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EXPRESSION AND ACTIVITY OF CYP2C8 AND 2C9 IN DIABETES MELLITUS AND NONALCOHOLIC FATTY LIVER DISEASE

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EXPRESSION AND ACTIVITY OF CYP2C8 AND 2C9 IN DIABETES MELLITUS AND NONALCOHOLIC FATTY LIVER DISEASE

BY

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ABSTRACT

Background and Objectives:

Diabetes mellitus and Non-Alcoholic Fatty Liver Disease (NAFLD) are two highly prevalent and related diseases. Research have shown that they can affect the expression and activity of enzymes integral in the clearance of xenobiotics. Such enzymes include the Cytochrome P-450 isoforms 2C8 and 2C9. Our objective is to study the effect of these diseases on CYP2C8 and CYP2C9 using in vitro tools.

Methods:

A bank of donated livers was utilized for in vitro studies. Hepatic microsomal incubations of S-warfarin, a (CYP2C9) probe substrate, were performed to measure CYP2C9 activity by product formation and S-warfarin's intrinsic clearance by in vitro half-life approach. Additionally, previously acquired data in our lab on mRNA and protein levels of CYP2C8 and CYP2C9 have been analyzed and compared among diabetic and NAFLD groups.

Results and Conclusions:

Analysis of CYP2C8 mRNA and protein expression in addition to CYP2C9 mRNA expression, activity, and warfarin's intrinsic clearance showed no significant alteration by NAFLD nor diabetes. CYP2C9 protein expression significantly varied between different diabetic and NAFLD populations ($p = 0.047$).

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TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

LIST OF ABBREVIATIONS

AUC: Area Under the Concentration-Time Curve Until the Last Quantifiable Value

CAR: Constitutive Androstane Receptor

CL: Clearance

Cmax: Maximum Observed Plasma Concentration

CYP: Cytochrome P450 Enzyme System

DMEs: Drug Metabolizing Enzymes

EETs: Epoxyeicosatrienoic Acids

g: grams

HLM: Human Liver Microsomes

IS: Internal Standard

Ke: Elimination Rate Constant

kg: kilograms

LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry

min: minutes

mRNA: Messenger Ribonucleic Acid

n: number of samples

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NAFL: Non-Alcoholic Fatty Liver

NAFLD: Non-Alcoholic Fatty Liver Disease

NASH: Non-Alcoholic Steatohepatitis

NSAID: Non-Steroidal Anti-Inflammatory Drug

PBPK: Physiologically-Based Pharmacokinetics

PK: Pharmacokinetics

PXR: Pregnane X Receptor

SD: Standard Deviation

T ½: Elimination Half-Life

T2DM: Type 2 Diabetes Mellitus

Tb: Tuberculosis

Tmax: Time to Reach Maximum Concentration (Cmax)

UGT: Uridine 5'-diphospho-glucuronosyltransferase enzyme system

Vd: Volume of Distribution

v: volume

Chapter 1

Literature Review

Pathological conditions can produce changes in the expression and activity of drug metabolizing enzymes (DMEs) causing altered pharmacokinetic (PK) profiles of their substrate drugs (Sane & Sinz, 2017). If an alteration is clinically significant, individualized pharmacotherapy for the specific patient population is needed to ensure adequate treatment and avoid adverse drug reactions. Therefore, it is crucial to perform additional studies on DME expression and activity in different disease populations.

1.1. Phases of Metabolism

Hepatic metabolism is broadly classified into two phases which act to increase a chemical's hydrophilicity facilitating its excretion (Cederbaum, 2015). Phase I involves oxidation, reduction, and hydrolysis reactions. Multiple enzyme families catalyze this phase. The most notable of which is the cytochrome P450 monooxygenation family (CYP). CYPs oxidize substrates by introducing one oxygen atom and utilizing nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for the reaction. Alternatively, phase II is a conjugation phase where polar endogenous cofactors are conjugated with the substrate. An example of a phase II catalyzer is the uridine 5'-diphospho-glucuronosyltransferase family (UGT) that conjugate their substrates with glucuronic acid. Endogenous and exogenous chemicals do not necessarily undergo both metabolic phases to be cleared. Some would undergo phase I or phase II only. Others

would undergo both. Compounds hydrophilic enough to be excreted from the body unchanged do not undergo metabolism at all.

1.2. CYP2C Subfamily

CYP2C is a subfamily of cytochrome P450 enzymes that has four isoforms in humans: CYP2C8, CYP2C9, CYP2C18, and CYP2C19 (Goldstein, 2001). Members of this subfamily are involved in the metabolism of endogenous compounds such as arachidonic acid in addition to xenobiotics.

Arachidonic acid is an endogenous fatty acid that is found in cellular membranes. It is metabolized to active products via three enzymatic systems one of which is the CYP system. Studies by Rifkind, Lee, Chang, & Waxman (1995) have shown that both CYP2C8 and CYP2C9 play an important role in arachidonic acid metabolism through the CYP epoxygenase pathway. The resulting metabolites, called epoxyeicosatrienoic acids (EETs), are involved in numerous biological activities such as vascular smooth muscle homeostasis, endothelial calcium signaling, and regulation of cardiac muscles' ion channels (Spector, Fang, Snyder, & Weintraub, 2004).

CYP2C8 is a CYP isoform that is involved in the hepatic clearance of nearly 5% of prescribed medications (Naraharisetti et al., 2010). The importance of this enzyme in drug development studies has not been recognized until the recent years (Backman, Filppula, Niemi, & Neuvonen, 2016). As a result, research on CYP2C8 phenotypes at Messenger Ribonucleic Acid (mRNA) level, protein expression, and enzyme activity, with respect to disease states is lacking. Since CYP2C8 is a major metabolic pathway for

several antidiabetic drugs, it is important to study its expression and activity in this population. For example, CYP2C8 is the primary metabolizing enzyme for the thiazolidinedione insulin sensitizers, rosiglitazone (Baldwin, Clarke, & Chenery, 1999) and pioglitazone (Jaakkola, Laitila, Neuvonen, & Backman, 2006), and the meglitinide insulin secretagogue, repaglinide (Bidstrup, Bjornsdottir, Sidelmann, Thomsen, & Hansen, 2003). In addition, CYP2C8 is involved in the metabolism of other antidiabetics as a minor pathway.

CYP2C9 is another enzyme that has an essential role in xenobiotic metabolism. It is the second most abundant CYP enzyme in the human liver, after CYP3A4, and is involved in phase I biotransformation of about 1/5 of all drugs. One of the widely prescribed CYP2C9 substrates is warfarin (Van Booven et al., 2010).

1.3. Effect of Diabetes Mellitus

According to the World Health Organization (WHO), the worldwide prevalence of diabetes has substantially increased to 8.5% of the population in 2014 (World Health Organization, 2017). A considerable number of patients with type 2 diabetes mellitus (T2DM) may have compromised hepatic function and other metabolic disorders. Although multiple research groups have studied the effects of T2DM on drug metabolism, the knowledge in this field is still limited to certain enzymes. For example, the in vivo activity of CYP2E1 is increased in obese T2DM patients using chlorzoxazone as a probe substrate (Lucas et al., 1998; Wang et al., 2003). CYP3A4 appears to be altered but there are conflicting results on the direction of its change (Dostalek, Court,

Yan, & Akhlaghi, 2011; Hu et al., 2014; Patoine et al., 2014). This conflict could be a result of species differences, variations in disease length, level of disease control and other factors.

To the best of our knowledge, there are no studies on the expression and activity of CYP2C8 and CYP2C9 in diabetic populations. However, there are studies showing altered pharmacokinetic profiles of clinically used drugs in diabetic states. Upon conducting a comprehensive review on PK in diabetes, thirty-five reports were found in the literature between January 1st, 2012 and September 4th, 2018 (Table 1). Reports prior to 2012 have been reviewed elsewhere (Dostalek, Akhlaghi, & Puzanovova, 2012). Several studies included drugs that are well known CYP2C8 or CYP2C9 substrates. For example, the non-steroidal anti-inflammatory drug (NSAID) diclofenac had altered PK parameters in diabetic animals as compared to non-diabetic controls (Ahmad, Iqbal, & Murtaza, 2012; Y. Li, Wei, Zhang, Wang, & Wu, 2012). An animal study published in 2016 by Zhou and colleagues, comparing diabetic to non-diabetic rats, reported five different drugs including glibenclamide. Glibenclamide (metabolized by CYP2C9 and CYP3A4) had alterations in all PK parameters measured: The area under the concentration-time curve until the last quantifiable value (AUC), clearance (CL), volume of distribution (Vd), and elimination half-life (t ½). Glibenclamide has also been reported to have different PK in diabetic rats by Y. Li, Wei, Zhang, Wang, and Wu (2012). The study included two routes of administration (oral and intravenous) and the PK differed under diabetic conditions for both routes. It is important to note however that not all substrates of CYP2C8 and 2C9 showed altered PK parameters in diabetics compared to non-diabetic groups. For

instance, the PK of rosiglitazone (Zhou et al., 2016) and losartan (Li et al., 2017) did not substantially differ between the groups. Involvement of alternative metabolic pathways and changes in absorption and/or distribution could be contributing to these mixed results.

Table 1. Influence of Diabetes Mellitus on Pharmacokinetics (Studies reported between 1/1/2012 – 9/4/2018)

Drug	Species	Major DMEs	PK parameters	Non-diabetic	Diabetic	p-value	Reference
Acetaminophen	Rabbit	UGTs and SULTs		(Mean ± SD)	(Mean ± SD)		Bienert et al. 2012
			ke (1/h)	0.55 ± 0.10	0.43 ± 0.15	0.07	
			t ½ (h)	1.31 ± 0.24	1.76 ± 0.49	0.03*	
			CL (L/h)	3.52 ± 0.54	4.99 ± 0.89	0.0008*	
			Vd (L/kg)	1.70 ± 0.49	3.83 ± 0.87	< 0.0001*	
			AUC (mg x h/L)	40.35 ± 7.18	23.11 ± 3.98	< 0.001*	
			Cmax (mg/L)	67.04 ± 9.11	50.96 ± 6.32	0.0008*	
			Tmax (h)	0.083 ± 0.00	0.083 ± 0.00	–	
			MRT (h)	0.89 ± 0.13	0.95 ± 0.28	0.65	
Caffeine	Mouse	Cyp1a2 (CYP1A2 in humans)		(Mean ± SEM)	(Mean ± SEM)		Li et al. 2017
			Cmax (µg/mL)	2.0 ± 1.1	4.9 ± 0.9	S	
			AUC (min*µg/mL)	491.1 ± 91.3	1418.0 ± 341.8	S	
			t ½ (min)	158 ± 71	342 ± 236	NS	
Canagliflozin	Rat	Oxidation and glucuronid-ation (humans: UGT1A9 and UGT2B4)		(Mean ± SD)	(Mean ± SD)		Zhou et al. 2016
			AUC (µM*h)	5.4 ± 0.6	8.6 ± 1.6	< 0.05*	
			AUCinf (µM*h)	5.8 ± 0.7	9.7 ± 1.8	< 0.05*	
			CL (mL/min per kg)	6.5 ± 0.8	4.0 ± 0.8	< 0.01*	
			Vdss (L/kg)				
			t ½ (h)	3.4 ± 0.3	2.4 ± 0.3	< 0.01*	
				6.2 ± 0.6	7.6 ± 0.5	< 0.01*	
Clopidogrel	Human	Activation: CYPs 3A4, 3A5, 2C19, 2C9, 2B6, 1A2 Clearance: Hydrolysis		(Mean ± SD)	(Mean ± SD)		Karaźniewi cz-Łada et al. 2014
			Cmax (ng/mL)	1.82 ± 1.86	2.34 ± 2.29	NS	
			Tmax (h)	1.39 ± 1.26	1.40 ± 0.79	NS	
			t ½ (h)	2.05 ± 1.54	1.33 ± 0.81	NS	
			AUC (ng x h/mL)	4.95 ± 3.70	5.01 ± 4.11	NS	
			AUCinf (ng x h/mL)	6.33 ± 4.32	6.07 ± 4.12	NS	

		and glucuronid-ation					
Clopidogrel	Human	Activation: CYPs 3A4, 3A5, 2C19, 2C9, 2B6, 1A2 Clearance: Hydrolysis and glucuronid-ation	Plasma active metabolite level (ng/mL) after 0.5 hour of the loading dose Plasma active metabolite level (ng/mL) after 3 hours of the loading dose	--- ---	--- ---	0.34 0.22	Niijima et al. 2018
Dexamethasone acetate (topical)	Rat	CYP3A	AUC (ng/m) Cmax (ng/mL) Tmax (h)	(Mean ± SD) 299.90 ± 93.73 61.76 ± 16.13 11.5 ± 0	(Mean ± SD) 319.88 ± 129.01 62.09 ± 10.51 11.5 ± 0	NS NS NS	Li et al. 2014
Dexamethasone sodium phosphate (topical)	Rat	CYP3A	AUC (ng/m) Cmax (ng/mL) Tmax (h)	(Mean ± SD) 164080 ± 68990 29330 ± 6410 11.5 ± 0	(Mean ± SD) 921880 ± 267750 131330 ± 41430 11.5 ± 0	< 0.01* < 0.01* NS	Li et al. 2014
Dextromethorphan	Mouse	Cyp2d22 (CYP2D6 in humans)	Cmax (µg/mL) AUC (min*µg/mL) t ½ (min)	(Mean ± SEM) 0.08 ± 0.03 19.1 ± 7.8 123 ± 30	(Mean ± SEM) 0.17 ± 0.10 37.0 ± 23.3 120 ± 35	NS NS NS	Li et al. 2017
Diclofenac	Rabbit	UGT2B7, CYP2C9, CYP3A4, CYP2C8	<u>Diclofenac sodium</u> AUCinf (µg.h/mL) Tmax (h) C max (µg/ml)	(Mean ± SEM) 37.4 ± 0.21 1.67 ± 1.50	(Mean ± SEM) 49.95 ± 0.22 2.15 ± 1.17	 < 0.05* NS	Ahmad et al. 2012

			Ka (1/h) MAT (1/h) t ½α (h) MRT (h) Vd (L/Kg) VSS (L/Kg) Ke (1/h) t ½ (h) CL (ml/h/Kg) <u>Diclofenac potassium</u> AUCinf (µg.h/mL) Tmax (h) C max (µg/ml) Ka (1/h) MAT (1/h) t ½α (h) MRT (h) Vd (L/Kg) VSS (L/Kg) Ke (1/h) t ½ (h) CL (ml/h/Kg)	13.64 ± 0.38 0.50 ± 2.48 2.18 ± 1.27 1.51 ± 1.52 2.68 ± 1.45 4.71 ± 0.63 0.68 ± 1.8 0.39 ± 3.11 1.86 ± 1.74 1.77 ± 1.09 80.73 ± 0.16 1.71 ± 1.97 26.83 ± 0.31 0.86 ± 2.49 1.2 ± 2.07 0.83 ± 2.49 2.91 ± 1.15 2.16 ± 0.995 0.29 ± 2.95 0.63 ± 3.69 2.02 ± 1.38 0.72 ± 2.02	12.84 ± 0.41 0.43 ± 1.96 3.91 ± 0.52 2.71 ± 0.62 2.92 ± 1.65 3.41 ± 0.85 0.4 ± 2.56 0.34 ± 5.07 2.06 ± 1.98 1.16 ± 1.51 50.73 ± 0.19 1.85 ± 1.38 18.13 ± 0.42 1.07 ± 4.46 0.94 ± 4.92 0.65 ± 5.92 2.99 ± 1.41 3.68 ± 0.72 0.47 ± 1.71 0.39 ± 4.16 2.07 ± 1.68 1.35 ± 1.11	S S S S NS NS NS < 0.05* NS NS S NS S S S S NS NS NS < 0.05* NS < 0.05*	
Diclofenac	Rat	CYP2C9 ortholog	t ½ (min) CL (x 10^3, L/min/kg) AUC (mg x min/L)	(Mean ± SD) 170.08 ± 24.73 11.29 ± 1.08 684.60 ± 63.47	(Mean ± SD) 288.93 ± 46.33 4.67 ± 0.58 1468.45 ± 45.06	< 0.05* < 0.05* < 0.05*	Li et al. 2012
Docetaxel	Rat	CYP3A (humans: CYP3A4)	(IV) AUCinf (µg.min/mL)	(Mean ± SD) 225 ± 44	(Mean ± SD) 206 ± 30	 NS	Lee et al. 2012

			t ½ (min) CL (mL/min/Kg) VSS (mL/Kg) (Oral) AUCinf (µg.min/mL) C max (µg/ml) Tmax (min) t ½ (min)	173 ± 24 23 ± 4 650 ± 130 18.5 ± 4.3 0.0760 ± 0.03 145 ± 101 156 ± 96	132 ± 43 25 ± 3 290 ± 130 25.2 ± 15.7 0.101 ± 0.06 138 ± 95 123 ± 34	< 0.05* NS < 0.01* NS NS NS NS	
Ertugliflozin	Human	UGT1A9 and UGT2B7	 AUC (ng * h/mL) CL/F (mL/min) Cmax (ng/mL) V/F (L) CLR (mL/min) Tmax (h) t ½ (h)	Geometric mean (%CV) 1236 (27) 202 (27) 219 (26) 305 (39) 1.68 (33) Median (range) 1.00 (1.00-2.00) (Mean ± SD) 17.7 ± 3.5	Geometric mean (%CV) 1199 (42) 209 (42) 216 (35) 240 (53) 2.09 (28) Median (range) 1.00 (1.00-1.50) (Mean ± SD) 14.6 ± 6.4	 NA NA NA NA NA NA NA	Sahasrabudhe et al. 2017
Ethambutol	Human	Mainly excreted unchanged in urine and feces	 Plasma concentration after 14 days (µg/mL) Plasma concentration after 30 days (µg/mL)	(Mean ± SEM) 2.81 ± 0.30 3.6 ± 0.3	(Mean ± SEM) 3.70 ± 0.64 4.3 ± 0.7	 NS NS	Babalik et al. 2013

Glibenclamide (Glyburide)	Rat	CYP2C9 and CYP3A4 orthologs	AUC (µM*h) AUCinf (µM*h) CL (mL/min per kg) Vdss (L/kg) t ½ (h)	(Mean ± SD) 5.1 ± 1.5 5.3 ± 1.6 6.8 ± 1.6 2.4 ± 0.2 4.8 ± 0.8	(Mean ± SD) 10 ± 1.5 24 ± 6.3 1.5 ± 0.5 3.6 ± 0.5 31 ± 9	 < 0.01* < 0.01* < 0.01* < 0.01* < 0.01*	Zhou et al. 2016
Glibenclamide (Glyburide)	Rat	CYP2C9 and CYP3A4 orthologs	(Oral) Tmax (min) Cmax (µg/mL) CL (L/min/kg) AUC (mg*min/L) (IV) t ½ (min) CL (x 10^3, L/min/kg) AUC (mg*min/L)	(Mean ± SD) 84.78 ± 15.96 0.26 ± 0.03 0.09 ± 0.01 57.75 ± 18.93 225.47 ± 25.34 7.61 ± 1.53 509.52 ± 56.14	(Mean ± SD) 255.43 ± 23.8 0.91 ± 0.14 0.02 ± 0.01 321.24 ± 130.37 560.90 ± 166.22 2.67 ± 0.70 1528.28 ± 214.49	 < 0.05* < 0.05* < 0.05* < 0.05* < 0.05* < 0.05* < 0.05*	Li et al. 2012
Glibenclamide (Glyburide)	Rat	CYP2C9 and CYP3A4 orthologs	Cmax (µg/mL) Tmax (h) AUC (µg/mL h) AUCinf (µg/mL h) t ½ (h) MRT (h) CL (mL/min) Vd (mL)	(Mean ± SD) 29.4 ± 0.7 2 109.3 ± 3.3 118.2 ± 3.5 1.67 ± 0.1 4.11 ± 0.1 82.1 ± 1.4 233.5 ± 7.5	(Mean ± SD) 68.0 ± 1.5 2 275.2 ± 6.2 305.2 ± 8.9 1.87 ± 0.1 4.34 ± 0.1 32.4 ± 1.1 87.1 ± 4.5	NA NA NA NA NA NA NA NA	Samala et al. 2016
Glimepiride	Rat	CYP2C9 ortholog (Humans: CYP2C9)	Cmax (µg/mL) Tmax (h) AUC (µg/mL h) AUCinf (µg/mL h) t ½ (h) MRT (h)	(Mean ± SD) 18.7 ± 0.8 4 161.7 ± 4.4 170.5 ± 6.7 5.6 ± 1.0 10.7 ± 0.9	(Mean ± SD) 21.4 ± 1.3 4 177.9 ± 9.7 198.2 ± 14.5 6.1 ± 0.7 10.8 ± 0.9	NA NA NA NA NA NA	Veeresha m et al. 2012

			CL (mL/min) Vd (mL)	325.1 ± 12.5 56.3 ± 5.6	306.2 ± 12.3 51.5 ± 7.6	NA NA	
IgG	Rat	Metabolism by phagocytic cells or by their target antigen-containing cells	 Vc (mL/kg) Vp (mL/kg) CL (mL/d/kg) Ka (1/d) F t ½ (d)	Mean (CV%) 53.2 (8.28) 96.7 (11.8) 5.83 (6.38) 0.0982 (11.8) 0.924 (6.78) 17.6	Mean (CV%) 53.2 (8.28) 46.2 (10.3) 13.1 (4.25) 0.116 (5.23) 0.808 (5.95) 5.29	 NS S S NS NS NA	Chadha et al. 2015
Isoniazid	Human	Acetylation and hydrolysis	Plasma concentration after 14 days (µg/mL) Plasma concentration after 30 days (µg/mL)	(Mean ± SEM) 3.2 ± 0.2 2.9 ± 0.2	(Mean ± SEM) 1.5 ± 0.2 1.2 ± 0.2	 < 0.05* < 0.05*	Babalik et al. 2013
Isoniazid	Human	Acetylation and hydrolysis	 Daily dosing Cmax (mg/L) AUC (mg*h/L) Twice weekly dosing Cmax (mg/L) AUC (mg*h/L)	TB alone (Median) 3 11.05 8.6 62.45	TB + DM (Median) 2.6 10.39 4.4 54.26	 1 0.6 0.3 0.2	Requena-Méndez et al. 2014
Itopride	Rat	FMO	 Cmax (µg/mL) Tmax (h)	(Mean ± SEM) 2.72 ± 0.03 2 ± 0	(Mean ± SEM) 3.92 ± 0.07 2 ± 0	 NS NS	Vunnam et al. 2015

			AUC (µg*h/mL) AUCinf (µg*h/mL) t ½ (h) CL/F (mL/h/kg) V/F (mL/kg)	21.9 ± 0.118 27.49 ± 0.808 12.03 ± 1.11 26.71 ± 0.18 497.65 ± 3.09	42.49 ± 11.1 59.53 ± 41.7 12.22 ± 15.1 15.475±12.2 459.16±2.1	NS NS NS NS NS	
Ketoconazole	Human	CYP3A4	 Tmax (h) Cmax (ng/mL) AUC (ng*h/mL per mg dose)	(Mean ± SD) 2.72 ± 2.45 0.75 ± 0.47 3.33 ± 0.47	(Mean ± SD) 2.93 ± 2.18 0.63 ± 0.39 3.06 ± 0.58	 NS NS NS	Akhlaghi et al. 2012
Lapatinib	Rat	Primarily CYP3A4 in humans (in addition to 3A5, 2C19 and 2C8	 Cmax (mg/L) Tmax (h) ke (1/h) t ½ (h) CL/F (L/h) V/F (L) AUC (mg*h/L) AUCinf (mg*h/L) MRT (h)	(Mean ± SD) 2.29 ± 0.52 3.17 ± 0.75 0.12 ± 0.09 11.67 ± 10.68 1.78 ± 0.99 20.67 ± 7.49 23.86 ± 7.74 37.12 ± 21.38 7.19 ± 3.15	(Mean ± SD) 4.15 ± 1.79 2.83 ± 1.60 0.14 ± 0.06 5.65 ± 1.99 1.17 ± 0.45 10.65 ± 5.95 43.28 ± 16.58 47.17 ± 14.87 6.55 ± 1.17	 0.0104* 0.2034 0.6857 0.1026 0.2623 0.0281* 0.0265* 0.3668 0.6604	Karbownik et al. 2018
Losartan	Mouse	Cyp2c29 (CYP2C9 in humans)	 Cmax (µg/mL) AUC (min*µg/mL) t ½ (min)	(Mean ± SEM) 0.15 ± 0.13 64.6 ± 115.1 114 ± 51	(Mean ± SEM) 0.94 ± 1.12 123.9 ± 109.0 90 ± 32	 NS NS NS	Li et al. 2017
Metformin	Rat	Mainly excreted unchanged in urine	 AUC (µM*h) AUCinf (µM*h) CL (mL/min per kg) Vdss (L/kg) t ½ (h)	(Mean ± SD) 1.9 ± 0.4 1.9 ± 0.4 71 ± 16 3.4 ± 2.3 2.6 ± 3.6	(Mean ± SD) 1.5 ± 0.2 1.5 ± 0.2 90 ± 14 3.7 ± 0.7 1.1 ± 0.4	 NS NS NS NS NS	Zhou et al. 2016

Metoprolol R (+) isomer	Human	CYP2D6 (preferred metabolism of R isomer)		(median) (95% CI)	(median) (95% CI)		Antunes et al. 2014
			Cmax (ng/mL)	22.89 (20.56, 64.26)	18.98 (12.53, 42.14)	NS	
			Tmax (h)	1.50 (1.16, 2.15)	2.50 (1.55, 3.94)	< 0.05*	
			AUCinf (ng/ml h)	62.65 (58.39, 225.03)	84.63 (41.48, 196.92)	NS	
			MRT (h)	6.71 (5.65, 10.14)	6.54 (4.78, 8.00)	NS	
			t ½ (h)	7.74 (6.38, 11.24)	3.57 (2.60, 7.38)	< 0.05*	
			ke (1/h)	0.09 (0.06, 0.13)	0.19 (0.12, 0.26)	< 0.05*	
			V/F (L/kg)	35.38 (23.81, 87.46)	25.60 (15.95, 66.19)	NS	
			CL/F (L/h*kg)	5.29 (3.19, 7.48)	4.85 (1.97, 12.62)	NS	
S (-) isomer			Cmax (ng/mL)	41.42 (30.16, 67.51)	22.90 (12.97, 49.54)	NS	
			Tmax (h)	1.50 (1.19, 2.15)	2.75 (1.94, 4.35)	< 0.05*	

			AUCinf (ng/ml h)	113.42 (89.73, 266.58)	125.32 (62.81, 238.7)	NS	
			MRT (h)	5.87 (4.85, 8.28)	6.37 (4.66, 9.01)	NS	
			t ½ (h)	7.01 (5.26, 9.77)	4.38 (3.08, 7.13)	NS	
			ke (1/h)	0.10 (0.08, 0.15)	0.16 (0.11, 0.24)	NS	
			V/F (L/kg)	26.87 (21.03, 62.98)	20.02 (14.58, 39.98)	NS	
			CL/F (L/h*kg)	3.19 (2.29, 4.95)	3.05 (1.88, 7.68)	NS	
Mexiletine	Rat	CYP2D6 and CYP1A2 in humans		Estimate (90% credible interval)	Estimate (90% credible interval)		Pardo et al. 2014
			<u>(-)-(R) Mexiletine</u> AUCinf (ng.h/mL)	154.2 (100.4–270.3)	84.8 (43.6–167.0)	NA	
			CL/F (L/h*kg)	32.4 (17.5–48.5)	58.6 (17.4–80.1)	NA	
			Vd/F (L/kg)	146.0 (95.3–244.3)	600.4 (378.4–1383.0)	NA	
			<u>(+)-(S) Mexiletine</u> AUCinf (ng.h/mL)	485.4 (302.9–856.0)	265.8 (213.9–381.1)	NA	

			CL/F (L/h*kg) Vd/F (L/kg)	10.3 (5.3–15.9) 38.4 (22.1–80.6)	18.8 (12.0–22.8) 86.8 (57.6–165.8)	NA NA	
Midazolam	Mouse	Cyp3a11 (CYP3A4 in humans)	Cmax (µg/mL) AUC (min*µg/mL) t ½ (min)	(Mean ± SEM) 0.30 ± 0.15 36.7 ± 11.8 88 ± 38	(Mean ± SEM) 0.26 ± 0.10 47.9 ± 18.5 140 ± 53	 NS NS NS	Li et al. 2017
Nifedipine	Human	CYP3A4 (also by CYP1A2 and CYP2A6)	 C max (ng/ml) Tmax (h) AUC (ng.h/mL) Ke (1/h) t ½ (h) Vd/F (L) CL/F (L/h)	Median (25th–75th percentiles) 26.41 (23.98–29.88) 1.79 (1.24–1.96) 235.99 (203.72–261.97) 0.16 (0.15–0.18) 4.34 (3.87–4.64) 560.96 (506.85–720.31) 84.77	Median (25th–75th percentiles) 23.52 (21.21–27.87) 1.48 (1.03–1.73) 202.23 (185.54–235.52) 0.14 (0.13–0.25) 5.00 (4.45–5.84) 609.40 (357.28–742.05) 98.94	 0.23 0.46 0.38 0.72 0.28 0.87 0.38	Filgueira et al. 2017

				(76.37–98.24)	(85.14–107.79)		
Omeprazole	Mouse	Cyp2c29 (CYP2C19 in humans)	Cmax (µg/mL) AUC (min*µg/mL) t ½ (min)	(Mean ± SEM) 0.6 ± 0.7 36.2 ± 31.7 70 ± 45	(Mean ± SEM) 3.1 ± 1.9 314.5 ± 195.4 56 ± 13	 NS NS NS	Li et al. 2017
Paclitaxel	Rat	CYP3A in rats (CYP3A4 and CYP2C8 in humans)	(IV) AUCinf (µg.min/mL) t ½ (min) CL (mL/min/Kg) VSS (mL/Kg) (Oral) AUCinf (µg.min/mL) C max (µg/ml) Tmax (min) t ½ (min)	(Mean ± SD) 475 ± 25 115 ± 10 11 ± 1 670 ± 40 17.2 ± 8.4 0.04 ± 0.03 261 ± 139 214 ± 66	(Mean ± SD) 523 ± 78 158 ± 46 10 ± 2 580 ± 22 27.0 ± 6.9 0.07 ± 0.03 206 ± 118 147 ± 36	 NS NS NS NS < 0.05* < 0.001* NS < 0.05*	Lee et al. 2012
Pantoprazole	Human	CYP2C19, 3A4, SULT	Plasma level (µg/mL)	(Mean ± SD) 0.34 ± 0.03	(Mean ± SD) 0.25 ± 0.03	< 0.001*	Sapmaz et al. 2015
Pioglitazone	Rat	CYP2C8, CYP2C9 and CYP3A4 orthologs	Cmax (µg/mL) Tmax (h) AUC (µg h/mL) t ½ (h) ke (1/h) MRT (h)	(Mean ± SEM) 5.55 ± 0.47 4.00 ± 0.00 42.42 ± 4.13 3.62 ± 0.22 0.192 ± 0.01 5.58 ± 0.14	(Mean ± SEM) 5.91 ± 0.52 4.00 ± 0.00 51.19 ± 13.5 3.86 ± 0.09 0.180 ± 0.004 5.84 ± 0.29	 NA NA NA NA NA NA	Singh et al. 2013

Prasugrel	Human	Activation: CYPs 3A4, 2B6, 2C19, 2C9 Clearance: Hydrolysis and glucuronide conjugation	Plasma active metabolite level (ng/mL) after 0.5 hour of the loading dose Plasma active metabolite level (ng/mL) after 3 hours of the loading dose	--- ---	--- ---	0.012* 0.70	Niijima et al. 2018
Prednisone (free fraction)	Human	CYP3A4		median [25th quartile, 75th quartile]	median [25th quartile, 75th quartile]		Ionita et al. 2014
			Tmax (h)	2.04 [1.30, 2.20]	3.00 [2.05, 4.08]	0.02*	
				geometric mean [95% CI]	geometric mean [95% CI]		
			Cmax/D (µg/L/mg)	0.738 [0.621, 0.877]	0.757 [0.655, 0.874]	0.82	
			AUC/D (µg*h/L/mg)	5.33 [4.53, 6.27]	6.31 [5.64, 7.06]	0.08	
			MTT (h)	5.97 [5.49, 6.49]	6.73 [6.22, 7.29]	0.04*	
			t ½ (h)	3.29 [2.86, 3.77]	3.49 [3.14, 3.88]	0.48	
			CL/F (L/h*kg)	2.15	1.78	0.20	

				[1.80, 2.57]	[1.57, 2.03]		
			V/F (L/kg)	10.2 [8.66, 12.0]	8.97 [7.91, 10.2]	0.08	
Prednisolone (free fraction)	Human	CYP3A4		median [25th quartile, 75th quartile]	Median [25th quartile, 75th quartile]		Ionita et al. 2014
			Tmax (h)	1.01 [0.558, 1.65]	1.80 [1.04, 2.44]	0.01*	
				Geometric mean [95% CI]	Geometric mean [95% CI]		
			Cmax/D (µg/L/mg)	3.90 [3.25, 4.68]	4.11 [3.46, 4.90]	0.66	
			AUC/D (µg*h/L/mg)	17.7 [15.2, 20.4]	21.7 [19.5, 24.1]	0.02*	
			MTT (h)	4.47 [4.09, 4.89]	5.16 [4.72, 5.65]	0.02*	
			t ½ (h)	2.83 [2.63, 3.04]	3.12 [2.90, 3.36]	0.05*	
Pyrazinamide	Human	Mainly excreted unchanged in urine Some hepatic metabolism	Plasma concentration after 14 days (µg/mL)	(Mean ± SEM) 23.4 ± 2.0	(Mean ± SEM) 25.9 ± 1.4	NS	Babalik et al. 2013

		by aldehyde oxidase	Plasma concentration after 30 days (µg/mL)	27.9 ± 1.6	23.4 ± 1.9	NS	
Remogliflozin etabonate	Human	Possibly CYP and UGT	Dose: 500 mg AUCinf (ng.h/mL) C max (ng/ml) t ½ (h) Tmax (h)	Geometric mean (CV%) 36.8 (67) 41.6 (81) 0.263 (27) Median (range) 1.25 (0.33 – 2.50)	Geometric mean (CV%) 91.9 (46) 83.9 (89) 0.82 (69) Median (range) 0.75 (0.33 – 2.50)	 NA NA NA NA	Kapur et al. 2013
Repaglinide	Rat	CYP3A4 and 2C8 in humans	 Cmax (µg/mL) Tmax (h) AUC (µg · h/mL) AUCinf (µg · h/mL) t ½ (h) MRT (h) CL (mL/h/kg) Vd (mL/kg)	(Mean ± SD) 3.78 ± 0.53 1 ± 0 23.06 ± 3.18 23.18 ± 3.14 3.05 ± 0.50 5.77 ± 0.40 9.13 ± 1.73 39.62 ± 6.31	(Mean ± SD) 7.4 ± 0.66 1.16 ± 0.41 41.39 ± 6.36 42.39 ± 6.54 3.91 ± 1.69 6.21 ± 1.83 4.58 ± 1.7 22.998 ± 2.37	 NA NA NA NA NA NA NA NA	Penta et al. 2017
Rifampin	Human	Deacetylation	 Plasma concentration after 14 days (µg/mL) Plasma concentration after 30 days (µg/mL)	(Mean ± SEM) 5.1 ± 0.5 5.4 ± 0.5	(Mean ± SEM) 2.9 ± 0.2 3.2 ± 0.5	 < 0.05* < 0.05*	Babalik et al. 2013

Rifampin	Human	Deacetylation	Non-compartmental PK Cmax (mg/L) Tmax (h) AUC (mg · h/L) AUCinf (mg · h/L) MRT (h) One-compartment open model Ka (1/h) t ½α (h) Vd (L/kg) ke (1/h) t ½ (h) CL (L/h)	TB patients 11.41 ± 3.8 2.32 ± 1.4 82.60 ± 35.5 87.07 ± 39.9 7.67 ± 2.7 1.79 (0.8–2.8) 0.39 (0.2–0.9) 0.84 ± 0.31 0.21 ± 0.1 4.31 ± 1.7 8.62 ± 3.9	TB + T2DM patients 12.10 ± 5.1 2.98 ± 1.9 97.52 ± 36.7 107.73 ± 56.3 9.07 ± 5.0 1.80 (0.4–3.4) 0.39 (0.2–1.8) 0.63 ± 0.21 0.19 ± 0.08 3.97 ± 1.5 6.04 ± 1.8	 NS < 0.05* NS NS NS NS NS < 0.05* NS NS < 0.05*	Medellín-Garibay et al. 2015
Rosiglitazone	Rat	CYP2C22 (ortholog of human CYP2C8)	AUC (µM*h) AUCinf (µM*h) CL (mL/ min per kg) Vdss (L/kg) t ½ (h)	(Mean ± SD) 40 ± 12 40 ± 12 1.2 ± 0.3 0.3 ± 0.0 2.9 ± 0.2	(Mean ± SD) 32 ± 7 32 ± 7 1.5 ± 0.3 0.2 ± 0.0 2.7 ± 0.1	 NS NS NS NS NS	Zhou et al. 2016
Salicylic acid	Rat	Mainly excreted unchanged in urine	CL/F (mL/h*kg)	Mean (CV%) 68.0 (6.57)	Mean (CV%) 94.6 (4.10)	NA	Cao et al. 2012
Sunitinib	Rabbit	CYP3A4	AUC (ng x h/mL) AUCinf (ng x h/mL)	(Mean ± SD) 2303.6 ± 1069.1 2425.0 ± 1077.5	(Mean ± SD) 3785.8 ± 1282.5 4699.4 ± 1338.3	NA < 0.01*	Szalek et al. 2014

			t ½ (h) CL (L/h) Vd (L) Cmax (ng/mL) Tmax (h) MRT (h)	23.2 ± 7.6 12.1 ± 5.4 418.2 ± 223.4 92.1 ± 22.1 7.2 ± 0.8 23.3 ± 2.8	31.6 ± 19.1 5.7 ± 1.6 242.3 ± 120.0 149.2 ± 33.7 8.8 ± 2.1 25.3 ± 5.8	NS NS NS NA NS NS	
Tacrolimus	Human	CYP3A4	 Tmax (h) Cmax (ng/mL per mg dose) AUC (ng*hour/ mL per mg dose) CL (L/hour/ kg)	median (interquartile range) 1.5 (1.4–2.0) 3.2 (2.3–5.2) 20.6 (12.9–41.9) 0.28 (0.13–0.58)	median (interquartile range) 3.0 (2.0–4.0) 5.8 (3.8–8.3) 37.7 (37.2–63.9) 0.13 (0.08–0.17)	 0.012* 0.044* 0.037* 0.037*	Chitnis et al. 2013
Tedizolid	Human	Sulfate conjugation	 Cmax (mg/L) Tmax (h) t ½ (h) AUC (mg*h/L) CL/F (L/h) Vd/F (L)	Healthy volunteers mean ± SD or median (range) 2.7 ± 1.1 2.5 (2.0–3.0) 8.9 ± 2.2 28.7 ± 9.6 11.4 ± 3.3 143.4 ± 50.4	diabetic foot patients mean ± SD or median (range) 1.5 ± 0.5 5.9 (1.2–8.0) 9.1 ± 3.6 18.5 ± 9.7 15.0 ± 6.8 177.3 ± 53.7	 0.005* 0.003* 0.932 0.004* 0.481 0.143	Stainton et al. 2018

Tramadol (+) isomer	Human	CYP3A4, 2B6, 2D6, then Sulfation or glucuronidation	AUCinf (ng*h/mL)	Median (25th–75th percentile) 1311.8 (1042.2–2166.6)	Median (25th–75th percentile) 977.5 (912.2–1432.3)	NS	De Moraes et al. 2014
			Cmax (ng/mL)	167.5 (137.5–253.1)	144.8 (139.2–149.0)	NS	
(-) isomer			Tmax (h)	1.5 (1.3–2.0)	1.3 (1.2–1.8)	NS	
			CL/F (L/h)	38.3 (23.8–48.3)	51.15 (35.1–54.8)	NS	
			t ½ (h)	7.6 (6.6–9.1)	7.5 (6.2–11.2)	NS	
			Vd/F (L)	357.3 (286.7–509.1)	384.2 (382.3–400.4)	NS	
			AUCinf (ng*h/mL)	1264.2 (851.5–1496.4)	1222.9 (870.8–2081.3)	NS	
			Cmax (ng/mL)	159.5 (119.7–223.6)	128.7 (123.7–156.8)	NS	
			Tmax (h)	1.5 (1.2–2.0)	1.3 (1.2–2.0)	NS	
			CL/F (L/h)	39.9	40.9	NS	

				(33.4–58.8)	(24.0–57.4)		
			t ½ (h)	7.1 (6.3–8.9)	7.3 (6.1–16.6)	NS	
			Vd/F (L)	368.4 (264.1–506.3)	446.8 (392.6–512.7)	NS	
Troglitazone	Rat	SULT (ortholog of human SULT1A1)		(Mean ± SD)	(Mean ± SD)		Zhou et al. 2016
			AUC (µM*h)	0.6 ± 0.1	1.1 ± 0.4	< 0.05*	
			AUCinf (µM*h)	0.6 ± 0.1	1.3 ± 0.6	< 0.05*	
			CL (mL/min per kg)	63 ± 10	35 ± 15	< 0.05*	
			Vdss (L/kg)	1.6 ± 0.6	4.7 ± 3.2	NS	
			t ½ (h)	2.0 ± 1.2	5.6 ± 4.2	NS	

DMEs = Drug Metabolizing Enzymes, PK = Pharmacokinetic, T2DM = Type 2 Diabetes Mellitus, TB = Tuberculosis, ke = elimination rate constant, Ka = absorption rate constant, t ½ = elimination half-life, t ½α = absorption half-life, AUC = area under the concentration-time curve until the last quantifiable value, AUCinf = AUC extrapolated from time zero to infinity, CL = clearance, CL/F = total apparent clearance, CLR = renal clearance, CLNR = non-renal clearance, Vd = volume of distribution, V/F = apparent volume of distribution, Vdss = volume of distribution at steady state, Vc = central volume of distribution, Vp = peripheral volume of distribution, Cmax = maximum observed plasma concentration, Tmax = time to reach maximum concentration (Cmax), MRT = mean residence time, MTT = mean transit time, MAT = mean absorption time, D = dose, DMIA = diabetes mellitus induced by alloxan, DMIS = diabetes mellitus induced by streptozotocin, IV = intravenous, CI = confidence interval, CV = coefficient of variance, NS = non-significant statistical difference, S = significant statistical difference but p-value not reported, NA = statistical analysis not reported.

1.4. Effect of Non-alcoholic Fatty Liver Disease

NAFLD is a health issue that has been on the rise globally. It is defined as an abnormal fat accumulation in the liver without significant alcohol consumption or other secondary causes. NAFLD is further sub-classified to non-alcoholic fatty liver (NAFL) where there is only simple steatosis and non-alcoholic steatohepatitis (NASH) where steatosis is accompanied by liver cell injury and inflammation and in some cases fibrosis (Chalasani et al., 2012). There are many inconsistent reports on NAFLD prevalence. This is mainly due to the different methods used for diagnosis. In a recent meta-analysis, the estimated global prevalence was approximately 25% when including patients diagnosed by imaging methods only (Younossi et al., 2016). The effect of NAFLD on DMEs has become a topic of interest. As in the case of T2DM, the effect seems to differ for each CYP450 enzyme. A clinical study in a pediatric population reported no change in CYP2C9 enzyme activity in the NASH group compared to the healthy group (H. Li et al., 2017). An in vitro study using human liver microsomes (HLM) from healthy and NAFLD adults showed a significant increase in CYP2C9 enzyme activity measured with two probe substrates, diclofenac and tolbutamide (Fisher et al., 2009). Concerning CYP2C8, the study by Fisher and his colleagues (2009) is the only study reporting this enzyme's phenotypes in NAFLD. Their results showed no change in CYP2C8 enzyme activity and mRNA level with disease progression. However, there was a non-statistically significant decrease in protein level. We believe that these results are not conclusive. More research is warranted for CYP2C8 and 2C9 expression and activity in NAFLD with a larger sample size.

1.5. Warfarin as a Probe Substrate

Warfarin is an anticoagulant that exerts its pharmacological action by interfering with the function of vitamin K as an essential component of the clotting cascade. The pharmacokinetics of warfarin has been extensively studied. Following oral administration, it is easily absorbed through the gastrointestinal tract and then highly bound to plasma proteins once in the main bloodstream which limits its distribution. Hepatic metabolism is the main route of clearance with little to no role of kidneys in excreting the parent compound (Park, 1988). The approved dosage form is a mixture of S- and R- enantiomers. These enantiomers have shown different potency and pharmacokinetic profiles (Choonara, Haynes, Cholerton, Breckenridge, & Park, 1986). S-warfarin is more potent as measured by the level of increase in prothrombin time (Park, 1988). Regarding PK differences, S-warfarin is majorly metabolized by CYP2C9 to 7-hydroxy warfarin while R-warfarin goes through multiple pathways and CYP2C9 plays a minor role in its biotransformation. Findings from pharmacogenetic research suggest that CYP2C9 is the main CYP enzyme implicated in the clearance of warfarin and the inter-individual variability to this drug ("COUMADIN Prescribing information," 2011). The clinical relevance of S-warfarin coupled with its sensitivity to CYP2C9 alterations makes it a suitable probe substrate for studies on CYP2C9 activity.

Chapter 2

Methodology

2.1 Chemicals and Supplies

S-warfarin, S-7-hydroxywarfarin, and S-7-hydroxywarfarin-d5 were obtained from Toronto Research Chemicals (North York, Ontario, Canada). Individual Human liver tissue samples and Xtreme pool of 200 human liver microsomes were purchased from Sekisui XenoTech (Kansas City, KS, USA). Tetrasodium salt of reduced nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from EMD Millipore (Burlington, MA, USA). LC/MS grade acetonitrile and methanol were obtained from ThermoFisher Scientific (Waltham, MA, USA). All other solvents were of analytical grade.

2.2 Liver Tissue Grading and Processing

Individual human liver tissue samples were commercially obtained from Sekisui XenoTech. Demographics and diabetic status of the liver donors were provided by the supplying company However, their identities were not known. Therefore, the research is exempt from review by The University of Rhode Island institutional review board (IRB) under exempt category # 4. Grading of liver tissues for NAFLD presence and severity was done by a histopathologist collaborating with our lab (Dr. Suzanne Delamonte, MD). Liver tissues were then categorized as: normal, NAFL, or NASH as described in detail by Jamwal et al. (2018).

Human liver microsomes were prepared in our lab by homogenization of the liver tissue samples using automated Omni Bead Ruptor 24 homogenizer (Omni International, Kennesaw, GA, USA). Homogenates were then differentially centrifuged using Eppendorf's 5810R Centrifuge (Eppendorf, Hamburg, Germany) then Beckman Coulter's ultracentrifuge (Beckman Coulter, Brea, CA) to obtain microsome pellets that were resuspended in glycerol buffer and stored in - 80 C^{o} for later use (Jamwal et al., 2017).

To account for the effects of enzyme inducers and inhibitors potentially taken by liver donors prior to death, our lab recently completed a project to detect several commonly used drugs which possess CYP450 induction or inhibition properties in the liver bank (Barlock & Jamwal, 2018. Manuscript in preparation). Three CYP2C8 inducers, two CYP2C9 inducers, and eleven CYP2C9 inhibitors were detected.

Table 2 shows the donors' demographics and the most frequently detected CYP2C8/2C9 inducers and inhibitors in the liver bank (n=106). CYP2C8's and CYP2C9's mRNA and protein levels were measured for all 106 livers in our lab using validated procedures (Jamwal et al., 2017). Table 3 shows the donor's demographics and the most frequently detected CYP2C9 inducers and inhibitors in a subset of the liver bank that was included in measuring CYP2C9 activity (n=76). Limited sample quantities prevented CYP2C9 activity measurement of the whole bank.

Table 2. Donors' demographics and detected CYP2C8/2C9 inducers and inhibitors in all livers (n=106)

	Non-diabetic/no NAFLD (n=21)	**Diabetic only (n=21)**	**NAFLD only (n=32)**	**Diabetic + NAFLD (n=32)**
Age (mean ± SD)	49.43 ± 16.14	48.62 ± 13.63	52.31 ± 10.87	53.28 ± 9.45
(minimum, maximum)	(21,73)	(21,78)	(33,76)	(38,74)
Gender (%)	Females (42.9) Males (57.1)	Females (33.3) Males (66.7)	Females (50.0) Males (50.0)	Females (56.3) Males (43.8)
Ethnicity (%)	Caucasians (81.0) African Americans (19.0) Hispanics (0.0)	Caucasians (76.2) African Americans (19.0) Hispanics (4.8)	Caucasians (90.6) African Americans (3.1) Hispanics (6.3)	Caucasians (90.6) African Americans (3.1) Hispanics (6.3)
BMI (%)	<30 (66.7) ≥30 (33.3)	<30 (52.4) ≥30 (47.6)	<30 (50.0) ≥30 (50.0)	<30 (34.4) ≥30 (65.6)
CYP2C8 Polymorphism (%)	*1/*1 (100.0) *1/*3 (0.0) *3/*3 (0.0)	*1/*1 (81.0) *1/*3 (19.0) *3/*3 (0.0)	*1/*1 (71.9) *1/*3 (28.1) *3/*3 (0.0)	*1/*1 (81.3) *1/*3 (15.6) *3/*3 (3.1)
CYP2C9 Polymorphism (%)	*1/*1 (100.0) *1/*2 (0.0) *2/*2 (0.0)	*1/*1 (81.0) *1/*2 (19.0) *2/*2 (0.0)	*1/*1 (68.8) *1/*2 (31.3) *2/*2 (0.0)	*1/*1 (81.3) *1/*2 (15.6) *2/*2 (3.1)
Dexamethasone (CYP2C8 inducer) (%)	Present (19.0) Absent (81.0)	Present (19.0) Absent (81.0)	Present (25.0) Absent (75.0)	Present (19.4) Absent (80.6)

Phenytoin (CYP2C8/2C9 inducer) (%)	Present (38.1) Absent (61.9)	Present (23.8) Absent (76.2)	Present (31.3) Absent (68.8)	Present (32.3) Absent (67.7)
Tetrahydrocannabinol (CYP2C9 inhibitor) (%)	Present (57.1) Absent (42.9)	Present (57.1) Absent (42.9)	Present (75.0) Absent (25.0)	Present (80.6) Absent (19.4)

Table 3. Donor's demographics and detected CYP2C9 inducers/inhibitors in livers included in CYP2C9 activity measurements (n=76)

	Non-diabetic/no NAFLD (n=16)	Diabetic only (n=16)	NAFLD only (n=25)	Diabetic + NAFLD (n=19)
Age (mean ± SD)	49.13 ± 16.42	45.44 ± 12.88	52.68 ± 11.8	52.47 ± 8.87
(minimum, maximum)	(21,73)	(21,70)	(33,76)	(38,74)
Gender (%)	Females (43.8) Males (56.3)	Females (31.3) Males (68.8)	Females (48.0) Males (52.0)	Females (68.4) Males (31.6)
Ethnicity (%)	Caucasians (75.0) African Americans (25.0) Hispanics (0.0)	Caucasians (75.0) African Americans (18.8) Hispanics (6.3)	Caucasians (88.0) African Americans (4.0) Hispanics (8.0)	Caucasians (94.7) African Americans (5.3) Hispanics (0.0)
BMI (%)	<30 (68.8) ≥30 (31.3)	<30 (50.0) ≥30 (50.0)	<30 (48.0) ≥30 (52.0)	<30 (31.6) ≥30 (68.4)
CYP2C9 Polymorphism (%)	*1/*1 (100.0) *1/*2 (0.0) *2/*2 (0.0)	*1/*1 (81.3) *1/*2 (18.8) *2/*2 (0.0)	*1/*1 (76.0) *1/*2 (24.0) *2/*2 (0.0)	*1/*1 (73.7) *1/*2 (21.1) *2/*2 (5.3)
Phenytoin (CYP2C9 inducer) (%)	Present (50.0) Absent (50.0)	Present (31.3) Absent (68.8)	Present (32.0) Absent (68.0)	Present (26.3) Absent (73.7)
Tetrahydrocannabinol (CYP2C9 inhibitor) (%)	Present (56.3) Absent (43.8)	Present (56.3) Absent (43.8)	Present (68.0) Absent (32.0)	Present (84.2) Absent (15.8)

2.3 Preliminary Experiments for CYP2C9 Activity Measurement

Detection system linearity was assessed by injecting a wide concentration range of S-warfarin (0.5 – 64 uM) and S-7OH-warfarin (0.25 – 64 uM) pure standards and plotting them against their peak areas as measured by LC-MS/MS.

Sekisui XenoTech Xtreme pool of 200 human liver microsomes was used to determine the optimal incubation settings prior to measurements in donor-specific liver microsomes.

Time and protein linearity experiments were performed to determine incubation times and microsomal protein concentrations where product formation is within the linear range.

In time linearity experiment, incubations were carried out in 100 mM Phosphate buffer containing 3 mM MgCl2 and 1 mM EDTA (pH 7.4). The incubation mixture consisted of 0.2 mg/mL HLM, 3.85 uM S-warfarin, and 1.3 mM NADPH. A water bath set at 37 ^{o}C was used to simulate body temperature. Aliquots were withdrawn from the incubation plate at 0, 20, 40, 60, 80, 100, 120, and 140 minutes and added to a quenching plate containing ice cold acetonitrile and internal standard (IS) (2 uM S-7-hydroxywarfarin-d5) to stop the reaction. Afterwards, the samples were centrifuged at 3200 g for 10 minutes and the resulting supernatants were injected into LC-MS/MS for metabolite quantification. Quantity of the metabolite S-7-hydroxywarfarin formed was plotted against incubation time to determine the linear range.

In protein linearity experiment, the same incubation mixture and settings were implemented except for HLM concentration where a range of (0.025 – 1 mg/mL) was used. The reaction was stopped after 50 minutes as this time point was found to be within the linear range in the previous experiment. Samples were then centrifuged, and the metabolite quantified by injecting the supernatants in LC-MS/MS.

After determining the optimal incubation times and HLM concentrations, an experiment to generate Michaelis-Menten plot was performed to find the Km and Vmax values of our system and ensure that the substrate concentration we use for the main experiments is below Km to prevent system saturation. A range of S-warfarin concentrations (0.5 – 64 uM) was incubated with 0.4 mg/mL HLM for 45 and 60 minutes (HLM concentration and incubation times were determined suitable by protein and time linearity experiments). The reaction was carried out in 100 mM Phosphate buffer as described earlier.

2.4 Measurement of S-Warfarin Depletion and S-7-Hydroxywarfarin Formation

After finalizing the protocol with the optimal experimental settings, the main experiments were commenced using HLM samples from the individual donors. Each HLM sample (n=95) was incubated in a separate well in the 96-well plate. The incubation mixture consisted of 0.4 mg/mL HLM, 8 uM S-warfarin, 100 mM Phosphate buffer, and 1.3 mM NADPH (total volume=180 uL). The mixture was preincubated in a water bath set at 37 ^{o}C for 5 minutes prior to adding NADPH. Following preincubation, the incubation was started by adding NADPH and immediately transferring a 20 uL aliquot

to a quenching plate containing 80 uL quenching solution (2 uM S-7-hydroxywarfarin-d5 as internal standard in ice cold acetonitrile) to measure the substrate at time zero. Four more 20 uL aliquots were taken at 12.5, 25, 37.5, and 50 minutes and added to the quenching plate. The quenching plate was then centrifuged, and the resulting supernatants were injected into LC-MS/MS.

Two calibration curves were created using known concentrations of S-warfarin (curve 1) and S-7OH-warfarin (curve 2) to interpolate concentrations of unknowns using linear regression. The remaining concentration of S-warfarin at different time points was used to measure its depletion and calculate the intrinsic clearance. The final aliquot at 50 minutes was used to measure S-7OH-warfarin formation to determine CYP2C9 activity.

2.5 Quantification of S-Warfarin and S-7-Hydroxywarfarin

The LC-MS/MS instrument consisted of ACQUITY UPLC System equipped with a Vanguard pre-column, an ACQUITY UPLC BEH C18 column, and an autosampler. Attached to the UPLC system is an API 3200™ Triple Quad mass spectrometer with an electrospray ionization probe set at positive ion mode.

Chromatographic separation using a gradient elution mode was performed. Target column temperature was 50 ^{o}C. The mobile phase constituted of 10 mM ammonium acetate with 5% acetonitrile at pH 4.85 (A) and 100% acetonitrile (B). The flow rate was set at 0.25 mL/min and the gradient of A and B solvents was started as

follows: A:B 95:5 (v/v) until 1 minute; A:B 75:25 (v/v) until 2.5 minutes; A:B 30:70 (v/v) until 3 minutes; A:B 10:90 (v/v) until 4 minutes; A:B 95:5 (v/v) until 5 minutes.

2.6 Intrinsic Clearance Calculation

As indicated earlier, S-warfarin's depletion over incubation time was used to calculate its intrinsic clearance in HLM samples. This method, known as in vitro t ½ approach, was performed as described by Obach et al. (1997). Briefly, resulting peak heights of the remaining substrate and the internal standard were recorded for each sample to calculate substrate peak height/IS peak height ratios. After that, the ratios were transformed to percentages using the ratio at time zero as %100 and then transformed again to the logarithmic form. The resulting values were plotted against their respective incubation times and slopes were calculated by doing linear regression analysis. Finally, the following formulas were utilized to calculate the intrinsic clearance (adapted from Obach, 1998):

slope = - k

$$t\,½ = \frac{-\,0.693}{k}$$

$$\mathrm{CLint} = \frac{0.693 \times incubation\ volume\ (mL) \times 45\ mg\ microsomes \times 20\ g\ liver}{in\ vitro\ t\,½ \times \mathrm{mg\ microsomes} \times \mathrm{liver\ weight\ (g)} \times body\ \mathrm{weight\ (kg)}}$$

2.7 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 24.0 for Windows (Released 2016. Armonk, NY: IBM Corp.). Normality was assessed using Shapiro-Wilk's test. Depending on normality results, one-way ANOVA or Kruskal-Wallis H test was chosen for testing the differences between more than two groups. The level of significance was set to be < 0.05. Calculation of Km and Vmax values and generation of graphs was done utilizing GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

Chapter 3

Results

3.1 Preliminary Experiments

LC-MS/MS system linearity was observed for injected concentrations versus responses up until the highest concentration used for both S-warfarin and S-7OH-warfarin (figures 1A and 1B respectively).

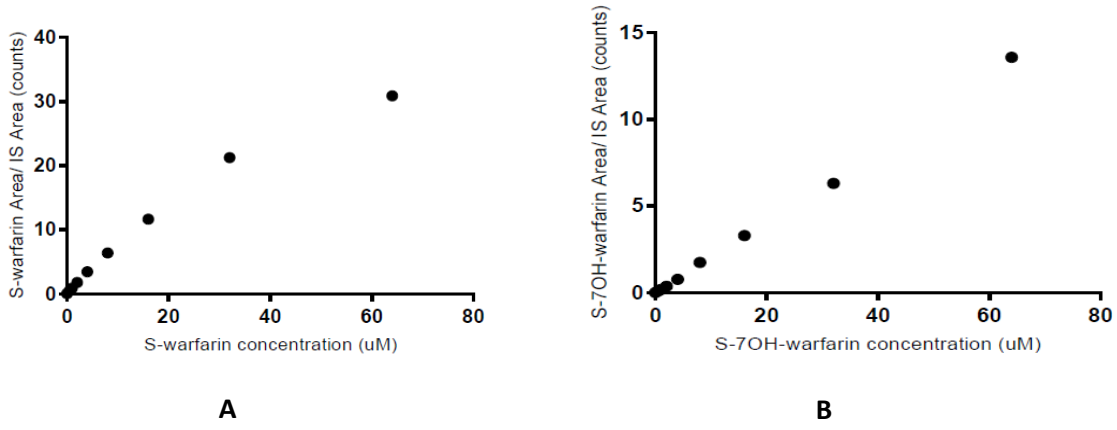

Figure 1. LC-MS/MS system linearity for injected analyte concentration versus detected response. A. S-warfarin B. S-7OH-warfarin

S-7OH-warfarin formation was within the linear range for incubation times between 20 and 60 minutes (figure 2A) and HLM concentrations between 0.1 mg/mL and 1 mg/mL (figure 2B).

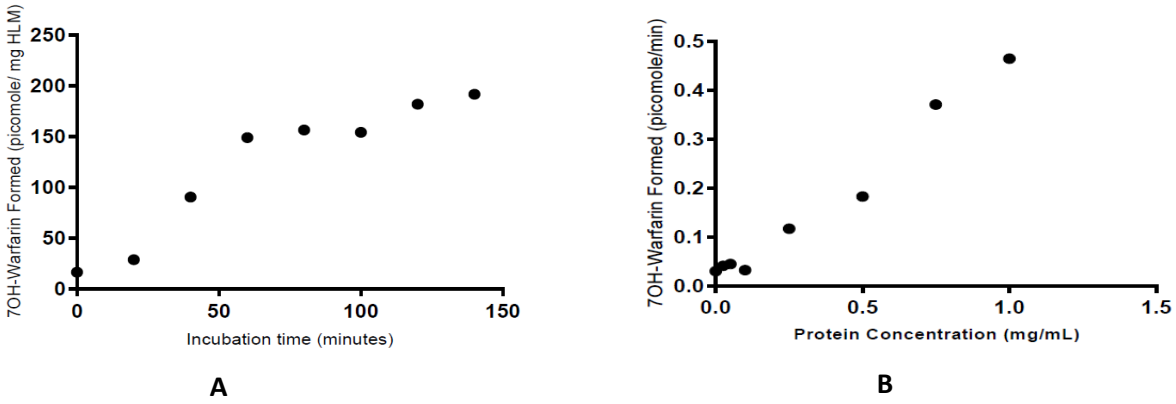

Figure 2. Range of linearity of S-7OH-warfarin formation with respect to A. incubation time B. microsomal concentration

Two Michaelis-Menten plots were generated (figure 4). Vmax was 6.188 picomole/min/mg HLM and 11.12 picomole/min/mg HLM after 45 and 60 minutes of incubation respectively. Km was approximately 13.12 uM.

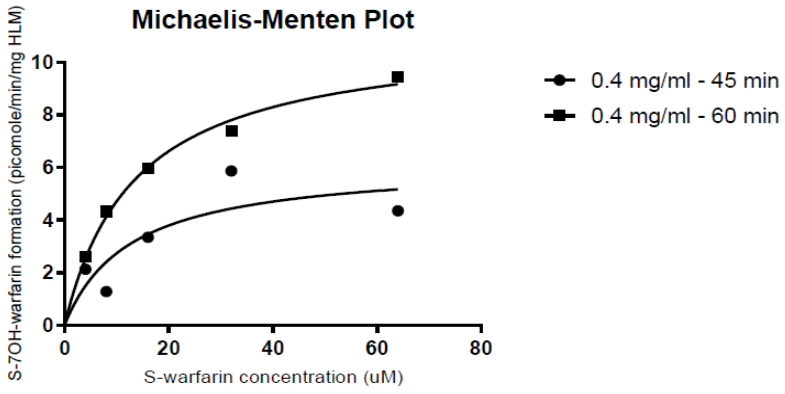

Figure 3. Michaelis-Menten Plots at 45 and 60 minutes incubation times

3.2 Effects of Diabetes and NAFLD on S-warfarin Intrinsic Clearance

Intrinsic clearance of S-warfarin per liver sample are shown in table 4. The distribution of CLint values did not differ significantly when compared between groups of different diabetic and NAFLD status (Table 5).

3.3 Effects of Diabetes and NAFLD on CYP2C8 and 2C9 Expression and Activity

CYP2C9 activity did not seem to be significantly affected by either diabetes nor NAFLD status (Table 6). CYP2C8 mRNA and protein expression in addition to CYP2C9 mRNA expression showed similar results of non-significant effect (Tables 7 and 8). Interestingly, however, CYP2C9 protein expression was significantly different between the groups (Figure 4 and Table 8).

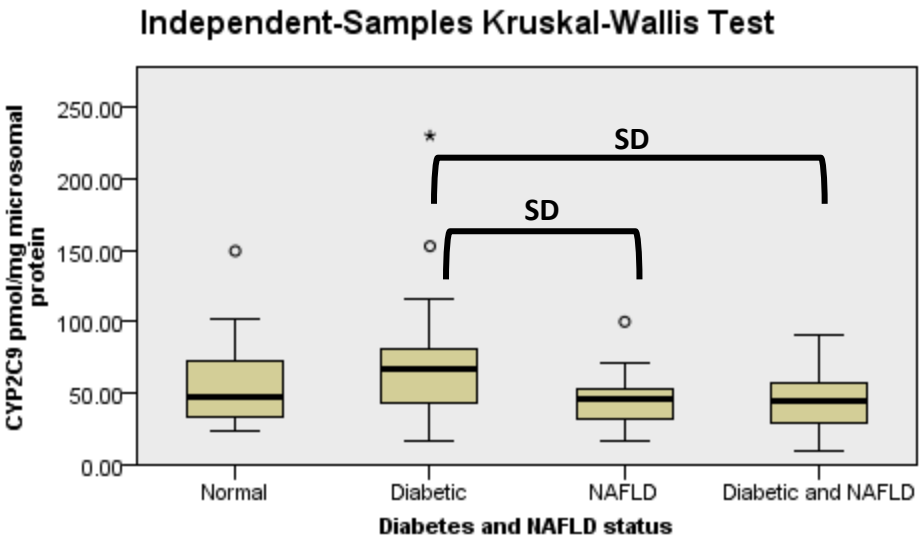

Figure 4. Boxplots representing the distribution of CYP2C9 protein levels across NAFLD subgroups within the diabetic group, SD = Significant difference

Table 4. Intrinsic clearance (mL/min/kg) values for individual liver samples

Liver ID	CLint	Liver ID	CLint	Liver ID	CLint	Liver ID	CLint	Liver ID	CLint
412	0.0000718	**490**	0.0000550	**540**	0.0000145	**743**	0.0000118	**815**	0.0000219
415	0.0000595	**493**	0.0000804	**542**	0.0000056	**749**	0.0000419	**816**	0.0000145
425	0.0000280	**495**	0.0000147	**543**	0.0000215	**750**	0.0000079	**818**	0.0000148
447	0.0000009	**498**	0.0000229	**545**	0.0000350	**752**	0.0000031	**821**	0.0001027
448	0.0000532	**502**	0.0000012	**546**	0.0000015	**762**	0.0000202	**847**	0.0000004
450	0.0000580	**508**	0.0000223	**548**	0.0000011	**768**	0.0000361	**850**	0.0000035
461	0.0000727	**510**	0.0000094	**715**	0.0000451	**772**	0.0000098	**879**	0.0000255
465	0.0000984	**527**	0.0000027	**717**	0.0000206	**791**	0.0000842	**892**	0.0000192
466	0.0000343	**531**	0.0000094	**723**	0.0000315	**799**	0.0000758	**944**	0.0000527
467	0.0000065	**533**	0.0000131	**740**	0.0000825	**802**	0.0000537	**981**	0.0000014
473	0.0000027	**536**	0.0000081	**741**	0.0001050	**803**	0.0000656		

Table 5. Effect of diabetes and NAFLD on S-warfarin's intrinsic clearance

	Non-diabetic/no NAFLD (n=12)	Diabetic only (n=9)	NAFLD only (n=20)	Diabetic + NAFLD (n=13)
CLint (mL/min/kg)				
Mean ± SD[a]	4.3 ± 2.68	3.5 ± 3.04	2.93 ± 2.54	2.61 ± 3.93
p-value		0.099		

p-values reported from data analysis using Kruskal-Wallis Test [a] all mean and SD values are multiplied by 10^5 for simplification (for example: 4.3 is actually 0.000043)

Table 6. Effect of diabetes and NAFLD on CYP2C9 activity

	Non-diabetic/no NAFLD (n=16)	Diabetic only (n=16)	NAFLD only (n=25)	Diabetic + NAFLD (n=19)
CYP2C9 activity (pmol/min/mg HLM)				
Mean ± SD	3.3 ± 2.66	3.54 ± 3.16	3.37 ± 3.43	2.14 ± 2.65
p-value		0.664		

p-values reported from data analysis using Kruskal-Wallis Test

Table 7. Effect of diabetes and NAFLD on CYP2C8 and CYP2C9 mRNA expression

	Non-diabetic/no NAFLD (n=21)	Diabetic only (n=21)	NAFLD only (n=32)	Diabetic + NAFLD (n=32)
CYP2C8 mRNA Mean ± SD	3.18 ± 5.19	2.87 ± 3.32	2.69 ± 3.82	1.82 ± 2.25
p-value		0.541		
CYP2C9 mRNA Mean ± SD	5.91 ± 11.53	3.59 ± 5.22	2.78 ± 4.03	2.02 ± 2.28
p-value		0.354		

p-values reported from data analysis using Kruskal-Wallis Test

Table 8. Effect of diabetes and NAFLD on CYP2C8 and CYP2C9 protein expression

	Non-diabetic/no NAFLD (n=20)	Diabetic only (n=21)	NAFLD only (n=32)	Diabetic + NAFLD (n=30)
CYP2C8 protein (pmol/mg HLM) Mean ± SD	47.66 ± 55.08	40.23 ± 32.65	29.08 ± 28.1	27.02 ± 21.56
p-value		0.341		
CYP2C9 protein (pmol/mg HLM) Mean ± SD	57.77 ± 30.5	70.28 ± 49.26	43.75 ± 17.28	42.51 ± 19.12
p-value		0.047*		

p-values reported from data analysis using Kruskal-Wallis Test, * significant result

Pairwise comparisons using the Dunn-Bonferroni approach was performed as post hoc analysis to determine which groups showed the significant difference found in CYP2C9 protein expression (Table 9). Both the Diabetic only/ NAFLD only and Diabetic only/ Diabetic + NAFLD pairwise comparisons were significant. Other comparisons were not significant.

Table 9. Pairwise comparisons of CYP2C9 protein levels distribution between disease groups

Pair	p-value
Diabetic + NAFLD/ NAFLD only	0.864
Diabetic + NAFLD/ Normal	0.100
Diabetic + NAFLD/ Diabetic only	0.019*
NAFLD only/ Normal	0.130
NAFLD only/ Diabetic only	0.026*
Normal/ Diabetic only	0.534

* significant result

To determine the magnitude of CYP2C9 protein level variability that is accounted by demographical data, multiple linear regression analysis was done. Age, gender, ethnicity, obesity, CYP2C9 polymorphism, diabetes/NAFLD status in addition to presence of phenytoin (CYP2C9 inducer) were all included as predictors in building the following regression model:

CYP2C9 protein level (pmol/mg HLM) = 59.436 - 0.077 (age) + 17.385 (gender) - 5.903 (ethnicity) + 6.14 (BMI) - 4.869 (polymorphism) - 7.058 (diabetes/NAFLD status) - 6.872 (phenytoin)

The regression model was significant (p-value: 0.015) which indicates that at least one of the predictors had a regression coefficient not equal to zero. When

analyzing each predictor, gender and diabetic/NAFLD status were the only significant predictors (p-values: 0.007 and 0.018 respectively). A newer model was created incorporating only these predictors:

CYP2C9 protein level (pmol/mg HLM) = 5.923 + 15.141 (gender) – 6.674 (diabetes/ NAFLD status)

The adjusted R-square value is 0.111. In other words, 11.1% of the variability in CYP2C9 protein expression can be explained by gender, diabetes, and NAFLD effects.

Multiple linear regression analysis was also performed to determine if any of the predictors mentioned above contribute to the variability in CYP2C8 mRNA, CYP2C8 protein, and CYP2C9 mRNA expression. Presence of phenytoin was the only significant predictor of CYP2C8 mRNA level (p-value = 0.001). Age and diabetic/NAFLD status were predictors of CYP2C8 expression (p-values: 0.035 and 0.046 respectively). As for CYP2C9 mRNA expression, presence of phenytoin and diabetic/NAFLD status were significant predictors (p-values: 0.01 and 0.026 respectively).

Although tetrahydrocannabinol (CYP2C9 inhibitor) was present in most of the livers. There was no notable influence of its presence on CYP2C9 activity and S-warfarin intrinsic clearance measured in HLM. This is not surprising when considering the process involved in hepatic microsomal preparation which is expected to remove much of the compounds present in the liver.

Chapter 4

Discussion and Conclusions

Our findings have shown that CYP2C8's mRNA and protein expression were not altered under the influence of two disease states: diabetes mellitus and NAFLD. Similarly, CYP2C9's mRNA expression, activity levels, and S-warfarin's intrinsic clearance showed no change. However, CYP2C9 protein levels significantly decreased in the NAFLD group and the combined diabetic and NAFLD group as compared to the diabetic only group. Multiple linear regression analysis showed that both diseases in addition to gender can explain about 11% of CYP2C9 protein level variability.

Several mechanisms have been proposed for the effect of diseases on hepatic drug metabolizing enzymes expression. Both diabetes mellitus (Sada et al., 2016; Norouzirad, Gonzalez-Muniesa, & Ghasemi, 2017) and NAFLD (Suzuki, Shinjo, Arai, Kanai, & Goda, 2014) are associated with a state of hypoxia. As reported by Simms & D'Amico (1996), there is a notable increased expression of cytokine receptors on cell surfaces in low oxygen states facilitating the intracellular effects of cytokines. Cytokines of interest to drug metabolism include Interleukin-1β and Interferon-γ which activate multiple intracellular signaling pathways that can eventually phosphorylate amino acid residues of some CYPs altering their activities (Fradette & Du Souich, 2004). Furthermore, these cytokines can activate intracellular cascades that lead to transcriptional modifications of CYP genes in the nucleus causing changes in protein expression. Increased activity of ubiquitin proteasome system in hypoxia has been

suggested as a post-transcriptional down-regulator of some CYPs (du Souich & Fradette, 2011). Apart from hypoxia, other conditions such as inflammation also play a role in CYPs alterations. Interleukin-6, which is a cytokine released in inflammation, was found to decrease mRNA levels of the nuclear receptors Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR). Both of which are crucial for induction of CYP2 family genes (Pascussi et al., 2000).

There are some limitations to this study. The limited samples' quantities prevented intrinsic clearance measurement using the metabolite formation approach which is a better-defined method than the in vitro half-life approach (Jones & Houston, 2004). Additionally, the lack of information on how long the enzyme inducers were taken may cause inaccuracies. This is due to the possibility that some liver donors took an inducer for a period long enough to exert its effect on enzyme levels while others were exposed to an inducer for a short period before death. Furthermore, the low representation of different ethnicities and people with polymorphic forms of CYP2C8 and CYP2C9 hinders the generalization of our findings. Finally, the nature of in vitro studies does not allow for direct clinical predictions as multiple factors in vivo can play a role in drug's clearance such as liver blood flow and plasma protein binding.

In conclusion, this study provides some insight into CYP2C8 and CYP2C9 expression and function in diabetic and/or NAFLD populations. Future studies in vivo are needed to determine the clinical significance of these findings.

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