: 800-812, 2009.

Joy MS, Frye RF, Nolin TD, Roberts BV, La MK, Wang J, Brouwer KL, Dooley MA, and Falk RJ. In vivo alterations in drug metabolism and transport pathways in patients with chronic kidney diseases. *Pharmacotherapy* **34**: 114-122, 2014.

Kambia NK, Dine T, Odou P, Bah S, Azar R, Gressier B, Dupin-Spriet T, Luyckx M, and Brunet C. Pharmacokinetics and dialysability of naltrexone in patients undergoing hemodialysis. *Eur J Drug Metab Pharmacokinet* **29**: 225-230, 2004.

Kaminsky LS, de Morais SM, Faletto MB, Dunbar DA, and Goldstein JA. Correlation of human cytochrome P4502C substrate specificities with primary structure: warfarin as a probe. *Mol Pharmacol* **43**: 234-239, 1993.

Kaminsky LS and Zhang ZY. Human P450 metabolism of warfarin. *Pharmacol Ther* **73**: 67-74, 1997.

Kang W and Weiss M. Modeling the metabolism of idarubicin to idarubicinol in rat heart: effect of rutin and phenobarbital. *Drug Metab Dispos* **31**: 462-468, 2003.

Kassner N, Huse K, Martin HJ, Godtel-Armbrust U, Metzger A, Meineke I, Brockmoller J, Klein K, Zanger UM, Maser E, and Wojnowski L. Carbonyl reductase 1 is a predominant doxorubicin reductase in the human liver. *Drug Metab Dispos* **36**: 2113-2120, 2008.

Kim MJ, Huang SM, Meyer UA, Rahman A, and Lesko LJ. A regulatory science perspective on warfarin therapy: a pharmacogenetic opportunity. *J Clin Pharmacol* **49**: 138-146, 2009.

Kim YG, Shin JG, Shin SG, Jang IJ, Kim S, Lee JS, Han JS, and Cha YN. Decreased acetylation of isoniazid in chronic renal failure. *Clin Pharmacol Ther* **54**: 612-620, 1993.

Kimmel SE, French B, Kasner SE, Johnson JA, Anderson JL, Gage BF, Rosenberg YD, Eby CS, Madigan RA, McBane RB, Abdel-Rahman SZ, Stevens SM, Yale S, Mohler ER, 3rd, Fang MC, Shah V, Horenstein RB, Limdi NA, Muldowney JA, 3rd, Gujral J, Delafontaine P, Desnick RJ, Ortel TL, Billett HH, Pendleton RC, Geller NL, Halperin JL, Goldhaber SZ, Caldwell MD, Califf RM, Ellenberg JH, and Investigators C. A pharmacogenetic versus a clinical algorithm for warfarin dosing. *N Engl J Med* **369**: 2283-2293, 2013.

Kinirons MT and O'Mahony MS. Drug metabolism and ageing. *Br J Clin Pharmacol* **57**: 540-544, 2004.

Klein TE, Altman RB, Eriksson N, Gage BF, Kimmel SE, Lee MT, Limdi NA, Page D, Roden DM, Wagner MJ, Caldwell MD, and Johnson JA. Estimation of the warfarin dose with clinical and pharmacogenetic data. *N Engl J Med* **360**: 753-764, 2009.

Kleinow ME, Garwood CL, Clemente JL, and Whittaker P. Effect of chronic kidney disease on warfarin management in a pharmacist-managed anticoagulation clinic. *J Manag Care Pharm* **17**: 523-530, 2011.

Lai HM, Aronow WS, Kalen P, Adapa S, Patel K, Goel A, Vinnakota R, Chugh S, and Garrick R. Incidence of thromboembolic stroke and of major bleeding in patients with atrial fibrillation and chronic kidney disease treated with and without warfarin. *Int J Nephrol Renovasc Dis* **2**: 33-37, 2009.

Lal S, Mahajan A, Chen WN, and Chowbay B. Pharmacogenetics of target genes across doxorubicin disposition pathway: a review. *Curr Drug Metab* **11**: 115-128, 2010.

Lalande L, Charpiat B, Leboucher G, and Tod M. Consequences of renal failure on non-renal clearance of drugs. *Clin Pharmacokinet* **53**: 521-532, 2014.

Lamba JK, Lin YS, Schuetz EG, and Thummel KE. Genetic contribution to variable human CYP3A-mediated metabolism. *Adv Drug Deliv Rev* **54**: 1271-1294, 2002.

Laouari D, Yang R, Veau C, Blanke I, and Friedlander G. Two apical multidrug transporters, P-gp and MRP2, are differently altered in chronic renal failure. *Am J Physiol Renal Physiol* **280**: F636-645, 2001.

Larsen MR, Trelle MB, Thingholm TE, and Jensen ON. Analysis of posttranslational modifications of proteins by tandem mass spectrometry. *Biotechniques* **40**: 790-798, 2006.

Leblond F, Guevin C, Demers C, Pellerin I, Gascon-Barre M, and Pichette V. Downregulation of hepatic cytochrome P450 in chronic renal failure. *J Am Soc Nephrol* **12**: 326-332, 2001.

Leblond FA, Giroux L, Villeneuve JP, and Pichette V. Decreased in vivo metabolism of drugs in chronic renal failure. *Drug Metab Dispos* **28**: 1317-1320, 2000.

Leblond FA, Petrucci M, Dube P, Bernier G, Bonnardeaux A, and Pichette V. Downregulation of intestinal cytochrome p450 in chronic renal failure. *J Am Soc Nephrol* **13**: 1579-1585, 2002.

Lehmann CR, Heironimus JD, Collins CB, O'Neil TJ, Pierson WP, Crowe JT, Melikian AP, and Wright GJ. Metoclopramide kinetics in patients with impaired renal function and clearance by hemodialysis. *Clin Pharmacol Ther* **37**: 284-289, 1985.

Lenzini P, Wadelius M, Kimmel S, Anderson JL, Jorgensen AL, Pirmohamed M, Caldwell MD, Limdi N, Burmester JK, Dowd MB, Angchaisuksiri P, Bass AR, Chen J, Eriksson N, Rane A, Lindh JD, Carlquist JF, Horne BD, Grice G, Milligan PE, Eby C, Shin J, Kim H, Kurnik D, Stein CM, McMillin G, Pendleton RC, Berg RL, Deloukas P, and Gage BF. Integration of genetic, clinical, and INR data to refine warfarin dosing. *Clin Pharmacol Ther* **87**: 572-578, 2010.

Levey AS and Coresh J. Chronic kidney disease. *Lancet* **379**: 165-180, 2012.

Levey AS, Coresh J, Balk E, Kausz AT, Levin A, Steffes MW, Hogg RJ, Perrone RD, Lau J, Eknoyan G, and National Kidney F. National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Ann Intern Med* **139**: 137-147, 2003.

Levey AS, Eckardt KU, Tsukamoto Y, Levin A, Coresh J, Rossert J, De Zeeuw D, Hostetter TH, Lameire N, and Eknoyan G. Definition and classification of chronic kidney disease: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). *Kidney Int* **67**: 2089-2100, 2005.

Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, 3rd, Feldman HI, Kusek JW, Eggers P, Van Lente F, Greene T, Coresh J, and Ckd EPI. A new equation to estimate glomerular filtration rate. *Ann Intern Med* **150**: 604-612, 2009.

Lewis RJ and Trager WF. The metabolic fate of warfarin: studies on the metabolites in plasma. *Ann N Y Acad Sci* **179**: 205-212, 1971.

Lewis RJ, Trager WF, Chan KK, Breckenridge A, Orme M, Roland M, and Schary W. Warfarin. Stereochemical aspects of its metabolism and the interaction with phenylbutazone. *J Clin Invest* **53**: 1607-1617, 1974.

Lewis RJ, Trager WF, Robinson AJ, and Chan KK. Warfarin metabolites: the anticoagulant activity and pharmacology of warfarin alcohols. *J Lab Clin Med* **81**: 925-931, 1973.

Liao Z, Feng S, Ling P, and Zhang G. Meta-analysis of randomized controlled trials reveals an improved clinical outcome of using genotype plus clinical algorithm for warfarin dosing. *J Thromb Thrombolysis*, 2014.

Limdi NA, Beasley TM, Baird MF, Goldstein JA, McGwin G, Arnett DK, Acton RT, and Allon M. Kidney function influences warfarin responsiveness and hemorrhagic complications. *J Am Soc Nephrol* **20**: 912-921, 2009.

Limdi NA, Limdi MA, Cavallari L, Anderson AM, Crowley MR, Baird MF, Allon M, and Beasley TM. Warfarin dosing in patients with impaired kidney function. *Am J Kidney Dis* **56**: 823-831, 2010.

Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402-408, 2001.

Lomonaco T, Ghimenti S, Piga I, Onor M, Melai B, Fuoco R, and Di Francesco F. Determination of total and unbound warfarin and warfarin alcohols in human plasma by high performance liquid chromatography with fluorescence detection. *J Chromatogr A* **1314**: 54-62, 2013.

Ma D, Mizurini DM, Assumpcao TC, Li Y, Qi Y, Kotsyfakis M, Ribeiro JM, Monteiro RQ, and Francischetti IM. Desmolaris, a novel factor XIa anticoagulant from the salivary gland

of the vampire bat (Desmodus rotundus) inhibits inflammation and thrombosis in vivo. *Blood* **122**: 4094-4106, 2013.

Maddison J, Somogyi AA, Jensen BP, James HM, Gentgall M, and Rolan PE. The pharmacokinetics and pharmacodynamics of single dose (R)- and (S)-warfarin administered separately and together: relationship to VKORC1 genotype. *Br J Clin Pharmacol* **75**: 208-216, 2013.

Malatkova P, Ebert B, Wsol V, and Maser E. Expression of human carbonyl reductase 3 (CBR3; SDR21C2) is inducible by pro-inflammatory stimuli. *Biochem Biophys Res Commun* **420**: 368-373, 2012.

Malatkova P, Maser E, and Wsol V. Human carbonyl reductases. *Curr Drug Metab* **11**: 639-658, 2010.

Malatkova P and Wsol V. Carbonyl reduction pathways in drug metabolism. *Drug Metab Rev* **46**: 96-123, 2014.

Martin HJ, Breyer-Pfaff U, Wsol V, Venz S, Block S, and Maser E. Purification and characterization of akr1b10 from human liver: role in carbonyl reduction of xenobiotics. *Drug Metab Dispos* **34**: 464-470, 2006.

Maser E, Wsol V, and Martin HJ. 11Beta-hydroxysteroid dehydrogenase type 1: purification from human liver and characterization as carbonyl reductase of xenobiotics. *Mol Cell Endocrinol* **248**: 34-37, 2006.

Masuzaki H and Flier JS. Tissue-specific glucocorticoid reactivating enzyme, 11 beta-hydroxysteroid dehydrogenase type 1 (11 beta-HSD1)--a promising drug target for the treatment of metabolic syndrome. *Curr Drug Targets Immune Endocr Metabol Disord* **3**: 255-262, 2003.

Matsunaga T, Shintani S, and Hara A. Multiplicity of mammalian reductases for xenobiotic carbonyl compounds. *Drug Metab Pharmacokinet* **21**: 1-18, 2006.

McAleer SD, Chrystyn H, and Foondun AS. Measurement of the (R)- and (S)-isomers of warfarin in patients undergoing anticoagulant therapy. *Chirality* **4**: 488-493, 1992.

McAleer SD, Foondun AS, Feely M, and Chrystyn H. Steady-state clearance rates of warfarin and its enantiomers in therapeutically dosed patients. *Chirality* **9**: 13-16, 1997.

Meyer A, Vuorinen A, Zielinska AE, Strajhar P, Lavery GG, Schuster D, and Odermatt A. Formation of threohydrobupropion from bupropion is dependent on 11beta-hydroxysteroid dehydrogenase 1. *Drug Metab Dispos* **41**: 1671-1678, 2013.

Meyer BR. Renal function in aging. *J Am Geriatr Soc* **37**: 791-800, 1989.

Michaud J, Dube P, Naud J, Leblond FA, Desbiens K, Bonnardeaux A, and Pichette V. Effects of serum from patients with chronic renal failure on rat hepatic cytochrome P450. *Br J Pharmacol* **144**: 1067-1077, 2005.

Michaud J, Nolin TD, Naud J, Dani M, Lafrance JP, Leblond FA, Himmelfarb J, and Pichette V. Effect of hemodialysis on hepatic cytochrome P450 functional expression. *J Pharmacol Sci* **108**: 157-163, 2008.

Miller LM, Hopman WM, Garland JS, Yeates KE, and Pilkey RM. Cardioprotective medication use in hemodialysis patients. *Can J Cardiol* **22**: 755-760, 2006.

Miners JO and Birkett DJ. Cytochrome P4502C9: an enzyme of major importance in human drug metabolism. *Br J Clin Pharmacol* **45**: 525-538, 1998.

Miura M, Okuyama S, Kato S, Kagaya H, Murata A, Komatsuda A, Wakui H, and Sawada K. Simultaneous determination of warfarin and 7-hydroxywarfarin enantiomers by high-performance liquid chromatography with ultraviolet detection. *Ther Drug Monit* **33**: 108-114, 2011.

Miura T, Nishinaka T, and Terada T. Different functions between human monomeric carbonyl reductase 3 and carbonyl reductase 1. *Mol Cell Biochem* **315**: 113-121, 2008.

Molnari JC and Myers AL. Carbonyl reduction of bupropion in human liver. *Xenobiotica* **42**: 550-561, 2012.

Momper JD, Venkataramanan R, and Nolin TD. Nonrenal drug clearance in CKD: Searching for the path less traveled. *Adv Chronic Kidney Dis* **17**: 384-391, 2010.

Moreland TA and Hewick DS. Studies on a ketone reductase in human and rat liver and kidney soluble fraction using warfarin as a substrate. *Biochem Pharmacol* **24**: 1953-1957, 1975.

Naud J, Michaud J, Beauchemin S, Hebert MJ, Roger M, Lefrancois S, Leblond FA, and Pichette V. Effects of chronic renal failure on kidney drug transporters and cytochrome P450 in rats. *Drug Metab Dispos* **39**: 1363-1369, 2011.

Naud J, Michaud J, Boisvert C, Desbiens K, Leblond FA, Mitchell A, Jones C, Bonnardeaux A, and Pichette V. Down-regulation of intestinal drug transporters in chronic renal failure in rats. *J Pharmacol Exp Ther* **320**: 978-985, 2007.

Naud J, Michaud J, Leblond FA, Lefrancois S, Bonnardeaux A, and Pichette V. Effects of chronic renal failure on liver drug transporters. *Drug Metab Dispos* **36**: 124-128, 2008.

Naud J, Nolin TD, Leblond FA, and Pichette V. Current understanding of drug disposition in kidney disease. *J Clin Pharmacol* **52**: 10S-22S, 2012.

Ngui JS, Chen Q, Shou M, Wang RW, Stearns RA, Baillie TA, and Tang W. In vitro stimulation of warfarin metabolism by quinidine: increases in the formation of 4'- and 10-hydroxywarfarin. *Drug Metab Dispos* **29**: 877-886, 2001.

Nolin TD, Appiah K, Kendrick SA, Le P, McMonagle E, and Himmelfarb J. Hemodialysis acutely improves hepatic CYP3A4 metabolic activity. *J Am Soc Nephrol* **17**: 2363-2367, 2006.

Nolin TD, Frye RF, Le P, Sadr H, Naud J, Leblond FA, Pichette V, and Himmelfarb J. ESRD impairs nonrenal clearance of fexofenadine but not midazolam. *J Am Soc Nephrol* **20**: 2269-2276, 2009.

Nolin TD, Naud J, Leblond FA, and Pichette V. Emerging evidence of the impact of kidney disease on drug metabolism and transport. *Clin Pharmacol Ther* **83**: 898-903, 2008.

O'Connor T, Ireland LS, Harrison DJ, and Hayes JD. Major differences exist in the function and tissue-specific expression of human aflatoxin B1 aldehyde reductase and the principal human aldo-keto reductase AKR1 family members. *Biochem J* **343 Pt 2**: 487-504, 1999.

Odermatt A and Kratschmar DV. Tissue-specific modulation of mineralocorticoid receptor function by 11beta-hydroxysteroid dehydrogenases: an overview. *Mol Cell Endocrinol* **350**: 168-186, 2012.

Okabe H, Hashimoto Y, and Inui KI. Pharmacokinetics and bioavailability of tacrolimus in rats with experimental renal dysfunction. *J Pharm Pharmacol* **52**: 1467-1472, 2000.

Olesen JB, Lip GY, Kamper AL, Hommel K, Kober L, Lane DA, Lindhardsen J, Gislason GH, and Torp-Pedersen C. Stroke and bleeding in atrial fibrillation with chronic kidney disease. *N Engl J Med* **367**: 625-635, 2012.

Oppermann U. Carbonyl reductases: the complex relationships of mammalian carbonyl- and quinone-reducing enzymes and their role in physiology. *Annu Rev Pharmacol Toxicol* **47**: 293-322, 2007.

Osborne R, Joel S, Grebenik K, Trew D, and Slevin M. The pharmacokinetics of morphine and morphine glucuronides in kidney failure. *Clin Pharmacol Ther* **54**: 158-167, 1993.

Paap CM, Simpson KS, Horton MW, Schaefer KL, Lassman HB, and Sack MR. Multiple-dose pharmacokinetics of pentoxifylline and its metabolites during renal insufficiency. *Ann Pharmacother* **30**: 724-729, 1996.

Patel MR, Mahaffey KW, Garg J, Pan G, Singer DE, Hacke W, Breithardt G, Halperin JL, Hankey GJ, Piccini JP, Becker RC, Nessel CC, Paolini JF, Berkowitz SD, Fox KA, and Califf RM. Rivaroxaban versus warfarin in nonvalvular atrial fibrillation. *N Engl J Med* **365**: 883-891, 2011.

Pawliczak R, Logun C, Madara P, Barb J, Suffredini AF, Munson PJ, Danner RL, and Shelhamer JH. Influence of IFN-gamma on gene expression in normal human bronchial epithelial cells: modulation of IFN-gamma effects by dexamethasone. *Physiol Genomics* **23**: 28-45, 2005.

Persson B, Kallberg Y, Bray JE, Bruford E, Dellaporta SL, Favia AD, Duarte RG, Jornvall H, Kavanagh KL, Kedishvili N, Kisiela M, Maser E, Mindnich R, Orchard S, Penning TM, Thornton JM, Adamski J, and Oppermann U. The SDR (short-chain dehydrogenase/reductase and related enzymes) nomenclature initiative. *Chem Biol Interact* **178**: 94-98, 2009.

Pilka ES, Niesen FH, Lee WH, El-Hawari Y, Dunford JE, Kochan G, Wsol V, Martin HJ, Maser E, and Oppermann U. Structural basis for substrate specificity in human monomeric carbonyl reductases. *PLoS One* **4**: e7113, 2009.

Pinto N and Dolan ME. Clinically relevant genetic variations in drug metabolizing enzymes. *Curr Drug Metab* **12**: 487-497, 2011.

Pisters R, Lane DA, Nieuwlaat R, de Vos CB, Crijns HJ, and Lip GY. A novel user-friendly score (HAS-BLED) to assess 1-year risk of major bleeding in patients with atrial fibrillation: the Euro Heart Survey. *Chest* **138**: 1093-1100, 2010.

Rege B, Krieg R, Gao N, and Sarkar MA. Down-regulation of hepatic CYP3A in chronic renal insufficiency. *Pharm Res* **20**: 1600-1606, 2003.

Reidenberg MM. The binding of drugs to plasma proteins and the interpretation of measurements of plasma concentrations of drugs in patients with poor renal function. *Am J Med* **62**: 466-470, 1977.

Reinecke H, Brand E, Mesters R, Schabitz WR, Fisher M, Pavenstadt H, and Breithardt G. Dilemmas in the management of atrial fibrillation in chronic kidney disease. *J Am Soc Nephrol* **20**: 705-711, 2009.

Reinecke H, Engelbertz C, and Schabitz WR. Preventing stroke in patients with chronic kidney disease and atrial fibrillation: benefit and risks of old and new oral anticoagulants. *Stroke* **44**: 2935-2941, 2013.

Rendic S. Summary of information on human CYP enzymes: human P450 metabolism data. *Drug Metab Rev* **34**: 83-448, 2002.

Rettie AE, Korzekwa KR, Kunze KL, Lawrence RF, Eddy AC, Aoyama T, Gelboin HV, Gonzalez FJ, and Trager WF. Hydroxylation of warfarin by human cDNA-expressed cytochrome P-450: a role for P-4502C9 in the etiology of (S)-warfarin-drug interactions. *Chem Res Toxicol* **5**: 54-59, 1992.

Reyes M and Benet LZ. Effects of uremic toxins on transport and metabolism of different biopharmaceutics drug disposition classification system xenobiotics. *J Pharm Sci* **100**: 3831-3842, 2011.

Rosemond MJ, St John-Williams L, Yamaguchi T, Fujishita T, and Walsh JS. Enzymology of a carbonyl reduction clearance pathway for the HIV integrase inhibitor, S-1360: role of human liver cytosolic aldo-keto reductases. *Chem Biol Interact* **147**: 129-139, 2004.

Rosemond MJ and Walsh JS. Human carbonyl reduction pathways and a strategy for their study in vitro. *Drug Metab Rev* **36**: 335-361, 2004.

Roth M, Obaidat A, and Hagenbuch B. OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *Br J Pharmacol* **165**: 1260-1287, 2012.

Rouan MC, Lecaillon JB, Godbillon J, Menard F, Darragon T, Meyer P, Kourilsky O, Hillion D, Aldigier JC, and Jungers P. The effect of renal impairment on the pharmacokinetics of oxcarbazepine and its metabolites. *Eur J Clin Pharmacol* **47**: 161-167, 1994.

Routledge PA, Chapman PH, Davies DM, and Rawlins MD. Pharmacokinetics and pharmacodynamics of warfarin at steady state. *Br J Clin Pharmacol* **8**: 243-247, 1979.

Rowland A, Miners JO, and Mackenzie PI. The UDP-glucuronosyltransferases: their role in drug metabolism and detoxification. *Int J Biochem Cell Biol* **45**: 1121-1132, 2013.

Sakaan SA, Hudson JQ, Oliphant CS, Tolley EA, Cummings C, Alabdan NA, and Self TH. Evaluation of Warfarin Dose Requirements in Patients with Chronic Kidney Disease and End-Stage Renal Disease. *Pharmacotherapy* **34**: 695-702, 2014.

Schieber A, Frank RW, and Ghisla S. Purification and properties of prostaglandin 9-ketoreductase from pig and human kidney. Identity with human carbonyl reductase. *Eur J Biochem* **206**: 491-502, 1992.

Schiffrin EL, Lipman ML, and Mann JF. Chronic kidney disease: effects on the cardiovascular system. *Circulation* **116**: 85-97, 2007.

Schwartz JB, Kane L, Moore K, and Wu AH. Failure of pharmacogenetic-based dosing algorithms to identify older patients requiring low daily doses of warfarin. *J Am Med Dir Assoc* **12**: 633-638, 2011.

Shah M, Avgil Tsadok M, Jackevicius CA, Essebag V, Eisenberg MJ, Rahme E, Humphries KH, Tu JV, Behlouli H, Guo H, and Pilote L. Warfarin use and the risk for stroke and bleeding in patients with atrial fibrillation undergoing dialysis. *Circulation* **129**: 1196-1203, 2014.

Shameem R and Ansell J. Disadvantages of VKA and requirements for novel anticoagulants. *Best Pract Res Clin Haematol* **26**: 103-114, 2013.

Sheweita SA. Drug-metabolizing enzymes: mechanisms and functions. *Curr Drug Metab* **1**: 107-132, 2000.

Siegel D, Gibson NW, Preusch PC, and Ross D. Metabolism of mitomycin C by DT-diaphorase: role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res* **50**: 7483-7489, 1990.

Siegel D and Ross D. Immunodetection of NAD(P)H:quinone oxidoreductase 1 (NQO1) in human tissues. *Free Radic Biol Med* **29**: 246-253, 2000.

Siegel D, Yan C, and Ross D. NAD(P)H:quinone oxidoreductase 1 (NQO1) in the sensitivity and resistance to antitumor quinones. *Biochem Pharmacol* **83**: 1033-1040, 2012.

Simard E, Naud J, Michaud J, Leblond FA, Bonnardeaux A, Guillemette C, Sim E, and Pichette V. Downregulation of hepatic acetylation of drugs in chronic renal failure. *J Am Soc Nephrol* **19**: 1352-1359, 2008.

Singlas E, Pioger JC, Taburet AM, Colin JN, and Fillastre JP. Zidovudine disposition in patients with severe renal impairment: influence of hemodialysis. *Clin Pharmacol Ther* **46**: 190-197, 1989.

Sjoholm I, Kober A, Odar-Cederlof I, and Borgaa O. Protein binding of drugs in uremic and normal serum: the role of endogenous binding inhibitors. *Biochem Pharmacol* **25**: 1205-1213, 1976.

Skarka A, Skarydova L, Stambergova H, and Wsol V. Anthracyclines and their metabolism in human liver microsomes and the participation of the new microsomal carbonyl reductase. *Chem Biol Interact* **191**: 66-74, 2011.

Skarydova L, Nobilis M, and Wsol V. Role of carbonyl reducing enzymes in the phase I biotransformation of the non-steroidal anti-inflammatory drug nabumetone in vitro. *Xenobiotica* **43**: 346-354, 2013.

Skarydova L, Tomanova R, Havlikova L, Stambergova H, Solich P, and Wsol V. Deeper insight into the reducing biotransformation of bupropion in the human liver. *Drug Metab Pharmacokinet* **29**: 177-184, 2014.

Skarydova L and Wsol V. Human microsomal carbonyl reducing enzymes in the metabolism of xenobiotics: well-known and promising members of the SDR superfamily. *Drug Metab Rev* **44**: 173-191, 2012.

Skarydova L, Zverinova M, Stambergova H, and Wsol V. A simple identification of novel carbonyl reducing enzymes in the metabolism of the tobacco specific carcinogen NNK. *Drug Metab Lett* **6**: 174-181, 2012.

Sood MM, Komenda P, Sood AR, Rigatto C, and Bueti J. The intersection of risk and benefit: is warfarin anticoagulation suitable for atrial fibrillation in patients on hemodialysis? *Chest* **136**: 1128-1133, 2009.

Stafanger G, Larsen HW, Hansen H, and Sorensen. Pharmacokinetics of ketoprofen in patients with chronic renal failure. *Scand J Rheumatol* **10**: 189-192, 1981.

Stergiopoulos K and Brown DL. Genotype-guided vs clinical dosing of warfarin and its analogues: meta-analysis of randomized clinical trials. *JAMA Intern Med* **174**: 1330-1338, 2014.

Sun H, Frassetto LA, Huang Y, and Benet LZ. Hepatic clearance, but not gut availability, of erythromycin is altered in patients with end-stage renal disease. *Clin Pharmacol Ther* **87**: 465-472, 2010.

Sun H, Huang Y, Frassetto L, and Benet LZ. Effects of uremic toxins on hepatic uptake and metabolism of erythromycin. *Drug Metab Dispos* **32**: 1239-1246, 2004.

Takahashi H, Kashima T, Kimura S, Muramoto N, Nakahata H, Kubo S, Shimoyama Y, Kajiwara M, and Echizen H. Determination of unbound warfarin enantiomers in human plasma and 7-hydroxywarfarin in human urine by chiral stationary-phase liquid chromatography with ultraviolet or fluorescence and on-line circular dichroism detection. *J Chromatogr B Biomed Sci Appl* **701**: 71-80, 1997.

Thomas R, Kanso A, and Sedor JR. Chronic kidney disease and its complications. *Prim Care* **35**: 329-344, vii, 2008.

Tong Z, Chandrasekaran A, Li H, Rotshteyn Y, Erve JC, Demaio W, Talaat R, Hultin T, and Scatina J. In vitro metabolism and identification of human enzymes involved in the metabolism of methylnaltrexone. *Drug Metab Dispos* **38**: 801-807, 2010.

Trager WF, Lewis RJ, and Garland WA. Mass spectral analysis in the identification of human metabolites of warfarin. *J Med Chem* **13**: 1196-1204, 1970.

Treitel M, Marbury T, Preston RA, Triantafyllou I, Feely W, O'Mara E, Kasserra C, Gupta S, and Hughes EA. Single-dose pharmacokinetics of boceprevir in subjects with impaired hepatic or renal function. *Clin Pharmacokinet* **51**: 619-628, 2012.

Tsujimoto M, Hatozaki D, Shima D, Yokota H, Furukubo T, Izumi S, Yamakawa T, Minegaki T, and Nishiguchi K. Influence of serum in hemodialysis patients on the expression of intestinal and hepatic transporters for the excretion of pravastatin. *Ther Apher Dial* **16**: 580-587, 2012.

Tsujimoto M, Nagano Y, Hosoda S, Shiraishi A, Miyoshi A, Hiraoka S, Furukubo T, Izumi S, Yamakawa T, Minegaki T, and Nishiguchi K. Effects of decreased vitamin D and accumulated uremic toxin on human CYP3A4 activity in patients with end-stage renal disease. *Toxins (Basel)* **5**: 1475-1485, 2013.

Tukey RH and Strassburg CP. Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annu Rev Pharmacol Toxicol* **40**: 581-616, 2000.

Turpeinen M, Koivuviita N, Tolonen A, Reponen P, Lundgren S, Miettunen J, Metsarinne K, Rane A, Pelkonen O, and Laine K. Effect of renal impairment on the pharmacokinetics of bupropion and its metabolites. *Br J Clin Pharmacol* **64**: 165-173, 2007.

Uchida N, Kurata N, Shimada K, Nishimura Y, Yasuda K, Hashimoto M, Uchida E, and Yasuhara H. Changes of hepatic microsomal oxidative drug metabolizing enzymes in chronic renal failure (CRF) rats by partial nephrectomy. *Jpn J Pharmacol* **68**: 431-439, 1995.

Uno T, Niioka T, Hayakari M, Sugawara K, and Tateishi T. Simultaneous determination of warfarin enantiomers and its metabolite in human plasma by column-switching high-performance liquid chromatography with chiral separation. *Ther Drug Monit* **29**: 333-339, 2007.

Usami N, Kitahara K, Ishikura S, Nagano M, Sakai S, and Hara A. Characterization of a major form of human isatin reductase and the reduced metabolite. *Eur J Biochem* **268**: 5755-5763, 2001.

van der Hoeven TA and Coon MJ. Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from rabbit liver microsomes. *J Biol Chem* **249**: 6302-6310, 1974.

Vanholder R, De Smet R, and Ringoir S. Factors influencing drug protein binding in patients with end stage renal failure. *Eur J Clin Pharmacol* **44 Suppl 1**: S17-21, 1993.

Vanholder R, Van Landschoot N, De Smet R, Schoots A, and Ringoir S. Drug protein binding in chronic renal failure: evaluation of nine drugs. *Kidney Int* **33**: 996-1004, 1988.

Vazquez E, Sanchez-Perales C, Garcia-Garcia F, Castellano P, Garcia-Cortes MJ, Liebana A, and Lozano C. Atrial fibrillation in incident dialysis patients. *Kidney Int* **76**: 324-330, 2009.

Vazquez E, Sanchez-Perales C, Lozano C, Garcia-Cortes MJ, Borrego F, Guzman M, Perez P, Pagola C, Borrego MJ, and Perez V. Comparison of prognostic value of atrial fibrillation versus sinus rhythm in patients on long-term hemodialysis. *Am J Cardiol* **92**: 868-871, 2003.

Veau C, Leroy C, Banide H, Auchere D, Tardivel S, Farinotti R, and Lacour B. Effect of chronic renal failure on the expression and function of rat intestinal P-glycoprotein in drug excretion. *Nephrol Dial Transplant* **16**: 1607-1614, 2001.

Velenosi TJ, Feere DA, Sohi G, Hardy DB, and Urquhart BL. Decreased nuclear receptor activity and epigenetic modulation associates with down-regulation of hepatic drug-metabolizing enzymes in chronic kidney disease. *FASEB J*, 2014.

Velenosi TJ, Fu AY, Luo S, Wang H, and Urquhart BL. Down-regulation of hepatic CYP3A and CYP2C mediated metabolism in rats with moderate chronic kidney disease. *Drug Metab Dispos* **40**: 1508-1514, 2012.

Vogel C and Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet* **13**: 227-232, 2012.

Volpe DA, Tobin GA, Tavakkoli F, Dowling TC, Light PD, and Parker RJ. Effect of uremic serum and uremic toxins on drug metabolism in human microsomes. *Regul Toxicol Pharmacol* **68**: 297-303, 2014.

Voon PJ, Yap HL, Ma CY, Lu F, Wong AL, Sapari NS, Soong R, Soh TI, Goh BC, Lee HS, and Lee SC. Correlation of aldo-ketoreductase (AKR) 1C3 genetic variant with doxorubicin pharmacodynamics in Asian breast cancer patients. *Br J Clin Pharmacol* **75**: 1497-1505, 2013.

Wadelius M, Chen LY, Eriksson N, Bumpstead S, Ghori J, Wadelius C, Bentley D, McGinnis R, and Deloukas P. Association of warfarin dose with genes involved in its action and metabolism. *Hum Genet* **121**: 23-34, 2007.

Wang X, Hou J, Jann M, Hon YY, and Shamsi SA. Development of a chiral micellar electrokinetic chromatography-tandem mass spectrometry assay for simultaneous analysis of warfarin and hydroxywarfarin metabolites: application to the analysis of patients serum samples. *J Chromatogr A* **1271**: 207-216, 2013.

Wattanakit K and Cushman M. Chronic kidney disease and venous thromboembolism: epidemiology and mechanisms. *Curr Opin Pulm Med* **15**: 408-412, 2009.

Weiner DE. Public health consequences of chronic kidney disease. *Clin Pharmacol Ther* **86**: 566-569, 2009.

Wetmore JB, Ellerbeck EF, Mahnken JD, Phadnis M, Rigler SK, Mukhopadhyay P, Spertus JA, Zhou X, Hou Q, and Shireman TI. Atrial fibrillation and risk of stroke in dialysis patients. *Ann Epidemiol* **23**: 112-118, 2013.

White PJ. Patient factors that influence warfarin dose response. *J Pharm Pract* **23**: 194-204, 2010.

Wienkers LC, Wurden CJ, Storch E, Kunze KL, Rettie AE, and Trager WF. Formation of (R)-8-hydroxywarfarin in human liver microsomes. A new metabolic marker for the (S)-mephenytoin hydroxylase, P4502C19. *Drug Metab Dispos* **24**: 610-614, 1996.

Winkelmayer WC, Liu J, Setoguchi S, and Choudhry NK. Effectiveness and safety of warfarin initiation in older hemodialysis patients with incident atrial fibrillation. *Clin J Am Soc Nephrol* **6**: 2662-2668, 2011a.

Winkelmayer WC, Patrick AR, Liu J, Brookhart MA, and Setoguchi S. The increasing prevalence of atrial fibrillation among hemodialysis patients. *J Am Soc Nephrol* **22**: 349-357, 2011b.

Winkelmayer WC and Turakhia MP. Warfarin treatment in patients with atrial fibrillation and advanced chronic kidney disease: sins of omission or commission? *JAMA* **311**: 913-915, 2014.

Wong PC, Crain EJ, Watson CA, and Schumacher WA. A small-molecule factor XIa inhibitor produces antithrombotic efficacy with minimal bleeding time prolongation in rabbits. *J Thromb Thrombolysis* **32**: 129-137, 2011.

Wong PC, Crain EJ, Xin B, Wexler RR, Lam PY, Pinto DJ, Luettgen JM, and Knabb RM. Apixaban, an oral, direct and highly selective factor Xa inhibitor: in vitro, antithrombotic and antihemostatic studies. *J Thromb Haemost* **6**: 820-829, 2008.

Wong YW and Davis PJ. Analysis of warfarin and its metabolites by reversed-phase ion-pair liquid chromatography with fluorescence detection. *J Chromatogr A* **469**: 281-291, 1989.

Xu C, Li CY, and Kong AN. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res* **28**: 249-268, 2005.

Yamaguchi K, Inoue S, Ohara O, and Nagase T. Pulse-chase experiment for the analysis of protein stability in cultured mammalian cells by covalent fluorescent labeling of fusion proteins. *Methods Mol Biol* **577**: 121-131, 2009.

Yang F, Chou D, Schweitzer P, and Hanon S. Warfarin in haemodialysis patients with atrial fibrillation: what benefit? *Europace* **12**: 1666-1672, 2010.

Yeung CK, Shen DD, Thummel KE, and Himmelfarb J. Effects of chronic kidney disease and uremia on hepatic drug metabolism and transport. *Kidney Int* **85**: 522-528, 2014.

Yoo HH, Kim NS, Kim MJ, Shin D, Shin JG, and Kim DH. Enantioselective carbonyl reduction of eperisone in human liver microsomes. *Xenobiotica* **41**: 758-763, 2011.

Yoshida H, Goto M, Honda A, Nabeshima T, Kumazawa T, Inagaki J, Yamanaka N, and Ota K. Pharmacokinetics of doxorubicin and its active metabolite in patients with normal renal function and in patients on hemodialysis. *Cancer Chemother Pharmacol* **33**: 450-454, 1994.

Yu C, Ritter JK, Krieg RJ, Rege B, Karnes TH, and Sarkar MA. Effect of chronic renal insufficiency on hepatic and renal udp-glucuronyltransferases in rats. *Drug Metab Dispos* **34**: 621-627, 2006.

Zanger UM and Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther* **138**: 103-141, 2013.

Zhang M, Lv XY, Li J, Xu ZG, and Chen L. Alteration of 11beta-hydroxysteroid dehydrogenase type 1 in skeletal muscle in a rat model of type 2 diabetes. *Mol Cell Biochem* **324**: 147-155, 2009.

Zhang QL and Rothenbacher D. Prevalence of chronic kidney disease in population-based studies: systematic review. *BMC Public Health* **8**: 117, 2008.

Zhang Z, Fasco MJ, Huang Z, Guengerich FP, and Kaminsky LS. Human cytochromes P4501A1 and P4501A2: R-warfarin metabolism as a probe. *Drug Metab Dispos* **23**: 1339-1346, 1995.

Zhou Q, Yau WP, and Chan E. Enantioseparation of warfarin and its metabolites by capillary zone electrophoresis. *Electrophoresis* **24**: 2617-2626, 2003.

Zielinska A, Lichti CF, Bratton S, Mitchell NC, Gallus-Zawada A, Le VH, Finel M, Miller GP, Radominska-Pandya A, and Moran JH. Glucuronidation of monohydroxylated warfarin metabolites by human liver microsomes and human recombinant UDP-glucuronosyltransferases. *J Pharmacol Exp Ther* **324**: 139-148, 2008.

Zuo Z, Wo SK, Lo CM, Zhou L, Cheng G, and You JH. Simultaneous measurement of S-warfarin, R-warfarin, S-7-hydroxywarfarin and R-7-hydroxywarfarin in human plasma by liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal* **52**: 305-310, 2010.