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In Vitro Drug Metabolism and Population Pharmacokinetics as Tools for Elucidating Pharmacokinetic Variability

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IN VITRO DRUG METABOLISM AND POPULATION PHARMACOKINETICS AS TOOLS FOR ELUCIDATING PHARMACOKINETIC VARIABILITY

BY

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ABSTRACT

Pharmacokinetic variability is an important consideration in pharmacotherapy to ensure safety and efficacy of medications, thus the understanding of the sources of variability in drug concentrations in the body is imperative. The goals of this dissertation were: (1) To use in vitro drug metabolism tools to characterize the influence of non-alcoholic fatty liver disease (NAFLD) on Cytochrome P450 2B6 (CYP2B6)-mediated hydroxylation of bupropion in human liver microsomes; and (2) To use population pharmacokinetics to characterize the pharmacokinetics of PF-5190457, an inverse agonist of the growth hormone secretagogue receptor (hGHS-R1a). This work has been organized in two parts. Part one is made up of manuscripts I-III; and addresses the first objective. Part two, on the other hand, is made up of manuscript IV, and addresses the second objective. The manuscripts are briefly described below:

Manuscript I: Non-alcoholic fatty liver disease (NAFLD) is a spectrum of liver disorders. It is defined by the presence of steatosis in more than 5 % of hepatocytes with little or no alcohol consumption. The physiological and biochemical changes associated with NAFLD may result in altered expression and activity of drug metabolizing enzymes (DMEs) or transporters. Existing evidence suggests that the effect of NAFLD on CYP3A4, CYP2E1 and MRP3 are more consistent across rodent and human studies. CYP3A4 activity is down-regulated in NASH whereas the activity of CYP2E1 and the efflux transporter MRP3 are up-regulated. However, it is not clear how the majority of CYPs, UGTs, SULTs and transporters are influenced by NAFLD

either in vivo or in vitro. The alterations associated with NAFLD could be a potential source of drug variability in patients and could have serious implications for the safety and efficacy of xenobiotics. In the first manuscript, we reviewed the effects of NAFLD on the regulation, expression and activity of major drug metabolizing enzymes and transporters. We also discussed the potential mechanisms underlying these alterations.

Manuscript II: Diabetes is strongly associated with NAFLD. However, tools for predicting the diabetic status of human liver tissues (HLTs) is lacking. Manuscript II was aimed to establish a model-based approach for predicting the diabetic status of donors of HLTs. The liver tissue as well as demographic and anthropometric information were supplied by Xenotech LLC. Histopathological examination was conducted to characterize NAFLD lesions. HLTs were homogenized and levels of feeding-related hepatic neuroendocrine peptides (active amylin, insulin, c-peptide, glucagon, ghrelin, active GLP-1, GIP, PP, PYY, leptin and MCP-1) determined. The association between diabetes, and these covariates was modeled using multiple logistic regression. The statistically validated model was used to predict new diabetic classes of HLTs. A multiple logistic regression model adequately described the association between diabetes, NAFLD lesions and the neuroendocrine peptides. Liver weight, c-peptide, leptin, PYY, Amylin (active) and steatosis were significant predictors of diabetes. The final model had an AROC curve of 0.89, accuracy of 80%, sensitivity of 82.4% and specificity of 77%. The new diabetic classes showed that hepatic GLP-1 (active) level was 1.4 higher in non-diabetic livers compared to diabetic ones. In addition, the logistic regression model can be used as a tool to verify the diabetic

status of HLTs which are used for drug metabolism studies.

Manuscript III: Despite the initial belief that Cytochrome P450 (CYP) 2B6 is of minor significance, it is now recognized as a clinically relevant drug metabolizing enzyme. The impact of non-alcoholic fatty liver disease (NAFLD) on drug metabolism has been identified; however, it is still unclear how it influences CYP2B6. We used in vitro approaches in human liver microsomes (HLM) and HepaRG cells to investigate the effect of NAFLD on CYP2B6-mediated formation of hydroxybupropion. The presence of NAFLD increased the km significantly ($p < 0.04$) and reduced CYP2B6 intrinsic clearance by 2-fold. The results from the HepaRG cells qualitatively recapitulated findings in the HLMs. Fatty acid accumulation in hepatocytes seems to be involved with the alteration. This investigation contributes to our current knowledge on the influence of NAFLD on CYP2B6 in vitro kinetics and offers a basis for clinical trial in this patient population.

Manuscript IV: PF-5190457 is an inverse agonist of the growth hormone secretagogue receptor (hGHS-R1a), that is undergoing clinical trial for treatment of alcohol use disorder. The purpose of this study was to describe the population pharmacokinetics (PK) of PF-5190457 and to identify demographic and biochemical characteristics that influence its PK variability. Data on drug dosage, sampling times and plasma concentrations were collected retrospectively from two studies: Phase 1a and Phase 1b. Thirty five (35) healthy volunteers were enrolled in the Phase 1a, and 12 non-treatment seeking alcoholic subjects in the Phase 1b trial. The log-transformed concentration and time points were modeled in NONMEM. The influence of patients'

demographic and biochemical characteristics were evaluated; and the accuracy and precision of the model parameters determined using bootstrapping. The predictive performance of the final model was checked using percentile visual predictive checks.

The pharmacokinetics of PF-5190457 was best described by a one-compartmental model with first order absorption after oral administration. The estimated typical pharmacokinetic parameters included the absorption rate constant (ka, 3.6 h^{-1}), oral clearance (CL/F, 80 Lh^{-1}) and apparent volume of distribution (V/F, 575 L). Inclusion of body weight and serum albumin as covariates on V/F reduced the interindividual variability (IIV) associated with V/F by ~28%. Increasing body weight increased V/F, whereas increasing serum albumin levels reduced it. We anticipate that this model would serve as a guide in designing dosage regimen for future clinical trials with PF-5190457.

Conclusion. This work demonstrates that in vitro drug metabolism in human liver microsome has the potential to explain the effect of NAFLD on CYP2B6-mediated hydroxybupropion formation. Similarly, population pharmacokinetic modeling in NONMEM has the capability to elucidate the influence of body weight and serum albumin on the pharmacokinetics of PF-5190457.

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I would like to acknowledge my major professor, Dr. Fatemeh Akhlaghi, for her continuous support and guidance throughout this dissertation. I am grateful she supported me to build my career around my interests. I am really impressed by her extensive network of experts and her ability to find the right person for every challenge. I am also grateful to my committee members Dr. Sara Rosenbaum, Liliana Gonzalez, Prabhani Kuruppumullage Don, and my defense committee chairperson Dr. Ingrid Lofgren. Dr. Gavino Puggioni from the Computer Science and Statistics department played tremendous roles in mentoring and guiding me in making key decisions during my PhD. I am grateful for his help and advice.

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DEDICATION

This work is dedicated to Grace Angela my dear wife, Olive Angela my adorable daughter, and Jeremiel Luther my auspicious son. With inner strength and wisdom from God, we will do exploits!

PREFACE

This dissertation titled "*In Vitro Drug Metabolism and Population Pharmacokinetics as Tools for Elucidating Pharmacokinetic Variability*" is presented in manuscript format.

The first chapter is an introduction of general concepts in Pharmacokinetics and Pharmacokinetic variability. The next four chapters (2-5) are four manuscripts. **Manuscript I** was published in Drug Metabolism Review (Drug Metab Rev. 2017 May;49(2):197-211). **Manuscript II** has been formatted for publication in Diabetes/Metabolism Research and Reviews Journal. **Manuscript III** has been prepared for submission to Diabetes, Obesity and Metabolism Journal. Finally, **Manuscript IV** has been prepared for submission to Clinical Pharmacokinetics Journal.

In all four manuscripts, Dr. Fatemeh Akhlaghi, my major Professor is the corresponding author. Any other contributors have been included as co-authors or acknowledged appropriately.

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BACKGROUND

Introduction

Pharmacokinetics is the study of the processes involved with the absorption (A), distribution (D), metabolism (M) and excretion (E) of drugs from the body. Thus, the body interacts with the drug to absorb it from the site of administration and transport to the site of action to exert its effect before it is finally removed from the body. The ADME processes control the concentration a drug achieves in body compartments after administration of a dose. This implies that changes in the ADME properties can alter pharmacokinetic properties of a drug and ultimately the efficacy and toxicity. To improve the safety and efficacy of drugs, the sources of pharmacokinetic variability must be characterized and applied to individualize dosing. Pharmacokinetic variability results from inter-individual differences that alter pharmacokinetic parameters that control absorption, distribution, metabolism and excretion.

Absorption.

Drugs administered by extravascular route, are absorbed into the systemic circulation. Some drugs may undergo first pass metabolism in the enterocytes of the small intestines and the liver before they finally reach the systemic circulation. The product of the fraction of the drug that is absorbed into the enterocytes (fa), the fraction that escapes metabolism in the enterocyte (fg) and the fraction that escapes first-pass hepatic metabolism (fh) is described by the bioavailability parameter, F.

$$F = fa*fg*fh \quad \text{.....(1)}$$

The rate of absorption of a drug usually follows a first-order kinetics. The important pharmacokinetic parameters of absorption are the absorption rate constant (ka) and the overall bioavailability. The absorption of a drug in the GIT may be affected by the motility of the GIT, pH changes, presence of food, concomitant medication, and transporters. Consequently, these conditions can alter the magnitude of ka and the bioavailability (Rosenbaum, 2011).

Distribution.

The apparent volume of distribution (Vd) is the pharmacokinetic parameter that quantitatively describes the distribution of a drug in the body. It is the ratio of the amount of drug in the body at equilibrium (Ab) and the drug plasma concentration (Cp). Though this parameter does not have a physiological significance, it gives a general idea about the extent of drug distribution in the body. The extent of the distribution of a drug depends on tissue binding and plasma protein binding. The magnitude of Vd depends on whether the drug binds strongly to the plasma proteins or to tissues. A high plasma protein binding may result in a small Vd, whereas a high tissue binding may yield a large Vd.

The rate of drug distribution on the other hand, depends on tissue perfusion and diffusion into cells. Drug distribution is faster in well-perfused tissues than in poorly-perfused ones. In addition, small lipophilic drugs are able to distribute into tissues faster through passive transcellular diffusion. It must also be borne in mind that transporters like p-gp, MRP4 and BCRP play some role in drug distribution (Urquhart and Kim, 2009). The extent of drug distribution is affected by conditions that affect

blood and tissue volume as well as the free fraction of drug in the plasma and tissues. It is anticipated that since tissue-perfusion and diffusion control the rate of distribution, conditions that affect perfusion and diffusion would also affect the rate of distribution.

Elimination.

Drugs are eliminated from the body through metabolism and excretion. The primary organ for metabolism is the liver, whereas the kidney is the cardinal organ for excretion. Metabolism is a process whereby drug molecules are biotransformed into their metabolites by addition of groups that make them more hydrophilic. Drug metabolizing enzymes (cytochrome P450 enzyme, flavin monooxygenase, and UDP-glucuronosyltransferases (UGTs)) and transporters (MRPs, OATPs etc) are involved in the metabolism of drugs.

Renal excretion results from glomerular filtration, active tubular secretion and passive tubular reabsorption. The capillaries of the glomerulus are very permeable and allows neutral molecules of less than 4 nm diameter to pass through into the renal tubules. The presence of transporters in the proximal tubular cells augment renal clearance through tubular secretion (Ho and Kim, 2005, Kusuhara, 2009, Choi and Song, 2008). The uptake transporters involved in this process include OAT1, OAT3 and OCT2 whereas the efflux transporters include P-gp, MRP2 and MRP4. At the distal tubule, drugs may be reabsorbed through passive diffusion depending on the lipophilicity, pH of the filtrate and the flow of the urine.

Elimination incorporates the processes of metabolism and excretion. The elimination

of most drugs follow first order kinetics. The constant of proportionality between the rate of elimination and the plasma concentration is called clearance. Clearance is the most important pharmacokinetic parameter and it is defined as the volume of blood that is completely cleared of the drug per unit time. It is a function of blood flow and the efficiency with which the organs of elimination extract the drug that passes through them. Hence, conditions that affect these organs and their function like diseases affect the clearance and eventually the pharmacokinetics of the drug.

The elimination half-life of a drug.

The ratio of the clearance (Cl) and the volume of distribution (Vd) is called the elimination rate constant (ke). Closely related to the elimination rate constant is the half-life of a drug. It is the time required for the amount of drug in the body to fall by half, and it is estimated as:

$$t_{1/2} = 0.693/ke \quad \text{.....(2)}$$

The elimination rate constant and the half-life are derived parameters and depend on the volume of distribution and clearance which are independent of each other.

The half-life of a drug is particularly important because it helps to determine the time it takes a drug to reach steady state. It also guides the selection of dosing interval in multiple dosing. Since $t_{1/2}$ depends on clearance and volume of distribution, any factor that alters the distribution or clearance of a drug may alter its half-life. This would alter the dosing interval, the time to reach steady state and the time it will take to clear the drug from the body.

Elucidating the sources of pharmacokinetic variability.

To understand the sources of pharmacokinetic variability, pharmacokinetic parameters are investigated under various conditions to elucidate how those conditions influence their magnitude. These conditions include food, gender, lifestyle, genetic polymorphisms, diseases and co-administered drugs.

In this work, two methodologies were employed to investigate pathological and non-pathological sources of PK variability. In the first part of the study (**manuscripts I, II and III**), the influence of non-alcoholic fatty liver disease was investigated *in vitro* using both human liver microsomes (HLM) and steatosis-induced HepaRG cell lines. By incubating each of the two in vitro systems with a CYP2B6 probe substrate, bupropion, we were able to estimate parameters related to the in vitro clearance of bupropion via the CYP2B6 drug metabolizing enzyme pathway. Comparing the values obtained among the control and treatment groups enabled us to establish the influence of NAFLD on CYP2B6-mediated clearance of bupropion. NAFLD was chosen as the disease of interest mainly because it affects the liver, the major organ of clearance; and also because NAFLD is prevalent in the population.

In the second part of the study (**manuscript IV**), population pharmacokinetics using nonlinear mixed effect modeling was employed to determine the pharmacokinetic parameters of a ghrelin inverse agonist, PF-5190457, administered to healthy and non-treatment seeking alcoholic adults. The influence of various covariates including demographic (age, BMI, and Gender, etc.), and biochemical (albumin, serum creatinine, etc.) variables were examined on the pharmacokinetic parameters - ka, CL and Vd.

By employing these two methodologies, we were able to demonstrate the effect of NAFLD on CYP2B6-mediated formation of hydroxybupropion and the factors that influence the disposition of PF-5190457.

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MANUSCRIPT I

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Non-Alcoholic Fatty Liver Disease (NAFLD) - Pathogenesis, Classification, and Effect on Drug Metabolizing Enzymes and Transporters

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Running head: Effect of NAFLD on drug metabolizing enzymes and transporters

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1 MANUSCRIPT I

Abstract

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of liver disorders. It is defined by the presence of steatosis in more than 5 % of hepatocytes with little or no alcohol consumption. Insulin resistance, the metabolic syndrome or type 2 diabetes and genetic variants of PNPLA3 or TM6SF2 seem to play a role in the pathogenesis of NAFLD. The pathological progression of NAFLD follows tentatively a 'three-hit' process namely steatosis, lipotoxicity and inflammation. The presence of steatosis, oxidative stress and inflammatory mediators like TNF-α and IL-6 have been implicated in the alterations of nuclear factors such as CAR, PXR, PPAR-α in NAFLD. These factors may result in altered expression and activity of drug metabolizing enzymes (DMEs) or transporters.

Existing evidence suggests that the effect of NAFLD on CYP3A4, CYP2E1 and MRP3 are more consistent across rodent and human studies. CYP3A4 activity is down-regulated in NASH whereas the activity of CYP2E1 and the efflux transporter MRP3 are up-regulated. However, it is not clear how the majority of CYPs, UGTs, SULTs and transporters are influenced by NAFLD either in vivo or in vitro. The alterations associated with NAFLD could be a potential source of pharmacokinetic variability in patients and could have serious implications for the safety and efficacy of xenobiotics. In this manuscript, we summarize the effects of NAFLD on the regulation, expression and activity of major drug metabolizing enzymes and transporters. We also discuss the potential mechanisms underlying these alterations.

Key Words:

Non-alcoholic fatty liver disease, steatosis, non-alcoholic steatohepatitis, diabetes, drug metabolizing enzymes, transporters, cytochrome P450

Abbreviations

3-DMU - 1, 3- dimethyluric acid; ABC - ATP-binding cassette; AhR - Aryl hydrocarbon receptor; ALT - Alanine transaminase; ARE - Antioxidant response element; AST - Aspartate transaminase; AUC - Area under the curve; BAAT - BMI, Age, ALT, Triglycerides; BCRP - Breast cancer resistance protein; BMI - Body Mass Index; C/EBPs - CCAAT-enhancer-binding proteins (or C/EBPs); CAR - Constitutive androstane receptor; chREBP - Carbohydrate-responsive element-binding protein; CT - Computerized tomographic; CYP - Cytochrome P450; DAGs - Diacyl glycerols; DME - Drug metabolizing enzyme; ER - Endoplasmic Reticulum; FAT/CD36 - Fatty acid translocase; FATPs - Fatty acid transport proteins; FGF21- Fibroblast growth factor 21; GR - Glucocorticoid receptor; GSTs - Glutathione -S-transferases; HAIR - Hypertension, ALT, Insulin resistance; HDL - High-density lipoprotein; HFD - High fat diet; HNF-4 - Hepatic nuclear factors 4; IL-1β - Interleukin-1 β; IL-6 - Interleukin-6; JAK/STAT - Janus kinase / Signal Transducer and Activator of Transcription; keapl - Kelch-like ECH-associated protein 1; LPS - Lipopolysaccharide; LXRα - Liver X receptor alpha; MAPK - Mitogen-activated protein kinase; MCD - Methionine choline deficient; MRI - Magnetic resonance imaging; MRP - Multidrug resistance-associated protein; NADPH - Nicotinamide adenine dinucleotide phosphate (reduced); NAFL - Non-alcoholic fatty liver; NAFLD - Non-alcoholic fatty liver disease; NAS - NAFLD activity score; NASH -

Non-alcoholic steatohepatitis; NASH CRN - NASH Clinical Research Network; NEFA - Nonesterified fatty acids; NF-κβ - Nuclear factor – kappaβ; NIDDK - National Institute of Diabetes & Digestive & Kidney Disease; Nrf2 - Nuclear factor erythroid 2–related factor 2; OATPs - Organic anion transporting polypeptides; OATs - Organic anion transporter; OCTs - organic cation transporter; P-gp – Permeability glycoprotein or P-glycoprotein; PNPLA3 - Patatin-like phospholipase domain containing 3; PPARα - Peroxisome proliferator-activated receptor alpha; PPAR-γ - Peroxisome proliferator-activated receptor gamma; PUFA - Polyunsaturated fatty acids; PXR - Pregnane X receptor; QUICKI - Quantitative insulin sensitivity check index; RXRα - Retinoid X receptor alpha; SAF - Steatosis, activity and fibrosis; SLC - Solute carrier superfamily; SOCS3 – Suppressor of cytokine signaling 3; SREBP1c - Sterol regulatory binding protein-1c; SULT - Sulfotransferases; TAG - Triacylglycerol; TM6SF2 - Transmembrane 6 superfamily member 2; TNF-α - Tumor necrosis factor - alpha; UGTs - Uridine diphosphate; (UDP) - glucuronosyl transferases

1.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of liver disorders (**Figure 1.1**). It is a condition defined by the presence of steatosis in more than 5 % of hepatocytes (Sanyal *et al.*, 2011) with little or no alcohol consumption. NAFLD consists of the benign non-alcoholic fatty liver (NAFL), and the more severe non-alcoholic steatohepatitis (NASH). NASH is a more progressive form of NAFLD and is characterized by steatosis, hepatocellular ballooning, lobular inflammation and almost always fibrosis (Kleiner and Makhlouf, 2016). In an effort to regenerate new cells, NASH progresses (Argo and Caldwell, 2009, Starley *et al.*, 2010) to cirrhosis with the hepatocytes replaced by scar tissues of type I collagen produced by stellate cells. Cirrhosis is an end stage organ failure that require liver transplantation or may lead to hepatocellular carcinoma (Sorensen *et al.*, 2003, Yasui *et al.*, 2011). With progression of NASH to full-blown cirrhosis, some of the histological characteristics of NASH might be lost (Yoshioka *et al.*, 2004).

The metabolic syndrome, formerly known as *Syndrome X*, underlies both non-alcoholic fatty liver disease (NAFLD) and diabetes. It is defined by the presence of at least three of the following (**Figure 1.2**): abdominal obesity, increased triglycerides, reduced high density lipoprotein (HDL) cholesterol, increased blood pressure and hyperglycemia (Alberti *et al.*, 2009). Insulin resistance appears to explain almost all situations of metabolic syndrome (Eckel *et al.*, 2010); and hence diabetes (Groop, 1999) and NAFLD (Marchesini *et al.*, 1999).

Though NAFLD is more prevalent in obese and diabetic patients, it is also present in lean and non-diabetic individuals (Vos *et al.*, 2011, Younossi *et al.*, 2012). It is the

most common cause of cryptogenic cirrhosis (Clark and Diehl, 2003) and approximately 30 -50 % of NASH patients may progress to cirrhosis within 10 years (Jou *et al.*, 2008). NAFLD is not only prevalent in industrialized countries, but also in developing ones. Global prevalence of NAFLD has been reviewed and ranges from 6 - 35 % (Fazel *et al.*, 2016, Sayiner *et al.*, 2016, Bellentani, 2017); and approximately 30% of the population of United States (90 million persons) are estimated to be affected by NAFLD (Fazel *et al.*, 2016). About 70% Americans with diagnosed type 2 diabetes are believed to have NAFLD while 63–87% of patients having both diabetes and NAFLD may have NASH. (Bazick *et al.*, 2015, Corey *et al.*, 2016). The economic burden of NAFLD in four European countries (Germany, France, Italy and the United Kingdom) was projected to be ~35 billion US dollars compared to the approximately 103 billion dollars in the United States (Younossi *et al.*, 2016).

Pharmacotherapy of NAFLD or NASH is an unmet clinical need. To date, no drug has received FDA approval for NASH (Sanyal *et al.*, 2015), thus a clinical or regulatory pathway has not yet been established. Current therapies like vitamin E (Rinella and Sanyal, 2016), pentoxifylline (Zein *et al.*, 2011) and insulin sensitizers such as pioglitazone in patients with diabetes (Cusi, 2016) have been used. Therapies in development include obeticholic acid, a semi-synthetic bile acid analogue undergoing development by Intercept Pharmaceuticals and elafibranor (formerly GFT505) a Peroxisome proliferator-activated receptor (PPAR) alpha and gamma agonist (Rinella and Sanyal, 2016). In view of the lack of standard therapy, international guidelines on NAFLD (European Association for the Study of the Liver (EASL), 2016) recommend lifestyle modifications particularly diet and exercise as

treatment options. Recently, the role of Mediterranean diet in prevention and treatment of NAFLD has been proposed (Abenavoli *et al.*, 2014, Godos *et al.*, 2017).

The main clearance mechanisms of xenobiotics from the body are hepatic, renal and biliary. It has been reported that more than 60 % of commonly prescribed drugs in the United States are cleared hepatically (Williams *et al.*, 2004), indicating the crucial role of the liver in drug metabolism. Hepatic clearance of drugs is achieved through the activities of drug metabolizing enzymes (DMEs) and transporters and hence factors that affect their regulation and activities eventually alter drug disposition.

In this manuscript, we summarize the effects of NAFLD on the regulation, expression and activity of major drug metabolizing enzymes and transporters. In addition, we discuss the various classification systems of NAFLD and the potential mechanisms underlying these alterations. This work however does not include a discussion on models of NAFLD and most findings published before 2011 since these have been reviewed by other groups (Merrell and Cherrington, 2011, Naik *et al.*, 2013).

1.2 Pathogenesis of NAFLD

The mechanisms leading to NAFLD is unclear to date. Several mechanisms have been proposed, but insulin resistance seems to be pivotal in the pathogenesis of both NAFLD and type 2 diabetes (Shulman, 2000, Tarantino and Finelli, 2013). The genetic variant of PNPLA3 (patatin-like phospholipase domain-containing protein 3), an enzyme encoding I148M (rs738409 C/G) and involved in the hydrolysis of triacylglycerols in adipocytes, has been reported to be associated with NAFLD

independent of the metabolic syndrome (Romeo *et al.*, 2008, Sookoian and Pirola, 2011). Similarly, the genetic variant of the lipid transporter located on ER (endoplasmic reticulum) and ER-Golgi compartments, TM6SF2 (transmembrane 6 superfamily member 2), encoding E167K (rs58542926 C/T), causes loss of function of the protein and increases hepatic deposition of triglycerides (Dongiovanni *et al.*, 2015). The pathological progression of NAFLD follows tentatively a 'three-hit' process (Jou *et al.*, 2008) namely steatosis, lipotoxicity and inflammation.

Steatosis results from the interplay between diet, gut microbiota (Jiang *et al.*, 2015, Kirpich *et al.*, 2015), genetic factors (Romeo *et al.*, 2008), and *de novo* lipogenesis via up-regulation of lipogenic transcription factors like sterol regulatory binding protein-1c (SREBP1c), carbohydrate-responsive element-binding protein (chREBP), and peroxisome proliferator-activated receptor gamma (PPAR-γ) (Anderson and Borlak, 2008). Primarily, fatty acid (FA) is stored in the adipose tissue as TAG (triacylglycerol). However, in obese subjects, fatty acids seem to be misrouted from their primary storage site to ectopic sites like skeletal and hepatic tissues for re-esterification into diacyl glycerols (DAGs), perhaps through increased adipocyte lipolysis. The uptake of fatty acid by these organs probably is facilitated by fatty acid transport proteins (FATPs) and FAT/CD36 (fatty acid translocase) which have been shown to be elevated in obese subjects and NAFLD patients (Greco *et al.*, 2008, Fabbrini *et al.*, 2009).

Steatosis leads to increased signaling of the transcription factor NF-κβ (nuclear factor – kappaβ) through the upstream activation of IKKβ (inhibitor of nuclear factor kappaB (NF-κB)). The activation of NF-κβ induces the production of pro-inflammatory

mediators like TNF-α (tumor necrosis factor - alpha), IL-6 (interleukin-6) and IL-1β (interleukin-1 β). These cytokines contribute to the recruitment and activation of Kupffer cells (resident hepatic macrophages) (Anderson and Borlak, 2008) to mediate inflammation in NASH (Ramadori and Armbrust, 2001, Joshi-Barve *et al.*, 2007). Additionally, TNF- α and IL-6 have been reported to play a role in hepatic insulin resistance through the up-regulation of SOCS3 (suppressor of cytokine signaling 3) (Persico *et al.*, 2007, Torisu *et al.*, 2007).

The excess fat in the liver causes lipotoxicity and leads to organelle failure mainly mitochondrial dysfunction and endoplasmic reticulum stress (Browning and Horton, 2004, Bell *et al.*, 2008). A dysfunctional mitochondrion has an elevated capacity to oxidize FA resulting in the production of ROS (reactive oxygen species) and causing oxidative stress due to an imbalance between the production of ROS and protective oxidants. Oxidative stress in NAFLD patients (Sanyal *et al.*, 2001, Tiniakos *et al.*, 2010) is regarded as the third insult that eventually leads to hepatocyte death. The pathogenesis of NAFLD seem to be a vicious cycle of steatosis, lipotoxicity and inflammation resulting in intricate alterations in the histopathological and biochemical features of the liver.

1.3 Diagnosis and Classification of NAFLD

The diagnosis of NAFLD is challenging, as the current available routine techniques (serological tests and imaging techniques) are unable to distinguish between steatosis and NASH. Liver biopsy is considered the gold standard in defining NAFLD and is capable of differentiating steatosis and NASH. It is however, not recommended for routine use due to increased risk of bleeding and complications. In the last decades,

many diagnostic non-invasive tools have been described (**Table 1.1**). Accurate diagnosis of NAFLD is important for its classification. Some of the classification systems available include the scoring systems by Matteoni (Matteoni *et al.*, 1999), Brunt (Brunt *et al.*, 1999), NASH CRN (Clinical Research Network) system (Kleiner *et al.*, 2005), and the SAF (steatosis, activity and fibrosis) system (Bedossa *et al.*, 2012). The different classification systems of NAFLD may thus yield different results and hence introduce variability into scientific investigations.

One of the pioneering works with the largest number of patients and longest follow-up for the stratification of NAFLD patients was carried out (Matteoni *et al.*, 1999). The *Matteoni's system* was based on fat accumulation, inflammation, ballooning degeneration, Mallory hyaline and fibrosis. NAFLD patients were put into four groups: Type I (simple fatty liver), Type II (steatohepatitis), Type III (steatonecrosis) and Type IV (steanecrosis plus either Mallory hyaline or fibrosis). Type I was relatively benign whereas the necrotic forms were considered aggressive. The aggressive forms have higher risk of cirrhosis and liver-related death. Though this system helps to identify patients at risk of cirrhosis and liver-related death, it does not take into account NAFLD in children.

The system developed by Brunt (Brunt *et al.*, 1999, Brunt *et al.*, 2004) is semi-quantitative and evaluates the unique lesions of NASH. It unifies steatosis and steatohepatitis into a 'grade' and fibrosis into a 'stage'(Angulo, 2002). Steatosis is graded on a scale of 1 to 3 depending on the percentage of hepatocytes affected (<33% =1; 33-66% = 2; >66% = 3). Steatohepatitis was similarly graded on a scale of 1 to 3 (1 = mild; 2 = moderate; 3 = severe) but based on the severity and extent of steatosis,

ballooning, lobular inflammation and portal inflammation. Fibrosis on the other hand was staged on a scale of 1 to 4. Brunt's system does not cover the entire spectrum of NAFLD as defined by *Matteoni's system*. Additionally, it was not designed to evaluate NAFLD in children (Kleiner *et al.*, 2005).

In 2005, the Pathology Committee of the NASH Clinical Research Network (NASH CRN) of the National Institute of Diabetes & Digestive & Kidney Disease (NIDDK) came up with a scoring system and NAFLD activity score (NAS) for use in clinical trial (Kleiner *et al.*, 2005). The scoring system was intended to address the full spectrum of lesions of NAFLD. The histological features considered were grouped into five broad categories each with a scoring scale. These features, which were independently associated with NASH, included steatosis (0-3), lobular inflammation (0-3), hepatocellular injury (0-2), fibrosis (0-4) and miscellaneous features like Mallory's hyaline and glycogenated nuclei. The NAS is the unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning scores. NAS of ≥ 5 was found to correlate with the diagnosis of NASH and biopsies with scores of less than 3 were classified as "not NASH". Notwithstanding, not all biopsies with NAS ≥ 5 meet the diagnostic criteria of definite NASH and should be used carefully in establishing the presence or absence of NASH (Brunt *et al.*, 2011). In a number of experimental work involving humans and rodents, a NAS score of at least 4 was considered as NASH (Canet *et al.*, 2014, Ferslew *et al.*, 2015).

Recently, the SAF (steatosis, activity and fibrosis) system has been proposed. The SAF considers steatosis, lobular inflammation and ballooning in defining NAFL and NASH. The activity is defined as the sum of the grades of lobular inflammation and

ballooning and ranges from 0-4. The presence of NAFLD is defined by steatosis in the presence of any degree of activity. This implies that the definition of either NAFL or NASH requires the presence of steatosis (1-3) and varying degree of activity (**NAF**L: *steatosis (1-3) + lobular inflammation (0) + ballooning (0-2),* or *steatosis (1-3) + lobular inflammation (1-2) + ballooning (0)*; and **NASH:** *steatosis (1-3) + lobular inflammation (1) + ballooning (1-2)* or *steatosis (1-3) + lobular inflammation (2) + ballooning (1-2)*) (Bedossa *et al.*, 2012, Kleiner and Makhlouf, 2016).

Clinicobiological scores have also been used in relation to NAFLD for several reasons including selection of patients needing biopsy and prediction of advanced forms of NASH. These clinicobiologial scores make use of indices like body mass index (BMI), Age, AST/ALT ratio, albumin, platelet count, diabetes, hyperglycemia, insulin resistance index, triglycerides, hypertension and others (Angulo *et al.*, 1999, Dixon *et al.*, 2001, Harrison *et al.*, 2003). For instance, 'BAAT' scoring (Ratziu *et al.*, 2000) uses BMI, age, ALT, and serum triglycerides. The BAAT score is calculated as the sum of categorical variables with a scale of 0 to 4. A score of 0 or 1 on the BAAT scale would indicate absence of septal fibrosis. 'HAIR' scoring (Dixon *et al.*, 2001) on the other hand utilizes hypertension, ALT and insulin resistance as an index with a scale of 0 to 3. A score of ≥2 is suggestive of NASH.

1.4 Mechanisms of the alteration of DMEs and Transporters in NAFLD

The influence of diseases on DMEs and transporters is complex due to the associated physiological and pathological changes. For instance, inflammatory conditions have been reported to cause the release of circulating pro-inflammatory cytokines like TNF-

α, IL-1**β,** and IL-6 which act as signaling molecules to mediate the down-regulation of drug metabolizing enzymes partly through the suppression of transcription (Aitken *et al.*, 2006, Aitken and Morgan, 2007). The inflammation models, bacteria endotoxemia (lipopolysaccharide (LPS)) and turpentine have been employed in rodents and hepatocytes to gain some insight into the role of cytokines on the regulation of DMEs and transporters. It seems that in majority of cases, inflammation and the associated cytokines down-regulate the expression and activity of DMEs and some transporters as described in these reviews (Aitken *et al.*, 2006, Morgan, 2009).

Oxidative stress in NAFLD and diabetes causes activation of Nrf2 (nuclear factor erythroid 2-related factor 2) in both experimental (Fisher *et al.*, 2008) and clinical studies (Hardwick *et al.*, 2010). Nrf2 is a specific transcription factor that controls the antioxidant response. It is released from keapl (Kelch-like ECH-associated protein 1) and is translocated to the nucleus where it binds to antioxidant response element (ARE) within promoters of target genes, and induces expression of DMEs and transporters central to the maintenance of oxidative stress inducing molecules (Jaiswal, 2004, Nakata *et al.*, 2006, Zhang, 2006).

Fatty acids regulate gene expression by controlling the activity or expression of key nuclear receptors. *In vitro* studies have identified many transcription factors as possible targets for fatty acid regulation, including hepatic nuclear factors (HNF-4α and γ), PPARα, β, γ1, and γ2, SREBP-1c, retinoid X receptor (RXRα), liver X receptor (LXRα), and others. Some nuclear receptors, PPAR, HNF4 (hepatic nuclear factor), RXRα, and LXRα, bind directly to non-esterified fatty acids (NEFA), but others like SREBP-1c and NF-κB are regulated by fatty acids through indirect

mechanisms (Jump *et al.*, 2005, Jump, 2008). In rodents, SREBP-1c inhibits PXR (pregnane X receptor) and CAR (constitutive androstane receptor) (Roth *et al.*, 2008), and has been shown to be up-regulated in obese insulin-resistant patients (Pettinelli *et al.*, 2009). The modulation of the activity of CAR and PXR by polyunsaturated fatty acids (PUFA) has also been reported (Finn *et al.*, 2009).

In addition, changes in the architecture of the liver in hepatic cirrhosis have been reported to cause reduced liver blood flow, reduced functional hepatocytes and diminished functional capacity of the liver to synthesize serum proteins including albumin (Elbekai et al., 2004, Edginton and Willmann, 2008, Johnson et al., 2010). Collectively, the changes mediated by excess fatty acids, cytokines, oxidative stress, and other mechanisms in NAFLD and diabetes may affect the hepatic metabolism of certain drugs possibly through the alteration of the expression and activity of DMEs and transporters. This could result from host defense mechanisms at the transcriptional as well as pre- and post-translational levels (George et al., 1995, Renton, 2004, Aitken et al., 2006). These aberrant signals disrupt the normal hepatic signaling pathways and eventually dysregulate major drug-metabolism-associated nuclear factors leading to altered drug metabolism in NAFLD and diabetic patients (Naik *et al.*, 2013).

1.5 Hepatic Drug Metabolism

Phase I reactions are mainly oxidative processes and are predominantly carried out by the cytochrome P450 (CYP) enzyme system (Guengerich and MacDonald, 1990, Guengerich, 2008, Guengerich and Munro, 2013). Of the 18 known families of CYP enzymes (Zanger and Schwab, 2013), only a few of the members belonging to families

1, 2 and 3 appear to be relevant to biotransformation of xenobiotics (Cholerton *et al.*, 1992, Zanger and Schwab, 2013). These include CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, and CYP3A5. Non-CYP enzymes involved in phase I reactions include monoamine oxidase, flavin-containing monooxygenase (Rettie *et al.*, 1995, Fisher *et al.*, 2002) and aldehyde oxidase (Johns, 1967).

Phase II biotransformation on the other hand are primarily conjugation reactions and it includes glucuronidation (Meech and Mackenzie, 1997), sulfation (Negishi *et al.*, 2001), and glutathione conjugation (Sofia *et al.*, 1997). The enzymes responsible for these processes are Uridine diphosphate (UDP) - glucuronosyl transferases (UGTs), Sulfotransferases (SULT1A), Glutathione -S-transferases (GSTs) respectively.

Drug transporters are crucial for metabolism of drugs and have been reviewed by several groups (Giacomini *et al.*, 2010). Hepatic transporters are classified into uptake and efflux transporters (Mizuno and Sugiyama, 2002, Mizuno *et al.*, 2003). The main uptake transporters belong to the solute carrier (SLC) superfamily and facilitate the movement of drugs into cells. These include OATPs (organic anion transporting polypeptides), OCTs (organic cation transporter), and OATs (organic anion transporter). The efflux transporters on the other hand belong to the ABC (ATP-binding cassette) superfamily and help move drugs out of cells (Mizuno *et al.*, 2003, Sugiura *et al.*, 2006). Examples include P-gp (P-glycoprotein), BCRP (Breast cancer resistance protein) and MRPs (Multidrug resistance-associated protein).

Several factors have been reported to affect DMEs and transporters. These include

genetic polymorphisms, epigenetic factors, and non-genetic factors. Genetic polymorphisms result in alterations in DNA sequence of genes that regulate the expression of DMEs and transporters; and have led to loss-of-function or gain-of-function variants. The association between genetic polymorphisms and variation of plasma concentration levels of drugs as well as response has been extensively studied (Koren *et al.*, 2006, Elens *et al.*, 2011). Epigenetic influences on drug metabolism have also been reported. These are heritable changes in gene function that are not based on DNA sequence variation, but covalent modification of DNA, modification of histones or microRNA regulation (Pan *et al.*, 2009, Mohri *et al.*, 2010). In addition to the above, non-genetic factors like sex (Schmidt *et al.*, 2001, Wolbold *et al.*, 2003), age (Cotreau *et al.*, 2005, Stevens *et al.*, 2008) and disease state like diabetes (Dostalek *et al.*, 2011 , Dostalek *et al.*, 2012a, Dostalek *et al.*, 2012b) affect the expression and activity of DMEs and transporters.

1.6 Effect of NAFLD on Phase I Drug Metabolizing Enzymes (DMEs)

1.6.1 CYP3A

This gene is part of a cluster of cytochrome P450 genes on chromosome 7q21.1 and includes four genes - 3A4, 3A5, 3A7 and 3A43 (Zanger and Schwab, 2013). It is the most abundant human cytochrome P450 isoform in the liver and is involved in the metabolism of about half of clinically useful drugs (Guengerich, 1999). The CYP3A5 isoform is expressed mostly in Africans (Diczfalusy *et al.*, 2011). It also exhibits wide inter-individual variability in its expression and activity through polymorphisms,

epigenetic and non-genetic influences.

The influence of NAFLD on the expression and activity of CYP3A has been studied using animal and cell culture models, human hepatic tissues, and human subjects (Woolsey *et al.*, 2015). Previous studies in rats and mice models are conflicting. However, a more consistent result have been emerging showing down-regulation of the mRNA and protein expressions, and the corresponding CYP3A activity in NAFLD (**Table 1.2**). This is perhaps due the use of models that are able to simulate better the metabolic and histological lesions of NAFLD. The activity of CYP3A decreased with severity of steatosis (Kolwankar *et al.*, 2007) and with the progression of NAFLD (Woolsey *et al.*, 2015). Dostalek et al. (2011) observed significantly lower protein levels, reduced enzymatic activity of CYP3A4 and unchanged mRNA levels in microsomal fractions of human diabetes mellitus livers (Dostalek *et al.*, 2011). Again, the plasma levels of atorvastatin, a substrate of CYP3A4 (Lennernäs, 2003), has been reported to be elevated in patients with diabetes mellitus (Dostalek *et al.*, 2012b). In view of the high prevalence of NAFLD in the diabetic population, it is likely that NAFLD could be involved in the down-regulation of CYP3A4 activity in the diabetic.

CYP3A genes seem to be regulated by a multiplicity of signaling pathways via CCAAT-enhancer-binding proteins (C/EBP) (Martínez-Jiménez *et al.*, 2005), HNF4 (Jover *et al.*, 2009), PXR (Liu *et al.*, 2008), and CAR (Timsit and Negishi, 2007). A reduced CYP3A4 luciferase reporter activity in steatotic mice suggested a reduced CYP3A4 transcription in NAFLD (Woolsey *et al.*, 2015). The cytokine-mediated down-regulation of CYP3A4 (Werk and Cascorbi, 2014) in the course of the inflammatory response via the JAK/STAT (Janus kinase / Signal Transducer and

Activator of Transcription) pathway (Jover *et al.*, 2002) seem to be clinically relevant in NAFLD and diabetic patients due to circulating cytokines. Additionally, it has been suggested that the hepatic CYP3A4 expression is probably down-regulated by FGF21 (fibroblast growth factor 21) through the receptor-mitogen-activated protein kinase (MAPK) pathway which leads to reduced gene transcription (Woolsey *et al.*, 2016).

1.6.2 CYP2

The CYP2 family contains several of the most important drug metabolizing CYPs including CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP2D6. Some of these members are highly polymorphic (Zanger and Schwab, 2013). The regulation of the subfamilies of CYP2 appears to involve nuclear factors like PXR, CAR, GR, and HNF4α. Conflicting results have been reported in NAFLD and diabetic models. This is perhaps due to differences in models used. Additionally, the polymorphic nature of some of the members of this family could be a source of discrepancy in findings especially where the genotypes involved are not considered. Several groups have studied the effect of NAFLD on CYP2 enzymes. Reduced activity and mRNA expression of CYP2A6, CYP2B6, CYP2C9 and CYP2D6 have been reported in primary human cultured hepatocytes exposed to increasing concentrations (0.25 to 3 mM) of mixture (2:1) of oleic and palmitic acids (Donato *et al.*, 2006). This study suggested probable alterations in some of the CYP2 enzymes in steatosis.

1.6.3 CYP2A6

CYP2A6 is clinically relevant for the hydroxylation of coumarin. The murine ortholog of CYP2A6, Cyp2a5, was found to be elevated in the presence of steatosis

(Li *et al.*, 2013, Cui *et al.*, 2016) similar to the observations made in human hepatic tissues (Fisher *et al.*, 2009). These observations however contradict the observations made by another group (Donato *et al.*, 2006).

1.6.4 CYP2B6

CYP2B6 is an emerging enzyme with significant importance. It is involved in the biotransformation of several clinically relevant drugs like bupropion, efavirenz and cyclophosphamide. It also plays a role in the inactivation of environmental toxins. Recently, *in vivo* and *in vitro* studies using male *Sprague Dawley* rats and rat hepatic tissues respectively showed down-regulation of rat Cyp2b1(rat ortholog of human CYP2B6) activity, mRNA and protein expressions. This observation was made in both steatotic (HF diet) and NASH (MCD-diet) models with pronounce effect in NASH. It appears progression of NAFLD to hepatocellular carcinoma aggravates the decrease in CYP2B6 activity (Gao *et al.*, 2016). Notwithstanding, Fisher and colleagues (Fisher *et al.*, 2009) observed a slight increase in the mRNA levels, but did not observe any change in the protein level and activity of CYP2B6 in steatotic and NASH human liver tissues. Since CYP2B6 is less abundant and highly variable, evaluating the effect of heterogeneous NAFLD on its expression and activity poses a challenge.

1.6.5 CYP2C

The CYP2C family of CYPs are responsible for the metabolism of about 12 % (Wang and Tompkins, 2008) of clinically useful drugs. These include CYP2C8 (paclitaxel, amodiaquine), CYP2C9 (warfarin, tolbutamide) and CYP2C19 (phenytoin,

omeperazole). There seems to be very little information about the CYP2Cs since the last reviews on NAFLD and DMEs (Merrell and Cherrington, 2011, Naik *et al.*, 2013). The available reports suggest alterations of CYP2C in NAFLD. However, the direction of change is not clear, as both increasing and decreasing trends have been observed (Fisher *et al.*, 2009, Li *et al.*, 2016). The AUC of rosiglitazone, an insulin sensitizer and a substrate of CYP2C8 and CYP2C9 (Baldwin *et al.*, 1999), was found to be significantly increased in male mice after high fat and high fructose NAFLD induction (Kulkarni *et al.*, 2016). Nevertheless, it is not clear whether this increase was mediated through down-regulation of the CYP2C8/9 or alteration in transport mechanisms.

1.6.6 CYP2D6

CYP2D6 constitutes about 4 % of total CYP content, yet it is involved in the biotransformation of more than 25 % (Wang and Tompkins, 2008) of clinically useful drugs including dextromethorphan and bufuralol. It is highly polymorphic (Ingelman-Sundberg, 2005) and the few reports are conflicting. In leptin-deficient (*ob/ob*) mice, the protein levels of Cyp2d22 (rat ortholog of human CYP2D6) (Li *et al.*, 2016) were decreased. Similarly, in human liver tissues, CYP2D6 protein levels and activity showed a decreasing trend in NASH (Fisher *et al.*, 2009).

1.6.7 CYP2E1

CYP2E1 is the most studied CYP enzyme in relation to NAFLD. CYP2E1 is involved in the biotransformation of acetaminophen, ethanol, acetone and fatty acid oxidation. It is known for the generation of ROS like hydrogen peroxide, and superoxide anion

radicals (Aubert *et al.*, 2011) due to uncoupling of oxygen consumption with NADPH (Nicotinamide adenine dinucleotide phosphate) oxidation and as a by-product of lipid peroxidation (Robertson *et al.*, 2001). It is therefore considered to probably worsen the oxidative stress associated with diabetes and NAFLD, and may play a key role in the progression of NAFLD (Aubert *et al.*, 2011). In fact, it is suspected to be a contributor to acetaminophen-induced liver injury in obesity and NAFLD (Michaut A1, 2014). There seem to be an increasing number of findings in the literature to support the enhancement of expression and activity of CYP2E1 in NAFLD in both humans and rodents (Chalasani *et al.*, 2003, Abdelmegeed *et al.*, 2012, Aljomah *et al.*, 2015). Results in rat studies have shown a consistent trend of increase in Cyp2e1 expression and activity in MCD (Methionine choline deficient) diet fed rats (Weltman *et al.*, 1996). Diabetes has also been reported to increase the mRNA and protein expressions of CYP2E1 (Lucas *et al.*, 1998, Wang *et al.*, 2003), and perhaps generating tissue-damaging hydroxyl radical in patients (Caro and Cederbaum, 2004).

1.7 CYP1A

The CYP1A subfamily has two functional members oriented head-to-head on chromosome 15q24.1. These are CYP1A1 and CYP1A2 (Zanger and Schwab, 2013). The two are highly inducible by ligands of CAR and AhR (aryl hydrocarbon receptor) (Zanger and Schwab, 2013). CYP1A2 constitutes approximately 15 % of total hepatic CYP enzymes (Wang and Tompkins, 2008). Its substrates include anticoagulants, antidepressants, antihistamines and anticancer agents (Zanger and Schwab, 2013). Reports from different groups about the down-regulation of CYP1A2 in NAFLD appears to be one of the most consistent despite some discrepancies (Merrell and

Cherrington, 2011). The levels of expression of mRNA and protein are decreased in different rodent models of NAFLD (Zhang *et al.*, 2007, Hanagama *et al.*, 2008). In human related tissues, down-regulation of mRNA, protein and activity have been observed (Donato *et al.*, 2006, Fisher *et al.*, 2009).

Significant increases in the systemic clearance of antipyrine and protein levels of hepatic CYP1A2 were observed in diabetic rats possibly due to the enhancement of hepatic CYP1A2-mediated metabolism (Ueyama *et al.*, 2007). Similarly, the metabolism of antipyrine was observed to be increased in patients with type 1 diabetes (Matzke *et al.*, 2000). The hepatic metabolism of theophylline into 1, 3- dimethyluric acid (3-DMU) by CYP1A2 and CYP2E1 were studied using diabetes mellitus rat models (alloxan-induced and streptozotocin-induced). A significant increase in the AUC of 1, 3-DMU was observed in the diabetic rats compared to the controls. Based on *in vitro* rat hepatic microsomal studies, the increased clearance of theophylline was confirmed in the diabetic rats (Kim *et al.*, 2005). Other studies in similar diabetic models have reported similar findings (Bae *et al.*, 2006, DY *et al.*, 2007).

1.8 Effect on Phase II Drug Metabolizing Enzymes (DMEs)

1.8.1 UDP-glucuronosyltransferases (UGTs)

Glucuronidation is the major route for phase II reactions catalyzed by the UDP-glucuronosyltransferases (UGTs). UGTs have been reported to be involved in the glucuronidation of more than 40 % of drugs in clinical use (Wells *et al.*, 2004). They are anchored in the endoplasmic reticulum. Members of the UGT1A and 2B subfamilies appear relevant in humans due to their roles in the elimination of

xenobiotics. In some reports, there was no change in Ugtb1 protein (rat) and UGT2B7 activity (humans) in NASH (Dzierlenga *et al.*, 2015, Ferslew *et al.*, 2015). An earlier work utilizing human liver and kidney microsomes, however, observed a decrease in the activity as well as reduction in the mRNA and protein expression of UGT2B7 in diabetes compared to control (Dostalek *et al.*, 2011). Again, it is not clear whether the presence of NASH in the diabetic livers contributed to this observation. Limited literature on this subject matter does not allow a clear understanding of how the expression and activities of UGTs are modified by diabetes and NAFLD.

1.8.2 Sulfotransferases

Sulfotransferases (SULTs) are cytosolic enzymes that catalyze the sulfonation reaction of xenobiotics and endogenous compounds by adding a sulfonate moiety to a compound to increase its water solubility and decrease its biological activity. In humans three SULT families, SULT1, SULT2, and SULT4 have been reported PPARα mediates the induction of human SULTs, thus implicating a role for fatty acids as endogenous regulators of hepatic sulfonation in humans (Runge-Morris and Kocarek, 2005). In human patients, SULT1A2 was found to be down-regulated in NASH (Younossi *et al.*, 2005); and resulted in decreased plasma levels of acetaminophen-sulfate (Canet *et al.*, 2015). Yalcin and colleagues (Yalcin *et al.*, 2013) also observed that sulfotransferase activity decreased significantly with severity of liver disease from steatosis to cirrhosis. Available reports therefore suggest that the activities of SULT1A1 and SULT1A3 were lower in disease states compared to non-steatotic tissues.

1.8.3 Glutathione-S-transferases

The Glutathione-S-transferases are present as different isoforms - α (A=alpha), μ (M=mu), π (P=pi), Θ (T=theta), and ζ (Z=zeta) (Hayes *et al.*, 2005). They are involved in the conjugation of glutathione (GSH) to reactive drug metabolites, though this reaction can be spontaneous without GST (Dragovic *et al.*, 2010). A number of studies into GST activity in NAFLD and diabetes have found decreased enzymatic activity in *ob/ob* mice (Barnett *et al.*, 1992, Roe *et al.*, 1999) and human liver samples (Hardwick *et al.*, 2010). GSTM2, M4 and M5 expressions were higher in African Americans with NASH than in Caucasians (Stepanova *et al.*, 2010).

1.9 Effect of NAFLD on efflux and uptake transporters

The down-regulation of uptake and up-regulation of efflux transporters in obese and NAFLD have been observed in studies involving rodents and human samples (Canet *et al.*, 2014, Canet *et al.*, 2015). Though interspecies variation limits the use of rodents in modeling human NAFLD, concordance analysis has suggested that both mouse and rat MCD models, as well as mouse *ob/ob* and *db/db* NASH models show some similarity to human transporter mRNA and protein expression, and hence may be useful for predicting altered drug disposition (Canet *et al.*, 2014). Canet et al. (2014) observed mainly up-regulation of mRNA and protein expressions of Mdr1 (multidrug resistance protein), Mrp1-4 (multidrug resistance-associated protein) and Bcrp (Breast cancer resistance protein) in rat and mouse NASH models. Conversely, the Oatps (organic anion transporting polypeptides) mainly showed a down-regulation (Canet *et al.*, 2014). The plasma concentrations of metformin, an anti-hyperglycemic agent, were slightly increased in the WT/MCD and *ob*/Control groups. In *ob*/MCD

mice compared to Wild Type, the plasma concentrations were 4.8-fold higher. These changes were attributed to decreases in the kidney mRNA expression of Oct2 and Mate1, the primary mediators of metformin elimination (Clarke *et al.*, 2015).

In the literature, the influence of NAFLD on MRP2-3 appears more obvious compared to other transporters (Hardwick *et al.*, 2012, Canet *et al.*, 2015). **Table 1.3** shows some of the published work on the effect of NAFLD on MRP3. In MCD diet-induced NASH male *Sprague-Dawley* rats, mislocalization of Mrp2, the canaliculi efflux transporter, was observed. Mrp2 appeared to pocket inward, resulting in a diminished function of effluxing substrates into bile. On the other hand, the sinusoidal Mrp3 efflux transporter increased with respect to protein expression leading to increased efflux of substrates into plasma (Dzierlenga *et al.*, 2015). These findings were consistent with human clinical studies involving MRP3 and its morphine glucuronide (morphine 3 and 6 glucuronides) substrate in NASH subjects (Ferslew *et al.*, 2015). The AUC of morphine glucuronide was 58 % higher in NASH subjects compared to healthy subjects. The Cmax also was also significantly higher in NASH subjects. In addition, fasting levels of total bile acids, glycocholate and taurocholate were also elevated in NASH subjects suggesting up-regulation of the basolateral efflux MRP-3 (Ferslew *et al.*, 2015). Clinical impact of NAFLD/NASH on pharmacotherapy

Though very few clinical studies have reported the impact of NAFLD on pharmacotherapy, they strongly highlight the potential of NAFLD to cause variable drug response, adverse drug reaction and eventually toxicity through alteration of pharmacokinetic profile. Midazolam (Woolsey *et al.*, 2015), morphine (Ferslew *et al.*, 2015) and acetaminophen (Canet *et al.*, 2015) have been evaluated in both healthy and

NAFLD patients. NAFLD seem to increase the AUC of midazolam by reducing the activity of CYP3A4; and similarly increase the AUC of the glucuronide metabolites of morphine and acetaminophen via the up-regulation of the MRP3 efflux transporter. Perhaps, the available evidence in the literature is the main motivation behind the emerging interest in drug disposition in NAFLD patients. Hopefully, additional clinical studies would be conducted to gain more insight into the nature and extent of impact of NAFLD on pharmacotherapy.

1.10 Challenges to studying the effect of NAFLD on DMEs and Transporters

Studying the effect of NAFLD on DMEs and transporters is challenging. First, the pathogenesis of NAFLD is not clearly understood, and is usually asymptotic requiring biopsy for definitive diagnosis. Due to ethical reasons, researchers are unable to routinely obtain biopsies from patients for studies. Also, the presence of co-morbidities particularly diabetes, which is highly prevalent in NAFLD patients, is not accounted for. For instance, it has been demonstrated that antipyrine elimination rate was dependent on the type of diabetes (type 1 versus type 2) and gender (Sotaniemi *et al.*, 2002). It was observed that insulipenia enhanced hepatic microsomal enzyme activity (probably through increased ketone bodies), whereas relative insulin deficiency was associated with decreased metabolic activity (Sotaniemi *et al.*, 2002). Since the presence of diabetes and other demographic characteristics could confound the effect of NAFLD on DMEs and transporters, it may be necessary to account for them. Finally, the absence of consensus on NASH models and NAFLD classification

system to use for experiments has permitted the use of different NASH models and classification systems. For instance, a mice diabetic model of NASH only recapitulated human CYP alterations in NAFLD partially (Li *et al.*, 2016); and hence may be inadequate for all CYPs. This has made comparison of results from some groups difficult. It is hoped that as research advance in this earlier, these procedures would be harmonized to allow comparability of results.

1.11 Conclusion

NAFLD and diabetes are gradually becoming pandemic globally. Limited options are available for the treatment of NASH; hence, several pharmaceutical companies are trying to develop new molecules for this condition. However, lack of knowledge on the effect of NAFLD or NASH on the expression and activity of hepatic DMEs and transporters can impede drug development in this area. Current research findings, though limited and sometimes conflicting, suggest alterations in DMEs and transporters in NAFLD. Few of the results however are consistent across studies and species and includes the down-regulation of CYP3A; and up-regulation of CYP2E1 and MRP3. Results from other DMEs and transporters are either lacking or conflicting. Investigating the influence of NAFLD on DMEs and transporters is challenging because NAFLD is heterogeneous and involves a spectrum of hepatic lesions. The challenges introduce another layer of variability to NAFLD experimental studies. The presence of steatosis, oxidative stress and inflammatory mediators like TNF-α and IL-6 have been implicated in the alterations of nuclear factors in NAFLD. Consequently, the regulation of transcription factors like CAR, PXR, PPAR-α, etc. may change and eventually alter the expression of DMEs and transporters. These

alterations could be potential sources of drug variability in patients and could have serious consequences on safety and efficacy. We recommend more studies in this area to augment our understanding on the effect of NAFLD on drug metabolism.

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Declaration of interest statement:

Author EC and Author FA declare that they have no conflict of interest.

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1.13 Tables

Table 1.1 Biomarkers and imaging techniques employed in diagnosis of NAFLD.

Diagnosis Tools	Technique / Principle	Features	References
Serological Tests	Aspartate aminotransferase (AST)	Raised levels not indicative of NAFLD because AST and ALTs are normal in some NAFLD patients.	(Mofrad *et al.*, 2003, Browning *et al.*, 2004, Bugianesi *et al.*, 2004)
	Alanine aminotransferase (ALT)		
	AST/ALT	> 1 is predictive of fibrosis	
Imaging Techniques	Ultrasonography	Sensitive when steatosis is > 30 % of hepatocytes; Does not distinguish between steatosis and NASH	(Wieckowska and Feldstein, 2008)
	Computerized Tomographic (CT) Scanning Magnetic Resonance Imaging (MRI)	More sensitive than ultrasonography Cannot distinguish between steatosis and NASH Expensive	
	Transient Elastography	Can detect fibrosis but expensive	
Liver Biopsy	Histological evaluation of hepatic tissues. Hepatic lesions like steatosis, inflammation and ballooning are graded; and fibrosis is staged.	Gold Standard but invasive and may be involved with complications and sampling variability Able to detect steatosis and inflammation	(Ratziu *et al.*, 2005, Wieckowska and Feldstein, 2008)

Table 1.2 The effect of NAFLD on CYP3A4/CYP3A5.

Overall, NAFLD progression seem to reduce the activity of CYP3A.

Study	NAFLD Model	NAFLD category	mRNA	Protein	Activity	Activity Probe
(Kolwankar *et al.*, 2007)	Human liver tissues (Ex vivo)	Steatosis	Decreased	Slight decrease	Decreased	Testosterone
(Fisher *et al.*, 2009)	Human liver tissues (Ex vivo)	Steatosis	No change	Slight increase	Decreased	Testosterone
		NASH (fatty)	No change	Decreased	Decreased	
		NASH (not fatty)	No change	Decreased	Decreased	
(Woolsey *et al.*, 2015)	Human Subjects (in vivo)	Steatosis	Not Reported	Not reported	Decreased (2.4 fold)	Midazolam
		NASH			Decreased (2.5 fold)	
	Human liver tissues (Ex vivo)	Steatosis	Decreased (60 %)	Not reported	Not reported	
		NASH	Decreased (69 %)			
	Female Mice (In vivo) HFD	Steatosis	Not reported	Not reported	Not reported	CYP3A4 Luciferase Reporter plasmid
	Huh7 hepatoma cells (In vitro)	Steatosis	Decreased (80 %)	Not reported	Decreased (38 %)	Midazolam
(Li *et al.*, 2016)	ob/ob male Mouse (In vivo) (MCD)	Diabetic	Increase	Slight decrease	Slight decrease	Midazolam
		Diabetic NASH	Increase	Decreased	Slight decrease	

Table 1.3 The effect of NAFLD on MRP3.

Overall, NAFLD progression seem to increase the expression and activity of MRP3

Study	Species	Ref/NAFLD	Endpoint	Change	Probe Substrate
(Hardwick *et al.*, 2012)	Rats (male Sprague-Dawley)	Control/NASH	mRNA level	Significantly increased	
			Protein	Significantly increased	
			Plasma Concentration	Significantly increased	Ezetimibe glucuronide
(Dzierlenga *et al.*, 2015)	Rats (male Sprague-Dawley)	Control/NASH	AUC	150 % increase	Morphine glucuronide
			Protein	Significantly increased	
			Activity	Significantly increased	
(Ferslew *et al.*, 2015)	Human	Healthy/NASH	Cmax	52 % increase in NASH	Morphine glucuronide
			AUC	58 % increase in NASH	Morphine glucuronide
(Canet *et al.*, 2015)	Human (Children)	Healthy/Steatosis /NASH	AUC	Increased	Acetaminophen glucuronide
	Human Liver Tissues	Healthy/Steatosis /NASH	MRP3 Protein	Significantly increased	

1.14 Figures

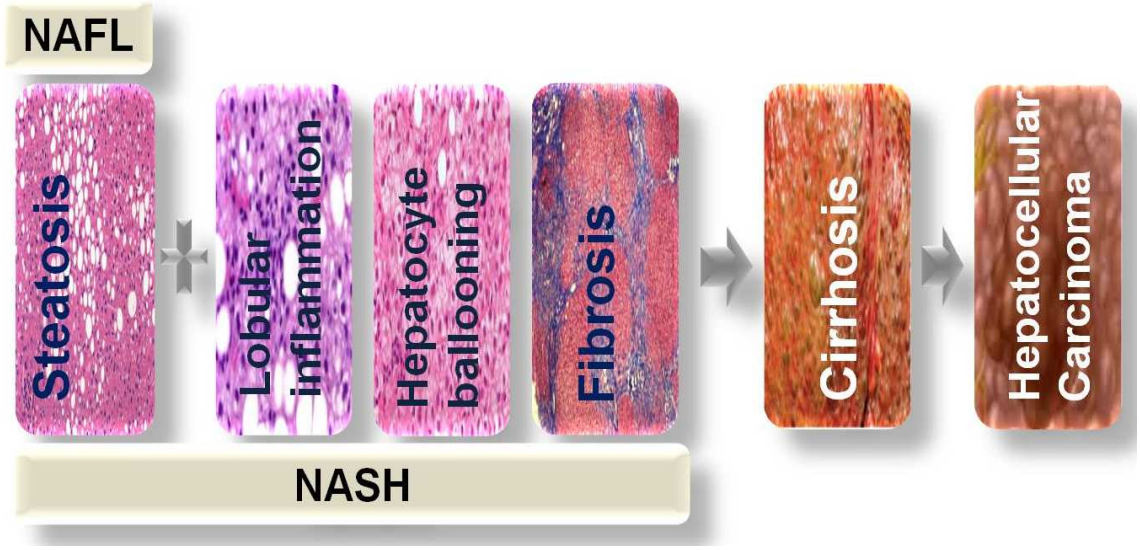

Figure 1.1 The progressive stages of NAFLD (non-alcoholic fatty liver disease)

The benign form of NAFLD, NAFL (non-alcoholic fatty liver), progresses to NASH (non-alcoholic steatohepatitis) with or without fibrosis. Subsequently, NASH leads to cirrhosis and eventually hepatocellular carcinoma.

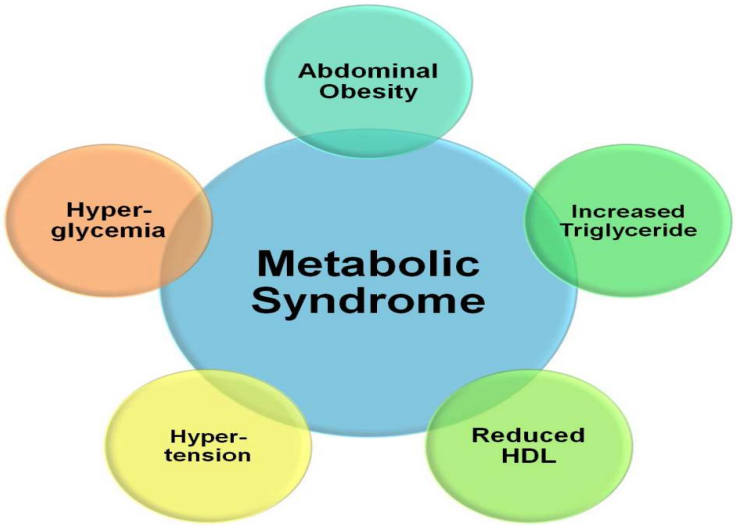

Figure 1.2 Major components of the metabolic syndrome.

Major components of the metabolic syndrome. The presence of at least three of these components define the presence of metabolic syndrome.

MANUSCRIPT II

(For submission to Diabetes/Metabolism Research and Reviews Journal)

Hepatic neuroendocrine peptides and fatty liver strongly predict the diabetic status of donors of human liver tissues

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Running Title: Prediction of the diabetic status of human liver tissues (HLTs)

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2 MANUSCRIPT II

Abstract

Aims: Tools for determination of the diabetic status of human liver tissues (HLTs) used to study the effect of diabetes on drug metabolizing enzymes (DMEs) is lacking. This study is aimed to establish a model-based approach for predicting the diabetic status of donors of HLTs.

Materials and Methods: HLTs, demographic and anthropometric information were supplied by Xenotech LLC. Histopathological examination was conducted to characterize non-alcoholic fatty liver disease (NAFLD) lesions. HLTs were homogenized and levels of feeding-related hepatic neuroendocrine peptides (amylin (active), insulin, c-peptide, glucagon, ghrelin, GLP-1 (active), GIP, PP, PYY, leptin and MCP-1) determined. The association between diabetes, and these covariates was modeled using multiple logistic regression. The statistically validated model was used to predict new diabetic classes of HLTs.

Results: A multiple logistic regression model adequately described the association between diabetes, NAFLD lesions and the neuroendocrine peptides. Liver weight, c-peptide, leptin, PYY, Amylin (active) and steatosis were significant predictors of diabetes. The final model had an AROC curve of 0.89, accuracy of 80%, sensitivity of 82.4% and specificity of 77%. The new diabetic classes showed that hepatic GLP-1 (active) level was 1.4 higher in non-diabetic livers compared to diabetic ones.

Conclusions: Hepatic neuroendocrine peptides and steatosis strongly predicted the diabetic status of HLT donors. The logistic regression model describing this

relationship can be used as a tool to predict the diabetic status of HLTs.

Key Words:

Diabetes, liver, model, neuroendocrine, non-alcoholic fatty liver disease

List of abbreviations

AIC: Akaike information criterion; AROC: Area under ROC curve; BAPP: Bias-adjusted predicted probabilities; BMI: Body mass index; DMEs: Drug metabolizing enzymes; FBG: Fasting blood glucose; GIP: Glucose-dependent insulinotropic peptide; GLP-1: Glucagon-like peptide-1; HbA1c: Hemoglobin A1c; HLTs: Human liver tissues; IGT: Impaired glucose tolerance.; NAFL: Non-alcoholic fatty liver; NAFLD: Non-alcoholic fatty liver disease; NAS: NAFLD activity score; NASH: Non-alcoholic steatohepatitis; OGTT: Oral glucose tolerance test; OR: Odds ratio; PP: Pancratic polypeptide; PYY: Peptide YY; ROC: Receiver operating characteristics; T2D: Type 2 Diabetes

2.1 Introduction

The liver is an important organ involved in the metabolism of many drugs [1]. Consequently, many drug metabolism studies are performed using human liver tissues (HLT) or human hepatocytes [2,3]. Subcellular fractions, mainly microsomes and cytosol, derived from HLTs have been used to study the metabolic pathways of new drugs and also to investigate the influence of demography, polymorphisms and diseases on drug metabolizing enzymes (DMEs) particularly Cytochrome P450 enzymes [4,5]. Unlike primary human hepatocytes and cell lines, human liver tissues are scarce and may not be well characterized with respect to demographic differences, disease conditions, medication use and environmental exposures. Despite these limitations, certain investigations including disease effect on the expression and activity of DMEs, are preferentially conducted in vitro using human liver tissues [6,7].

In recent times, efforts have been made to study the effect of non-alcoholic fatty liver disease on DMEs using HLTs [6,7]. Nonalcoholic fatty liver disease (NAFLD) is a common liver disease with global prevalence ranging from 6-35 % [8-10]. It progresses from simple non-alcoholic fatty liver (NAFL) or steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis, and eventually hepatocellular carcinoma [11]. To delineate the effects of NAFLD on DMEs, HLTs are characterized with respect to steatosis and other NAFLD lesions (inflammations, ballooning, fibrosis, etc.) using histopathological techniques. NAFLD is strongly associated with Type 2 diabetes (T2D) via the metabolic syndrome [12,13.] Unlike NAFLD however, methods to characterize HLTs with respect to T2D is still lacking.

Type 2 diabetes affects over 400 million people globally [14], and like NAFLD, may

influence the expression and activity of DMEs. However, this influence is not clearly understood. To study the influence of diabetes on DMEs, it is similarly important to correctly classify the HLTs with respect to the presence or absence of diabetes. This is important because information on the diabetic status of human livers may be lacking or unreliable. For instance, liver donors with prediabetes or undiagnosed diabetes may be identified as non-diabetic, although their biochemical profile may be diabetic. Conversely, well-managed diabetic donors may be labeled as "diabetic" but may have a more non-diabetic biochemical profile. The mismatch between diabetic labels and the biochemical profile of donor livers can adversely affect the reliability of investigations aimed at studying the effect of disease state on protein expression or enzyme activity. To predict the diabetic status of HLTs, we propose a novel approach that combines vendor-provided information and the biochemical state of the liver tissues to confirm the diabetic status of donors.

In this study, we measured the hepatic concentrations of neuroendocrine peptides associated with feeding and diabetes [15]. They included amylin (active), insulin, c-peptide, glucagon, ghrelin, GLP1-active, GIP (Glucose-dependent insulinotropic peptide), PP (pancreatic polypeptide), PYY (peptide YY), leptin, and MCP-1 (monocyte chemoattractant protein 1). These peptides are essential to glucose homeostasis and some others such as GLP1 and ghrelin, etc. have been targeted for the treatment of diabetes [16,17]. Together with anthropometric information and NAFLD lesions, a multiple logistic regression model was developed and used to predict new diabetic status of the HLT. It is hoped that findings in this study would help in future prediction of HLTs with unknown diabetic status; and pave the way for further

investigations to advance approaches to characterizing the true diabetic status of HLTs used for drug metabolism studies.

2.2 Materials and Methods

2.2.1 Human Liver Tissues Characterization.

Diabetic (n = 51) and non-diabetic (n = 52) human livers were obtained from XenoTech LLC (Lenexa, KS, USA) and were carefully selected based on their similarity in demographic data. The NAFLD lesions were determined at the Liver Research Center of Brown University Medical School according to a previously described scoring system [18]. This scoring system comprises a semi-quantitative grading of steatosis (0-3), lobular inflammation (0-2), hepatocellular ballooning (0-2), and fibrosis stage (0-4). These grades generated a NAFLD Activity Score (NAS) ranging from 0 to 8 excluding the score from fibrosis that is less reversible. Using established algorithms for NAFLD classification (Kleiner et al., 2005, Kleiner and Makhlouf, 2016) the livers were grouped into NoNAFLD (n=29), NAFL (n=34) and NASH (n=27).

2.2.2 Biochemical Analysis.

The concentration of amylin (active), insulin, c-peptide, glucagon, ghrelin, GLP1-active, GIP, PP, PYY, leptin and MCP-1 in liver homogenate were determined using the Milliplex MAP Kit, HMHEMAG-34K, (EMD Millipore Corporation, Billerica, MA) according to manufacturer's instructions with slight modification to the sample preparation. Briefly, liver homogenate was prepared from 200 mg human liver in

phosphate buffer containing recommended protease inhibitors at pH=7.4 using Omni Bead Ruptor 24 (NW Kennesaw, GA, US). The homogenate was first centrifuged to remove debris and the concentration determined on a MagPix (Luminex, Chicago). The concentration of the peptides were expressed in nanogram per gram of liver tissue ($ng.g^{-1}$).

2.2.3 Statistical methods

Statistical analyses. Descriptive statistics was used to summarize the demographic and anthropometric information, NAFLD lesions and neuroendocrine data. Correlation between hepatic neuroendocrine peptides was also obtained. Graphs were generated using GraphPad Prism version 7.0 (GraphPad Software Inc., La Jolla, CA). All statistical analyses and modeling were done in SAS version 9.3 (SAS Institute, Cary, NC). Differences between groups were evaluated using Mann-Whitney U and Kruskal-Wallis tests (PROC NPAR1WAY) for groups with two and greater than two categories respectively. Both tests are nonparametric and were used because they do not require the normality distribution assumption of data required by parametric approaches like T-test and ANOVA (analysis of variance). Statistical differences were considered significant at the $P < 0.05$ level.

Dependent variable. The vendor-provided information about the diabetic status of the HLTs were used. Hence, livers were identified as either diabetic or non-diabetic with no additional information about the type of diabetes. The diabetic status (Y) of the livers was thus a dichotomous variable as shown below:

$$Y = \begin{cases} 1: if\ diabetic \\ 0: if\ nondiabetic \end{cases}$$

The dependent variable was modeled as the logit of (Y), i.e., the logit of a diabetic liver. This is summarized below:

$$\text{logit}(Y) = log\left(\frac{Y}{1-Y}\right)$$

Independent variables. Demographic (age, ethnicity) and anthropometric information (body weight, body mass index (BMI), liver weight) from Xenotech LLC, the NAFLD lesions and concentration levels of the neuroendocrine peptides were used as covariates to examine their effect on the probability of the liver donated by a diabetic individual. Categorical variables were included as dummy variables (0 or 1), whereas continuous variables were modeled without transformation. The continuous variables included the hepatic levels of all the neuroendocrine peptides, age, body weight, BMI and liver weight. The remaining variables - ethnicity, NAFLD lesions were all considered as categorical.

2.2.4 Modeling.

A multiple logistic model was implemented using PROC LOGISTIC to establish the association between the logit of a diabetic liver and the covariates. The model was of the form:

$$\text{logit}(Y) = a + b_1X_1 + \cdots + b_nX_n$$

Where "*a"* is the intercept; "b_1" and "b_n" are coefficients associated with the 1st and nth covariate "X_1" and "X_n". A logistic model was used because the dependent variable was dichotomous. Model results are presented as odds ratios (OR) with 95% confidence intervals.

Model selection and validation. Parsimonious logistic regression models were selected using forward, backward and stepwise procedures in SAS. The adequacy of the logistic model was examined using multiple criteria: residuals from the diagnostics statistics, the model fit statistics (Akaike Information Criterion (AIC)), and the Hosmer-Lemeshow goodness-of-fit test. This approach was intended to minimize the criterion-bias associated with each technique on the model selection.

The model was validated by classification using bias-adjusted predicted probabilities (BAPP) implemented using the CTABLE option of the PROC LOGISTIC model statement [19]. The cut-off probability for deciding whether an HLT donor is diabetic or not was established using a decision probability. Decision rule probabilities ranging from 0.40 to 0.95 were explored. The optimal decision probability was chosen based on accuracy, sensitivity and specificity of the classification. This approach approximates the unbiased method where a training dataset is modeled and the resulting model is used to classify a validation set [19]. The area under the receiver operating characteristic (AROC) curve, accuracy, sensitivity, and specificity of the final models were computed.

Prediction of the new diabetic status of the HLTs. Using the final validated logistic model, and the optimal decision rule from the BAPP validation, new diabetic classes

of the human livers were predicted.

Biological validation of model. The model-based diabetic classes of the HLTs were applied to investigate the differences between the concentration levels of GLP1 whose plasma concentration levels between diabetic and non-diabetic populations have been studied. Secondly, the hepatic levels of leptin, PYY, amylin (active) and C-peptide between the diabetic and non-diabetic groups were compared with corresponding plasma levels reported in literature.

2.3 Results

2.3.1 Demographic, anthropometric and HLT characteristics.

A total of 103 human liver tissues (Diabetic = 51, non-diabetic = 52) were used in this work. The covariates included demographic information, NAFLD lesions characterization, and concentration of neuroendocrine peptides. The covariates were grouped into categorical (**Table 2.1**) and continuous variables (**Table 2.2** and **Table 2.4)** for easy statistical description. The median age of donors were similar; however, the median BMI and liver weight were significantly higher for the diabetic donors compared to the non-diabetic ($p<0.06$). The median of all the neuroendocrine peptides were significantly higher in the non-diabetic group compared to the diabetic, except insulin, c-peptide and leptin. Leptin level was significantly higher in the diabetic group. The levels of insulin and c-peptide were however not different between the two groups.

2.3.2 Correlation among neuroendocrine peptides.

With the exception of insulin, MCP-1 and leptin, the hepatic neuroendocrine peptides investigated in this work correlated with each other significantly **Table 2.5.** PYY showed very strong correlation (correlation coefficient > 0.75, p-value <0.001) with ghrelin, GLP-1 (active), and GIP.

2.3.3 Predictors of HLT diabetic status.

The univariate analyses showed that weight, BMI and liver weight were predictive of the diabetic status of the HLTs (AROC curve = 0.60 and 0.64 respectively). PYY and leptin were the most predictive neuroendocrine peptides (AROC curve = 0.73 and 0.69 respectively). The remaining neuroendocrine peptides were also predictive of diabetic status of the HLT, except c-peptide, and insulin. None of the NAFLD lesions was statistically significant predictor of diabetes, though NAS gave a high AROC curve of 0.56. The bivariate analyses however showed that in the presence of PYY and c-peptide, steatosis and NAS were significant predictors. Similarly, steatosis was predictive in the presence of amylin (active), suggesting a dependence of NAS and steatosis on levels of PYY and amylin (active).

2.3.4 Multiple logistic regression model.

Two final multiple logistic regression models: model 1 and model 2 (**Table 2.3)** were selected. In both models, liver weight, c-peptide and leptin had positive effect on the diabetic status of the HLTs. PYY and amylin (active) on the other hand, had a negative influence, suggesting reduced risk of diabetes with raised hepatic levels. NAS and steatosis positively influenced the diabetic status of livers according to

models 1 and 2 respectively. The AROC curve of model 1 was 0.87, Hosmer-Lemeshow p-value, 0.80; AIC 106.60; and provided optimal classification at a decision probability of 0.55 (accuracy=75%, sensitivity=72.5%, and specificity=77%), **Figure 2.1**. Model 2 on the other hand, had AROC curve of 0.89, a Hosmer-Lemeshow p-value of 0.16; AIC 102.60; and a decision probability of 0.5 provided optimal classification (accuracy = 80 %, sensitivity = 82.4 % and specificity = 77 %) of the liver tissues, **Figure 2.1**. On the basis of a better AIC, AROC curve and classification indices (accuracy, sensitivity and specificity), model 2 was selected as the best model that adequately demonstrated the relationship between the logit of diabetes and the liver weight, c-peptide, leptin, PYY, amylin (active) and steatosis. The graphical representation of ROC (receiver operating characteristic) curve of models 1 and 2 have been presented in **Figure 2.1**. The cross tabulation of vendor-supplied and predicted diabetic labels presented in **Table 2.6,** summarizes the number of livers correctly and incorrectly classified.

2.3.5 Biological validation of model.

GLP-1 is an incretin involved in postprandial insulin regulation [20]. The plasma levels in diabetic and non-diabetic subjects [21,22] have been reported; and hence was used as a marker to test the validity of the predicted classes. We observed a 1.4 fold (non-diabetic/diabetic: 0.58 ± 0.19 / 0.41 ± 0.20 ng/g of liver) decrease in the hepatic levels of GLP1 in diabetic compared to non-diabetic HLTs. This reduction was similar to the 1.4 fold (control/T2D: Fasting levels = 2.87 ± 0.67 / 2.06 ± 0.43; Postprandial levels = 3.42 ± 0.85 / 2.49 ± 0.60 pg/mL) [21] and 1.6 fold (control/T2D: Fasting levels = 0.32 (0.18 - 0.53) / 0.20 (0.13-0.43) pM) [22] decrease in plasma GLP1 levels in T2D

diabetic human subjects.

In the diabetic HLTs, leptin level was higher, whereas amylin (active), PYY, and c-peptide were lower when compared to the non-diabetic HLTs. Leptin plasma levels were elevated in Type 2 diabetes [23], and amylin (active) plasma was lower [24,25]. Pre-prandial plasma level of PYY was reported to be higher in diabetic subjects, however after eating, it did not rise significantly compared to the non-diabetic group where there was a 63.6 % increase in plasma PYY [26]. Another group also showed reduced postprandial plasma PYY level in subjects with a strong history of Type 2 diabetes. These results suggested a defect in the functioning of PYY in diabetic subjects and corroborated with the hepatic levels shown in Figure 2.2. Plasma c-peptide has been used as a measure of the current functioning of pancreatic β-cells, and also to distinguish between Type 1 from Type 2 diabetes [27]. The level we observed suggested a better functioning of pancreatic β-cells in the non-diabetic group compared to diabetic, however excessive increase may increase the risk of diabetes in accordance with the logistic model. In addition, we detected c-peptide in all the livers suggesting that donors may not be Type I diabetic subjects. Though the plasma levels of these peptides may fluctuate with fasting and food intake, the established effects on diabetes and glucose homeostasis [28,29] seem to be adequately described by the logistic regression model described in this work.

2.4 Discussions

Human liver tissues are important *in vitro* tools for drug metabolism studies particularly to investigate the influence of diseases like diabetes and NAFLD on drug

metabolizing enzymes. The correct detection of the influence of each of these pathologies on DMEs depends on the accurate characterization of each HLT with respect to the disease. Currently the techniques to predict or confirm the diabetic status of HLT is limited. To our knowledge, this is the first study to investigate the hepatic levels of feeding-related neuroendocrine peptides, and to model the relationship between diabetes, liver weight, steatosis and the neuroendocrine peptides.

In this study, we used vendor-provided information, hepatic neuroendocrine levels and NAFLD lesions to establish a logistic regression model for prediction of the diabetic classes of HLTs. This study confirmed that neuroendocrine peptides: PYY, leptin, amylin (active) and c-peptides are significant predictors of diabetes. Though steatosis, an important component of the NAFLD spectrum, and NAS were not significant predictors of the diabetic status of HLTs independently, they became significant in the presence of PYY and amylin (active), corroborating current knowledge about the intricate association of diabetes and NAFLD through the metabolic syndrome [10]. Additionally, our findings showed that liver weight was also a significant predictor with or without the presence of neuroendocrine peptides. This is not surprising since the liver weight is proportional to body weight [30] which is a predictor of diabetes. Overall, this study presented a multiple logistic regression model that was capable of predicting new diabetic classes of HLTs.

The most common markers used in clinics for diagnosis and monitoring of diabetes include fasting blood glucose (FBG), oral glucose tolerance test (OGTT), hemoglobin A1c (HbA1c) and c-peptide [31-33]. The FBG, OGTT and HbA1c tests give indication about the plasma glucose concentration, where chronically elevated levels suggest

impaired glucose tolerance (IGT) resulting from defective insulin secretion or function. Plasma c-peptide levels directly measures the functionality of the pancreatic β-cells, which secrete insulin and hence have been used to aid the differentiation between Type 1 and Type 2 diabetes. C-peptide thus serves as a good surrogate for insulin secretion since insulin undergoes significant first pass metabolism [34], and in patients receiving exogenously administered insulin, levels may not be accurate as both exogenous and endogenous insulin are detected together by assays [27]. Our findings, at the hepatic level was in line with the use of c-peptide as a marker for diabetes.

Furthermore, recent drug development efforts for T2D therapies have targeted the incretin system [35,36] and other feeding-related neuroendocrine peptides like amylin [37], leptin and PYY [38]. This is based on their effect on glucose homeostasis and hence association with diabetes. More importantly is the GLP-1 receptor agonists like exenatide [17], liraglutide [16] and semaglutide [39,40], which have been successfully developed for the treatment of T2D. The evidence available in literature thus support that our model is pharmacologically plausible.

The choice of model 2 over model 1 was done on the basis of the improved accuracy, sensitivity and specificity when NALFD was incorporated as steatosis instead of NAS. Notwithstanding, both emphasize the strong association between diabetes, NAFLD and feeding-related neuropeptides at the hepatic level. To prevent autocorrelation in the models, PYY which was a superior predictor was selected instead of GLP-(active), Ghrelin and GIP which correlated strongly. Our model will thus be particularly useful for predicting the diabetic classes of HLT, particularly T2D (since we detected c-

peptide in all the liver tissues), for studying effect of diabetes on DMEs. This model is not meant to be used for clinical diagnosis but for *in vitro* experiments.

Some of the limitations of this work include the lack of information about the presence of prediabetes and the type of diabetes of the liver donors. Secondly, we were unable to ascertain whether the livers were donated preprandially or postprandially. Finally, we did not have the full record of the medications taken by the donors. Notwithstanding, our model was adequate to recapitulate the known relationship between diabetes, feeding-related neuroendocrine peptides, NAFLD and liver weight.

In conclusion, we have presented a pharmacologically plausible model that shows the intricate relationship between diabetes, steatosis and feeding-related peptides. We have also demonstrated the ability of this model to predict the diabetic status of human liver tissues. It is hoped that findings in this study would pave the way for further investigations to develop more approaches to characterizing the diabetic status of human liver tissues used for drug metabolism studies.

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2.6 Tables

Table 2.1 Description of Human Liver Tissue (HLT) donors.
Categorical variables from demographic data (Gender and Ethnicity) and non-alcoholic fatty liver disease characterization (NAFLD).

Variable	Definition	Category	Frequency (D/ND)
Diabetic status	Diabetic (D)	0	52
	Non-diabetic (ND)	1	51
Gender	Female	0	49 (25/24)
	Male	1	54 (26/28)
Ethnicity	Caucasian (0)	0	88 (43/45)
	African American (1)	1	10 (5/5)
	Hispanics (2)	2	5 (3/2)
Steatosis (% of hepatocytes fatty)	< 5	0	39 (19/20)
	5 – 33	1	24 (15/9)
	> 33 - 66	2	20 (8/12)
	> 66	3	20 (9/11)
Lobular_inf (Overall assessment of all inflammatory foci)	No foci	0	26 (11/15)
	2 foci per 200X field	1	60 (31/29)
	2-4 foci per 200X field	2	15 (9/6)
	> 4 foci per 200X field	3	2 (0/2)
Hepatocyte_Ballooning	Non	0	64 (28/36)
	Few balloon cells	1	30 (18/12)
	Prominent ballooning	2	9 (5/4)
Fibrosis	None	0	42 (19/23)
	Perisinusoidal or periportal	1	45 (24/21)
	Perisinusoidal and portal/periportal	2	10 (6/4)
	Bridging fibrosis	3	6 (2/4)
NAFLD Activity Score (NAS)	The unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning scores	0	14 (4/10)
		1	24 (14/10)
		2	16 (10/6)
		3	15 (6/9)
		4	17 (9/8)
		5	9 (5/4)
		6	4 (1/3)
		7	4 (2/2)

Table 2.2 Description of characteristics of Human Liver Tissues (Continuous variables).

Continuous variables from demographic (Age), anthropometric data (Weight, BMI, Liver weight) and hepatic concentration of neuroendocrine peptides (ng/g of liver). Data summarized according to diabetes category.

Variable	Diabetic Status	N	Median (min-max)	p-value
Age (years)	D	51	51 (21-78)	0.7
	ND	52	53.5 (21-76)	
Weight (kg)	D	51	94 (51-213)	0.06
	ND	52	85 (48-159)	
BMI (kg/m^2)	D	51	34 (18-89)	0.008
	ND	52	28.5 (17-54)	
Liver Weight (g)	D	51	1719 (1016-4375)	0.04
	ND	52	1522 (884-3181)	
C-Peptide	D	51	1.3 (0.5-2.7)	0.09
	ND	52	1.6 (0.5-2.9)	
Ghrelin	D	51	0.19 (0.09-0.39)	0.0003
	ND	52	0.27 (0.1-0.62)	
GIP	D	51	0.15 (0.03-0.32)	0.004
	ND	52	0.19 (0.06-0.34)	
GLP1 (Active)	D	51	0.4 (0.15-0.91)	0.002
	ND	52	0.54 (0.09-1.02)	
Glucagon	D	51	0.75 (0.36-2.1)	0.005
	ND	52	1.1 (0.28-2.75)	
Insulin	D	51	5.56 (1.33-24.68)	0.9
	ND	52	5.5 (1.02-23.16)	
Leptin	D	51	95.38 (28.04-276.98)	0.0007
	ND	52	68.68 (15.46-183.5)	
MCP2	D	51	1.67 (0.33-13.18)	0.8
	ND	52	1.72 (0.42-22.38)	
PP	D	51	0.22 (0.05-0.51)	0.008
	ND	52	0.27 (0.1-0.66)	
PYY	D	51	1.46 (0.76-2.17)	0.0001
	ND	52	1.87 (1.04-2.83)	
Amylin_Active	D	51	0.89 (0.27-1.66)	0.007
	ND	52	1.14 (0.51-2.8)	

ND: Non-diabetic; D: Diabetic.

*P-values were obtained using nonparametric Mann-Whitney U test.

Table 2.3 Multiple logistic regression model predicting the diabetic status of human liver tissues.

Model 1 (NAFLD was incorporated as NAFLD activity score (ref=0, when NAS <2));

and Model 2 (NAFLD was incorporated as steatosis, (ref=0, when steatosis <33 %)).

MODEL1

Parameter	Beta Coefficient	Standard Error	p-value	Odds Ratio	95 % Confidence Limit
Intercept	0.5481	1.7122	0.7489		
Liver Weight	0.00122	0.0006	0.0285	1.001	1.000 - 1.002
PYY	-4.2113	1.124	0.0002	0.015	0.002 - 0.134
C-Peptide	2.8297	0.9721	0.0036	16.941	2.52 - 113.872
Leptin	0.0166	0.00607	0.0061	1.017	1.005 - 1.029
Amylin (active)	-2.2311	0.8877	0.012	0.107	0.019 - 0.612
NAS (ref=0)	1.8378	0.6558	0.0051	6.283	1.737 - 22.718

MODEL 2

Parameter	Beta coefficient	Standard error	p-value	Odds Ratio	95 % CI
Intercept	0.85	1.7982	0.6364		
Liver Weight	0.00137	0.0006	0.0206	1.001	1.000 -1.003
PYY	-4.7323	1.2311	0.0001	0.009	0.001 - 0.098
C-Peptide	2.7635	0.9953	0.0055	15.855	2.254 - 111.53
Leptin	0.0152	0.0059	0.0099	1.015	1.004 - 1.027
Amylin (active)	-2.2536	0.8892	0.0113	0.105	0.018 - 0.600
Steatosis (ref=0)	2.3874	0.7327	0.0011	10.885	2.589 - 45.766

2.7 Figures

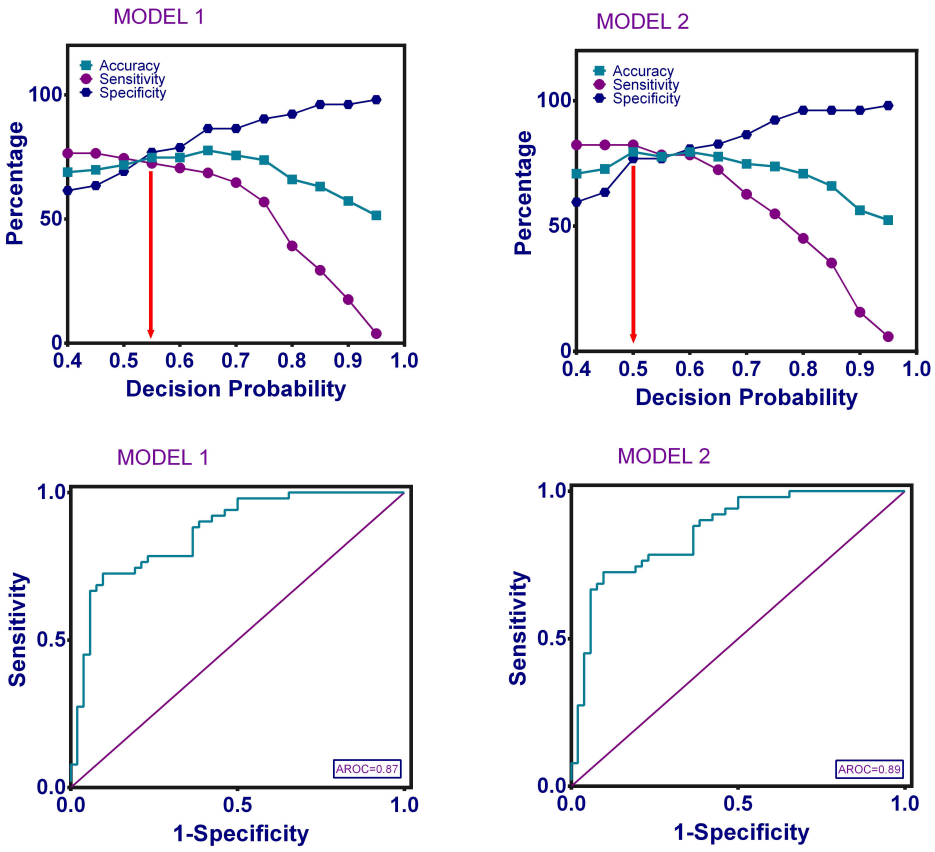

Figure 2.1 Classification indices of logistic regression models.

Graphical representation of classification indices of logistic regression models used for predicting diabetic classes of human liver tissues. Upper graphs: ROC curves for Models 1 and 2; Lower graphs : accuracy, sensitivity and specificity graphs for Models 1 and 2. On the basis of accuracy, sensitivity and specificity a decision probability of 0.55 was selected for Model 1, and 0.5 for model 2. The chosen decision probabilities are shown by arrows on respective graphs. In model 1, NAFLD was incorporated as NAS whereas in Model 2 it was added as steatosis.

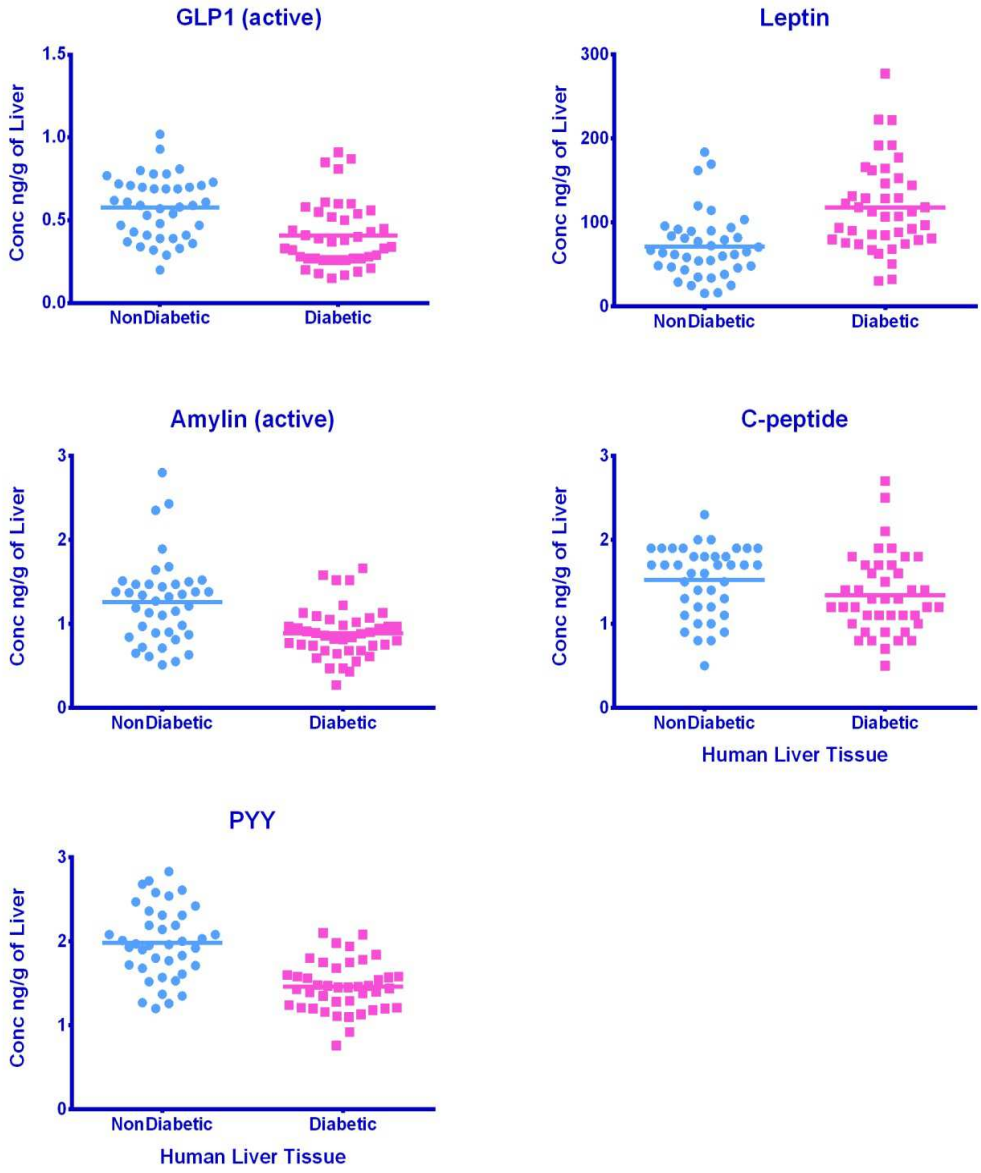

Figure 2.2 Comparison of hepatic levels of feeding related-neuroendocrine peptides.

Feeding related-neuroendocrine peptides used for biological validation of the model (GLP1 (active), leptin, amylin (active), c-peptide and PYY). The hepatic levels were statistically significant ($p < 0.01$) between the non-diabetic (n=42) and the diabetic group (n=40)

2.8 Supplementary Information

Table 2.4 Description of characteristics of HLTs (Continuous variables).

Continuous variables from demographic (Age), anthropometric data (Weight, BMI, Liver weight) and hepatic concentration of neuroendocrine peptides (ng/g of liver). Data summarized according to nonalcoholic fatty liver disease (NAFLD) grouping.

Variable	Category	N	Median (min - max)	P-value
Age	NoNAFLD	39	49 (21 - 78)	0.2
	NAFL	35	54 (33 - 76)	
	NASH	29	51 (33 - 74)	
Weight (kg)	NoNAFLD	39	78 (55 - 213)	0.1
	NAFL	35	93 (48 - 191)	
	NASH	29	91 (69 - 158)	
BMI (kg/m^2)	NoNAFLD	39	28 (18 - 89)	0.2
	NAFL	35	31 (17 - 70)	
	NASH	29	32 (22 - 52)	
Liver Weight (g)	NoNAFLD	39	1465 (884 - 2300)	0.006
	NAFL	35	1902 (1053 - 4375)	
	NASH	29	1819 (1180 - 3685)	
C-Peptide	NoNAFLD	39	1.7 (0.9 - 2.9)	0.0001
	NAFL	35	1.2 (0.5 - 1.9)	
	NASH	29	1.1 (0.8 - 2)	
Ghrelin	NoNAFLD	39	0.31 (0.17 - 0.51)	0.0001
	NAFL	35	0.19 (0.09 - 0.58)	
	NASH	29	0.19 (0.09 - 0.62)	
GIP	NoNAFLD	39	0.21 (0.09 - 0.34)	0.001
	NAFL	35	0.15 (0.03 - 0.29)	
	NASH	29	0.15 (0.06 - 0.27)	
GLP (active)	NoNAFLD	39	0.55 (0.27 - 1.02)	0.0001
	NAFL	35	0.39 (0.15 - 0.93)	
	NASH	29	0.37 (0.09 - 0.81)	
Glucagon	NoNAFLD	39	1.09 (0.4 - 2.17)	0.03
	NAFL	35	0.74 (0.36 - 2.75)	
	NASH	29	0.83 (0.28 - 1.76)	
Insulin	NoNAFLD	39	6.23 (1.96 - 23.16)	0.2
	NAFL	35	5.56 (1.33 - 24.68)	
	NASH	29	4.77 (1.02 - 22.99)	
Leptin	NoNAFLD	39	74.24 (15.46 - 191.78)	0.3
	NAFL	35	81.01 (30.24 - 276.98)	

	NASH	29	96.62 (24.65 - 222.36)	
MCP1	NoNAFLD	39	1.66 (0.33 - 9.09)	0.6
	NAFL	35	1.63 (0.51 - 13.18)	
	NASH	29	2.42 (0.42 - 22.38)	
PP	NoNAFLD	39	0.32 (0.18 - 0.66)	0.0001
	NAFL	35	0.22 (0.1 - 0.42)	
	NASH	29	0.2 (0.05 - 0.41)	
PYY	NoNAFLD	39	1.95 (1.35 - 2.68)	0.0001
	NAFL	35	1.45 (0.76 - 2.83)	
	NASH	29	1.47 (0.92 - 2.47)	
Amylin (Active)	NoNAFLD	39	1.19 (0.47 - 2.8)	0.008
	NAFL	35	0.87 (0.27 - 1.89)	
	NASH	29	0.9 (0.55 - 1.52)	

*P-values were obtained using nonparametric Kruskal-Wallis test.

Table 2.5 Correlation between the hepatic neuroendocrine peptides.
Correlation between the hepatic neuroendocrine peptide concentrations (ng/g of liver).

	C-Peptide	Ghrelin	GIP	GLP-1 (Active)	Glucagon	Insulin	Leptin	MCP-1	PP	PYY	Amylin (active)
C-Peptide	1.00										
Ghrelin	0.71	1.00									
	<.0001										
GIP	0.67	0.74	1.00								
	<.0001	<.0001									
GLP-1 (Active)	0.71	0.74	0.63	1.00							
	<.0001	<.0001	<.0001								
Glucagon	0.33	0.60	0.52	0.48	1.00						
	0.0007	<.0001	<.0001	<.0001							
Insulin	0.09	0.11	0.04	0.20	0.05	1.00					
	0.4	0.3	0.7	0.0	0.6						
Leptin	-0.17	-0.28	-0.25	-0.25	-0.14	-0.06	1.00				
	0.09	0.004	0.01	0.010	0.1	0.6					
MCP-1	0.02	-0.04	-0.01	0.08	-0.12	0.03	-0.12	1.00			
	0.9	0.7	0.9	0.4	0.2	0.7	0.2				
PP	0.68	0.62	0.74	0.62	0.42	0.02	-0.23	-0.01	1.00		
	<.0001	<.0001	<.0001	<.0001	<.0001	0.8533	0.0188	0.9002			
PYY	0.68	0.83	0.79	0.75	0.59	0.10	-0.26	-0.12	0.72	1.00	
	<.0001	<.0001	<.0001	<.0001	<.0001	0.3	0.01	0.2	<.0001		
Amylin (active)	0.61	0.51	0.52	0.45	0.26	-0.08	-0.12	0.08	0.46	0.52	1.00
	<.0001	<.0001	<.0001	<.0001	0.007	0.4	0.2	0.4	<.0001	<.0001	

Correlation coefficient are aligned to the left (shaded rows); and p-values associated with correlation coefficients aligned to the right (unshaded rows).

Table 2.6 Cross tabulation.

Cross tabulation of vendor-provided diabetic labels and predicted diabetic classes.

Vendor-provided Diabetic label	Predicted Diabetic Class		
	ND	D	Total
ND	40	12	52
D	9	42	51
Total	49	54	103

ND: Non-diabetic; D: Diabetic; Shaded cells are correctly predicted livers.

40 out of 52 livers were predicted correctly as non-diabetic. 42 out of 51 liver tissues were predicted correctly as diabetic.

CHAPTER 4: MANUSCRIPT III

(For publication in Diabetes Obesity and Metabolism Journal)

Nonalcoholic fatty liver disease (NAFLD) alters the *in vitro* kinetics of the CYP2B6-mediated formation of hydroxybupropion in human liver tissues and HepaRG cells.

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Running Title: NAFLD alters CYP2B6-mediated hydroxylation of bupropion

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3 MANUSCRIPT III

Abstract

Despite the initial belief that Cytochrome P450 (CYP) 2B6 is of minor significance, it is now recognized as a clinically relevant drug metabolizing enzyme. The impact of non-alcoholic fatty liver disease (NAFLD) on drug metabolism has been identified; however, it is still unclear how it influences CYP2B6. We used *in vitro* approaches in human liver microsomes (HLM) and HepaRG cells to investigate the effect of NAFLD on CYP2B6-mediated formation of hydroxybupropion. The presence of NAFLD increased the km significantly ($p < 0.04$) and reduced CYP2B6 intrinsic clearance 2-fold. The results from the HepaRG cells qualitatively recapitulated findings in the HLMs. Fatty acid accumulation in hepatocytes seems to be involved with the alteration. This investigation is hoped to contribute to our current knowledge on the influence of NAFLD on CYP2B6 *in vitro* kinetics and offers the opportunity for further studies in a clinical trial.

Keywords

Bupropion, Hydroxybupropion, Cytochrome P450 2B6, fatty acids, NAFLD, NASH, Steatosis,

Abbreviations:

AUC - Area Under (the plasma concentration) Curve

BSA - Bovine Serum Albumin

CAR/NR1I3 - Constitutive Androstane Receptor; nuclear factor subfamily 1, group I, member 3

CLint - Intrinsic Clearance

CYP - Cytochrome P450

FAF - Fatty Acid Free

EMEM -Eagle's Minimum Essential Medium

HBUP - Hydroxy Bupropion

HF - High-fat

HLT - Human Liver Tissue

HLM - Human Liver Microsome

km - Michaelis-Menten constant

MCD - Methionine-Choline Deficient

MPPGL - Microsomal Protein Per Gram of Liver

NAFLD - Non-Alcoholic Fatty Liver Disease

NAS - NAFLD activity score

NASH - Non-Alcoholic Steatohepatitis

PBPK - Physiologically-based Pharmacokinetics

PBS – Phosphate Buffer Saline

PXR/NR1I2 - Pregnane X receptor; Nuclear factor subfamily 1, group I, member 2

ROC - Receiver Operating Characteristic

UPLC/ MS/MS - Ultra Performance Liquid Chromatography tandem Mass Spectrometry

Vmax - Maximum Velocity

3.1 Introduction

Cytochrome P450 (CYP) 2B6 is one of the drug metabolizing enzymes (DMEs) mainly expressed in the liver (Mimura et al., 1993). Though it is considered a minor DME, it is involved in the biotransformation of clinically relevant drugs like bupropion (Hesse et al., 2000), efavirenz (Ward et al., 2003), and cyclophosphamide (Xie et al., 2003). Bupropion and efavirenz are also used as sensitive probe substrates for phenotyping CYP2B6 activity via the formation of hydroxybupropion (Hesse et al., 2000) and 8-hydroxyefavirenz (Ward et al., 2003) respectively.

The expression and activity of CYP2B6 is highly variable among individuals. The sources of variability include polymorphisms in the CYP2B6 gene, induction via the constitutive androstane receptor (CAR, NR1I3) and pregnane X receptor (PXR, RN1I2) (Faucette et al., 2006), inhibition by potent agents like ticlopidine (Turpeinen et al., 2005), and disease state. The study of hepatic diseases as a source of variability in DMEs is crucial because, hepatic clearance constitutes about 60% of major clearance mechanisms of clinically relevant drugs (Williams et al., 2004).

The effect of alcoholic liver disease (ALD) on the disposition of a single dose (200 mg) bupropion was studied in human subjects (healthy (n=8) and ALD (n=8)) (DeVane et al., 1990). Compared to healthy subjects, the ALD subjects had elevated AUC of bupropion (~57 %) and hydroxybupropion (~53 %). The changes in the other metabolites of bupropion, erythrohydrobupropion (EB) and threohydrobupropion (TB), were however minimal. The apparent clearance of bupropion reduced from 187 to 145 L/hr in ALD subjects. There was also a large intersubject variability in the pharmacokinetics of bupropion, especially in the ALD subjects, where bupropion half-

life ranged from 2.2 - 29.9 hours. Though the study was conducted in a limited number of subjects, the findings highlighted the potential influence of hepatic diseases on the disposition of bupropion, hence on CYP2B6 that is responsible for the formation of hydroxybupropion.

Similar to ALD is nonalcoholic fatty liver disease (NAFLD) (Toshikuni et al., 2014). NAFLD is emerging as one of the commonest liver diseases worldwide. Its global prevalence is estimated to range from 6-35 % (Bellentani, 2017, Cobbina and Akhlaghi, 2017). NAFLD progresses from steatosis, also known as nonalcoholic fatty liver (NAFL), to fibrosis, non-alcoholic steatohepatitis (NASH), cirrhosis, and eventually hepatocellular carcinoma (HCC) (Angulo, 2002). These lesions cause alterations in inflammatory and biochemical pathways which subsequently interfere with normal hepatic regulation of drug metabolism and glucose homeostasis (Cobbina and Akhlaghi, 2017). NAFLD is strongly associated with the metabolic syndrome and is common in obesity (Bellentani et al., 2010), Type 2 diabetes (Anstee et al., 2013) and HIV/AIDs patients (Verna, 2017). In view of the high prevalence of NAFLD in the population, several groups have evaluated the impact of NAFLD on drug metabolizing enzymes (Fisher et al., 2009, Woolsey et al., 2015, Canet et al., 2015, Ferslew et al., 2015).

Though a number of the studies that examined the impact of NAFLD on DMEs did not observe alterations in the expression and activity of CYP2B6, others have highlighted the potential impact of NAFLD on CYP2B6 where the presence of NASH reduced gene expression (Yoneda et al., 2008, Stepanova et al., 2010). In high-fat induced steatotic male C57/BL6 mice, the expression of Cyp2b10, (the mouse

ortholog of the human CYP2B6), was reduced (Kirpich et al., 2011). Similarly, in human liver tissues the gene expression of CYP2B6 was reduced in the Non-NASH group (Stepanova et al., 2010, Yoneda et al., 2008). A more recent study in Sprague−Dawley rats fed with a methionine/choline deficient (MCD) diet to induce NASH, showed marked reduction in activity, mRNA and protein expression of Cyp2b1 (rat ortholog of human CYP2B6) (Cho et al., 2016). Additionally, the *in vitro* kinetic parameters determined using the rat liver microsomes showed 26 % reduction in Vmax and 2.4 fold reduction in Km of Cyp2b1. Consequently, the bupropion AUC in the rat NASH model was about 1.9-fold higher compared to control. Conversely, the same study reported no change in the activity, mRNA and protein expression of Cyp2b1 in high-fat diet induced steatotic rats (Cho et al., 2016). Another key group observed increasing trend ($p=0.003$) in the relative mRNA expression of CYP2B6 in NAFLD human liver tissues compared to control. Relative protein expression, however, showed a decreasing trend from steatosis to NASH (no longer fatty) (Fisher et al., 2009). The current evidence in the literature do not agree very well. This lack of consensus may emanate from the variableness of the expression and activity of CYP2B6; and disparity in the different models used for the investigations (Li et al., 2018).

Despite the important contributions by other groups to this field, the activity together with *in vitro* kinetics of CYP2B6 in human liver tissues have not been evaluated with respect to NAFLD. Though, gene and protein expression information helps us gain mechanistic insight into the expression of enzymes, for the purposes of translational pharmacokinetics (*in-vitro-in-vivo* extrapolation (IVIVE)), information about the

activity and kinetics of an enzyme is more useful. Hence, in this study, we investigated the influence of NALFD on the activity and kinetics of CYP2B6 using bupropion hydroxylation as probe. To reduce the heterogeneity in the human liver tissues, the livers were characterized with respect to demography, genetic polymorphisms, and diabetes. We examined further the effect of NAFLD on CYP2B6 using fatty-acid-treated HepaRG cell lines. With the aid of a physiologically-based pharmacokinetic (PBPK) model (Rowland et al., 2011) implemented in Simcyp simulator (Jamei et al., 2009), we simulated the potential effect of NAFLD on the biotransformation of bupropion to hydroxybupropion. This investigation is hoped to contribute to our current knowledge on the influence of NAFLD on CYP2B6 *in vitro* kinetics and offers the opportunity for further studies in a clinical trial.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents.

Bupropion hydrochloride (BUP), hydroxybupropion (HBUP), hydroxybupropion-d6, chlorzoxazone (CZ), 6-hydroxy chlorzoxazone (6-OH CZ), and 6-hydroxy chlorzoxazone-d2 were purchased from Toronto Research Chemicals Inc. (North York, Canada); LC/MS-grade, acetonitrile, ammonium acetate and formic acid were from Fisher Scientific (Fair Lawn, NJ); potassium phosphate monobasic and potassium phosphate dibasic were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase was obtained from EMD Millipore (Billerica, MA). All other reagents and solvents were obtained from general commercial suppliers and used without further purification. Solutions were prepared in accordance with manufacturers' instructions.

3.2.2 Experiments in Human Liver Tissues

Characterization of human liver tissues (HLT). Human livers from donors with diabetes (n=53) and without diabetes (n=53) were obtained from XenoTech LLC (Lenexa, KS, USA). These were matched based on age, gender and degree of liver fat. The presence of NAFLD was established by histological evaluation of the livers in a blinded fashion. A semi-quantitative grading of steatosis (0-3), lobular inflammation (0-2), hepatocellular ballooning (0-2), and fibrosis staging (0-4) permitted the classification of the livers into NoNAFLD, NAFL and NASH according to the SAF (steatosis, activity and fibrosis) algorithm (Bedossa et al., 2012, Kleiner and Makhlouf, 2016, Cobbina and Akhlaghi, 2017). The diabetic status of livers were confirmed using a logistic regression model established by our lab [manuscript in preparation]. The model had an AROC curve of 0.89 and was accurate (80 %), sensitive (82.4%) and specific (77%). Predicted probability of each liver being diabetic was determined. Using a previously established decision probability of 0.5, liver was categorized nondiabetic if predicted probability was less than 0.5 and diabetic if greater than 0.5. The predicted diabetic class was compared with the original vendor-provided labels to determine the correctly classified and the misclassified (false positives and negatives) livers. This approach was carried out to reduce intrinsic experimental error due to misclassification of the diabetic status of HLTs. Misclassified livers were removed from analyses when CYP2B6 activity and expression were compared between diabetic and nondiabetic groups.

Human liver microsome (HLM) isolation: The human liver microsomes were prepared by differential ultracentrifugation. Briefly, 200 mg of HLT was homogenized in 100 mg liver tissue/ 300 uL of homogenization buffer (100 mM potassium phosphate buffer, pH=7.4; 1 mM EDTA; 0.1 mM potassium chloride; 8.55 g of sucrose; and 0.02 mM butylated hydroxytoluene) using Omni Bead Ruptor 24 (NW Kennesaw, GA, US). Homogenate was centrifuged at 10,000g for 30 min at 4 ºC and the supernatant was collected and centrifuged at 100,000g for 60 min at 4 ºC. The supernatant, cytosolic fraction, was collected and stored at -80°C. The microsomal pellet was then washed with 100 mM potassium pyrophosphate buffer (pH=7.4) and re-suspended in 1000 mg liver tissue/ 660 uL of storage buffer (100 mM phosphate buffer, pH=7.4; 20% glycerol and 1 mM EDTA). Prepared microsomes were stored at -80°C until analysis. Total protein concentrations were determined using a bicinchoninic acid (BCA) assay in accordance with manufacturer's instructions (Pierce-Fisher, Rockford, IL, USA).

Human Liver Microsomal Incubation*:* The activity of CYP2B6 was measured using bupropion hydroxylation, by examining varying concentrations of bupropion (0, 10, 50, 100, 400, 800, 1600 uM) in an incubation buffer (100 mM potassium phosphate buffer (pH=7.4), and ~3 mM magnesium chloride, and 1 mM EDTA) containing 0.05 $mg.mL^{-1}$ of HLM. All incubations were carried out in accordance with previously described methods with slight modification (Faucette et al., 2000, Walsky and Obach, 2009). The incubation mixture containing bupropion was pre-incubated for 5 mins in a water bath at 37 ºC; and reaction initiated by addition of ~1.3 mM NADPH. This

was incubated for 10 mins and then terminated with 20 uL of ice cold 5:92:3 acetonitrile/water/formic acid containing hydroxy bupropion-d6 internal standard. The final incubation mixture was 200 uL. After centrifugation at 3000 rpm for 10 mins at 4 ^{o}C, the supernatant was collected and the amount of hydroxybupropion formed was quantified using liquid chromatography/ tandem mass spectrometry methodologies (UPLC-MS/MS). The rate of hydroxybupropion formation was then calculated and the in vitro kinetics estimated.

RNA Content determination using Reverse transcription-polymerase chain reaction (qRT-PCR)*:* Total RNA was isolated from the HLTs using the RNeasy Mini Kit (Qiagen, Valencia, CA). The total RNA was reverse-transcribed, and the single stranded DNA was used for real-time PCR. The mRNA expression of hepatic CYP was quantified by real-time PCR using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems) at least two times according to the manufacturer's instructions. Primer sequences for the CYP2B6, CAR, and PXR are reported in supplementary **Table 3.5.** 18S ribosomal RNA (rRNA) was also quantified as an internal control.

CYP2B6 Genotyping of HLTs*:* Genomic DNA was isolated from the HLTs using QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions and genotyped for two single nucleotide polymorphisms (SNPs) in the P450 CYP2B6: CYP2B6*5 (rs3211371; 25505C>T) and CYP2B6*6 (rs3745274; 15631G>T). The CYP2B6 SNPs were determined by Taqman® Allelic

Discrimination Assays and Taqman Genotyping Mastermix (Applied Biosystems, Foster, CA) on ViiA™ 7 Real-Time PCR System (Applied Biosystems).

3.2.3 Experiments in HepaRG Cell Lines

Cell Culture: HepaRG cell lines have been used for drug metabolism studies because of its ability to express various Cytochrome P450 enzymes including CYP2B6 (Kanebratt and Andersson, 2008). The HepaRG cells (passages 2-5) were obtained from Biopredic International (Rennes, France), and were cultured and differentiated in accordance with manufacturer's instructions. Steatosis was induced with 500 μM fatty acids (1:2 palmitate : oleate) conjugated to albumin in 30% essential fatty acid free (FAF) bovine serum albumin (BSA) solution (Invitrogen, Grand Island, NY) by modifying a previously described method (Brown et al., 2013). The cells were treated for 72 h and media replenished after 48 h.

Oil Red O Staining: Fatty-acid-treated and control HepaRG Cells were washed twice with PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich, Saint Louis, MO) for 30 min at room temperature. Cells were washed with PBS and subsequently incubated with Oil Red O (Sigma-Aldrich, Saint Louis, MO) in PBS for 20 mins at room temperature. After three washes, cells were incubated with hematoxylin (Sigma-Aldrich, Saint Louis, MO) for 1 min to stain the nuclei and imaged to examine intracellular lipid accumulation using EVOS cells imaging systems (Life technologies, Foster City, CA). Subsequently, the cells were examined spectrophotometrically in 100% isopropanol using Spectromax (Molecular Devices, Sunnyvale, CA) at 492 nm.

***RNA Extraction*:** For mRNA measurements, total RNA was isolated from both control and fatty-acid-treated HepaRG cells using the RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The quantity and quality of the RNA were determined using Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The cDNA was prepared from 1 μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. DNAse treatment (Qiagen, Valencia, CA) was included to avoid genomic DNA contamination.

***Real-Time PCR*.** Quantitative real-time PCR analyses of both fatty-acid-treated and control were performed with specific sets of Taqman primers and Taqman probes for CYP2B6 (Hs03044634_m1), PXR (Hs01114267_m1), CAR (Hs00901571_m1), 18S (Hs03003631_g1), GAPDH (Hs99999905_m1) and β-actin (Hs99999903_m1) using a ViiA™ 7 Real-Time PCR system in accordance with the manufacturer's instructions. GAPDH, 18 S and β-actin were used as endogenous controls to normalize the data. All runs were carried out in triplicate; and data analyzed using DataAssist software (Thermo Fisher Scientific, Waltham, MA).

Insulin-stimulated phosphorylation of AKT: Following 72 h treatment, HepaRG cells were placed in serum free EMEM media for 12 h followed by stimulation with recombinant human insulin (Roche Diagnostics, Indianapolis, IN) at 1, 10 or 100 nM for 10 minutes at 37^0C. Samples were rinsed in PBS and lysed with RIPA buffer (150mM NaCl, 50mM Tris, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA) supplemented with complete protease inhibitor cocktail (Roche Diagnostics) and HALT^{TM} phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA) for 30

minutes at 4^0C. The lysate was run on sandwich ELISA kit that detected both pAkt (Ser473) and total Akt (Abcam, Cambridge, MA). The assay was performed in accordance with the manufacturer's protocol.

Activity studies: Both control and fatty-acid-loaded HepaRG Cells were incubated with varying concentrations of bupropion (100, 250, 500, 750, 1000, 2500 uM), and chlorzoxazone (500, 750, 1000 μM) for 1 and 8 h in Williams' medium E in triplicates. The compounds were dissolved in DMSO in such a way that the final concentration was less than 0.1%. The reaction was terminated with equal volume of ice-cold acetonitrile containing 0.1% formic acid and deuterated internal standards (hydroxybupropion-d6 for bupropion and chlorzoxazone-d2 for chlorzoxazone respectively). The formation of hydroxybupropion and 6-hydroxy chlorzoxazone were determined using UPLC-MS/MS.

3.2.4 UPLC-MS/MS measurements.

Hydroxybupropion and 6-hydroxy chlorzoxazone were quantified using ACQUITY UPLC™ chromatographic system (Waters Corp., MA, USA) and an API 3200 triple quadrupole mass spectrophotometer (Applied Biosystems, Foster City, CA) with positive electrospray ionization. Chromatographic separation was achieved using Waters ACQUITY C-18 column (50×2.1 mm; 1.7 μm particle size) and waters ACQUITY UPLC C-18 Vanguard pre-column. Mobile phase A (10 mM aqueous ammonium acetate, 5% acetonitrile) and B (100% acetonitrile) were pumped at a flow rate of 0.45 $mL.min^{-1}$ using a gradient ranging from 2% B at 0 to 0.5 mins, 32% B at 2.4 to 2.7 mins, 2% B at 2.9 to 4.5 mins. The column temperature was maintained at

32 ºC.

For hydroxybupropion, optimal conditions for the MRM scan in the positive ionization mode were: ion spray voltage, 5500 V; curtain gas, 35 L/h; ion source Gas1, 55 $L.h^{-1}$; ion source Gas2, 50 $L.h^{-1}$; temperature, 450 ºC; collision Gas, 6 $L.h^{-1}$. The MRM transitions of hydroxybupropion were m/z 256→238 and 256→130; and those of the hydroxy bupropion - d6 were 262 → 139 and 262 → 244. The assay was linear from 0.001 to 10 uM (R^2 < 0.99), precise (Coefficient of variation = 1-8%) and accurate (94 - 105%).

For 6-hydroxy chlorzoxazone, optimal conditions for the multiple reaction monitoring (MRM) scan in the negative ionization mode were: ion spray voltage, -4500 V; curtain gas, 35 L/h; ion source Gas1, 55 $L.h^{-1}$; ion source Gas2, 50 $L.h^{-1}$; temperature, 450 ºC; collision Gas 6 $L.h^{-1}$. The MRM transitions of 6-hydroxy chlorzoxazone were m/z 183.8→119.8 and 183.8→148.1; and those of 6-hydroxy chlorzoxazone-d2 were 187.6 → 121.9 and 187.6 → 149.8. The assay was also linear, precise and accurate.

3.2.5 Determination of in vitro kinetics of HBUP formation.

Following the determination of the concentration of HBUP in HLMs and HepaRG cells, the rate of formation of HBUP was calculated. The *in vitro* kinetic parameters of HBUP formation, km (apparent Michaelis-Menten constant), Vmax (the maximal velocity) and CLint (intrinsic clearance), were estimated using GraphPad Prism version 7.0 (GraphPad Software Inc., La Jolla, CA). The appropriateness of fit was assessed by the sum of squares of residuals, and the standard errors of the parameter estimates.

3.2.6 PBPK Simulation

IVIVE of HBUP exposure after single and multiple doses of 150-mg oral bupropion in obese volunteers were performed using Simcyp® simulator version 15.1. The demographic characteristics of donors **Table 3.1** were used to match the donors of livers with respect to age, BMI and gender. The input parameters including physicochemical properties of HBUP and CLint have been presented in **Table 3.6.** All other parameters were set to the Simcyp default values of SV-bupropion compound file. Bupropion was used as the substrate and the CYP2B6-mediated formation of HBUP was added as the only metabolite. The other pathways leading to the biotransformation of BUP to EB and TB were not included, hence changes in BUP exposure was not reflective of BUP total clearance. Ten trials in 10 virtual subjects were simulated and the overall means of Tmax, Cmax and AUC compared between NoNAFLD and the NAFLD groups. The first order absorption model with a full PBPK model was used to perform the simulations.

3.2.7 Statistical Analysis

Statistical analyses were carried out using SAS software version 9.3 (SAS Institute Inc., Cary, NC) and graphs were generated using GraphPad Prism version 7.0. The median together with the minimum and maximum values were expressed as: median (minimum - maximum). Differences between groups were evaluated using Mann-Whitney U and Kruskal-Wallis tests for groups with two and greater than two categories respectively. Both tests, which are nonparametric, were used because they do not require the normality distribution assumption of data required by parametric

approaches like T-test and ANOVA (analysis of variance). Statistical differences were considered significant at the $P < 0.05$ level.

3.3 Results

3.3.1 Donor demographics and liver characterization.

Table 3.1 shows the demographics of 90 donors of the liver tissues, vendor-supplied diabetic labels, results of the NAFLD characterization, and results of genotyping. The median age and BMI were respectively 49 years and 31 $kg.m^{-2}$ suggesting an obese adult population. The demographic distribution was approximately equal in all categories of diabetes and NAFLD. Using generally accepted algorithms for NAFLD classification (Kleiner et al., 2005, Kleiner and Makhlouf, 2016) the livers were categorized into NoNAFLD (n=29), NAFL (n=34) and NASH (n=27). The NoNAFLD group was therefore designated as the control group. The NoNAFLD group had steatosis level of < 5% in the hepatocytes and a median NAS score of 1, compared with the NAFL and NASH groups with higher hepatic content of fat and > 1 median NAS score.

With the aid of our previously developed logistic model, the diabetic status of the livers were confirmed: True nondiabetic (n = 36), True diabetic (n = 38), false nondiabetic (n=8) and false diabetic (n=8). In all analyses where diabetic and non-diabetic groups were compared, the misclassified ones were excluded.

3.3.2 Activity of CYP2B6 in HLM.

The rate of formation of HBUP was used as a probe to measure the activity of CYP2B6. Though other pathways like the carbonyl reductase are involved in the metabolism of BUP (Connarn et al., 2015), the formation of HBUP is mainly mediated via the CYP2B6 pathway (Faucette et al., 2000, Hesse et al., 2000) and hence used for phenotyping its activity. The activity of CYP2B6 in the 90 HLMs were determined at 10, 50, 100, 400, 800 and 1600 uM of bupropion. Initial analysis was however done with the activity measures at 10, 100 and 400 uM bupropion in line with reported km values **Table 3.7**. Activities were compared without the CYP2B6*5 and CYP2B6*6 variants. Though their impact on BUP disposition is not significant, this was done to minimize confounding by their reduced in vitro activity and to balance the proportion in the three NAFLD groups.

The activity of CYP2B6 was higher in the nondiabetic group compared with the diabetic group. Similarly, the false diabetic group had higher CYP2B6 activity compared to the false nondiabetic group (**Table 3.8**). These differences were however not significant. On the other hand, the activity was significantly different among the NALD groups ($p < 0.01$), with the NoNAFLD group having higher activity.

In the next step of the analyses, the HLMs with misclassified diabetes status were removed to prevent them from confounding the analysis. Then the effect of NAFLD was compared in the diabetic and nondiabetic groups (**Table 3.9**). Again, activity was significant ($p<0.03$) among the NAFLD group. The trend of decrease in activity for NAFLD was: NoNAFLD > NAFL > NASH in the nondiabetic group, but NoNAFLD > NASH > NAFL in the diabetic group. Consequently, the highest activity was

observed in the nondiabetic-NoNAFLD group and lowest was found in the diabetic-NAFL group. This suggested that the presence of diabetes could be a potential aggravator of NAFLD and could confound the detection of the influence of NAFLD on CYP2B6.

3.3.3 Kinetics of CYP2B6-mediated HBUP in HLMs.

The kinetics of HBUP in the HLMs were determined using the Michaelis-Menten equation. The km, Vmax and CLint were estimated. Graphical inspection showed that some of the HLMs did not attain saturable rates of HBUP formation as observed in previous study(Faucette et al., 2000). Those HLMs had very high km values > 1000 uM. To improve the reliability of our analysis, a subset of the livers (**Table 3.2**) with km ≤ 2x130 uM were compared (Faucette et al., 2000). The results of the kinetic analysis (**Table 3.3**) showed that the Vmax decreased in the fashion: NoNAFLD>NAFL>NASH; whereas the km increased in the order: NoNAFLD<NAFL<NASH. The decrease in Vmax was not statistically significant, but the increase in km was ($p<0.041$). The CLint therefore was approximately 2-fold lower in both NAFL and NASH groups.

3.3.4 mRNA Expression of CYP2B6, CAR and PXR in HLM.

The mRNA level of CYP2B6 decreased in the fashion: NoNAFLD > NAFL > NASH, but was not statistically significant (**Table 3.9**). The fold change in NoNAFLD/NAFLD, did not correspond with the changes in the kinetic parameters. Similarly, changes in mRNA of CAR and PXR did not correspond with changes in CYP2B6 mRNA and kinetics. This suggests that mRNA levels may not reflect

CYP2B6 kinetic parameters adequately and may mislead the detection of the influence of NAFLD on CYP2B6 if solely relied upon.

3.3.5 HepaRG Cell Lines.

In view of the heterogeneity of the HLMs, the HepaRG cell lines was used as a homogenous system to confirm qualitatively and quantitatively the effect of fat content on CYP2B6 expression and activity. Steatosis was induced in the HepaRG, and the accumulation of fatty acids was confirmed using Oil Red O staining and UV-spectrophotometry (**Figure 3.1**). The measurement of the Total AKT/Phospho AKT (results not shown) showed a reduced insulin response in the FA-treated cells. Because CYP2E1 enzyme expression and activity is elevated under steatotic conditions (Chalasani et al., 2003, Aljomah et al., 2015), it was used as an endogenous control to confirm induction of steatosis by monitoring the formation of 6-hydroxychlorzoxazone (6-OH CZ). In the FA-treated cells, the chlorzoxazone hydroxylase activity was about 4-times higher compared to the control suggesting a viable steatotic tool for further investigation.

Qualitatively consistent with the HLM data, the fatty-acid-loaded HepaRG cells showed significantly lower activity compared to control (**Figure 3.2**). The Vmax was 47% lower in the FA-treated group. However, the km was 3.5-fold higher in the FA-treated. Consequently, CLint was 5-fold lower in the FA-treated group. Thus, quantitatively, the HepaRG cells amplified the effect of steatosis in the HLMs. Furthermore, the mRNA expression of CYP2B6, CAR and PXR were also significantly reduced in fatty-acid-loaded HepaRG cells compared to control (results

not shown).

3.3.6 Physiologically based pharmacokinetic (PBPK) Simulation.

The simulated exposure parameters of HBUP via CYP2B6 are presented in **Table 3.4** and the concentration profiles in **Figure 3.3**. The Cmax was slightly higher in NoNAFLD group compared to NAFL and NASH. AUC of HBUP was however similar among groups. Despite the limitations of our simulations (no NAFLD population, no data on clearance of HBUP), the results show that a completely different findings from our in vitro work is possible in complete human subjects. Hence, clinical studies in human subjects would greatly enhance our understanding on the influence of NAFLD on CYP2B6.

3.4 Discussion

CYP2B6 was originally considered an enzyme of minor significance. However, further investigations have shown that it plays both major and minor roles in the metabolism of clinically relevant drugs. Bupropion is one of its major substrates. Though bupropion is metabolized into erythrohydrobupropion (EB) and threohydrobupropion (TB) by carbonyl reductases, hydroxybupropion is the main metabolite and it is formed via the CYP2B6 pathway (Faucette et al., 2000, Hesse et al., 2000, Connarn et al., 2015). It is used in the treatment of depression and smoking cessation, and to ensure safety and efficacy, various factors capable of influencing its pharmacokinetics like gender, polymorphisms, renal impairment and alcoholic liver

diseases (Turpeinen et al., 2007, DeVane et al., 1990, Ilic et al., 2013) have been studied. Other groups have also studied the influence of NAFLD on the activity and expression of CYP2B6 in human liver tissues. The aim of this study, however, was to determine the influence of NAFLD on the activity of CYP2B6 and the in vitro kinetics of HBUP formation. To our knowledge, this is the first report of the influence of NAFLD on the in vitro kinetics of HBUP formation by CYP2B6.

In this work, we used two in vitro systems, Human Liver tissues and HepaRG cell lines, to show that both Vmax and km of CYP2B6-mediated HBUP formation are altered in NAFLD. In the HLMs, the presence of NAFLD caused a modest reduction in the Vmax, but a significant increase in the km. Eventually, the CLint was about 2-fold lower in the NASH group. Qualitatively, the findings in the HepaRG system was similar to the HLM system, but alteration was more pronounced quantitatively. This is not surprising as cell culture systems have the tendency to exaggerate effects. Notwithstanding, our work is in agreement with another study in Sprague−Dawley rats fed with a methionine/choline deficient (MCD) diet to induce NASH (Cho et al., 2016). The *in vitro* kinetic parameters determined using the rat liver microsomes showed 26 % reduction in Vmax and 2.4 fold increase in Km for the formation of HBUP by Cyp2b1 (rat orthologue of human CYP2B6) in the NASH group.

Contrary to the findings in the HLMs, HepaRG, and the rat studies by Cho et al., 2016, the differences in CLint between the NoNAFLD and the NAFLD groups did not translate into differences in AUC except a slight decrease after simulating single and multiple doses of 150-mg BUP. CLint is one of the parameters that could change in

NAFLD. However, changes in the function and structure of the heart, kidneys and other organs (Cassidy et al., 2015, Machado et al., 2012) may also influence the overall disposition of a drug. Therefore, the combined effect of changes in the organs and enzymatic activity are relevant to determine the overall disposition of BUP in NAFLD. Our PBPK model attempted to do that, but we did not find enough data in the literature to recapitulate NAFLD in the virtual population. This did not allow us to account for possible changes in organs in NALF and NASH. Notwithstanding, our *in vitro* findings highlight the potential of NALFD to alter CYP2B6-mediated HBUP kinetics like ALD (Toshikuni et al., 2014, DeVane et al., 1990), which is similar to NAFLD, but caused by significant alcohol consumption.

In addition to the above, our work shows that the heterogeneity of NAFLD and the variableness of expression and activity of CYP2B6 may impair detection of the influence of NAFLD on CYP2B6. Hence, the need to account for reasonable amount of the variability in CYP2B6. We accounted for diabetes because it is closely associated with NAFLD (Anstee et al., 2013) and could potentially confound it. HLMs showing non-saturable kinetics were excluded from analysis to prevent confounding (Faucette et al., 2000).

This work had some limitations. Donors of HLMs were predominantly obese adults, so we could not evaluate the influence of NAFLD in lean subjects. Secondly, we could not account for all polymorphisms in our dataset, as this was impractical. However, the high frequency variants with potential to alter *in vitro* kinetics were removed. Lastly, we did not investigate the carbonyl reductase pathways for the formation of EB and TB, hence, the CLint determined was more relevant to the

CYP2B6-mediated formation of HBUP.

In summary, we investigated the effect of NAFLD on CYP2B6 using HLMs and HepaRG cell lines. We observed that the Vmax was modestly reduced, and km significantly increased, and CLint reduced in NAFL and NASH HLMs. Qualitatively, the HepaRG findings corroborated the finding in the HLM in the NAFL group. This investigation is hoped to contribute to our current knowledge on the influence of NAFLD on CYP2B6-mediated HBUP *in vitro* kinetics; and offers some basis for further studies in a clinical trials.

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3.6 Tables

Table 3.1 Demographic characteristics, diabetes status, liver biopsy grade.

Demographic characteristics, diabetes status, liver biopsy grade, and CYP2B6 genotypes of the donors of human liver tissues.

Variable	Category	NoNAFLD (n=29)	NAFL (n = 34)	NASH (n = 27)	Overall (n=90)
Age	N/A	49 (21-78)	53.5 (33-56)	49 (33-74)	51 (21-78)
BMI	N/A	29 (19-89)	31 (17-70)	32 (22-52)	31 (17-89)
Gender	Female	13 (14.4)	19 (21.1)	14 (15.6)	46 (51.1)
	Male	16 (17.8)	15 (16.7)	13 (14.4)	44 (48.9)
Ethnicity	Caucasian	21 (23.3)	30 (33.3)	25 (27.8)	76 (84.4)
	African American	7 (7.8)	1 (1.1)	1 (1.1)	9 (10.0)
	Hispanics	1 (1.1)	3 (3.3)	1 (1.1)	5 (5.6)
Diabetes	Nondiabetic	13 (14.4)	19 (21.1)	12 (13.3)	44 (48.9)
	Diabetic	16 (17.8)	15 (16.7)	15 (16.7)	46 (51.1)
Liver histology	Steatosis	0 (0-0)	1.5 (1-3)	2 (1-3)	1 (0-3)
	Lobular inflammation	1 (0-2)	1 (0-2)	1 (1-3)	1 (0-3)
	Hepatocyte ballooning	0 (0-1)	0 (0-2)	1 (1-2)	0 (0-2)
	NAS score	1 (0-3)	3 (1-5)	5 (3-7)	3 (0-7)
CYP2B6*5	*1*1	26 (28.9)	26 (28.9)	22 (24.4)	74 (82.2)
	*1*5	2 (2.2)	8 (8.9)	4 (4.4)	14 (15.6)
	*5*5	1 (1.1)	0	1 (1.1)	2 (2.2)
CYP2B6*6	*1*1	11 (12.2)	18 (20.0)	13 (14.4)	42 (46.7)
	*1*6	15 (16.7)	15 (16.7)	12 (13.3)	42 (46.7)
	*6*6	3 (3.3)	1 (1.1)	2 (2.2)	6 (6.7)

Data presented as median (minimum - maximum) or frequency (percentage of total number of liver tissues). NAS, nonalcoholic fatty liver disease activity score. N/A, not applicable.

Table 3.2 Demographic characteristics of a subset of donor livers.

Demographic characteristics of a subset of donor livers used to determine final in vitro kinetic parameters (km, Vmax, CLint) of hydroxybupropion (HBUP) formation CYP2B6-mediated hydroxylation of bupropion (BUP).

Variable	Category	NoNAFLD	NAFL	NASH
Age	NA	41 (28-62)	53 (36-69)	48 (39-68)
BMI	NA	27 (19-50)	31 (17-37)	34 (26-50)
Gender	Female	3 (27.27)	2 (28.57)	3 (42.86)
	Male	8 (72.73)	5 (71.43)	4 (57.14)
Ethnicity	Caucasian	8 (72.73)	6 (85.71)	6 (85.71)
	African American (AA)	2 (18.18)*	0	0
	Hispanics	1 (9.09)	1 (14.29)	1 (14.29)
Diabetic	Nondiabetic	3 (27.27)	5 (71.43)	2 (28.57)
	Diabetic	8 (72.73)	2 (28.57)	5 (71.43)
Liver histology	Steatosis	0 (0-0)	1 (1-3)	2 (1-3)
	Lobular inflammation	1 (0-2)	1 (0-2)	1 (1-3)
	Hepatocyte ballooning	0 (0-0)	0 (0-1)	1 (1-2)
	Fibrosis	1 (0-2)	1 (0-3)	0 (0-1)
	NAS score	1 (0-2)	3 (2-4)	4 (3-7)

*Only the NoNAFLD group had African Americans (AA). However, removing AA did not alter the kinetics, maintained in the final analyses.

Table 3.3 In vitro kinetic parameters of CYP2B6.

In vitro kinetic parameters of CYP2B6-mediated hydroxybupropion (HBUP) formation in human liver microsomes. Values reported as median (min - max).

NAFLD Lesion	**Vmax[a]**	**Km[b]***	**Clint[c]**	**Fold Change in Clint (NoNAFLD/NASH)**
NoNAFLD (n=11)	109.2 (26.41-350.8)	142.7 (66.38-240.2)	0.77	1
NAFL (n=7)	89.41 (22.97-349.8)	239.3 (128.2-247.7)	0.37	2.05
NASH (n=7)	73.38 (18.17-172.4)	218.1 (160.9-244.2)	0.34	2.27

* Kruskal Wallis , p-value <0.041; a, Vmax pmoles.min^{-1}.mg microsomal protein^{-1}; b, Km in units of micromolar (uM); c, CLint = Vmax/km in units of uL.min^{-1}.mg microsomal protein^{-1}

Table 3.4 Simulated exposure parameters of hydroxybupropion.

Simulated exposure parameters of hydroxybupropion after single and multiple doses of 150-mg oral bupropion using Simcyp simulator.

Dosing	Substrate		T_{Max} (h)	C_{Max} (mg/L)	AUC (mg/L.h)
Single	Hydroxybupropion	NoNAFLD	4.32	0.10	2.79
		NAFL	5.53	0.09	2.68
		NASH	6.03	0.09	2.67
Multiple	Hydroxybupropion	NoNAFLD	5.50	0.19	6.52
		NAFL	8.25	0.18	6.74
		NASH	8.25	0.18	6.78

The $AUC_{0\text{-}inf}$ was estimated for single doses; and $AUC_{0\text{-}504\ hrs}$ for multiple doses.

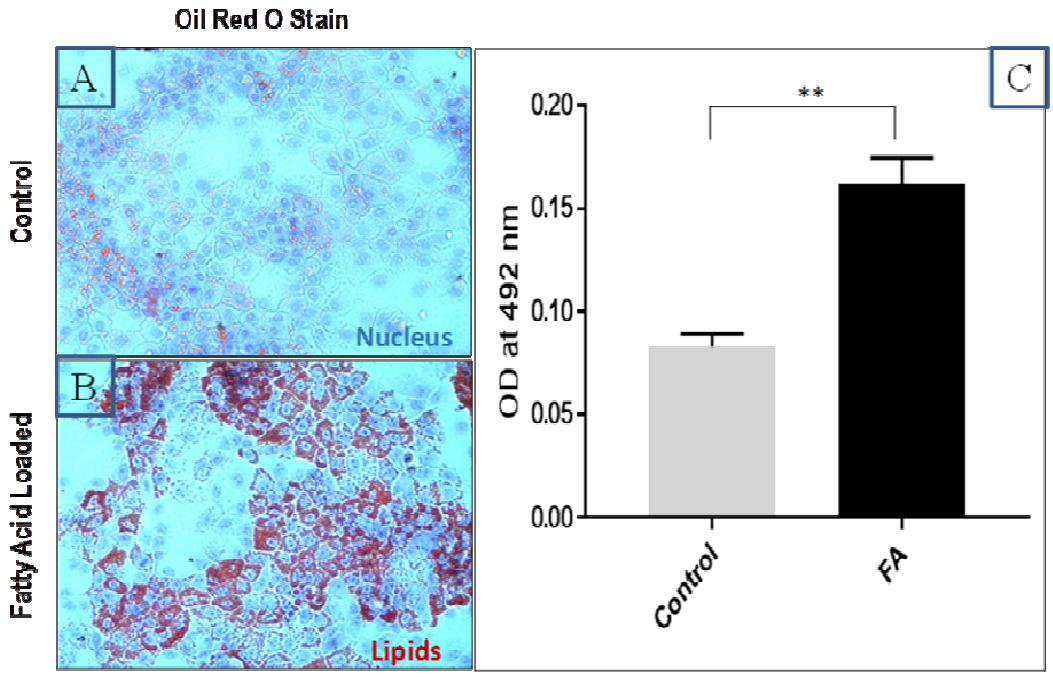

Figure 3.1 Lipid content of control and fatty acid (FA) loaded HepaRG cells.

Evaluation of the lipid content of control and fatty acid (FA) loaded HepaRG cells

(A and B) Oil Red O (ORO) staining. Intracellular lipid accumulation was not observed in control (A). However, because the treatment group (B) was loaded with palmitate and oleate (1:2), lipid accumulation was observed after ORO staining. (C) Spectrophotometric assessment of intracellular lipid content (** TTest, $p < 0.001$).

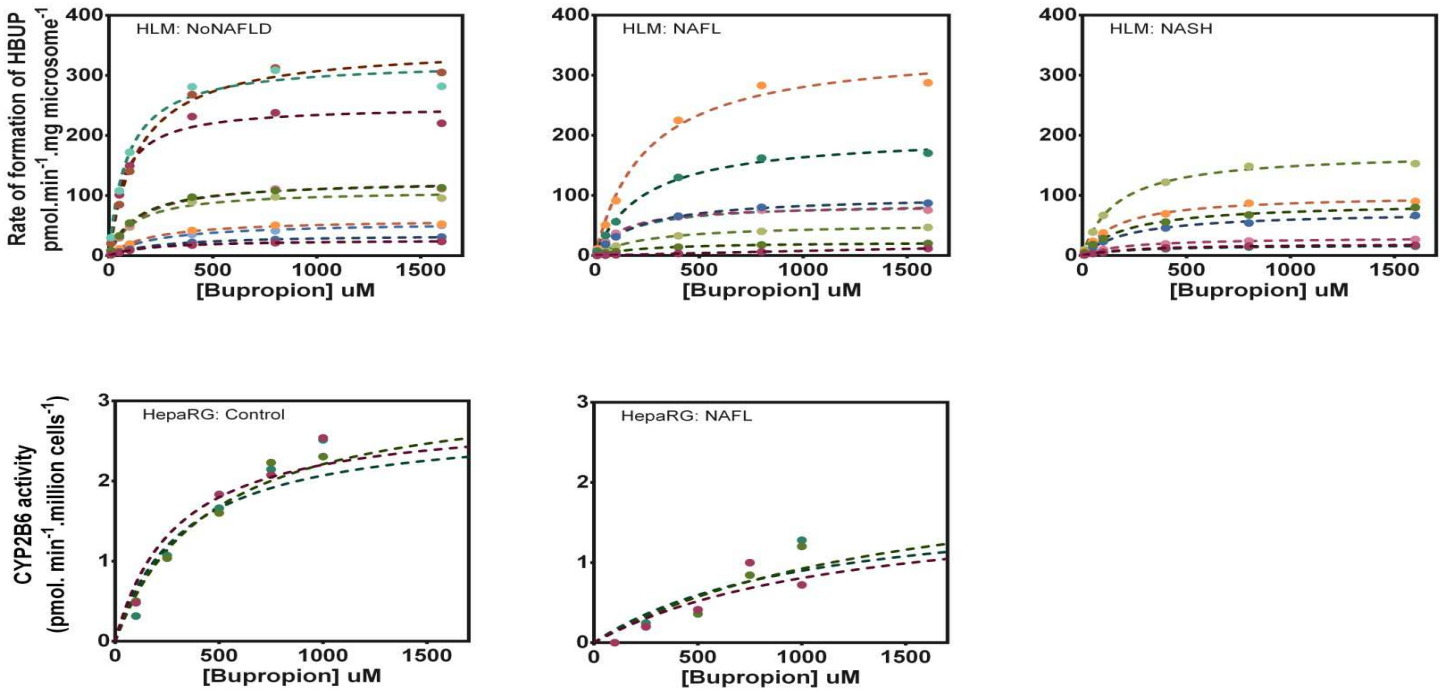

Figure 3.2 Representation of kinetic profile of CYP2B6.

Representation of kinetic profile of CYP2B6-mediated formation of hydroxybupropion:

Upper panel: In NoNALFD, NAFL and NASH human liver microsomes (HLMs). Lower panel: In control and steatotic HepaRG cell lines.

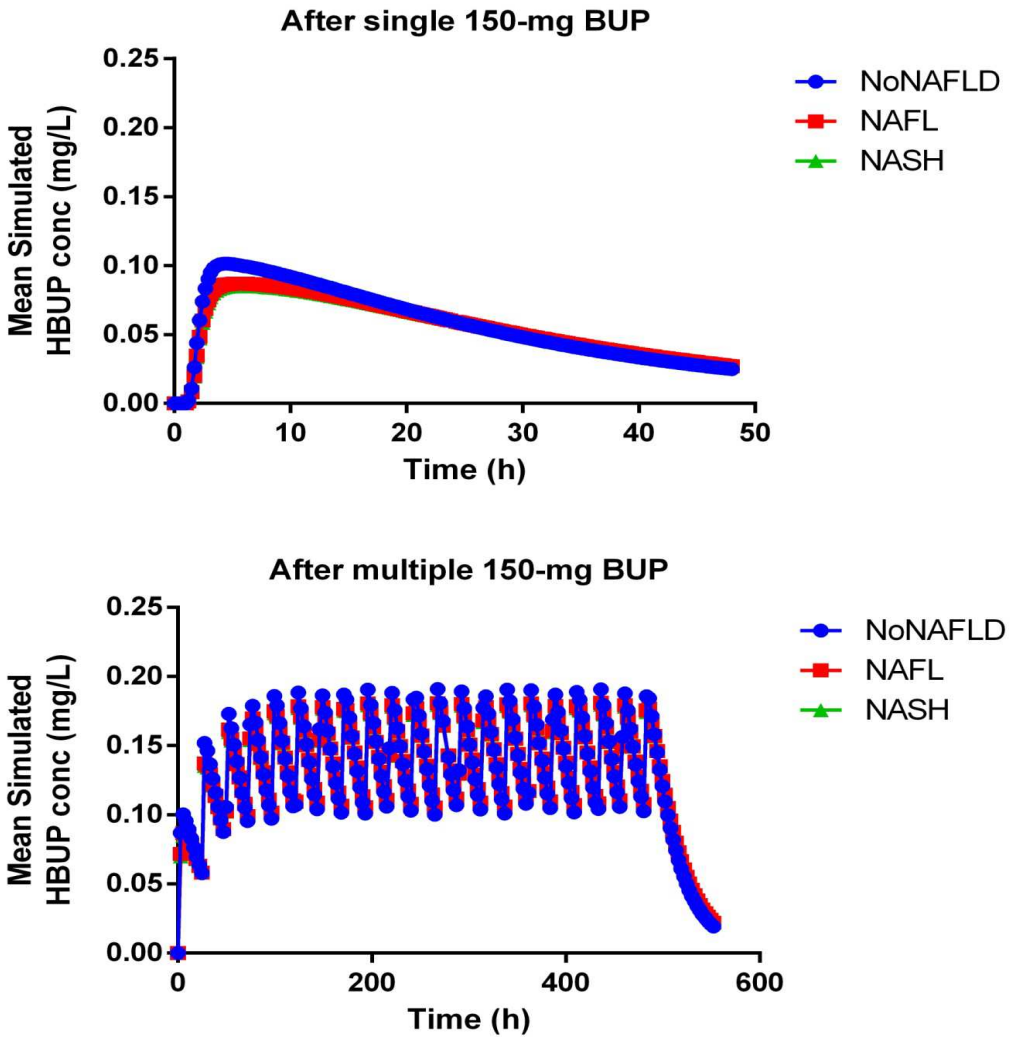

Figure 3.3 Simulation of the plasma concentration time curve of HBUP.

Simulation of the plasma concentration time curve of Hydroxybupropion (HBUP) in NoNAFLD, NAFL and NASH donors after administration of 150-mg of bupropion single (upper graph) and multiple (lower graph) doses.

3.7 Supplementary Information

Table 3.5 Sequences of primers for qRT-PCR

Gene	Forward primer	Reverse Primer
CYP2B6	ATGGGGCACTGAAAAAGACTGA	AGAGGCGGGGACACTGAATGAC
CAR	AGATGGAGCCCGTGTGGG	GGTAACTCCAGGTCGGTCAGG
PXR	GCTGACAGAGGAGCAGCGGATGA	CCCTGGCAGCCGGAAATTCTT

Table 3.6 PBPK input parameters.

Summary of parameters for simulating exposure of Hydroxybupropion (HBUP) after administering 150-mg of bupropion (BUP).

Category	Parameter	NoNAFLD	NAFL	NASH	Reference
Physicochemical properties (BUP)	Molecular weight	239.74	239.74	239.74	Simcyp
	log P	3.4	3.4	3.4	Simcyp
	Compound type	monoprotic base	monoprotic base	monoprotic base	Simcyp
	pKa	8.02	8.02	8.02	Simcyp
	B/P	0.82	0.82	0.82	Simcyp
Physicochemical properties (HBUP)	Molecular weight	255.74	255.74	255.74	Drugbank*
	log P	2.22	2.22	2.22	Drugbank*
	Compound type	monoprotic base	monoprotic base	monoprotic base	
	pKa	7.65	7.65	7.65	Drugbank*
	B/P	0.55	0.55	0.55	Simcyp Predicted
Absorption	Absorption type	First-order absorption model	First-order absorption model	First-order absorption model	
Distribution	Distribution model	Full PBPK model	Full PBPK model	Full PBPK model	
Elimination (Bupropion)	CYP2B6 CLint ($nL.min^{-1}$. mg microsomal $protein^{-1}$)	770	370	340	Experimental
Elimination (HBUP)	CLiv (L/h)	37.05	37.05	37.05	Simcyp**

*Parameters were obtained from Drugbank: https://www.drugbank.ca/metabolites/DBMET00277 (accessed on 3/09/2018); ** Value obtained by dividing the default Simcyp value (74.1 L/h) by 2. This was done to obtain half-life of approximately 20 hrs for HBUP

Table 3.7 Reported kinetics of CYP2B6.

Reported kinetics of CYP2B6-mediated formation of hydroxybupropion (HBUP) from bupropion (BUP) in human liver microsomes (HLM).

No	N	Vmax	Km	Clint	Study
1	HLM	105±3.4	198±18	0.53	(Skarydova et al., 2014)
2	HLM (n=105)	53.3 (12.8 - 333.5)	73.4 (17.1-393.3)	0.77 (0.13-5.22)	(Gao et al., 2017)
3	HLM	131.2±5.8	87.9±20.2	1.49	(Connarn et al., 2015)
4	HLM (n=5)	739.5 ± 440.6	130.2±22.0	5.68	(Faucette et al., 2000)
5	HLM (n=4)	3623±1520	89±14	40.7	(Hesse et al., 2000)

n= number of human liver tissues used in study.

Table 3.8 Summary of mRNA expression and activity of CYP2B6 (I)

A

Variable	NonDiabetic (ND) (n=31)	Diabetic (D) (n=34)	False nondiabetic (n=6)	False Diabetic (n=8)	Fold change (ND/D)
CYPB6 activity (10 uM)	1.18 (0.2-45.85)	0.77 (0.27-30.2)	1.04 (0.28-8.16)	1.01 (0.25-92.6)	1.53
CYPB6 activity (100 uM)	6.49 (0.45-256.5)	4.41 (0.83-172)	6.24 (0.87-47.6)	6.69 (1.08-467.5)	1.47
CYPB6 activity (400 uM)	14.65 (1.19-459.5)	9.98 (2.11-281)	13.85 (2.41-89.55)	17.3 (3.33-726)	1.47
CYP2B6 mRNA	1.76 (0.04-58.15)	0.52 (0.02-47.56)	0.26 (0.08-7.9)	0.3 (0.01-68.68)	3.38
PXR mRNA	1.49 (0.23-7.74)	1.36 (0.12-6.18)	1.89 (0.45-4.83)	1.1 (0.01-4.8)	1.10
CAR mRNA	1.67 (0.03-4.61)	1.67 (0.04-23.48)	1.3 (0.38-4.33)	1.22 (0.01-5.99)	1.00

B

Variable	NoNAFLD (n=24)	NAFL (n=32)	NASH (n=23)	Fold Change (NoNAFLD/NASH)
CYPB6 activity (10 uM)*	1.78 (0.33-45.85)	0.6 (0.2-10.95)	0.92 (0.28-92.6)	1.93
CYPB6 activity (100 uM)*	11.62 (1.22-256.5)	3.41 (0.45-91.3)	5.62 (0.87-467.5)	2.07
CYPB6 activity (400 uM)*	27.93 (2.81-459.5)	7.33 (1.19-225)	12.65 (2.41-726)	2.21
CYP2B6 mRNA	1.36 (0.1-59.17)	0.63 (0.01-30.21)	0.45 (0.02-68.68)	3.02
PXR mRNA	1.53 (0.16-6.18)	1.41 (0.01-7.74)	1.09 (0.12-4.83)	1.40
CAR mRNA	1.98 (0.21-23.48)	1.94 (0.01-6.67)	1.11 (0.04-22.84)	1.78

A: The diabetic group was compared.

B: The NAFLD group was compared. * Kruskal Wallis, p-value <0.01;

Table 3.9 Summary of mRNA expression and activity of CYP2B6 (II).
Values reported as median (min - max). Summary statistics of overall levels of mRNA expression and activity of CYP2B6.

Values reported as median (min - max).

	Variable	NONAFLD (n=11/9)	NAFL (n =13/13)	NASH (n=10/9)	Fold Change (NONAFLD/NASH)
Diabetic	CYPB6 activity (10 uM)*	1.74 (0.33-30.2)	0.46 (0.27-2.38)	1.02 (0.33-9.66)	1.71
	CYPB6 activity (100 uM)*	10.48 (1.22-172)	2.73 (0.83-15.7)	5.85 (1.72-66.7)	1.8
	CYPB6 activity (400 uM)*	22.2 (2.81-281)	5.65 (2.11-32.75)	12.7 (6.39-122)	1.75
	CYP2B6 mRNA	1.63 (0.12-47.56)	0.45 (0.03-7.98)	0.19 (0.02-4.02)	8.58
	PXR mRNA	2.02 (0.16-6.18)	1.63 (0.29-2.78)	1.09 (0.12-2.5)	1.85
	CAR mRNA	2.08 (0.46-23.48)	2.09 (0.24-6.67)	1.02 (0.04-22.84)	2.04
Nondiabetic	CYPB6 activity (10 uM)*	1.81 (0.36-45.85)	1.18 (0.2-10.95)	0.72 (0.41-6.22)	2.51
	CYPB6 activity (100 uM)*	12.75 (1.93-256.5)	6.49 (0.45-91.3)	4.78 (1.74-37.7)	2.67
	CYPB6 activity (400 uM)	33.65 (4.28-459.5)	14.65 (1.19-225)	12.45 (4.44-69.55)	2.7
	CYP2B6 mRNA	0.38 (0.1-58.15)	2.37 (0.05-30.21)	0.83 (0.04-5.41)	0.45
	PXR mRNA	1.04 (0.23-3.84)	1.94 (0.61-7.74)	1.46 (0.3-2.41)	0.71
	CAR mRNA	2.26 (0.21-4.61)	2.3 (0.03-4.31)	0.86 (0.29-3.63)	2.63

*Kruskal Wallis, p-value <0.03

The diabetic and NAFLD groups were simultaneously compared.

Table 3.10 Summary of mRNA expression and activity of CYP2B6

Summary of mRNA expression and activity of CYP2B6 in the subset of human liver microsomes (HLM) used to determine the in vitro kinetics of CYP2B6-mediated hydroxybupropion (HBUP) formation. Values reported as median (min - max).

Variable	NoNAFLD (n=11)	NAFL (n=7)	NASH (n=7)	Fold Change (NoNAFLD/NASH)
CYPB6 activity (10 uM)	7.89 (1.43-30.2)	4.28 (0.25-10.95)	3.59 (0.92-9.66)	2.20
CYPB6 activity (100 uM)	47.6 (8.99-172)	31.15 (1.08-91.3)	23.2 (5.11-66.7)	2.05
CYPB6 activity (400 uM)	89.55 (17.55-281)	64.35 (3.33-225)	45.85 (11.25-122)	1.95
CYP2B6 mRNA	3.15 (0.1-47.56)	2.37 (0.04-27.51)	0.11 (0.02-5.41)	28.64
PXR mRNA	0.86 (0.23-4.02)	1.86 (0.29-3.32)	1.49 (0.52-2.14)	0.58
CAR mRNA	1.65 (0.39-23.48)	2.62 (0.31-6.67)	1.36 (0.04-22.84)	1.21

MANUSCRIPT IV

(Prepared for submission to Clinical Pharmacokinetics)

Population Pharmacokinetics of a Ghrelin Receptor Inverse Agonist (PF-5190457) in Healthy and Non-Treatment Seeking Alcoholic Adults

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4 MANUSCRIPT IV

Abstract

Background and Objectives: PF-5190457 is an inverse agonist of the growth hormone secretagogue receptor (hGHS-R1a), that is undergoing clinical trial for treatment of alcohol use disorder. The purpose of this study was to describe the population pharmacokinetics (PK) of PF-5190457 and to identify clinical and demographic characteristics that influence its PK variability.

Subjects and Methods. Data on drug dosage, sampling times and plasma concentrations were collected retrospectively from two studies: Phase 1a and Phase 1b. Thirty five (35) healthy volunteers were enrolled in the Phase 1a, and 12 non-treatment seeking alcoholic subjects in the Phase 1b trial. The log-transformed concentration-time points were modeled in NONMEM. The influence of patients' demographic and biochemical characteristics were evaluated; and the accuracy and precision of the model parameters determined using bootstrapping. The predictive performance of the final model was checked using percentile visual predictive check.

Results. The pharmacokinetics of PF-5190457 was best described by a one-compartmental model with first order absorption after oral administration. The estimated typical pharmacokinetic parameters included the absorption rate constant (ka, 3.6 h^{-1}), oral clearance (CL/F, 80 Lh^{-1}) and apparent volume of distribution (V/F, 575 L). Body weight and serum albumin on V/F reduced the interindividual variability (IIV) associated with V/F by ~28%. Increasing body weight increased V/F, whereas increasing serum albumin levels reduced it.

Conclusion. PF-5190457 is rapidly cleared from the body. The V/F of PF-5190457 is influenced by body weight and albumin. We anticipate that this model would serve as a guide in designing dosage regimen for future clinical trials with PF-5190457.

Key words:

Albumin, Alcoholism, Clinical trial, Ghrelin, Growth Hormone, Pharmacokinetics,

Abbreviations:

ALT: alanine aminotransferase

AST: aspartate aminotransferase

AUDs: Alcohol Use Disorders

CL/F: oral clearance

CrCL: Creatinine clearance

CWRES: conditional weighted residuals

DSGRP: Dose group

DV: observed plasma concentration

GAM: generalized additive model

GH: growth hormone

GOAT: ghrelin O-acyl-transferase

hGHS-R1a: human growth hormone secretagogue receptor 1a

IIV: interindividual variability

IPRED: individual predicted concentration

IRB: Institutional Review Board

ka: absorption rate constant

MDRD: modification of diet in renal disease

NCA: non-compartmental analysis

OFV: objective function value

PK: Pharmacokinetics

PRED: predicted plasma concentration

UPLC-MS/MS: UPLC/tandem mass spectrometry

V/F: apparent volume of distribution

VPC: visual predictive check

4.1 Introduction

Alcohol Use Disorders (AUDs), i.e., alcohol abuse and alcohol dependence (alcoholism) is a global concern [1, 2]. Despite its effects on health, economy and society, only few medications are approved for treatment in both the United States (US) and Europe. The available medications including naltrexone, disulfiram, and acamprosate [3, 4] have not adequately met patients' needs due to side effects and moderate efficacy. Consequently, there is a crucial need to identify novel pharmacological targets to effectively treat AUDs.

Several drugs including sertraline [5, 6], topiramate [7-9] and baclofen [10, 11] are under investigation for the treatment of AUD. More recently, the ghrelinergic system (ghrelin receptor also known as growth hormone secretagogue receptor (hGHS-R1a), ghrelin, and ghrelin O-acyl-transferase (GOAT)) is studied due to its involvement in alcohol craving [12, 13] and offers a potential for the treatment of AUDs.

Ghrelin is a gastrointestinal peptide hormone produced mainly in the oxyntic glands of the gastric fundus [14]. It serves as the endogenous agonist of the ghrelin receptor. The activation of this receptor by ghrelin requires the acylation of its serine-3 residue [15, 16] to acyl-ghrelin by GOAT. The physiological roles of the ghrelin system in humans include stimulating the release of growth hormone (GH) [14], regulation of food intake, body weight, adiposity, and glucose metabolism [17-19]. In view of its roles, the ghrelinergic system is increasingly becoming an attractive pharmacological target not only for the treatment of drug addiction [13, 20-22]; but also diabetes [23, 24], obesity [25], and Parkinson's disease [26].

PF-5190457, a spiro-azetidino piperidine compound, is an inverse agonist of hGHS-R1a [27, 18]. Originally developed for the treatment of Type 2 diabetes and obesity, PF-5190457 is being repurposed for the treatment of alcoholism. Due to its promising pharmacological and safety profile, it has advanced into human clinical trials. To maximize efficacy and minimize toxicity in subsequent phases of clinical trials of PF-5190457, a thorough understanding of the clinical PK is essential. Therefore, the goal of this work was to characterize the PK of PF-5190457 in healthy and non-treatment seeking alcoholic subjects, and to investigate the covariates that influence PK variability. We hope that the findings in this work will serve as a guide in designing subsequent clinical trials in humans.

4.2 Methods

4.2.1 Study Design and Patients

Two Phase 1 studies, in healthy humans (Phase 1a) and in non-treatment seeking alcoholic subjects (Phase 1b) were conducted by Pfizer and NIH at l'Hôpital Erasme (Brussels, Belgium) and NIH Clinical Center (Bethesda, Maryland) respectively. Institutional Review Board (IRB) approval was obtained prior to the beginning of each study. All participants provided written informed consent before enrolment. Full details of the Phase 1a study has been described previously [28]; whereas the Phase 1b study has been accepted for publication (Manuscript 2017MP001300RR, Mol. Psychiatry).

Phase 1a was a randomized, double-blind, placebo-controlled study of four cohorts of single and divided doses of PF-5190457 or placebo: Cohorts 1 and 2 (9 subjects each)

participated in a three-sequence crossover dose-escalation leg with doses of 2, 10, 50, 100, 300 mg and placebo substitution; Cohort 3 (8 subjects) underwent a standard two-sequence crossover investigating gastric emptying after a single 150 mg dose or placebo; and Cohort 4 (9 subjects) underwent a two-period, three-sequence incomplete block receiving 40 or 300 mg divided dose regimens (with breakfast, 2 h post breakfast, with dinner, and 2 h post dinner) [28]. In the dose-escalating, single-blind, placebo-controlled, crossover Phase 1b study, 12 subjects received b.i.d doses of PF-5190457 (placebo, 50 and 100 mg b.i.d) for 3 days. PF-5190457 was administered as oral suspension in both studies.

4.2.2 Inclusion and Exclusion criteria for patient enrollment

A total of 47 adult subjects (age ≥ 18 years) with no clinically relevant abnormalities identified in medical history, physical examinations, vital signs, electrocardiograms or clinical laboratory tests were selected for both studies. Enrolled subjects in the Phase 1a study were healthy whereas those in Phase 1b were non-treatment seeking alcoholics. Women enrolled in the studies were non-childbearing.

4.2.3 Data Collection

Patient data collected included demographics (e.g., age, race, sex, and body weight) and relevant laboratory findings (e.g., alanine aminotransferase, aspartate aminotransferase, serum albumin, serum creatinine, total bilirubin). Additional study related characteristics like dose group (DSGRP), alcohol and nicotine use status of subjects were included as covariates. Creatinine clearance was determined from serum creatinine using the MDRD (modification of diet in renal disease) equation [29]

which accounts for racial and gender differences in serum creatinine levels.

4.2.4 PK sampling schedule and PF-5190457 Assay

Plasma concentration samples were collected up to 48 h (non-steady state PF-5190457 concentrations) for the Phase 1a study; and up to 73.5 h (Steady state PF-5190457 concentrations) from day 1 to 3 (immediately prior to dosing, 1-2 h. after dosing, and approximately 30 mins after day 2) for Phase 1b. Each sample was quantified using a validated UPLC/tandem mass spectrometry (UPLC-MS/MS) method described previously. The limit of quantification of the two assays was 1 ng/mL [28, 30].

4.2.5 Population PK modeling

PopPK analysis of the concentration-time data of PF-5190457 was performed using the computer program NONMEM (Version 7.3; ICON Development Solution, MD). NONMEM allows the implementation of mixed effects (fixed and random) non-linear regression models to estimate population means and variance of the population pharmacokinetic parameters. PLTTOOLS (Version 5.5.1, San Francisco, CA) was used as an interface throughout the entire modeling process and covariate selection; while R (version 3.4.0) and R Studio (Version 1.0.153) were used for graphical evaluations. A stepwise procedure was used to find the model that adequately fit the data. Parameter estimation was done with first order conditional estimation with interaction (FOCE -I). Natural logarithmic-transformed data were used for the analysis. Both one-compartment (ADVAN2, TRANS2); and two-compartments (ADVAN4, TRANS4) structural models with or without lag-time were explored.

Inter-individual variability was modeled using exponential error model:

Pi = TVP*EXP(ETA(i)) ..... (1);

Where Pi represents the parameter estimate of the ith subject, and TVP is the typical parameter estimate in the population, and ETA(i) the random inter-individual variability (IIV) in the parameter for the ith participant.

Residual variability was modeled using an additive proportional log error model:

```
$ERROR
PRED=F
IPRED=0
IF(F.GT.0) IPRED=LOG(F)
Yij = IPRED +SQRT(THETA(4)**2 + THETA(5)**2/F**2)*EPS(1)
```

Where Yij is the observed jth concentration in the ith subject; F is the model prediction for jth concentration on the ith subject; and EPS(1) is the random residual effect for the jth concentration; THETA(4) and THETA(5) are fixed effect additive and proportional components of the error model respectively.

4.2.6 Covariate analysis

Candidate covariates presented in **Table 4.1** were selected based on the trial design, graphical inspection, significant change in OFV, changes in variance of IIV, and physiological plausibility. The influence of covariates were conducted sequentially using forward selection followed by backward elimination by comparing changes in objective function value (OFV) of base and covariate models. Continuous covariates

were centred to their median (med) values and modeled using additive (3), multiplicative (4) and exponential (5) models in PLTTOOLS:

TVPi = ((THETA(i) + (THETA(x)-1) * (COV-medCOV)) * EXP(ETAi) .....(3)

TVPi = ((THETA(i) * (1+THETA(x)-1) * (COV-medCOV)) * EXP(ETAi) ...(4)

TVPi = ((THETA(i) * (COV/medCOV) * *(THETA(x)-1) * EXP(ETAi) .....(5)

Where COV is the covariate, medCOV is the median value of that covariate; and

x = 1 + (the number of THETAs in the $THETA block in the control stream).

4.2.7 Model Selection and evaluation

Comparison among the structural models as well as models containing covariates was based on the OFV, goodness-of-fit plots, estimates of parameters, and visual predictive checks. A $p < 0.01$, representing a decrease in OFV greater than 6.635 was considered statistically significant (degrees of freedom = 1) for selection of a structural model and covariates. The basis of the critical values for model and covariate selection is derived from the approximate χ^2 distribution of the difference between the OFV of two models.

The statistical significance and accuracy of the model parameters were assessed using bootstrapping. Bootstrap datasets (n=1000, stratified by study population) were generated by random sampling with replacement from the original dataset; and parameters were estimated from each dataset. A 95 % confidence interval (2.5th and

97.5th percentile) of each parameter distribution was then computed.

Finally, percentile visual predictive check (VPC) was conducted to examine adequacy of the model in predicting the original data. We simulated 1000 replicates of the original dataset, and compared the lower quartile, median and upper quartiles of the observed and simulated data.

4.3 Results

4.3.1 Study Population

Forty-seven subjects were enrolled in both studies. All subjects in Phase 1a trial were males (Caucasian = 90%); whereas in Phase 1b, 90 % were males (Black = 90%). Body weight differed significantly between the two studies (t-test, p-value < 0.04), but age did not. The demographic details of all subjects are summarized in **Table 4.1.** Alcohol consumption per week and serum albumin concentration were significantly higher in Phase 1b subjects (Mann Whitney, p <0.001). There was no major differences in the distribution of other laboratory tests **Table 4.1** between study subjects.

4.3.2 Pharmacokinetic Population Model

A total of 1354 data points were included in the analysis. A one-compartment model with first-order absorption from the gastrointestinal tract adequately described the data. Addition of a two-compartment model did not improve fit (increased OFV > 6.635). The overall model fitting to the data was satisfactory (r^2=0.95, p-value < 0.001), as shown in **Figure 4.1**. The best-fit population PK parameter estimates and

the bootstrap median (95 % confidence intervals) of the base model are shown in **Table 4.2**. The oral clearance was ~80 Lhr^{-1}, oral volume of distribution 575 L, and $t_{1/2}$ approximately 5 h. The inter-individual variability (IIV) of CL/F and Vd/F were less than 30%. The rate of absorption was however imprecise because few concentration data points were available during the absorption phase of PF-5190457.

4.3.3 Covariate Analysis

The influence of each covariate on the PK parameters was tested. Based on the trial design, the sex covariate was not considered though it appeared significant. This is because the study population was predominantly males (~98 %). Also, since the Phase 1a study was carried out in healthy subjects (who were predominantly white); and Phase 1b in non-treatment seeking alcoholic subjects (who were predominantly blacks), RACE was not considered as a potential covariate due to the possible confounding by the differences in alcohol consumption by the two groups.

Graphical inspection and evaluation using a generalized additive model (GAM) showed a significant association ($p < 0.03$) between interindividual variability (ETAs of CL/F, V/F and Ka), body weight (WEIGHTKG) albumin (ALBUMIN) (**Figure 4.2**). In a univariate analysis, DSGRP on V/F significantly reduced the OFV (ΔOFV = 30) along with ALBUMIN and body WEIGHTKG (**Supplementary Table 4.4 : Table 4.6**). Further evaluation of covariates resulted in no significant change in OFV after addition of ALBUMIN and WEIGHTKG (on V/F) to the base model (with DSGRP on V/F). The final covariate model based on forward selection was DSGRP, ALBUMIN and WEIGHTKG on V/F. Backward elimination and examination of

changes in IIV showed that a base model with ALBUMIN and WEIGHTKG on V/F significantly reduced the OFV and the IIV associated with V/F by ~28% compared to one containing DSGRP which changed OFV significantly, but reduced the IIV on V/F by ~21% (**Supplementary Table 4.7**). In addition, since the covariates (WEIGHTKG and ALBUMIN) were physiologically relevant to V/F, this model was selected as the final covariate model and presented below (**model 6**):

$$\frac{\mathrm{V}}{\mathrm{F}} = \mathrm{THETA(2)} * \left(\left(\frac{\mathrm{WEIGHTKG}}{78.3}\right)^{0.85} + \left(\frac{\mathrm{ALBUMIN}}{4.32}\right)^{-4.48}\right) \ldots\ldots\ldots\ldots\ldots\ldots (6)$$

Where V/F is the apparent volume of distribution (L); THETA(2) is 279.53 L; WEIGHTKG is body weight in kg; and ALBUMIN is serum albumin in g/dL. The final PK parameters for the full model are presented in **Table 4.3**.

This model suggests that the typical value of V/F of a median weight (78.3 kg) individual with median albumin level of 4.32 g/dL is 559 L. This value will increase with increase body weight (when albumin is 4.32 g/L). On the other hand, the V/F will decrease with increasing serum albumin levels (when body weight is 78.3 kg).

4.3.4 Model Evaluation

The goodness of fit plots for the base and full models are shown in **Figure 4.1**. The plot of predicted (PRED) and observed (DV) shows symmetry of points about the line of unit slope. The relative tightness of points was improved when individual predicted values (IPRED) were plotted against DV. The conditional weighted residuals (CWRES) [31] were approximately distributed around CWRES=0, but showed a

slight trend towards an additional compartment. Notwithstanding, the one-compartmental model was adequate for the model.

The median estimates and nonparametric 95 % CIs from the bootstrap analyses for fixed effect and IIV parameters were in a 1:1 ratio indicating that the parameter estimates in the final models were accurate, precise and statistically significant.

The results from the VPC evaluations **Figure 4.3** suggested that there was good agreement on the time course and central tendency (median) between distributions of observed and simulated data. The lower quartile, median and upper quartile of the observed concentration data were in agreement with the simulated data.

4.4 Discussion

PF-5190457 is an inverse agonist of the growth hormone secretagogue receptor (hGHS-R1a), believed to reduce alcohol craving. The purpose of this study was to describe the population pharmacokinetics of PF-5190457 and to identify clinical and demographic patient characteristics that influence PK variability.

Two studies (Phase 1a and Phase 1b) have been conducted recently to characterize the pharmacokinetics of PF-5190457 in healthy and alcoholic subjects separately [28]. In both studies, non-compartmental analysis (NCA) was employed to estimate the pharmacokinetic parameters. NCA is relatively fast and does not require any assumption of compartments. However, it has the tendency to under-estimate the ascending absorption phase of the plasma-concentration time curve after oral administration, or over-estimate the descending elimination phase, especially where the sampling interval is larger than the half-life of the drug [32]. Population

pharmacokinetics on the other hand, is model-based and is dependent on assumptions that drugs move into hypothetical body compartments and permits the incorporation of influential covariates on the PK parameters. PopPK is thus superior and used for comprehensive characterization of the PK profile of a drug. To the best of our knowledge, this is the first PopPK work for PF-5190457. This work presents an early characterization of the PK profile of PF-5190457 using combined data from both healthy and alcoholic subjects.

A one-compartment model with first-order absorption from the gastrointestinal tract adequately described the data. Though the CWRES showed a slight trend towards an additional compartment, a two-compartmental model did not improve the goodness of fit. This is because the sampling interval between 57 and 72 h was large for the Phase 1b study, and introduced a pseudo compartment. The typical PopPK estimates of the base model (CL/F, Vd/F, Ka and $t_{1/2}$: 80 Lhr^{-1}, 559 L, 3.7 hr^{-1} and 5 hrs respectively) were in agreement with the bootstrap values; thus indicating the stability of the model under 1000 bootstrap data sets. The reported half-life from the previous works however was higher (5.5 - 9.8 hrs) [28]. In NCA, the estimation of the half-life is heavily dependent on 3-4 observations which determines the terminal slope. Notwithstanding, visual inspections of the concentration-time curves (**Supplementary**) shows that plasma concentration falls by 50 % approximately every ~5 hours. Overall, PF-5190457 is rapidly absorbed and rapidly cleared from the body, supporting a washout period of 72 hrs for the removal of the parent drug from the body during a crossover clinical trial.

We evaluated patient demographics (e.g., age, race, sex, and body weight) and

relevant laboratory findings (e.g., alanine aminotransferase, aspartate aminotransferase, serum albumin, serum creatinine, total bilirubin) on the PK-parameters. Though initially many covariates came important; after further optimization of the model, body weight (kg) and serum albumin (g/dL) remained significantly important predictors on V/F. An increase in body weight will result in increase in V/F; but an increase in serum albumin will decrease V/F. This implies that, with oral clearance of ~80 Lh^{-1} and body weight of 78.3 kg, a subject with serum albumin level of <3.5 gdL-1 (hypoalbuinemia) will have a V/F > 997 L and a $t_{1/2}$ of > 8.6 hrs. Thus, the V/F may change from 559 L to more than 990 L in hypoalbuminemic subjects weighing 78.3 kg. In future trials and use of PF-5190457, it may be necessary to dose subjects based on body weight and serum albumin levels; and monitor patients for conditions that could alter serum albumin levels. This may be more necessary in alcoholic individuals with alcoholic liver disease where serum albumin level may be altered.

One limitation of this work was the absence of data points between 57 and 72 hrs for the Phase 1b study. This resulted in a pseudo bi-exponential profile. Also, because the studies were carried out in mainly males (98% of subjects), we could not evaluate the differences in PK between males and females. Lastly, since the distribution of race wasn't uniform in both studies, we could not evaluate the influence of race without confounding from alcohol consumption.

Despite the limitations, our work provides the first PopPK characterization of PF-5190457 and adequately shows that body weight and serum albumin are important predictors of V/F. We anticipate that our model would serve as a guide in designing

dosage regimen for participants of future clinical trials with PF-5190457.

Acknowledgement

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4.6 Tables

Table 4.1 Demographic and biochemical covariates of subjects

Characteristics	Subjects (N)	Median	Range
Sex (Male/Female)			
Overall	46/1		
Study 1	35/0		
Study 2	1-Nov		
Race(White/Black/Others)			
Overall	33/13/1		
Study 1	32/2/1		
Study 2	1/11/2000		
Age (YEARS)			
Overall	47	37	19-58
Study 1	35	37	19-55
Study 2	12	40	23-58
Weight (kg)			
Overall	47	78.3	56.60-120.50
Study 1	35	75.8	56.60-98.20
Study 2	12	82.9	58.10-120.50
BMI (kg/m^2)			
Overall	47	25.6	18.60 - 38.03
Study 1	35	25.4	18.60 - 30.30
Study 2	12	26.09	21.60 - 38.03
ALT (IU/L)			
Overall		20.165	Jul-59
Study 1		19.75	9.5 - 59
Study 2		21.165	7 - 56.67
ALBUMIN (g/dL)			
Overall		4.32	3.76 - 4.90
Study 1		4.23	3.76 - 4.79
Study 2		4.65	4.00 - 4.90
AST (IU/L)			
Overall		21.665	11.33 - 43
Study 1		21	12.5 - 43
Study 2		24.5	11.33 - 41
Serum Creatinine (mg/dL)			

Overall	0.9	0.53 - 1.2
Study 1	0.88	0.68 - 1.18
Study 2	0.945	0.53 - 1.20
CRCL (mL/min)		
Overall	125.528	73.97 - 246.64
Study 1	130.761	76.15 - 172.27
Study 2	119.884	73.97 - 246.63
Total Bilirubin (mg/dL)		
Overall	0.64	0.27 - 1.55
Study 1	0.7	0.3 - 1.55
Study 2	0.515	0.27 - 1.03
Alcohol per week (Glass)		
Overall	5	0 - 136.60
Study 1	3	0 - 12
Study 2	65.8	37.87 - 136.36
Nicotine Use		
Overall	1	0 - 6
Study 1	1	1 - 6
Study 2	2	0 - 6

Table 4.2 Population pharmacokinetic parameter estimates of the base model for PF-5190457

Parameter	Estimate (Final Model)	Bootstrap: median (95 % CI)
Fixed Effects		
CL/F (L/hr)	79.53	79.80 (73.47 - 131.17)
V/F (L)	575.00	577.00 (521.38 - 985.83)
Ka (hr^{-1})	3.62	3.65 (2.58 - 28.70)
Theta(4); additive component	0.31	0.31 (0.28 - 0.33)
Theta(5); proportional component	0.56	0.56 (0.43 - 0.85)
Inter-individual Variability (IIV)		
IIV CL/F, %	26.00	26.18 (18.46 - 54.78)
IIV V/F, %	29.00	29.12 (18.96 - 67.74)
IIV Ka, %	102.00	102.00 (74.11 - 454.78)
Residual Variability (Epsilon)		
EPS (σ1), %	1, FIXED	

CL/F, apparent clearance; V/F, apparent volume of distribution; Ka, absorption rate constant; Theta(4), additive component of error model; Theta(5), proportional component of error model weighting factor in the residual error; σ1is the residual error variability.

Table 4.3 Population pharmacokinetic parameter estimates of the full model (base+covariate) for PF-5190457

Parameter	Estimate (Final Model)	Bootstrap: median (95 % CI)
Fixed Effects		
CL/F (L/hr)	79.58	80.00 (73.49 - 101.00)
Theta(2)	279.53	280.77 (257.36 - 497.32)
Ka (hr^{-1})	3.60	3.66 (2.56 - 19.11)
Theta(4)	0.31	0.31 (0.28 - 0.33)
Theta(5)	0.56	0.56 (0.43 - 0.84)
Theta(6); on body weight (WT)	0.85	
Theta(7); on serum albumin (ALB)	-4.48	
Inter-individual Variability (IIV)		
IIV CL/F, %	26.05	26.24 (18.42 - 67.34)
IIV V/F, %	21.00	21.27 (13.37 - 82.21)
IIV Ka, %	104.19	103.50 (76.13 - 192.38)
Residual Variability (Epsilon)		
EPS (σ1), %	1, FIXED	
Covariate model		
V/F (L)	Theta(2)*[(WT/78.3)**0.85 + (ALB/4.32)**(-4.48)]	

CL/F, apparent clearance; V/F, apparent volume of distribution; Ka, absorption rate constant; Theta(4), additive component of error model; Theta(5), proportional component of error model weighting factor in the residual error; σ1is the residual error variability.

4.7 Figures

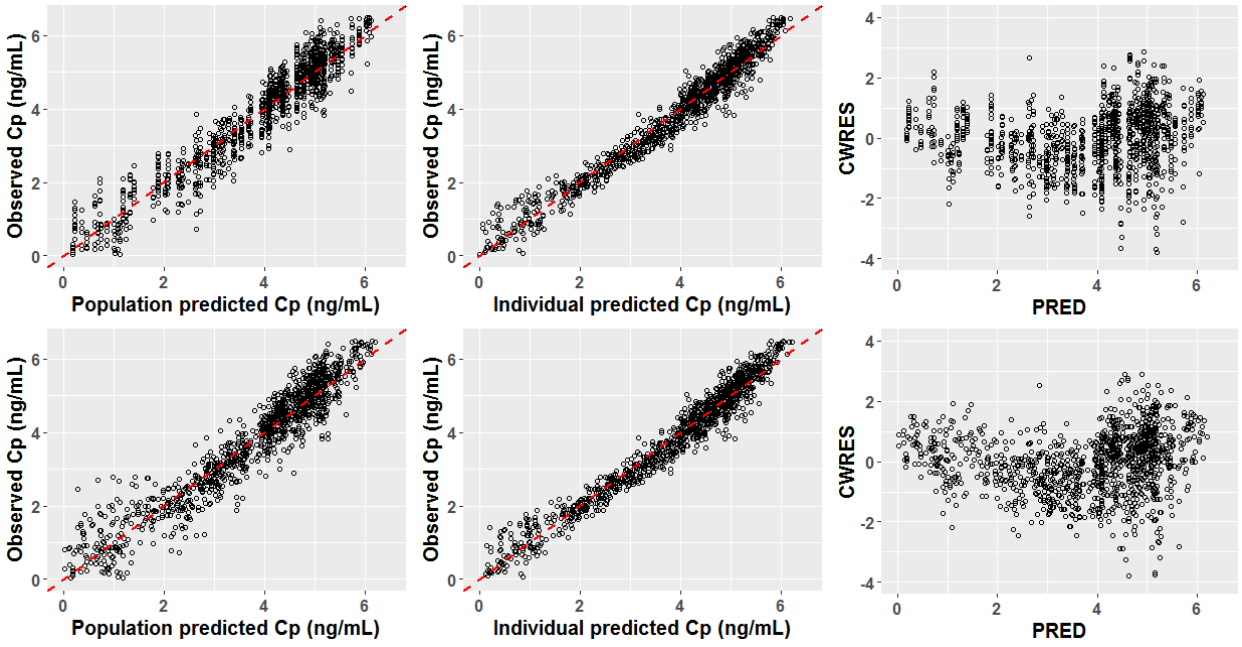


Figure 4.1 Goodness of fit plots for base (top panel) and full model (bottom panel)

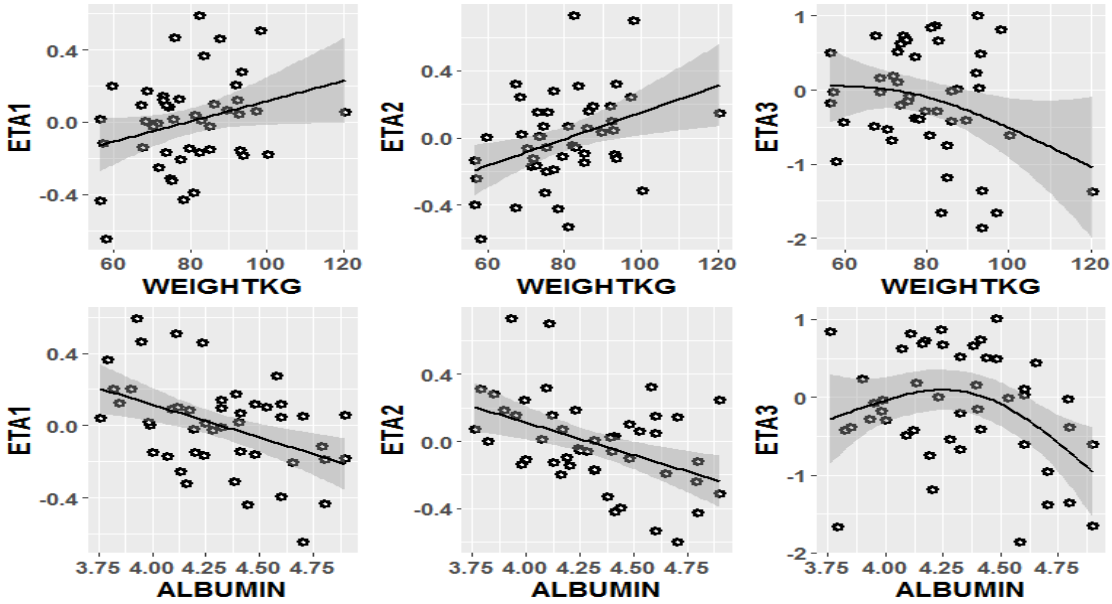


Figure 4.2 Relationship between ETAs and covariates: Body weight (WEIGHTKG) and serum albumin.

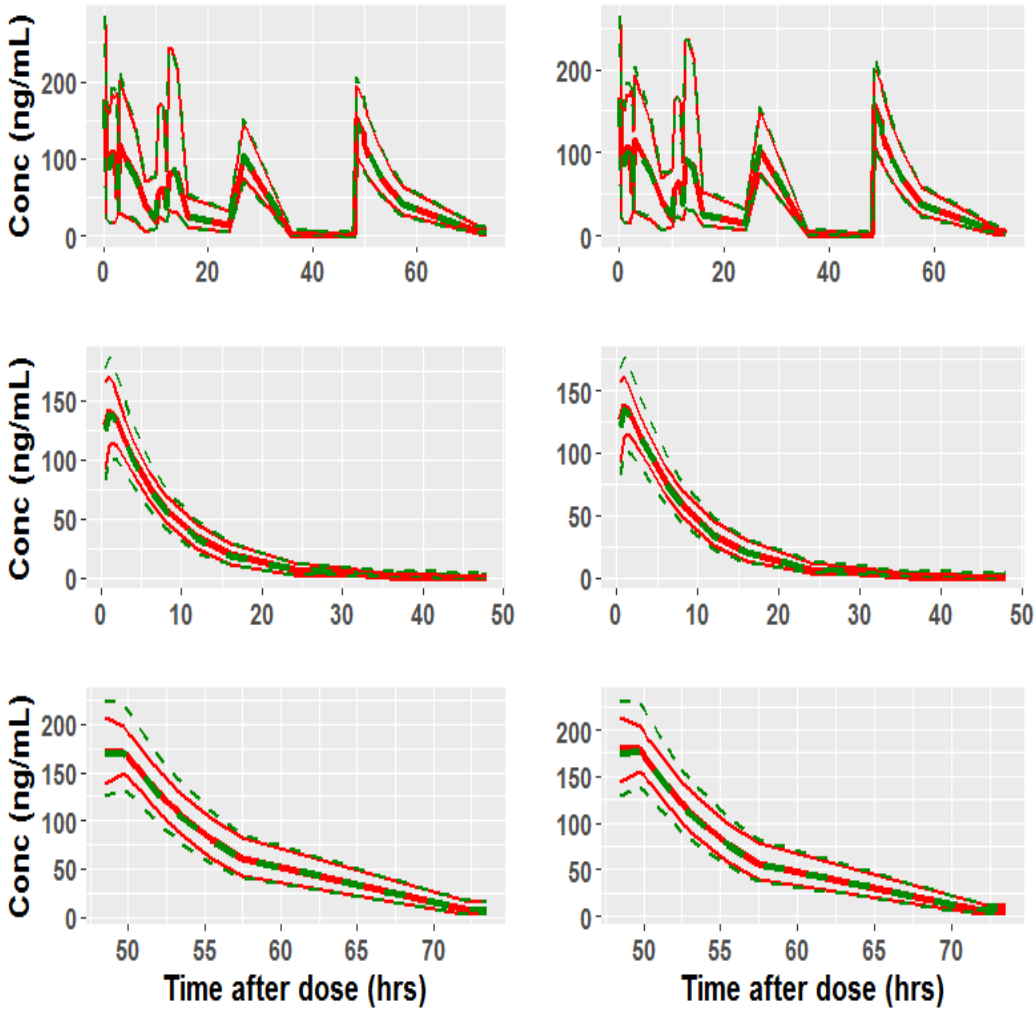
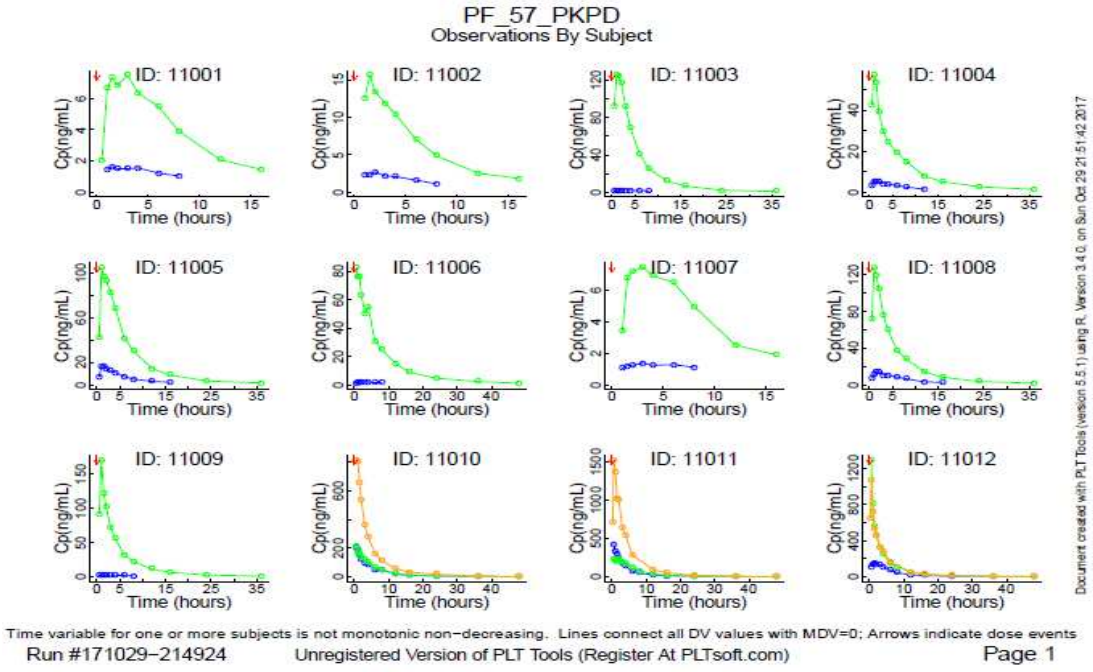


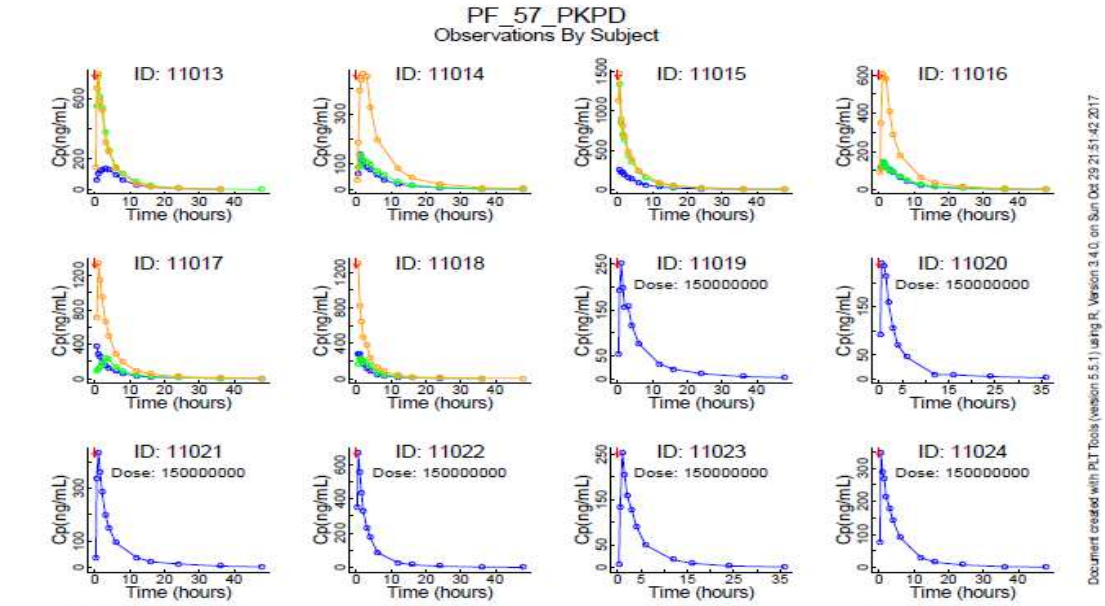
Figure 4.3 Percentile visual predictive checks.
Percentile visual predictive checks for base (left hand side) and full (right hand side) models. The lower quantile, median and upper quantile of observations (solid lines) and simulations (dashed lines) of all data (top panel), data corresponding to 100 mg dose in STUDY 1 (middle panel), and data corresponding to 100 mg dose in STUDY 2 (bottom panel).

4.8 Supplementary



Time variable for one or more subjects is not monotonic non−decreasing. Lines connect all DV values with MDV=0; Arrows indicate dose events

Run #171029−214924 Unregistered Version of PLT Tools (Register At PLTsoft.com) Page 1



Time variable for one or more subjects is not monotonic non−decreasing. Lines connect all DV values with MDV=0; Arrows indicate dose events

Run #171029−214924 Unregistered Version of PLT Tools (Register At PLTsoft.com) Page 2

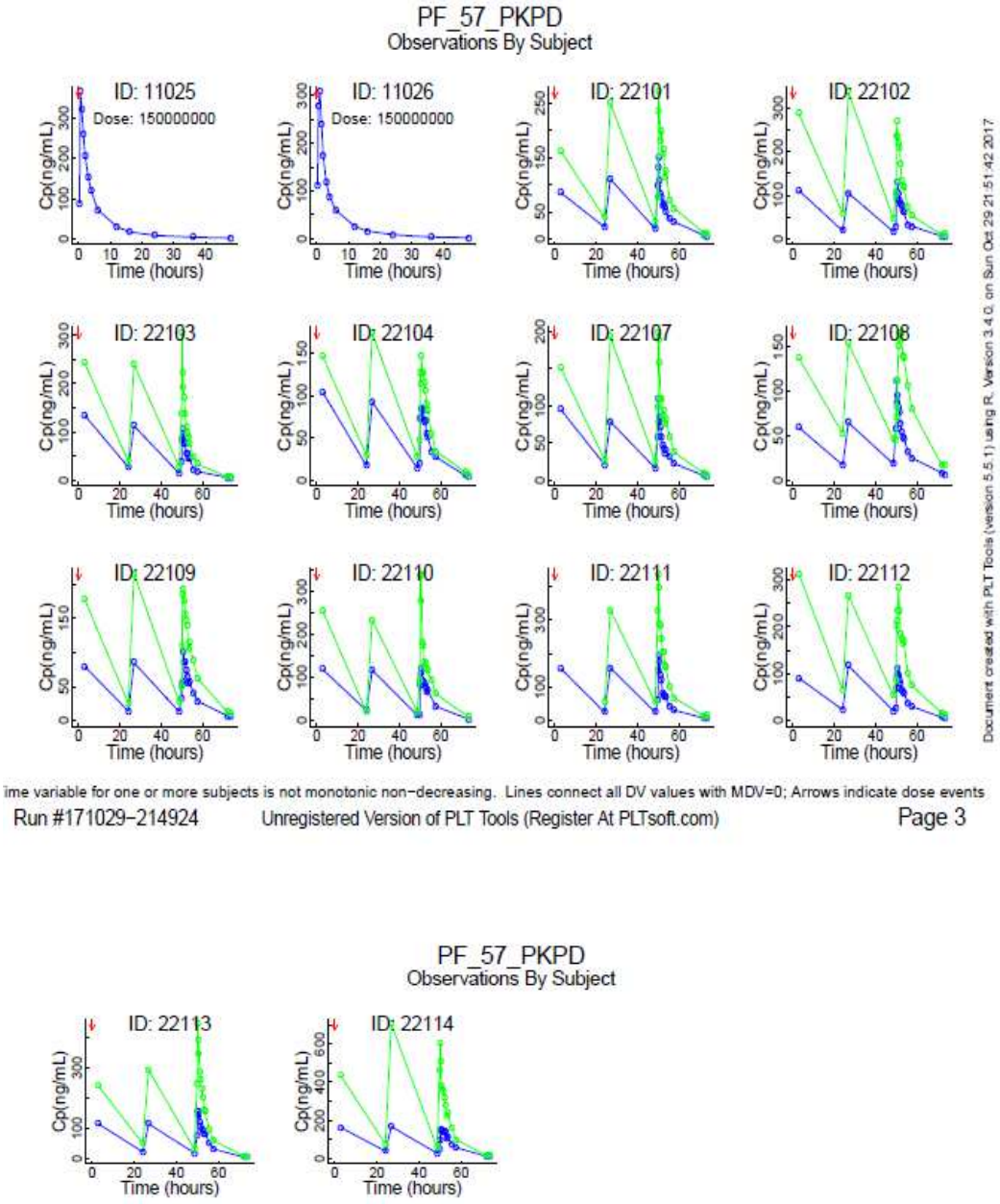
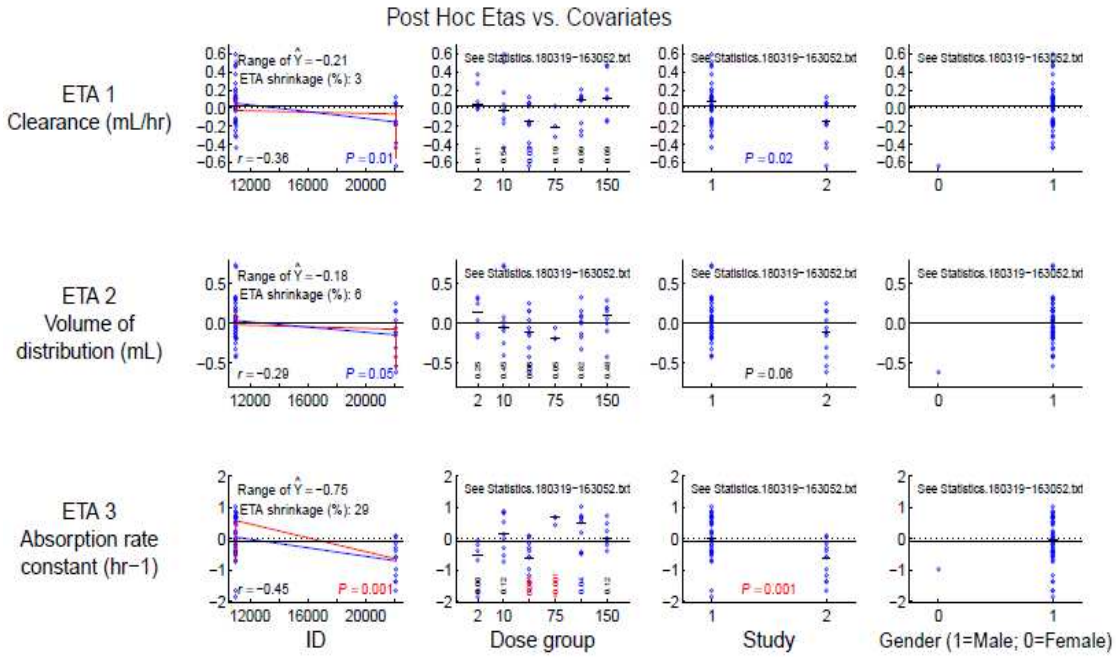
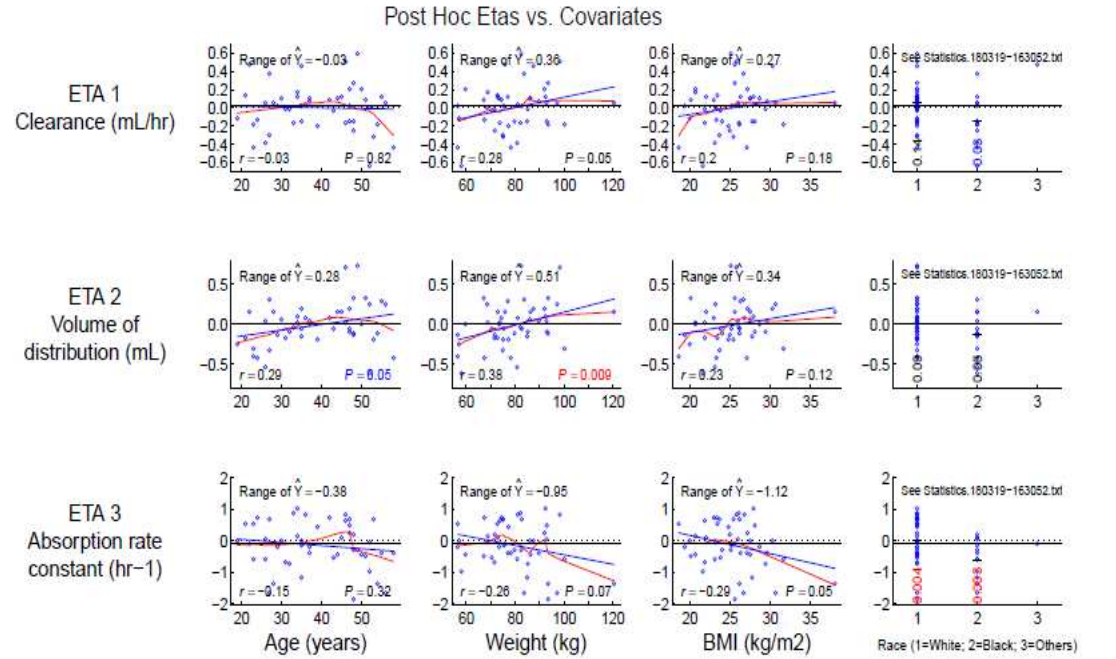


Figure 4.4 Graphs showing the plasma concentration-time plot of PF-5190457


Post Hoc Etas vs. Covariates
ETA 1
Clearance (mL/hr)
ETA 2
Volume of
distribution (mL)
ETA 3
Absorption rate
constant (hr−1)
Range of Ŷ = −0.21
ETA shrinkage (%): 3
r = −0.36
P = 0.01
Range of Ŷ = −0.18
ETA shrinkage (%): 6
r = −0.29
P = 0.05
Range of Ŷ = −0.75
ETA shrinkage (%): 29
r = −0.45
P = 0.001
See Statistics.180319−163052.txt
P = 0.02
P = 0.06
P = 0.001
ID
Dose group
Study
Gender (1=Male; 0=Female)


Post Hoc Etas vs. Covariates
ETA 1
Clearance (mL/hr)
ETA 2
Volume of
distribution (mL)
ETA 3
Absorption rate
constant (hr−1)
Range of Ŷ = −0.03
r = −0.03
P = 0.82
Range of Ŷ = 0.36
r = 0.28
P = 0.05
Range of Ŷ = 0.27
r = 0.2
P = 0.18
Range of Ŷ = 0.28
r = 0.29
P = 0.05
Range of Ŷ = 0.51
r = 0.38
P = 0.009
Range of Ŷ = 0.34
r = 0.23
P = 0.12
Range of Ŷ = −0.38
r = −0.15
P = 0.32
Range of Ŷ = −0.95
r = −0.26
P = 0.07
Range of Ŷ = −1.12
r = −0.29
P = 0.05
See Statistics.180319−163052.txt
Age (years)
Weight (kg)
BMI (kg/m2)
Race (1=White; 2=Black; 3=Others)

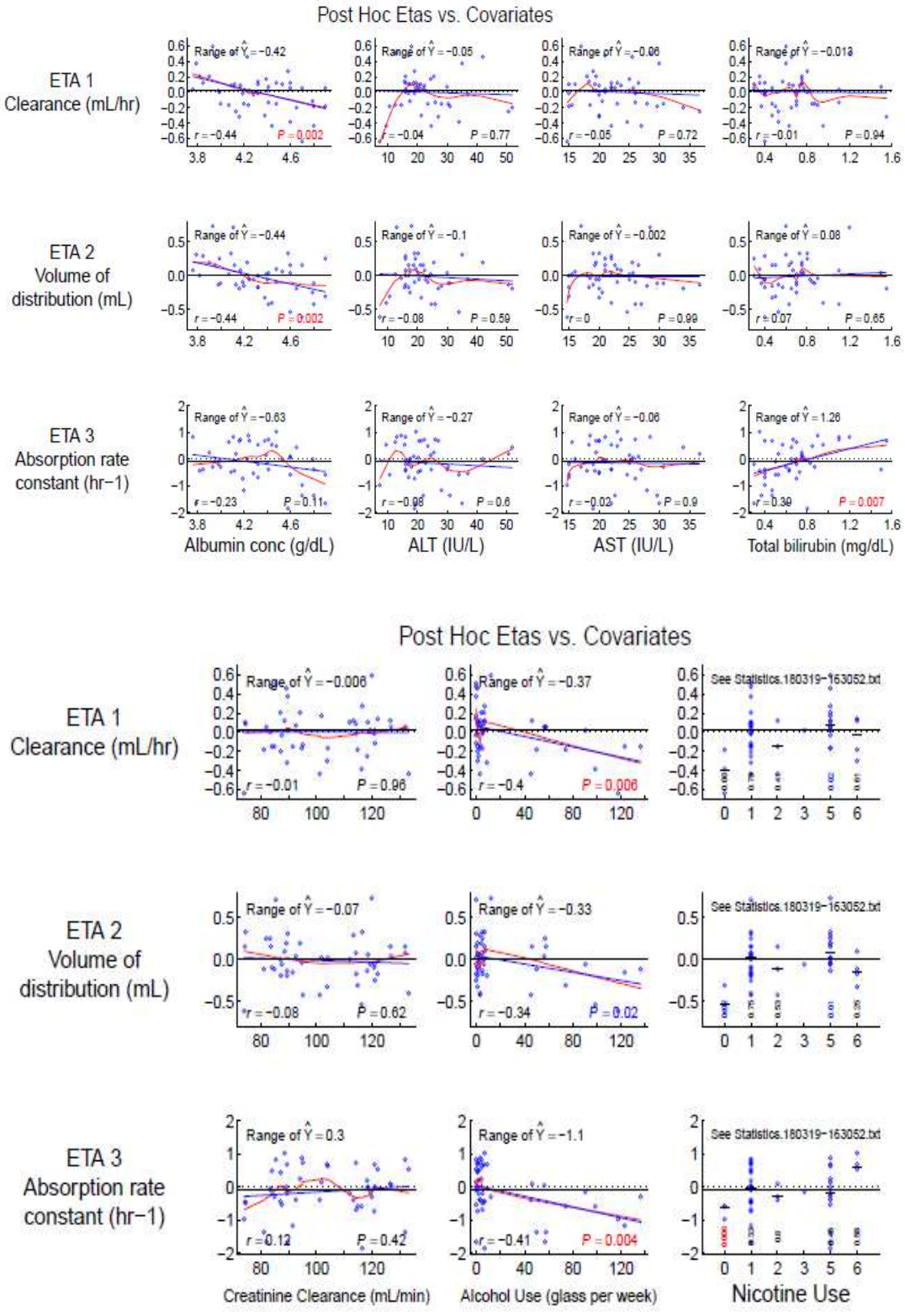


Figure 4.5 Graphs showing the relationship between ETAs and all covariates.

Covariate selection (forward selection) based on change in OFV and improvement in variance associated with interindividual variability.

Table 4.4 Step 2: Base model + single covariate

Covariate	Parameter	OF	ΔOF	CutOff	OM-VAR1	OM-VAR2	OM-VAR3	Functional Form	p-value
BASE		-1071.89	0.00		0.069	0.084	1.046		
DSGRP	**V/F**	**-1101.93**	**30.04**	**> 6.63**	**0.068**	**0.087**	**1.051**	**Exponential**	**0.00**
DSGRP	ka	-1086.79	14.90	> 6.63	0.068	0.084	0.872	Exponential	0.00
ALBUMIN	V/F	-1085.55	13.66	> 6.63	0.069	0.063	1.103	Multiplicative	0.000
ALBUMIN	V/F	-1085.55	13.66	> 6.63	0.069	0.063	1.103	Additive	0.000
STUDY	ka	-1081.23	9.34	> 6.63	0.067	0.087	0.693	Additive	0.002
STUDY	ka	-1081.23	9.34	> 6.63	0.067	0.087	0.693	Exponential	0.00
STUDY	ka	-1081.23	9.34	> 6.63	0.067	0.087	0.693	Multiplicative	0.002
BMI	ka	-1080.99	9.10	> 6.63	0.055	0.085	1.040	Exponential	0.00
ALBUMIN	CL/F	-1080.09	8.20	> 6.63	0.055	0.085	1.040	Additive	0.004
ALBUMIN	CL/F	-1080.09	8.20	> 6.63	0.055	0.085	1.040	Multiplicative	0.004
ALCOHOLPERWEEK	CL/F	-1080.05	8.16	> 6.63	0.057	0.084	1.050	Additive	0.004
DSGRP	V/F	-1079.45	7.56	> 6.63	0.069	0.086	1.051	Additive	0.006
AGEYRS	V/F	-1079.43	7.54	> 6.63	0.068	0.071	1.012	Exponential	0.01
WEIGHTKG	V/F	-1079.33	7.44	> 6.63	0.068	0.071	1.012	Additive	0.006
STUDY	CL/F	-1078.45	6.56	< 6.63	0.059	0.084	1.054	Exponential	0.01
STUDY	CL/F	-1078.45	6.56	< 6.63	0.059	0.084	1.054	Additive	0.010
STUDY	CL/F	-1078.45	6.56	< 6.63	0.059	0.084	1.054	Multiplicative	0.010
DSGRP	ka	-1078.38	6.49	< 6.63	0.068	0.083	0.869	Additive	0.011
ALCOHOLPERWEEK	V/F	-1077.91	6.02	< 6.63	0.069	0.072	1.069	Additive	0.014
CRCL_MDRD	ka	-1077.88	5.99	< 6.63	0.068	0.085	1.103	Multiplicative	0.014
AGEYRS	V/F	-1076.34	4.45	< 6.63	0.068	0.075	1.044	Additive	0.035
STUDY	V/F	-1076.34	4.45	< 6.63	0.069	0.076	1.067	Multiplicative	0.035
STUDY	V/F	-1076.34	4.45	< 6.63	0.069	0.076	1.067	Additive	0.035
AGEYRS	CL/F	-1076.20	4.31	< 6.63	0.062	0.084	1.047	Exponential	0.04
WEIGHTKG	CL/F	-1075.98	4.10	< 6.63	0.062	0.084	1.047	Multiplicative	0.043
WEIGHTKG	CL/F	-1075.98	4.10	< 6.63	0.062	0.084	1.047	Additive	0.043
TOTBILIRUB	V/F	-1075.85	3.96	< 6.63	0.068	0.085	1.066	Exponential	0.05

OF is objective function; ΔOF is change in OF; OM-VAR variance associated with CL/F (1), V/F (2), and Ka (3); DSGRP is dose group. Covariate was considered for the next step of the analysis if p-value < 0.01.

Table 4.5 Step 3. Base model + DSGRP on V/F + single covariate

Covariate	Parameter	OF	ΔOF	Cut off	OM-VAR1	OM-VAR2	OM-VAR3	Functional form	P-value
Base		-1101.93	0.00	< 6.63	0.068	0.087	1.051	Exponential	1.000
ALBUMIN	V/F	-1112.56	10.63	> 6.63	0.069	0.067	1.090	Additive	0.001
WEIGHTKG	**V/F**	**-1111.56**	**9.63**	**> 6.63**	**0.068**	**0.070**	**1.012**	**Exponential**	**0.002**
WEIGHTKG	V/F	-1111.44	9.51	> 6.63	0.068	0.070	1.011	Additive	0.002
ALBUMIN	CL/F	-1110.69	8.76	> 6.63	0.055	0.088	1.048	Multiplicative	0.003
ALBUMIN	CL/F	-1110.69	8.76	> 6.63	0.055	0.088	1.048	Additive	0.003
ALCOHOLPERWEEK	CL/F	-1109.70	7.77	> 6.63	0.057	0.088	1.055	Additive	0.005
DSGRP	CL/F	-1108.81	6.88	> 6.63	0.069	0.088	1.055	Exponential	0.009
WEIGHTKG	CL/F	-1106.68	4.75	< 6.63	0.062	0.087	1.050	Exponential	0.029
WEIGHTKG	CL/F	-1106.47	4.54	< 6.63	0.062	0.087	1.050	Additive	0.033
ALCOHOLPERWEEK	V/F	-1106.39	4.46	< 6.63	0.069	0.078	1.070	Additive	0.035
DSGRP	CL/F	-1102.09	0.16	< 6.63	0.069	0.087	1.052	Additive	0.688
ALCOHOLPERWEEK	CL/F	1166.90	-2268.83	< 6.63	0.072	0.284	1.515	Multiplicative	
ALBUMIN	V/F	-924.68	-177.25	< 6.63	0.109	0.798	20.458	Multiplicative	
ALBUMIN	CL/F	-911.14	-190.78	< 6.63	0.106	1.636	26.595	Exponential	
ALBUMIN	V/F	-859.56	-242.37	< 6.63	0.112	1.303	39.615	Exponential	
WEIGHTKG	V/F	-865.94	-235.99	< 6.63	0.038	0.130	137.206	Multiplicative	
WEIGHTKG	CL/F	-486.88	-615.05	< 6.63	0.003	4.543	620.837	Multiplicative	
DSGRP	CL/F	-759.80	-342.13	< 6.63	0.039	0.642	203346.000	Multiplicative	

OF is objective function; ΔOF is change in OF; OM-VAR variance associated with CL/F (1), V/F (2), and Ka (3); DSGRP is dose group. Covariate was considered for the next step of the analysis if p-value < 0.01.

Table 4.6 Step 4. Base model + DSGRP on V/F + Body weight on V/F + single covariate

Covariate	Parmeter	OF	ΔOF	Cutoff	OM-VAR1	OM-VAR2	OM-VAR3	Functional Form	p-value
Base		-1102.6	0	< 6.63	0.068	0.072	1.035		1
ALBUMIN	**V/F**	**-1124.8**	**22.21**	**> 6.63**	**0.067**	**0.043**	**1.085**	**Exponential**	**0.0000**
ALBUMIN	V/F	-1123.3	20.65	> 6.63	0.067	0.045	1.094	Multiplicative	0.0000
ALBUMIN	V/F	-1123.3	20.65	> 6.63	0.067	0.045	1.094	Additive	0.0000
ALBUMIN	CL/F	-1111.5	8.89	> 6.63	0.055	0.073	1.032	Exponential	0.0029
ALBUMIN	CL/F	-1110.7	8.09	> 6.63	0.055	0.073	1.031	Additive	0.0044
ALBUMIN	CL/F	-1110.7	8.09	> 6.63	0.055	0.073	1.031	Multiplicative	0.0044
ALCOHOLPERWEEK	CL/F	-1110.4	7.79	> 6.63	0.056	0.073	1.038	Additive	0.0052
ALCOHOLPERWEEK	V/F	-1109.8	7.16	> 6.63	0.068	0.060	1.059	Additive	0.0074
WEIGHTKG	CL/F	-1107.5	4.85	< 6.63	0.061	0.071	1.035	Exponential	0.0276
WEIGHTKG	CL/F	-1107.2	4.64	< 6.63	0.061	0.071	1.036	Additive	0.0313
DSGRP	CL/F	-1103.2	0.60	< 6.63	0.067	0.072	1.035	Additive	0.4391
ALCOHOLPERWEEK	CL/F	-985.01	-117.61	< 6.63	0.053	0.081	1.131	Multiplicative	
WEIGHTKG	CL/F	-984.83	-117.78	< 6.63	0.054	0.078	1.145	Multiplicative	
DSGRP	CL/F	-980.46	-122.15	< 6.63	0.062	0.079	1.148	Exponential	
DSGRP	CL/F	-979.76	-122.85	< 6.63	0.062	0.080	1.150	Multiplicative	
ALCOHOLPERWEEK	V/F	-851.57	-251.04	< 6.63	0.504	0.125	4.051	Multiplicative	

OF is objective function; ΔOF is change in OF; OM-VAR variance associated with CL/F (1), V/F (2), and Ka (3); DSGRP is dose group. Covariate was considered for the next step of the analysis if p-value < 0.01.

Covariate selection (backward elimination) based on change in OFV and improvement in variance associated with interindividual variability of V/F (ETA(2)).

Table 4.7 Backward elimination

Covariate	OF	ΔOF	DF	IIV [ETA(2)]	p-value
BASE MODEL	-1071.89	0	0	29	
WEIGHT+ALBUMIN	**-1098.15**	**26.26**	**2**	**21**	**> 0.0001**
DSGRP+WEIGHTKG	-1102.55	30.66	2	27	> 0.0001
DSGRP+ALBUMIN	-1105.14	33.25	2	25	> 0.0001
DSGRP+WEIGHTKG+ALBUMIN	-1109.44	37.55	3	23	> 0.0001

WEIGHTKG is body weight in kg; DSGRP is dose group; IIV [ETA(2)] is interindividual variability associated with volume of distribution (V/F).

5 CONCLUSION

This work demonstrates the utility of in vitro drug metabolism and population pharmacokinetics to elucidate the sources of pharmacokinetic variability.

In the first part of this work (Manuscripts I, II and III), we demonstrated that NAFLD influences the in vitro kinetics of CYP2B6-mediated hydroxylation of bupropion. It reduced the Vmax by ~32% and increased the km by ~2 fold. Consiquently, the intrinsic clearance was reduced in NASH human liver microsomes (HLM) by 2.3 fold compared to the NoNAFLD HLM. The HepaRG system, together with available findings in Sprague-Dawley rats corroborrated the findings in the HLM. However, the Simcyp simulation suggested a possibly different scenario in vivo. This may be due to lack of an appropriate NAFLD population model for PBPK simulation. A well-controlled clinical trial may therefore be necessary to confirm the findings of this study.

In the second part of this work (Manuscript IV), we used population pharmacokinetics to characterize PF-5190457, and to identify potential covariates that influence the PK variability of PF-5190457. A one-compartmental model with first order absorption after oral administration best described the PK profile of PF-5190457. The estimated typical PK parameters of the base model, including the absorption rate constant (3.6 h^{-1}), oral clearance (79.53 Lh^{-1}) and apparent volume of distribution (575 L), suggested that PF-5190457 is rapidly absorbed and rapidly cleared from the body. Additionally, body weight and serum albumin reduced the IIV associated with V/F by ~28%. Body

weight and serum albumin were thus identified to be potential sources of PF-5190457 PK variability.

We believe this work presents new insights into the influence of NAFLD on CYP2B6-mediated hydroxylation of bupropion. Similarly, it gives new information about the PK of PF-5190457. It is hoped that the information will be useful for future studies involving CYP2B6 and NAFLD. We also anticipate that our model for PF-5190457 would serve as a guide in designing future clinical trials with PF-5190457.

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6 APPENDIX

6.1 NONMEM codes for Manuscript IV

BASE MODEL

```
;PF-05190457 was given as oral suspension

$PROBLEM 1CMT WITH ABSORPT PF-05190457 (BASE MODEL)

$DATA
C:/Users/ENOCH/Documents/4_PKPD_Model/step_5_final_models/DATAFILES/Fi
nalData_PK_COV_ALLSUBJ_V2_orem1a.csv IGNORE=C

$INPUT C SUBJID=DROP ID=PTID TIMEMNS=DROP TIME=TIMHRS ODV
DV=LNDV MDV CMT EVID AMT=AMTNG ADDL II DSGRP STUDY COHORT
CPEVENT ALBUMIN ALT AST SCr=DROP CRCL_CG=DROP CRCL_MDRD
TOTBILIRUB MALE1 AGEYRS WEIGHTKG BMI RACEW
ALCOHOLPERWEEK NICTINUSE

$SUBROUTINE ADVAN2 TRANS2

$PK

 TVCL = THETA(1) ; specifying the clearance
 CL = TVCL*EXP(ETA(1)); exponential IIV


 TVV = THETA(2)
 V = TVV*EXP(ETA(2)) exponential IIV

 TVKA = THETA(3) ; specifying the absorption rate constant
 KA = TVKA*EXP(ETA(3)) ; exponential IIV

 S2=V ; scale paramenter of 1


$ERROR (OBSERVATIONS ONLY)
      PRED=F
         IPRED=0
         IF(F.GT.0) IPRED=LOG(F)
```

```
Y= IPRED +SQRT(THETA(4)**2 + THETA(5)**2/F**2)*EPS(1)


$THETA
  (0.1,6000,);initial estimate for CL [CL]
  (0.1,50000,); initial estimate for Vd [V]
  (0.01,1,) ; initial estimate for KA [KA]
  (0, 0.1);
  (0, 0.1);
 ;(,0.01,);


$OMEGA 0.1 0.1 1; diagonal matrix for variance of omegas 1, 2, and 3

$SIGMA 1 FIX


$ESTIMATION
      METHOD=1       PRINT=1        MAXEVALS=9999    NOABORT
      SIGDIGITS=6    POSTHOC        INTER          MSFO=MSFO.OUTPUTFILE

$COVARIANCE
$TABLE  ID  EVID  AMT TIME DSGRP STUDY IPRED CWRES NOPRINT
FILE=ALLRECORDS.TXT
$TABLE  ID  DSGRP  STUDY  CL V KA ETA1 ETA2 ETA3 FIRSTONLY
NOPRINT NOAPPEND FILE=FIRSTRECORDS.TXT
```

FULL MODEL (Base + Covariates)

```
;PF-05190457 was given as oral suspension

$PROBLEM 1CMT WITH ABSORPT PF-05190457 (Full model)

$DATA
C:/Users/ENOCH/Documents/4_PKPD_Model/step_5_final_models/DATAFILES/Fi
nalData_PK_COV_ALLSUBJ_V2_orem1a.csv IGNORE=C
```

```
$INPUT C SUBJID=DROP ID=PTID TIMEMNS=DROP TIME=TIMHRS ODV
DV=LNDV MDV CMT EVID AMT=AMTNG ADDL II DSGRP STUDY COHORT
CPEVENT ALBUMIN ALT AST SCr=DROP CRCL_CG=DROP CRCL_MDRD
TOTBILIRUB MALE1 AGEYRS WEIGHTKG BMI RACEW
ALCOHOLPERWEEK NICTINUSE


$SUBROUTINE ADVAN2 TRANS2


$PK

  TVCL = THETA(1) ; specifying the clearance
  CL = TVCL*EXP(ETA(1)); exponential IIV

 TVV = (THETA(2) * (WEIGHTKG / 78.3) ** (0.85)) + (THETA(2) * (ALBUMIN /
4.32) ** (-4.48))
  V = TVV*EXP(ETA(2)) exponential IIV

  TVKA = THETA(3) ; specifying the absorption rate constant
  KA = TVKA*EXP(ETA(3)) ; exponential IIV

S2=V ; scale paramenter of 1

$ERROR (OBSERVATIONS ONLY)
         PRED=F
          IPRED=0
          IF(F.GT.0) IPRED=LOG(F)
          Y= IPRED +SQRT(THETA(4)**2 + THETA(5)**2/F**2)*EPS(1)


$THETA
  (0.1,6000,);initial estimate for CL [CL]
  (0.1,50000,); initial estimate for Vd [V]
  (0.01,1,) ; initial estimate for KA [KA]
  (0, 0.1);
  (0, 0.1);


$OMEGA 0.1 0.1 1; diagonal matrix for variance of omegas 1, 2, and 3
```

```
$SIGMA 1 FIX


$ESTIMATION
      METHOD=1       PRINT=1        MAXEVALS=9999    NOABORT
      SIGDIGITS=6    POSTHOC        INTER          MSFO=MSFO.OUTPUTFILE

$COVARIANCE
$TABLE  ID  EVID  AMT TIME DSGRP STUDY IPRED CWRES NOPRINT
FILE=ALLRECORDS.TXT
$TABLE  ID  DSGRP  STUDY  CL V KA ETA1 ETA2 ETA3 FIRSTONLY
NOPRINT NOAPPEND FILE=FIRSTRECORDS.TXT
```