2019

USE OF HYPHENATED MASS SPECTROMETRY TO UNCOVER TRUE NAFLD EFFECT ON HUMAN DRUG DISPOSITION PROTEINS

Benjamin Joseph Barlock
University of Rhode Island, Bbarlock@uri.edu

Follow this and additional works at: https://digitalcommons.uri.edu/oa_diss

Recommended Citation

Barlock, Benjamin Joseph, "USE OF HYPHENATED MASS SPECTROMETRY TO UNCOVER TRUE NAFLD EFFECT ON HUMAN DRUG DISPOSITION PROTEINS" (2019). *Open Access Dissertations*. Paper 1096.
https://digitalcommons.uri.edu/oa_diss/1096

This Dissertation is brought to you for free and open access by DigitalCommons@URI. It has been accepted for inclusion in Open Access Dissertations by an authorized administrator of DigitalCommons@URI. For more information, please contact digitalcommons-group@uri.edu.

USE OF HYPHENATED MASS SPECTROMETRY TO UNCOVER TRUE NAFLD
EFFECT ON HUMAN DRUG DISPOSITION PROTEINS

BY

BENJAMIN JOSEPH BARLOCK

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

PHARMACEUTICAL SCIENCES

UNIVERSITY OF RHODE ISLAND

2019

DOCTOR OF PHILOSOPHY DISSERTATION

OF

BENJAMIN J. BARLOCK

APPROVED:

Dissertation Committee

Major Professor

Fatemeh Akhlaghi

Angela L. Slitt

Kathleen J. Melanson

Nasser H. Zawia
Dean, The Graduate School – URI

UNIVERSITY OF RHODE ISLAND
2019

ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is growing in prevalence across the world and is associated with a significant economic cost. In addition to life style modification, no pharmacotherapy method is available to treat NAFLD. As the histology of the liver changes over the course of the disease, it can be expected that the liver proteome will change too. Included in the liver proteome are drug disposition proteins comprised of drug metabolizing enzymes and transporters. These proteins are responsible for the removal of drugs and endogenous compounds from the body. The goals of this dissertation were: (1) Develop methodology to accurately and reproducibly measure drug metabolizing enzymes and transporters and (2) determine the alterations in these proteins as a result of the progression of NAFLD and diabetes. Manuscripts I and II are used to develop the methodology required to measure the proteins of interest and determine other variables that may be affecting protein expression changes besides disease. Manuscripts III and IV bring biological effect into the equation to determine how NAFLD and diabetes change the proteome and compare the results to a mouse model of NASH to assess its validity. The manuscripts are briefly described below:

Manuscript I: Hepatic xenobiotic transporters in the human liver play an important role in the elimination of drugs or toxins and significantly contribute to variability in drug response. We have developed a label-free mass spectrometry-based method to study the protein expression of 13 of 25 clinically relevant transporter proteins (6 ABC and 7 SLC) in liver tissue from 20 donors (9 female, 11 male). Whole tissue homogenate was used, and in-solution trypsin digestion was performed using pressure-

cycling technology. Data was acquired in data-dependent and sequential window acquisition of all theoretical mass spectra (SWATH-MS) mode. Bovine serum albumin (BSA) was spiked in each sample before digestion and monitored for batch to batch variability. Spectronaut™ was used for peptide identification and data extraction from SWATH files. Na⁺/K⁺ transporting subunit alpha 1 (ATP1A1), was quantified as a cell membrane marker and its coefficient of variation was 16.6% across different liver samples. The work highlights the suitability of SWATH-MS for large-scale simultaneous quantification of several xenobiotic transporters important in drug disposition. We found that average differential expression of transporters proteins was similar and much smaller than the inter-individual variability observed between the genders.

Manuscript II: Human liver tissue utilized in drug metabolism (DM) studies is retrieved from brain-dead individuals receiving an array of therapeutic agents during hospitalization. The anticonvulsant agent phenytoin, a potent cytochrome P450 (CYP) 3A4 inducer, is among such agents that potentially can impact the expression and activity of DM enzymes. Here we identify and highlight the need to screen human liver tissues for the presence of phenytoin. One-hundred and six samples from an in-house repository of human livers were screened for the presence of phenytoin and 1,702 other compounds utilizing AB SCIEX forensic library and a Triple TOF-instrument. Phenytoin concentration were then quantified in phenytoin positive tissue using LC-MS/MS. Activity levels for CYP3A4 were collected in-house as well as provided by the vendor. Expression of CYP proteins were analyzed by an untargeted proteomic analysis. mRNA levels for all CYPs and certain nuclear transcription

factors were also measured. Non-parametric independent sample tests were run in SPSS and comparisons with a p-value <0.05 were considered significant. Phenytoin was present in 34 of the 106 human livers (32%) and the functional activity and protein level of CYP3A4 were significantly higher in these samples. Caution should be taken when performing studies with commercially available liver sample and microsomes prepared thereof. The use of ambiguous protein expression values due to induction from phenytoin may decrease the accuracy of PBPK models developed using such data. Whenever possible, we suggest that livers tissue, primary hepatocytes and liver microsomes be screened for phenytoin to prevent altered results due to the induction of DMEs.

Manuscript III: Differential basal expression of drug metabolizing enzymes (DMEs) and transporters in disease state can contribute to significant changes in systemic exposure of xenobiotics. To gain insight into the alterations of these proteins during non-alcoholic fatty liver disease (NAFLD) and diabetes, we investigated the protein abundance and activity in 106 human liver samples. In-solution trypsin digestion of proteins in whole tissue lysate was carried out using pressure-cycling technology. Data were acquired in data-dependent acquisition (DDA) and sequential window acquisition of all theoretical ion mass spectra (SWATH-MS) mode on a time of flight (TOF) mass spectrometer, and absolute protein levels were determined using total protein approach. Functional activity data from the tissue vendor were used to evaluate the effect of disease on cytochrome P450 (CYP450) activity and further correlated with protein abundance. It was found that NAFLD altered CYP20A1, CYP27A1, CYP2B6, CYP2E1, CYP3A4, CYP2J2, UGT1A3, UGT1A4, SULT2A1, BSEP and

MRP2 protein expression. Diabetes was found to significantly alter the protein expression of CYP4F11, CYP20A1, CYP27A1, CYP2B6, CYP2E1, CYP3A4, CYP2J2, SULT1B1, SULT1A1, UGT1A3, UGT1A4, OATP1B3 and OATP2B1. It is important to point out that when livers containing the anticonvulsant, phenytoin, were excluded from the analysis, the changes seen in CYP3A4 were no longer significant. In some cases, both diseases altered the expression of the same protein leading to potentially exacerbated implications. It is important to study these special populations as patients with NAFLD and diabetes tend to be prescribed more medications than healthy individuals. The alteration to drug disposition proteins could lead to an altered pharmacokinetic profile and sub therapeutic or even toxic results.

Manuscript IV: Non-alcoholic fatty liver disease (NAFLD) is a highly prevalent disease in our modern society. It is important to develop an accurate mouse model that is representative of the disease. The American Lifestyle Induced Obesity Syndrome (ALIOS) model aims to cause mice to develop NAFLD through diet and lifestyle management simulating western culture. Determining the proteomic changes occurring to the drug metabolizing profile is critical to assess if the model accurately translates to humans. Pressure-cycling technology aided trypsin digestion was carried out on the whole tissue lysate from human and mouse livers. Both a data-dependent and data-independent acquisition method was used to acquire data on a time of flight (TOF) mass spectrometer. Absolute protein quantification was determined and fold change analysis in each species was performed between the healthy and disease state. It was found that about 75% of cytochrome P450s (CYPs) related to xenobiotic metabolism showed the same change from mouse to humans. Phase II metabolic

enzymes were accurate 50% of the time and transporter differences were accurate in the two proteins that were measured. The enzymes that did not properly match up were mainly proteins that had poor orthology between the species.

Conclusions: The ALIOS model looks to be an accurate representation for the majority of drug metabolism related CYPs.

Conclusion: This work demonstrates the hepatic proteomic changes that occur as NAFLD and diabetes progress. Numerous drug disposition proteins are altered in both disease states with some overlap. This indicates that diabetes can exacerbate the changes seen in NAFLD. These changes can result in altered metabolic profiles and allow for a better understanding of how to better treat individuals with NAFLD.

ACKNOWLEDGMENTS

First, I would like to thank my major professor, Dr. Fatemeh Akhlaghi, for all your guidance and support beginning way back in my junior year as an undergraduate where I began my pharmaceutical research career. You have provided me with all the tools and expertise that I have needed over the past few years. I would also like to thank my committee members, Drs. Angela Slitt, Kathleen Melanson and Anis Triki for your guidance and contributions to my dissertation and growth as a scientist.

I would also like to thank my wife, Amy, for pushing me to be the best I can be. Without your support, I do not know if I would have even pursued graduate studies. To my parents, John and Deborah, thank you for raising me to be the person I am today and for continuing to support me day in and day out. Thank you Will, my brother, for always being around to talk and take my mind off things. To my grandmother, Bubbe, thank you for always being supportive and interested in my work even if you didn't understand it. I know, and you always remind me, that Zayde would be extremely proud of me. To my new parents in law, Faina and Lenny, thank you for always being supportive and asking questions about my work.

To all the friends that I have made along the way, thank you for the community that you have helped me develop. You made graduate school a fun and enjoyable experience and I expect our friendships to flourish in the years to come.

I would finally like to thank my dog, Mishka, for being the best study buddy, writing companion and emotional support dog through this whole process. I'm sorry I won't be able to bring you to work with me anymore and I know everyone in lab will miss you more greatly.

PREFACE

This dissertation has been written in manuscript style format. The first chapter is a basic background and introduction to NAFLD and proteomics. Chapters 2-5 are the manuscripts. **Manuscript I** entitled, “SWATH-MS Based Method for Simultaneous Absolute Quantification of 13 Clinically Important Drug Transporters in Human Liver” is relatively simple method for measuring the absolute protein expression of certain clinically relevant drug transporters and demonstrates the differences that arise due to gender. **Manuscript II**, entitled “Presence of Drug Metabolizing Enzymes Inducers in Commercially Available Human Liver Samples Results in Altered Metabolic Profiles” is about to be submitted for review in Drug Metabolism and Disposition. It shows the importance of screening human liver samples for medications prior to performing enzyme studies due to the potential for altered metabolic profiles. **Manuscript III**, entitled “Non-Alcoholic Fatty Liver Disease (NAFLD) and Diabetes – Effect on the Protein Abundance and Activity of Human Hepatic Drug Metabolizing Enzymes and Transporters” demonstrates the effect that nonalcoholic fatty liver disease has on multiple phase I and phase II drug metabolizing enzymes and xenobiotic transporters. **Manuscript IV**, entitled “Evaluation of The American Lifestyle Induced Obesity Syndrome (ALIOS) Mouse as a Model for Human Drug Metabolizing Enzymes Using SWATH-MS Proteomics” compares the drug metabolizing proteome of human livers that are affected by NASH to a mouse model that induces NASH by diet modification. All manuscripts have my major professor, Dr. Fatemeh Akhlaghi, as the corresponding author. All other contributors have been acknowledged appropriately and included as co-authors.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGMENTS	vii
PREFACE.....	viii
TABLE OF CONTENTS.....	ix
LIST OF TABLES	xiii
LIST OF FIGURES	xv
BACKGROUND	1
Introduction	1
Significance of This Work.....	3
Objectives of the Research	4
Organization of Dissertation.....	6
MANUSCRIPT 1.....	11
1.1 Abstract	12
1.2 Introduction	13
1.2 Materials and methods	16
1.2.1 Chemicals and reagents	16
1.2.2 Human liver bank.....	16
1.2.3 Human liver tissue preparation	17
1.2.4 Pressure-cycling technology (PCT) aided trypsin digestion	17
1.2.5 LC-MS/MS Analysis	18
1.2.6 Data-dependent and SWATH-MS acquisition parameters	19
1.2.7 Data processing and extraction	19
1.2.8 Data normalization	20
1.2.9 Statistical analysis	20
1.3 Results.....	21
1.3.1 Yield of protein per gram of liver tissue	21
1.3.2 Effect of gender on drug efflux transporters.....	22

1.3.3 Effect of gender on drug uptake transporters.....	22
1.4 Discussion	22
1.5 References.....	27
1.6 Tables	33
1.7 Figures	37
MANUSCRIPT 2.....	42
2.1 Abstract	43
2.2 Introduction	45
2.3 Materials and Methods.....	46
2.3.1 Chemicals and reagents.....	46
2.3.2 Human Liver Bank.....	47
2.3.3 Forensic sample preparation	47
2.3.4 Screening for drugs.....	47
2.3.5 Quantification of phenytoin by LC-MS/MS.....	48
2.3.6 Proteomic and mRNA studies	50
2.3.7 PBPK modeling.....	50
2.3.8 Statistical analysis	50
2.4 Results.....	51
2.4.1 Drugs found in forensic library	51
2.4.2 Phenytoin concentration	51
2.4.3 Effect of phenytoin on CYP450 expression and activity	52
2.4.4 PBPK model.....	53
2.5 Discussion	53
2.6 References.....	58
2.7 Tables	62
2.8 Figures	68
MANUSCRIPT 3.....	72
3.1 Abstract	73
3.2 Introduction	74
3.3 Materials and Methods.....	76
3.3.1 Chemicals and reagents.....	76

3.3.2 Human liver and homogenate preparation	76
3.3.3 In-solution trypsin digestion	77
3.3.4 LC-MS/MS analysis.....	77
3.3.5 Data processing	79
3.3.6 Statistical analysis	80
3.4 Results.....	80
3.4.1 NAFLD and CYP450 protein expression	81
3.4.2 Diabetes and CYP450 protein expression	81
3.4.3 NAFLD and CYP450 enzymatic activity	81
3.4.4 Diabetes and CYP450 enzymatic activity	82
3.4.5 Correlation of CYP450 enzymatic activity and protein expression.....	82
3.4.6 NAFLD and phase II drug metabolizing enzymes.....	82
3.4.7 Diabetes and phase II drug metabolizing enzymes	82
3.4.8 NAFLD and drug transporters	83
3.4.9 Diabetes and drug transporters.....	83
3.4.10 Effect of phenytoin	83
3.5 Discussion	84
3.6 References.....	92
3.7 Tables	97
3.8 Figures	107
MANUSCRIPT 4.....	119
4.1 Abstract	120
4.2 Introduction	121
4.3 Materials and methods	123
4.3.1 Chemicals and reagents	123
4.3.2 Human liver bank.....	123
4.3.3 Animal Treatment and Study Design	124
4.3.4 Mouse Diet Selection.....	124
4.3.5 Tissue preparation	125
4.3.6 In-solution trypsin digestion	125
4.3.7 LC-MS/MS analysis.....	126
4.3.8 Mass spectrometer acquisition parameters.....	127

4.3.9 Data processing	128
4.3.10 Data normalization	128
4.3.11 Statistical analysis	129
4.4 Results.....	129
4.4.1 Human liver histology	129
4.4.2 Mouse liver histology.....	129
4.4.3 Total protein extracted	130
4.4.4 Effect of NASH on human drug metabolizing enzymes and transporters	130
4.4.5 Effect of NASH on mouse drug metabolizing enzymes and transporters	131
4.4.6 Comparison of species effect.....	131
4.5 Discussion	131
4.6 References.....	137
4.7 Tables	143
4.8 Figures	149
CONCLUSION.....	152

LIST OF TABLES

Table 1.1 Demographics characteristics of liver donors	33
Table 1.2 Gender-specific expression levels of hepatic efflux transporters	34
Table 1.3 Gender-specific expression levels of hepatic uptake transporters	35
Table 1.4 Comparison of transporter expression between in-house measurements and reported literature values	36
Table 2.1 List of drugs found in 3 or more human liver samples	62
Table 2.2 Demographic data for the phenytoin containing and phenytoin free livers	63
Table 2.3 Analyte specific mass spectrometry parameters of phenytoin and phenytoin D ₁₀	64
Table 2.4 Effect of phenytoin on protein expression of various CYP450 proteins	65
Table 2.5 Effect of phenytoin on mRNA expression of various CYP450 proteins	66
Table 2.6 Effect of phenytoin on enzymatic activity of various CYP450 proteins	67
Table 3.1 Demographic characteristics of control, NAFL and NASH groups	99
Table 3.2 Effect of NAFLD on protein expression of various CYP450 proteins	100
Table 3.3 Effect of NAFLD on protein expression of various phase II metabolic proteins	101
Table 3.4 Effect of NAFLD on protein expression of various transporter proteins	102
Table 3.5 Effect of diabetes on protein expression of various CYP450 proteins	103

Table 3.6 Effect of diabetes on protein expression of various UGT proteins	104
Table 3.7 Effect of diabetes on protein expression of various SULT proteins	105
Table 3.8 Effect of diabetes on protein expression of various transporter proteins	106
Table 4.1 Comparison of human DME gene names and their orthologous gene in mice	145
Table 4.2 Demographic characteristics of control, NAFL and NASH groups.	146
Table 4.3 Demographic characteristics of mouse low-fat diet (LFD) and high-fat diet (HFD) groups.	147
Table 4.4 Effect of NASH on protein expression of various human DMES and transporter proteins.	148
Table 4.5 Effect of NASH on protein expression of various mouse orthologs of DMEs and transporter proteins.	149
Table 4.6 Comparison of alteration to DMEs and transporters in mouse and human livers	150

LIST OF FIGURES

Figure 1.1: Graphical abstract of transporter extraction, digestion and LC-MS/MS analysis	37
Figure 1.2: Gender difference on liver protein abundance and ATP1A1 (a membrane protein marker).....	38
Figure 1.3: Effect of gender on hepatic efflux transporters	39
Figure 1.4: Effect of gender on hepatic uptake transporters	40
Figure 1.5: Comparison of transporter expression between in-house measurements and Groen et al. 2018 and Wang et al. 2016.....	41
Figure 2.1: Effect of phenytoin on protein expression of various CYP450 proteins	68
Figure 2.2: Effect of phenytoin on mRNA expression of various CYP450 proteins	69
Figure 2.3: Effect of phenytoin on enzymatic activity of various CYP450 proteins	70
Figure 2.4: Effect of phenytoin on PB/PK modeling simulation of IV midazolam dose	71
Figure 3.1: Correlation of protein expression and functional activity of CYP enzymes	107
Figure 3.2: Effect of NAFLD on protein expression of various CYP450 proteins	108
Figure 3.3: Effect of NAFLD on protein expression of various CYP450 proteins	109
Figure 3.4: Effect of NAFLD on protein expression of various SULT proteins	110
Figure 3.5: Effect of NAFLD on protein expression of various UGT proteins	111

Figure 3.6: Effect of NAFLD on protein expression of various drug transporter proteins	112
Figure 3.7: Effect of diabetes on protein expression of various CYP proteins	114
Figure 3.8: Effect of diabetes on protein expression of various CYP proteins	115
Figure 3.9: Effect of diabetes on protein expression of various UGT proteins	116
Figure 3.10: Effect of diabetes on protein expression of various SULT proteins	117
Figure 3.11: Effect of diabetes on protein expression of various drug transporter proteins	118
Figure 3.12: Effect of phenytoin on CYP3A4 protein expression and enzymatic metabolism of testosterone in regard to NAFLD and diabetes.....	119
Figure 4.1 Effect of NASH on protein expression of various CYP450 proteins in human and mice.....	151
Figure 4.2 Effect of NASH on protein expression of various CYP450 proteins in human and mice.....	152
Figure 4.3 Effect of NASH on protein expression of various UGT and drug transporter proteins in human and mice.....	153

BACKGROUND

Introduction

Nonalcoholic fatty liver disease (NAFLD), a chronic liver condition that is increasing in prevalence around the world, can range from reversible simple steatosis, to irreversible and life-threatening cirrhosis and liver cancer. Along with lifestyle management, patients with NAFLD are generally prescribed numerous medications to treat their metabolic syndrome. These drugs must be cleared from the body and the liver, the main site for drug metabolism, contains drug metabolizing enzymes and transporters that facilitate the elimination of many compounds and their ultimate removal from the body. Any change to these proteins can result in altered exposure to systemic circulation and effects on a target. Considering the human liver tissues that are used in research come from donors who were hospitalized prior to death, it is also important to determine if they were receiving medications that may induce or inhibit drug metabolism. Alterations to these important proteins can cause changes to a person's pharmacokinetic and pharmacodynamic profile but it is also imperative to make sure these changes are not a result of an exogenous compound rather than NAFLD itself.

Nonalcoholic fatty liver disease (NAFLD) is a growing concern around the world. In the United States alone, prevalence of NAFLD has been found to be 30% with 10.3% of these patients having advanced fibrosis ¹. It is estimated that over 64 million people in the US have NAFLD and this is associated with a significant economic burden of approximately \$103 billion or about \$1,600 per patient ². NAFLD is defined by the presence of >5% macrovascular steatosis without high alcohol intake. There

are multiple stages of NAFLD starting with benign fatty liver which can lead to nonalcoholic steatohepatitis (NASH) and even liver cancer or cirrhosis ³. Hepatocyte ballooning, fibrosis and lobular inflammation along with steatosis are associated with the later stages of this disease ⁴. This disease is also commonly associated with insulin resistance, obesity, cardiovascular diseases, lipotoxicity and hepatic fat accumulation⁵.

Currently, there is a lack of consensus and little information regarding the effect of NAFLD on important drug metabolizing enzymes (DMEs) and transporters found in the liver ⁶. Of the few studies on DMEs in humans with NAFLD, it has been found that significant downregulation of CYP3A4 and CYP1A2 occurs in the progression of the disease ^{7,8}. DMEs and transporters in the liver are responsible for the elimination of both exogenous and endogenous compounds found in the body. Specifically, the cytochrome P450 (CYP) family of enzymes are responsible for 70-80% of the metabolism of currently marketed drugs ⁹. Drug transporters are responsible for the active transport of drugs into and out of cells that would not normally move across passively ¹⁰. Together, these proteins are moderators of the absorption, distribution, metabolism and elimination (ADME) of compounds both foreign and endogenous to the human body.

Certain commonly prescribed drugs can be classified as either inducers or inhibitors of CYPs which can affect their ability to metabolize drugs. CYPs have the ability to metabolize multiple drugs so whenever two drugs are given concurrently, interactions can possibly exist ¹¹. Inhibition can occur as either irreversible inhibition, reversible inhibition or quasi-irreversible inhibition ¹¹. Induction can occur when a drug has an activation effect on nuclear receptors responsible for regulating the

production of certain CYPs. For example, phenobarbital activates the nuclear receptor CAR which then upregulates CYP2B, CYP3A and CYP2C family of enzymes ¹². Transporters are also known to be induced by some xenobiotics. P-glycoprotein (P-gp), along with CYP3A4, was found to be significantly induced by rifampin ¹³. Similarly, efavirenz, an HIV-1 drug, was found to induce the expression of both P-gp and CYP3A4 ^{14,15}. It is important to perform a thorough screening of donor liver tissues prior to metabolic and proteomic studies as inducers and inhibitors could affect results. For example, dexamethasone, a known weak inducer of CYP3A4 ¹⁶, is used at a concentration of 16 mg/L in University of Wisconsin (UW) solution when preserving tissue ¹⁷. Additionally, methylprednisolone, a CYP2C8 inhibitor, may be administered to patients after brain death to stabilize lung function ^{18,19}. The use of dexamethasone or methylprednisolone could result in alteration to CYP metabolism which in turn would show false activity and proteomic data, possibly resulting in the masking of the true effect of NAFLD. It is important to discover the true effect of NAFLD on drug metabolism in order to discover new treatment and early detection methods.

Significance of This Work

The manuscripts in this dissertation are centered around the continued exploration of the effect that NAFLD has on the human liver and other factors that may be masking its true effect. The significance of this research is listed below.

1. Certain medications can remain in the liver after donation. These compounds have the potential to induce or inhibit certain drug metabolizing enzymes.
2. Drug transporters are a large part of the metabolic profile and effectiveness of many drugs. Historically, they have been difficult to measure, and new methods need to be discovered to make their measurement a simpler process.
3. Current research in the effect of Non-Alcoholic Fatty Liver Disease on drug metabolizing enzymes and transporters is currently sparse and variable. It is imperative to better understand the effect this disease is having on a significant portion of the population in order to better treat patients with the disease itself or a combination of other diseases.

Objectives of the Research

The overall objective of this dissertation was to develop new methodology for the measurement of drug metabolizing enzymes and low abundance drug transporters in order to determine the effect that NAFLD has on them. Additionally, as a donor medication list is not provided when obtaining human liver samples, it was important to determine what small molecules were remaining and the effect these drugs were having on certain protein, enzymatic and mRNA studies. In this regard, each manuscript has its own specific objectives:

1. “SWATH-MS based method for simultaneous absolute quantification of 13 clinically important drug transporters in human liver”:
 - a. Develop a simple method of the quantification of drug transporters in healthy human liver samples.
 - b. Evaluate the accuracy of the measurements when compared to literature values using complex methodology.
 - c. Determine if there was an effect on transporter expression levels due to gender differences.

2. “Presence of Drug Metabolizing Enzymes Inducers in Commercially Available Human Liver Samples Results in Altered Metabolic Profiles”
 - a. Create a method to successfully screening human liver samples against a known forensic compound identification library.
 - b. Determine if any compounds identified were ones that are known to cause induction or inhibition of drug metabolism.
 - c. Evaluate the effect that the found inducers or inhibitors were having on the enzymatic activity, protein expression and mRNA expression of specific DMEs.
 - d. Evaluate the changes observed using a PBPK model to simulate the effect being seen.

3. “Non-alcoholic fatty liver disease (NAFLD) and diabetes – Effect on the protein abundance and activity of hepatic drug metabolizing enzymes in human”
 - a. Measure protein expression levels for a wide variety of phase I and phase II drug metabolizing enzymes and drug transporters.
 - b. Determine the effect that different stages of NAFLD were having on the specific protein levels.
4. “Evaluation of The American Lifestyle Induced Obesity Syndrome (Allos) Mouse as a Model for Human Drug Metabolizing Enzymes Using SWATH-MS Proteomics”
 - a. Measure protein expression levels of phase I and phase II drug metabolizing enzymes and drug transporters in human and mouse livers.
 - b. Compare the expression level between healthy livers and those with nonalcoholic steatohepatitis (NASH).
 - c. Determine if the changes are similar between mouse and human liver samples.

Organization of Dissertation

The remainder of this dissertation will be organized as follows:

Manuscript 1 describes the work in the manuscript “SWATH-MS based method for simultaneous relative quantification of 13 clinically important drug transporters in human liver,” which provides novel methodology used for the rest of the experiments

throughout the other Manuscripts. This work involved the development of new protein digestion and mass spectrometry analytical methods in order to accurately quantify drug metabolizing enzymes and transporters in a single sample. The Manuscript additionally compared certain quantified transporters to reported literature values and does a general comparison to discover gender differences.

Manuscript 2 describes the work related to the manuscript, “Presence of Drug Metabolizing Enzymes Inducers in Commercially Available Human Liver Samples Results in Altered Metabolic Profiles,” which involves the screening of human liver samples for drugs or medications present. It also describes the further investigation of the drug, phenytoin, and the affect seen in the alteration of drug metabolizing enzymes due to the induction potential of phenytoin.

Manuscript 3 describes the work related to the manuscript, “Non-alcoholic fatty liver disease (NAFLD) and diabetes – Effect on the protein abundance and activity of hepatic drug metabolizing enzymes in human,” which involves the analysis of phase I and II drug metabolizing enzymes and xenobiotic transporters and the affect seen due to nonalcoholic fatty liver disease.

Manuscript 4 describes the work related to the manuscript, “Working Title,” which involves the comparison in proteomic changes of drug metabolizing enzymes and transporters in human livers and the American Lifestyle Induced Obesity Syndrome mouse model.

1. Le MH, Devaki P, Ha NB, et al. Prevalence of non-alcoholic fatty liver disease and risk factors for advanced fibrosis and mortality in the United States. *PLoS One*. 2017;12(3):e0173499.
2. Younossi ZM, Blissett D, Blissett R, et al. The economic and clinical burden of nonalcoholic fatty liver disease in the United States and Europe. *Hepatology*. 2016;64(5):1577-1586.
3. Vuppalanchi R, Chalasani N. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: Selected practical issues in their evaluation and management. *Hepatology*. 2009;49(1):306-317.
4. Younossi Z, Henry L. Contribution of Alcoholic and Nonalcoholic Fatty Liver Disease to the Burden of Liver-Related Morbidity and Mortality. *Gastroenterology*. 2016;150(8):1778-1785.
5. Brunt EM, Tiniakos DG. Histopathology of nonalcoholic fatty liver disease. *World J Gastroenterol*. 2010;16(42):5286-5296.
6. Cobbina E, Akhlaghi F. Non-alcoholic fatty liver disease (NAFLD) - pathogenesis, classification, and effect on drug metabolizing enzymes and transporters. *Drug Metab Rev*. 2017;49(2):197-211.
7. Jamwal R, de la Monte SM, Ogasawara K, Adusumalli S, Barlock BB, Akhlaghi F. Nonalcoholic Fatty Liver Disease and Diabetes Are Associated with Decreased CYP3A4 Protein Expression and Activity in Human Liver. *Mol Pharm*. 2018;15(7):2621-2632.

8. Fisher CD, Lickteig AJ, Augustine LM, et al. Hepatic cytochrome P450 enzyme alterations in humans with progressive stages of nonalcoholic fatty liver disease. *Drug Metab Dispos.* 2009;37(10):2087-2094.
9. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther.* 2013;138(1):103-141.
10. Liang Y, Li S, Chen L. The physiological role of drug transporters. *Protein Cell.* 2015;6(5):334-350.
11. Lin JH, Lu AY. Inhibition and induction of cytochrome P450 and the clinical implications. *Clin Pharmacokinet.* 1998;35(5):361-390.
12. Zelko I, Negishi M. Phenobarbital-elicited activation of nuclear receptor CAR in induction of cytochrome P450 genes. *Biochem Biophys Res Commun.* 2000;277(1):1-6.
13. Williamson B, Dooley KE, Zhang Y, Back DJ, Owen A. Induction of influx and efflux transporters and cytochrome P450 3A4 in primary human hepatocytes by rifampin, rifabutin, and rifapentine. *Antimicrob Agents Chemother.* 2013;57(12):6366-6369.
14. Weiss J, Herzog M, Konig S, Storch CH, Ketabi-Kiyanvash N, Haefeli WE. Induction of multiple drug transporters by efavirenz. *J Pharmacol Sci.* 2009;109(2):242-250.

15. Hariparsad N, Nallani SC, Sane RS, Buckley DJ, Buckley AR, Desai PB. Induction of CYP3A4 by efavirenz in primary human hepatocytes: comparison with rifampin and phenobarbital. *J Clin Pharmacol*. 2004;44(11):1273-1281.
16. McCune JS, Hawke RL, LeCluyse EL, et al. In vivo and in vitro induction of human cytochrome P4503A4 by dexamethasone. *Clin Pharmacol Ther*. 2000;68(4):356-366.
17. Guibert EE, Petrenko AY, Balaban CL, Somov AY, Rodriguez JV, Fuller BJ. Organ Preservation: Current Concepts and New Strategies for the Next Decade. *Transfus Med Hemother*. 2011;38(2):125-142.
18. Korte C, Garber JL, Descourouez JL, Richards KR, Hardinger K. Pharmacists' guide to the management of organ donors after brain death. *Am J Health Syst Pharm*. 2016;73(22):1829-1839.
19. Walsky RL, Gaman EA, Obach RS. Examination of 209 drugs for inhibition of cytochrome P450 2C8. *J Clin Pharmacol*. 2005;45(1):68-78.

MANUSCRIPT 1

**SWATH-MS BASED METHOD FOR SIMULTANEOUS ABSOLUTE
QUANTIFICATION OF 13 CLINICALLY IMPORTANT DRUG TRANSPORTERS
IN HUMAN LIVER**

Prepared for submission in Journal of Proteome Research

Benjamin J. Barlock, Rohitash Jamwal, Noah E. Steinberg, Fatemeh Akhlaghi

Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode
Island, Kingston, RI, 02881, USA

1.1 Abstract

Hepatic xenobiotic transporters in the human liver play an important role in the elimination of drugs or toxins and significantly contribute to variability in drug response. We developed a label-free mass spectrometry-based method to study the protein expression of 13 of 25 clinically relevant transporter proteins (6 ABC and 7 SLC) in liver tissue from 20 donors (9 female, 11 male). Whole tissue homogenate and in-solution trypsin digestion was performed using pressure-cycling technology. Data was acquired in data-dependent and sequential window acquisition of all theoretical mass spectra (SWATH-MS) mode. Chromatographic separation was achieved over a 180-min gradient on Acquity UPLC BEH C18 peptide column, and mass spectrometer was operated in positive ionization mode. Bovine serum albumin (BSA) was spiked in each sample before digestion and monitored for batch to batch variability. Spectronaut™ was used for peptide identification and data extraction from DDA and SWATH-MS files. Na⁺/K⁺ transporting subunit alpha 1 (ATP1A1), was quantified as a cell membrane marker and its coefficient of variation was 16.6% across different liver samples.

Significance: The work highlights the suitability of SWATH-MS for large-scale simultaneous quantification of several xenobiotic transporters important in drug disposition. We found that average differential expression of transporters proteins was similar and much smaller than the inter-individual variability observed between the genders.

1.2 Introduction

Xenobiotic transporters play a crucial role in drug disposition by mediating drug uptake and efflux. These proteins govern the absorption, distribution, metabolism, and elimination of drugs, toxins and endogenous molecules across the cell membrane. Thus, transporters can govern the rate-limiting step in systemic and tissue exposure of drugs. Biologically, transporters are membrane-bound proteins are ubiquitously expressed throughout the body. These proteins are mostly localized on the apical or basolateral membranes of various organs including intestine, liver, kidney, and brain and facilitate the efflux and uptake of xenobiotics ¹. The ATP-binding cassette (ABC) family and the solute carrier (SLC) family represent the two significant superfamilies of membrane transporters in humans ²⁻⁴. ABC transporters utilize ATP for transport of substrates across the membrane, and most efflux transporters belong to this family. In contrast, SLC transporters mainly facilitate uptake can be active or facilitated. Some SLC transporters also mediate bidirectional movement of molecules. Altered systemic exposure and organ toxicity can be related to transporters mediated drug interactions ^{1,5}. Therefore, the last decade has seen a significant amount of research focusing on quantification and deorphanization of these transporters as well as elucidation of their functionality ^{6,7}. Inter-individual variability in the expression and drug response of xenobiotic transporters may arise from non-genetic (age and gender), genetic (polymorphism), and regulatory factors ⁸. While more than 400 different transporters have been annotated in the human genome, the function of many of these remains unknown ^{2,9}.

Efflux transporters localized on the canalicular membrane (e.g., P-gp/MDR1, MRP2, BCRP, BSEP, MDR3, and MATE1) facilitate excretion of molecules from hepatocytes into bile ^{4,6,10}. Meanwhile, basolateral hepatic efflux transporters (e.g., MRP1, MRP3, MRP4, MRP5, and MRP6) return molecules to hepatic blood from the hepatocyte ^{4,6,11}. Prominent uptake transporters that are localized on the sinusoidal membrane of hepatocytes include NTCP, OATP1B1, OATP1B3, OATP2B1, OAT2, and OCT1 ^{12,13}. These uptake transporters facilitate uptake of drugs from the blood into the hepatocyte thereby facilitating metabolic clearance of drugs. Moreover, OAT7 on the sinusoidal membrane and sterolin-1 and sterolin-2 on basolateral membrane mediate bidirectional (efflux and uptake) transport of various substrates ^{14,15}.

Traditionally, researchers have relied on quantification of mRNA expression of transporters as a surrogate to the functional activity in the tissue. However, a weak correlation was observed between mRNA and protein expression in human livers ^{16,17}. Protein abundance levels of these transporters are usually estimated by cumbersome Western blot analyses that is semi-quantitative at best. Last decade has seen a rise in the use of mass-spectrometry (MS) based techniques for quantification of the protein expression ^{10,18}. Available MS methods for quantification of liver transporters are based on targeted quantitative proteomics approaches ^{10,16,17,19,20}. A comprehensive cost comparison of different mass spectrometry-based techniques reported significant cost savings with label-free based quantitative (LFQ) proteomics ²¹. LFQ approaches are relatively inexpensive as compared to targeted MRM methods as there is no need

to synthesize unique peptides for each protein and isotopically labeled isoforms of these peptide as the internal standard.

LFQ using the sequential window acquisition of all theoretical ion mass spectra (SWATH-MS) technique provides an alternative to the targeted approaches for protein quantification²². SWATH-MS is a data-independent acquisition (DIA) technique in which all the precursors within a predefined m/z are fragmented, and product ions of these precursors are recorded as a digital repository²³. The data are further deconvoluted and extracted using software like OpenSWATH, SWATH 2.0, Spectronaut and Skyline^{24,25}. A significant advantage of SWATH-MS over the other mass spectrometry methods is related to the ability to perform retrospective mining of the data. For instance, if the researcher comes up with a new hypothesis in the future, SWATH-MS data would allow interrogation of the existing data for additional protein/s of interest without the need for additional sample digestion or data reacquisition. Such a strategy offers a tremendous benefit concerning saving of sample, time and money. A high linear association between MRM based methods with SWATH-MS has been shown in the past making it a suitable and reliable technique for proteomics-based studies^{26,27}. Nakamura et al. also described a SWATH-MS for quantification of drug-related transporter proteins in human liver microsomes, however no gender specific differences were reported due to use of small sample size (n=4) [25].

In this work, we report the development of a SWATH-MS based method to study the gender-specific differential expression of important drug and xenobiotic transporters in human liver (6 ABC and 7 SLC family). The transporter proteins were

shortlisted based on the recommendation made by the International Transporter Consortium (ITC), the US Food and Drug Administration (USFDA) and the European Medicines Agency (EMA), as human xenobiotic transporters that play a significant role in drug discovery^{6,7,28}.

1.2 Materials and methods

1.2.1 Chemicals and reagents

Protein preparation kit, TPCK-treated trypsin, and MS tuning solution was obtained from SCIEX (Framingham, MA). Acquity UPLC Peptide BEH C18 analytical column and VanGuard pre-columns were procured from Waters Corp. (Waltham, MA). 1,4-Dithiothreitol (DTT) was obtained from Roche Diagnostics (Indianapolis, IN). Sodium deoxycholate and iodoacetamide (IAA) were procured from Sigma Aldrich (St. Louis, MO). MS grade acetonitrile, bovine serum albumin (BSA) and formic acid were purchased from ThermoFisher (Waltham, MA).

1.2.2 Human liver bank

Frozen human liver samples from brain dead donors were purchased from Sekisui XenoTech LLC, Kansas City, KS. All the livers are from organ donors involved in automobile accidents and therefore are IRB-exempt. All livers were from non-diabetics and did not have any stage of non-alcoholic fatty liver disease. The age of liver donors ranged from 21 to 73 years, with 11 males and 9 females. The detailed demographics of the donors is given in **Table 1.1**.

1.2.3 Human liver tissue preparation

Liver tissue (~50 mg) was homogenized using a bead homogenizer (Omni Bead Ruptor, Kennesaw GA) in 1000 μ l of homogenization buffer (8 M Urea, 50 mM Triethylammonium Bicarbonate (TEAB), 10 mM DTT w/v) ²⁹. Samples were then spun at 1,000 g for 5 min. Supernatant was collected and Pierce BCA protein assay kit (ThermoFisher Scientific, Waltham, MA) was used to estimate protein concentration.

1.2.4 Pressure-cycling technology (PCT) aided trypsin digestion

Protein digestion was performed as previously described with modifications ^{29,30}. Homogenate samples (250 μ g) were spiked with 2 μ g undigested bovine serum albumin (BSA). Samples were denatured with 25 μ L DTT (100mM) at 35°C for 30 min at 120 rpm followed by alkylation in the dark with 25 μ L IAA (200 mM) for 30 min at room temperature. Protein was then concentrated using ice cold water, methanol and chloroform (1:2:1) followed by centrifugation at 10,000 rpm for 5 min at 10°C. The resulting pellet was washed with ice cold methanol and resuspended in 100 μ L of 50 mM ammonium bicarbonate (pH ~8) containing 3% w/v sodium deoxycholate (DOC). TPCK-treated trypsin (12.5 μ g) was then added at a ratio of 1:20 (trypsin:protein) and transferred into digestion tubes (PCT MicroTubes, Pressure Biosciences Inc., Easton, MA). The barocycler was run for 75 cycles at 35°C with 60 sec pressure cycles (50 sec at 25 kpsi, 10 sec at ambient pressure). Samples were removed, the same amount of trypsin was added and the barocycler was run again as above. After digestion, 110 μ L of digested peptides were added to 10 μ L acetonitrile/water (1:1 v/v, containing 5% formic acid) to precipitate DOC and stop the

trypsin digestion. Samples were centrifuged for 5 min at 10,000 *g* and 10°C to pellet the DOC. One hundred microliters of the supernatant were reserved for LC-MS/MS analysis. Subsequently, 25 µL of the digested peptides were injected onto the analytical column and used for the LC-MS/MS method described below.

1.2.5 LC-MS/MS Analysis

Mass spectrometry analysis was performed as described previously with modifications ³¹. All experiments were performed on a SCIEX 5600 TripleTOF® mass spectrometer equipped with a DuoSpray™ ion source (SCIEX, Concord, Canada) coupled to Acquity UHPLC HClass system (Waters Corp., Milford, MA, USA). Sample separation was achieved on an Acquity UPLC Peptide BEH C18 (2.1 X 150 mm², 300 Å, 1.7 µm) attached to an Acquity VanGuard pre-column (2.1 X 5 mm², 300 Å, 1.7 µm). Autosampler and analytical column were maintained at 10°C and 50°C, respectively. The chromatographic separation was achieved over a 180-min gradient at 100 µL/min. A linear gradient was used for chromatographic separation using mobile phase A (Water, 0.1% formic acid) and mobile phase B (Acetonitrile, 0.1% formic acid). The solvent composition was 98% A from 0 to 5 min, 98% to 70% A from 5 to 155 min, 70% to 50% A from 155 to 160 min, 50% to 5% from 160 to 170 min, 5% A held from 170 to 175 min. The gradient was held at initial conditions from 175 min until the end of the run to equilibrate the column before the start of next run. Mass calibration of the TOF detector was monitored by injecting trypsin-digested β- galactosidase peptides every 12 hours during the analysis.

1.2.6 Data-dependent and SWATH-MS acquisition parameters

Data-dependent, as well as data independent (SWATH) analysis, was performed in positive ionization mode. The method specific parameters were as follows: gas 1 (GS1) 55 psi, gas 2 (GS2) 60 psi, curtain gas (CUR) 25 psi. The source-specific parameters were as follows: temperature (TEM) 450°C, ion spray voltage floating (ISVF) 5500 V, declustering potential (DP): 100, collision energy (CE) 10, collision energy spread (CES) 15.

A maximum of 50 candidate ions with a charge state 2 to 4 was monitored every survey scan cycle. All the ions between m/z 300-1250 which exceeded 25 cps were subjected to MS/MS analysis. Rolling collision energy dependent on the m/z of the ion and dynamic accumulation were used. The mass tolerance was set at 50 mDa during the initial 0.75 sec survey scan (total cycle time: 3.50 sec).

All the parameters for SWATH acquisition were similar as described above except the following: TOF masses were collected from m/z 300 to 1500 and SWATH data was acquired (m/z 400-1100) over 70 SWATH windows per cycles with a window size of m/z 10.

1.2.7 Data processing and extraction

DDA samples were searched against reviewed Swiss-Prot identifiers (October 2016) using Spectronaut (Biognosys, Schlieren, Switzerland). The search was performed using Pulsar algorithm for identification of peptides and proteins from DDA and DIA data. BGS Factory settings were used for the search which included specific trypsin/P digestion with a minimum of 7 amino acids, maximum of 52 amino acids and 2 missed cleavages. Fixed modifications included carbamidomethyl (C) and

variable modifications included acetyl (protein N-terminus) and oxidation (M). All other options were kept as the BGS factory settings as well. Raw data files and search results are available at Japan Proteome Standard Repository (*jPOSTrepo* JPST000372, *ProteomeXchange* PXD008593), a publicly available data repository for proteomics data ³².

The spectral library was then used to analyze DIA data in a DIA analysis in Spectronaut. Samples were first converted to HTRMS files using the Spectronaut Converter (Biognosys, Schlieren, Switzerland). All settings were run as the BGS factory settings except “Used Biognosys’ iRT kit” was unselected and “PTM localization” was unselected. All other settings were left the same. Raw intensity after normalization was exported and bovine serum albumin (BSA) was monitored for variability.

1.2.8 Data normalization

Raw normalized intensity from Spectronaut was further converted to picomoles of protein per gram of liver tissue (pmol/g liver) use the total protein approach (TPA) and protein yield (protein per gram of liver or PPGL) ³³.

$$\text{Protein} \left(\frac{\text{pmol}}{\text{mg protein}} \right) = \frac{\text{Protein intensity}}{\text{Total intensity} \times \text{MW (g/mol)}} \times 10^6$$

$$\text{Protein} \left(\frac{\text{pmol}}{\text{g liver}} \right) = \text{Protein} \left(\frac{\text{pmol}}{\text{mg protein}} \right) \times \text{protein per gram of liver (mg)}$$

1.2.9 Statistical analysis

Non-parametric Mann Whitney U test was used to compare the differences in demographic data and Chi-square test was used to compare the proportionality

(GraphPad Software Inc. La Jolla, Ca). The geometric mean was calculated from non-transformed data using SPSS v26 (IBM Analytics, Armonk, NY). Gender differences in the absolute expression of major xenobiotic and drug transporters in human liver were carried out using a non-parametric Mann Whitney U test in Prism (GraphPad Software Inc. La Jolla, CA). Non-parametric Spearman analysis was used for correlation between different transporter proteins. $P < 0.05$ was considered significant throughout the analysis. Demographic data were reported as mean $\pm$ SEM unless stated otherwise.

1.3 Results

SWATH-MS was used to estimate the protein expression of 13 hepatic drug transporters (6 ABC and 7 SLC family) in membrane fractions from 20 human liver donors. Range for all expression values fell within the range reported by Groen et al. 2018 and Wang et al. 2016 except for OATP1B3, which was reported to be ~10-fold lower than what was measured. These data can be seen in **Figure 1.5** and **Table 1.4** ^{34,35}.

1.3.1 Yield of protein per gram of liver tissue

The average protein yield (mg/g liver tissue) for all the livers was 92.56 ± 3.97 , $n=20$ (**Figure 1.2(a)**). Protein yield was no different in females (92.23 ± 6.46 , $n=9$) as compared to males (92.84 ± 5.21 , $n=11$). The variability in sodium/potassium-transporting ATPase subunit alpha-1 (ATP1A1, an integral membrane marker) levels was used to gauge the quality of the membrane fractions and % coefficient of variation among the 20 samples was 16.62% (**Figure 1.2(b)**).

1.3.2 Effect of gender on drug efflux transporters

We found that none of the investigated efflux transporters was differentially expressed in males and females (**Figure 1.3**). All efflux transporters studied in this work were found in all the liver samples. Three MRP transporters, BSEP and MDR1 (P-gp) were quantified using this assay are given in **Figure 1.3**. No significant gender difference was seen in transporter proteins with efflux/uptake function as well (**Figure 1.3 and 1.4**).

1.3.3 Effect of gender on drug uptake transporters

Gender was found to only influence the protein abundance of OATP1B1 and OAT7 and no other uptake transporters in this study (**Figure 1.4**). Males were found to have 65.3 ± 9.61 pmol/g liver of OATP1B1 while females were found to have 111.1 ± 28.26 pmol/g liver ($p < 0.05$). OAT7 was also found to be slightly significantly lower in males (77.29 ± 7.04 pmol/g liver) when compared to females (96.26 ± 6.80 pmol/g liver) ($p < 0.05$). All other uptake transporters included in the study were detectable in all the liver samples except for OAT2 in one male sample. Gender-specific expression level and male/female ratio of transporter proteins is available at **Tables 1.2 and 1.3**.

1.4 Discussion

Drug response, efficacy, and toxicity are dependent on the expression of drug metabolizing enzymes and transporters in the intestine and liver. Differences in the basal expression of these proteins in the human liver can contribute to significant changes in systemic exposure of a drug. Extensive research in human has shown the gender-related differences, which partly explain the interindividual variability in drug

disposition, toxicity and therapeutic response ^{36,37}. Gender is an essential underlying biological factor for the development of personalized medicine. A study by the US General Accounting Office found that 80% of FDA-approved prescription drugs withdrawn from the market between 1997-2000 were due to higher adverse drug-related events in women ³⁸. While a considerable amount of data is available on the gender differences in transporter protein expression in rat and mice, studies are lacking in humans ^{39,40}. Also, limited data are available for the gender differences in expression of transporter proteins in humans since most available information is based on gene expression ³⁷. Interestingly, a growing body of research is suggesting that mRNA serves as a poorer surrogate than protein expression for prediction of transporter activity ^{17,30,41}.

The availability of human liver tissue for studying the hepatic xenobiotic transporters remains a challenge for researchers due to ethical consideration and the availability of tissue with adequate clinical information. Most of the samples available from healthy people come from donors who have sadly passed away in automobile accidents. Therefore, when possible, a judicious and parsimonious use of such tissue is warranted to obtain maximum data. MRM-based methods of protein quantification rely on customization of processes for each target protein before quantification. Though such means can be adapted to quantify more than one protein at a time, the cost and method complexity increases exponentially with an increase in the number of target proteins ²¹. Despite the benefits offered by SWATH-MS, there are some limitations to the methodology. As the window size in SWATH increases, the noise level increases drastically and impedes the quantification of low abundance proteins.

Second, the runtime for sample acquisition can become time consuming. Large batches of samples can take weeks to run and increase the chance mass spec variability. Nevertheless, the advantages of this technique outweigh its drawbacks.

We observed that the inter-individual variability seen in this study was much higher than the average differences between the two genders. While a study with a large sample set is desired to completely address the cofactors contributing to this variability, such studies are often limited due to lack of availability of the liver tissue. Most of the tissue that is available for commercial purchase is obtained from motor vehicle accidents or other brain-dead donors and a detailed medical history is often lacking. Therefore, the potential of conflicting effects from obesity or any other undiagnosed disease cannot be ruled out. Interestingly, there are currently no reports for any significant alteration of transporter proteins in human liver. Our findings are also limited as we do not have a large enough sample size to separately study the effect of obesity (BMI>30) in our sample set. Therefore, future studies with a larger sample size are currently being planned to address the high inter-individual variability and differential effect of obesity and diabetes.

Permeability glycoprotein 1 (P-gp)/ multidrug resistance protein (MDR1) is predominantly responsible for efflux of cations from hepatocytes into the bile duct. Prasad et al. found no association between age and gender with an expression of P-gp in human livers (n=64) ³⁰. Cheng et al. also found no significant gender-specific differences in human hepatic BSEP expression ⁴⁰.

Multidrug resistance-associated protein (MRP2) plays an important role in the efflux of lyophilic conjugates (glutathione, glucuronate, sulfate) ⁴². MRP2 is localized

on the apical membrane of polarized hepatocytes and expressed in kidney and intestine among other tissues⁴³. MRP3 has been found to transport similar drugs to MDR1 and 3 such as anionic drugs and conjugates⁴⁴. It has been associated with the resistance to traditional anti-cancer drugs such as methotrexate, etoposide and teniposide. MRP3 is localized to the basolateral side of the cell⁴⁵. There are currently very little data available on MRP6. It is predominantly in the liver and kidneys on the basolateral membrane and has been found to be upregulated similarly to MRP1 in certain cancer cell lines⁴⁶. Recently it has been found that MRP6 is involved with the transport of tyrosine kinase inhibitors, such as nilotinib and dasatinib, leading to resistance *in vitro*⁴⁷.

Organic anion transporting polypeptides (OATPs) facilitate uptake of large hydrophobic organic anions while smaller and hydrophilic organic anions are transported by the organic anion transporters (OATs)⁴⁸. OATP1B1, OATP1B3, and OATP2B1 are the most notable transporters for drug uptake in the liver. OATP1B1 and OATP1B2 are expressed predominantly in liver whereas OAT2B1 is ubiquitously expressed⁴⁹. We found OATP1B1 to be significantly upregulated in females. This matches previously published research by Yang et al. They found that OATP1B1 gene expression was increased by 1.29-fold in females⁵⁰. No gender-specific expression differences of OATP1B3 or OATP2B1 were found in previous or this current study^{16,30,51}.

OAT2 is highly expressed in the sinusoidal membrane of hepatocytes with lower expression also seen in the kidney⁴⁸. OAT7 is exclusively expressed in the basolateral membrane of human hepatocytes in the liver and participate in the

transport of anionic substances in exchange for butyrate ¹⁴. We found OAT7 to be slightly increased in females but not significant. Other groups report no statistical difference in OAT7 between genders although there are very little reported data available ⁵². Slight differences may arise as we are measuring in liver homogenate, not a membrane fraction. There will generally be less error due to the decreased complexity in sample preparation, which may result in slightly different results. There may be other factors within the demographics of the livers or the difference in quantification methods that is the cause for the conflicting data as well. Members of the OCT family transport organic cations down their electrochemical gradients ⁴⁸. OCT1 and OCT3 are predominantly expressed in human liver and are localized to the basolateral membrane of hepatocytes ⁵³. Prasad et al. also reported no gender differences in expression of OATPs, OATs and OCTs in human liver ⁵⁴.

We developed a SWATH-MS based method for label-free, relative quantitative proteomics analyses of drug and xenobiotic transporters in human liver. SWATH-MS based studies can be used for comparative global proteomics analysis and large-scale relative protein quantification, especially for studies that a limited quantity of tissue is available. We successfully showed that the current approach could be directly applied to estimate the protein expression of many target proteins at once and can be further applied to study the difference in expression in relation to demographic characteristics or disease state. Future studies with a larger sample size are in progress to address other factors (disease, alcohol consumption, and smoking), which may contribute to differential expression of these transporter proteins.

1.5 References

1. Hillgren KM, Keppler D, Zur AA, et al. Emerging transporters of clinical importance: an update from the International Transporter Consortium. *Clin Pharmacol Ther.* 2013;94(1):52-63.
2. Liang Y, Li S, Chen L. The physiological role of drug transporters. *Protein Cell.* 2015;6(5):334-350.
3. Lin L, Yee SW, Kim RB, Giacomini KM. SLC transporters as therapeutic targets: emerging opportunities. *Nat Rev Drug Discov.* 2015;14(8):543-560.
4. Pfeifer ND, Hardwick RN, Brouwer KL. Role of hepatic efflux transporters in regulating systemic and hepatocyte exposure to xenobiotics. *Annu Rev Pharmacol Toxicol.* 2014;54:509-535.
5. Muller F, Fromm MF. Transporter-mediated drug-drug interactions. *Pharmacogenomics.* 2011;12(7):1017-1037.
6. International Transporter C, Giacomini KM, Huang SM, et al. Membrane transporters in drug development. *Nat Rev Drug Discov.* 2010;9(3):215-236.
7. Huang SM, Zhang L, Giacomini KM. The International Transporter Consortium: a collaborative group of scientists from academia, industry, and the FDA. *Clin Pharmacol Ther.* 2010;87(1):32-36.
8. Fisel P, Nies AT, Schaeffeler E, Schwab M. The importance of drug transporter characterization to precision medicine. *Expert Opin Drug Metab Toxicol.* 2017;13(4):361-365.
9. Stieger B, Hagenbuch B. Recent advances in understanding hepatic drug transport. *F1000Res.* 2016;5:2465.
10. Fallon JK, Smith PC, Xia CQ, Kim MS. Quantification of Four Efflux Drug Transporters in Liver and Kidney Across Species Using Targeted Quantitative Proteomics by Isotope Dilution NanoLC-MS/MS. *Pharm Res.* 2016;33(9):2280-2288.
11. Jackson JP, Freeman KM, Friley WW, St. Claire RL, Black C, Brouwer KR. Basolateral Efflux Transporters: A Potentially Important Pathway for the Prevention of Cholestatic Hepatotoxicity. *Applied In Vitro Toxicology.* 2016;2(4):207-216.

12. Fahrmayr C, Fromm MF, König J. Hepatic OATP and OCT uptake transporters: their role for drug-drug interactions and pharmacogenetic aspects. *Drug Metab Rev.* 2010;42(3):380-401.
13. Kalliokoski A, Niemi M. Impact of OATP transporters on pharmacokinetics. *Br J Pharmacol.* 2009;158(3):693-705.
14. Shin HJ, Anzai N, Enomoto A, et al. Novel liver-specific organic anion transporter OAT7 that operates the exchange of sulfate conjugates for short chain fatty acid butyrate. *Hepatology.* 2007;45(4):1046-1055.
15. Ballatori N, Li N, Fang F, Boyer JL, Christian WV, Hammond CL. OST alpha-OST beta: a key membrane transporter of bile acids and conjugated steroids. *Front Biosci (Landmark Ed).* 2009;14:2829-2844.
16. Ohtsuki S, Schaefer O, Kawakami H, et al. Simultaneous absolute protein quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases as a novel approach for the characterization of individual human liver: comparison with mRNA levels and activities. *Drug Metab Dispos.* 2012;40(1):83-92.
17. Prasad B, Lai Y, Lin Y, Unadkat JD. Interindividual variability in the hepatic expression of the human breast cancer resistance protein (BCRP/ABCG2): effect of age, sex, and genotype. *J Pharm Sci.* 2013;102(3):787-793.
18. Prasad B, Unadkat JD. Optimized approaches for quantification of drug transporters in tissues and cells by MRM proteomics. *AAPS J.* 2014;16(4):634-648.
19. Kumar V, Prasad B, Patilea G, et al. Quantitative transporter proteomics by liquid chromatography with tandem mass spectrometry: addressing methodologic issues of plasma membrane isolation and expression-activity relationship. *Drug Metab Dispos.* 2015;43(2):284-288.
20. Deo AK, Prasad B, Balogh L, Lai Y, Unadkat JD. Interindividual variability in hepatic expression of the multidrug resistance-associated protein 2 (MRP2/ABCC2): quantification by liquid chromatography/tandem mass spectrometry. *Drug Metab Dispos.* 2012;40(5):852-855.
21. Al Feteisi H, Achour B, Barber J, Rostami-Hodjegan A. Choice of LC-MS methods for the absolute quantification of drug-metabolizing enzymes and transporters in human tissue: a comparative cost analysis. *AAPS J.* 2015;17(2):438-446.

22. Gillet LC, Navarro P, Tate S, et al. Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol Cell Proteomics*. 2012;11(6):O111 016717.
23. Rosenberger G, Koh CC, Guo T, et al. A repository of assays to quantify 10,000 human proteins by SWATH-MS. *Sci Data*. 2014;1:140031.
24. Navarro P, Kuharev J, Gillet LC, et al. A multicenter study benchmarks software tools for label-free proteome quantification. *Nat Biotechnol*. 2016;34(11):1130-1136.
25. Bruderer R, Bernhardt OM, Gandhi T, et al. Extending the limits of quantitative proteome profiling with data-independent acquisition and application to acetaminophen-treated three-dimensional liver microtissues. *Mol Cell Proteomics*. 2015;14(5):1400-1410.
26. Liu Y, Huttenhain R, Surinova S, et al. Quantitative measurements of N-linked glycoproteins in human plasma by SWATH-MS. *Proteomics*. 2013;13(8):1247-1256.
27. Nakamura K, Hirayama-Kurogi M, Ito S, et al. Large-scale multiplex absolute protein quantification of drug-metabolizing enzymes and transporters in human intestine, liver, and kidney microsomes by SWATH-MS: Comparison with MRM/SRM and HR-MRM/PRM. *Proteomics*. 2016;16(15-16):2106-2117.
28. Prueksaritanont T, Chu X, Gibson C, et al. Drug-drug interaction studies: regulatory guidance and an industry perspective. *AAPS J*. 2013;15(3):629-645.
29. Jamwal R, Barlock BJ, Adusumalli S, Ogasawara K, Simons BL, Akhlaghi F. Multiplex and Label-Free Relative Quantification Approach for Studying Protein Abundance of Drug Metabolizing Enzymes in Human Liver Microsomes Using SWATH-MS. *J Proteome Res*. 2017;16(11):4134-4143.
30. Prasad B, Evers R, Gupta A, et al. Interindividual variability in hepatic organic anion-transporting polypeptides and P-glycoprotein (ABCB1) protein expression: quantification by liquid chromatography tandem mass spectroscopy and influence of genotype, age, and sex. *Drug Metab Dispos*. 2014;42(1):78-88.
31. Jamwal R, Barlock BJ, Adusumalli S, Ogasawara K, Simons BL, Akhlaghi F. Multiplex and Label-Free Relative Quantification Approach for Studying

Protein Abundance of Drug Metabolizing Enzymes in Human Liver Microsomes Using SWATH-MS. *J Proteome Res.* 2017.

32. Okuda S, Watanabe Y, Moriya Y, et al. jPOSTrepo: an international standard data repository for proteomes. *Nucleic Acids Res.* 2017;45(D1):D1107-D1111.
33. Wisniewski JR, Rakus D. Multi-enzyme digestion FASP and the 'Total Protein Approach'-based absolute quantification of the Escherichia coli proteome. *J Proteomics.* 2014;109:322-331.
34. van Groen BD, van de Steeg E, Mooij MG, et al. Proteomics of human liver membrane transporters: a focus on fetuses and newborn infants. *Eur J Pharm Sci.* 2018;124:217-227.
35. Wang L, Collins C, Kelly EJ, et al. Transporter Expression in Liver Tissue from Subjects with Alcoholic or Hepatitis C Cirrhosis Quantified by Targeted Quantitative Proteomics. *Drug Metab Dispos.* 2016;44(11):1752-1758.
36. Waxman DJ, Holloway MG. Sex differences in the expression of hepatic drug metabolizing enzymes. *Mol Pharmacol.* 2009;76(2):215-228.
37. Yang L, Price ET, Chang CW, et al. Gene expression variability in human hepatic drug metabolizing enzymes and transporters. *PLoS One.* 2013;8(4):e60368.
38. Gahart MT, Rowe EJ, Bradley L. *Most Drugs Withdrawn in Recent Years Had Greater Health Risks for Women.* US Government Accountability Office;2000.
39. Buist SC, Cherrington NJ, Choudhuri S, Hartley DP, Klaassen CD. Gender-specific and developmental influences on the expression of rat organic anion transporters. *J Pharmacol Exp Ther.* 2002;301(1):145-151.
40. Cheng X, Buckley D, Klaassen CD. Regulation of hepatic bile acid transporters Ntcp and Bsep expression. *Biochem Pharmacol.* 2007;74(11):1665-1676.
41. Wang L, Prasad B, Salphati L, et al. Interspecies variability in expression of hepatobiliary transporters across human, dog, monkey, and rat as determined by quantitative proteomics. *Drug Metab Dispos.* 2015;43(3):367-374.
42. Jedlitschky G, Hoffmann U, Kroemer HK. Structure and function of the MRP2 (ABCC2) protein and its role in drug disposition. *Expert Opin Drug Metab Toxicol.* 2006;2(3):351-366.

43. Nies AT, Keppler D. The apical conjugate efflux pump ABCC2 (MRP2). *Pflugers Arch.* 2007;453(5):643-659.
44. Keppler D. The roles of MRP2, MRP3, OATP1B1, and OATP1B3 in conjugated hyperbilirubinemia. *Drug Metab Dispos.* 2014;42(4):561-565.
45. Kool M, van der Linden M, de Haas M, et al. MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci U S A.* 1999;96(12):6914-6919.
46. Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst.* 2000;92(16):1295-1302.
47. Eadie LN, Dang P, Goyne JM, Hughes TP, White DL. ABCC6 plays a significant role in the transport of nilotinib and dasatinib, and contributes to TKI resistance in vitro, in both cell lines and primary patient mononuclear cells. *PLoS One.* 2018;13(1):e0192180.
48. Roth M, Obaidat A, Hagenbuch B. OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *Br J Pharmacol.* 2012;165(5):1260-1287.
49. Hagenbuch B, Meier PJ. The superfamily of organic anion transporting polypeptides. *Biochim Biophys Acta.* 2003;1609(1):1-18.
50. Yang L, Li Y, Hong H, et al. Sex Differences in the Expression of Drug-Metabolizing and Transporter Genes in Human Liver. *J Drug Metab Toxicol.* 2012;3(3):1000119.
51. Badee J, Achour B, Rostami-Hodjegan A, Galetin A. Meta-analysis of expression of hepatic organic anion-transporting polypeptide (OATP) transporters in cellular systems relative to human liver tissue. *Drug Metab Dispos.* 2015;43(4):424-432.
52. Vildhede A, Kimoto E, Rodrigues AD, Varma MVS. Quantification of Hepatic Organic Anion Transport Proteins OAT2 and OAT7 in Human Liver Tissue and Primary Hepatocytes. *Mol Pharm.* 2018;15(8):3227-3235.
53. Nies AT, Herrmann E, Brom M, Keppler D. Vectorial transport of the plant alkaloid berberine by double-transfected cells expressing the human organic cation transporter 1 (OCT1, SLC22A1) and the efflux pump MDR1 P-

glycoprotein (ABCB1). *Naunyn Schmiedebergs Arch Pharmacol.* 2008;376(6):449-461.

54. Prasad B, Gaedigk A, Vrana M, et al. Ontogeny of Hepatic Drug Transporters as Quantified by LC-MS/MS Proteomics. *Clin Pharmacol Ther.* 2016;100(4):362-370.

1.6 Tables

	Female	Male	p-value
N (%)	9 (45%)	11 (55%)	-
Age (years)	48.11 ± 5.13	49.45 ± 5.36	<i>p=0.86</i>
Body weight (kg)	82.89 ± 8.7	80.82 ± 5.8	<i>p=0.84</i>
Height (cm)	162.56 ± 2.71	173.42 ± 2.66	<i>p<0.01*</i>
Body mass index (kg/m²)	31.22 ± 3.08	27.00 ± 1.92	<i>p=0.24</i>
Liver weight (kg)	1.33 ± 0.12	1.48 ± 0.09	<i>p=0.32</i>
Ethnicity (C, AA, H)	8, 1, 0	8, 3, 0	
Cause of death			
Anoxia	6	4	
Cerebrovascular aneurysm	2	3	
Head trauma	1	4	
Smoking status			
Non-smoker	5	4	
Smoker	4	7	
Alcohol consumption			
No	5	5	
Yes	4	6	

Table 1.1 Demographics characteristics of liver donors. P value represent the significance from Mann-Whitney U test and * represent $p<0.01$; C: Caucasian, AA: Afro-American, H: Hispanic.

Pmol protein per gram liver ¹				
Transporter protein	Female	Male	Female/Male ratio	p-value ²
MRP2	25.53 (15.14-43.15)	20.27 (12.47-32.96)	1.26	0.476
MRP3	20.8 (14.19-26.67)	17.4 (11.35-21.98)	1.2	0.324
MRP6	41.73 (37.76-46.13)	39.89 (34.59-45.92)	1.05	0.582
BSEP	-31.33)	24.62 (19.45-31.12)	0.92	0.616
MDR1	23.23 (16.52-32.66)	24.78 (17.66-34.75)	0.94	0.766
MDR3	30.20 (22.70-40.18)	30.90 (24.38-39.17)	0.98	0.892

Table 1.2 Gender-specific expression levels of hepatic efflux transporters.

¹Geometric mean (95% Confidence Interval), ²Student's t-test

Pmol protein per gram liver ¹				
Transporter protein	Female	Male	Female/Male ratio	p-value ²
OATP1B1	111.1 (71.78-171.8)	65.30 (49.66-85.70)	1.70	0.025*
OATP1B3	112.5 (95.06-133.4)	96.12 (74.82-123.6)	1.17	0.280
OATP2B1	41.84 (29.79-58.75)	39.57 (33.42-46.88)	1.06	0.726
OCT1	239.5 (151.4-379.3)	179.3 (134.3-239.3)	1.34	0.225
OCT3	25.96 (21.33-31.62)	19.91 (13.97-28.38)	1.30	0.150
OAT2	137.7 (102.3-185.4)	150.1 (104.5-215.8)	0.92	0.694
OAT7	96.26 (82.60-112.2)	77.29 (64.27-92.90)	1.25	0.06

Table 1.3 Gender-specific expression levels of hepatic uptake transporters.

¹Geometric mean (95% Confidence Interval), ²Student's t-test

Transporter Protein Expression (pmol/g liver)			
Transporter protein	In-house mean (range)	Groen mean (range)	Wang approx. mean (approx. range)
OATP1B1	82.95 (40.69-331.39)	17.7 (9.1-53.7)	150 (50-300)
OATP1B3	103.2 (54.37-202.8)	16 (7.9-28.1)	60 (10-140)
OATP2B1	40.57 (20.31-90.05)	61.3 (34.5-77.8)	60 (25-125)
OCT1	204.3 (74.54-482.5)	124.3 (60.1-181.4)	175 (25-325)
MRP2	22.49 (5.93-55.53)	29.5 (19.6-39.6)	50 (15-100)
MRP3	17.35 (6.86-29.91)	6.8 (4.2-9.9)	20 (7.5-30)
MDR1	24.07 (11.09-53.04)	20.5 (12.3-25.7)	20 (8-35)
ATP1A1	232.6 (155.4-301.9)	279.3 (193.8-391.3)	N/A
BSEP	23.66 (12.13-50.98)	60.1 (25.7-92.4)	75 (25-125)

Table 1.4: Comparison of transporter expression between in-house measurements and reported literature values. Data was taken from Groen et al. 2018 and Wang et al. 2016 and all values are expressed as pmol/g liver.

1.7 Figures

Figure 1.1 Graphical abstract of transporter extraction, digestion and LC-MS/MS analysis: Workflow for quantification of 13 clinically relevant transporter proteins in human liver tissue using SWATH-MS

Figure 1.2: Gender difference on liver protein abundance and ATP1A1 (a membrane protein marker). Absolute protein expression values expressed as picomole per gram of liver tissue. The line represents the geometric mean for the respective group and individual values are shown as aligned dots plot.

Figure 1.3: Effect of gender on hepatic efflux transporters. Absolute protein expression values expressed as picomole per gram of liver tissue. The line represents the geometric mean for the respective group and individual values are shown as aligned dots plot.

Figure 1.4: Effect of gender on hepatic uptake transporters. Absolute protein expression values expressed as picomole per gram of liver tissue. The line represents the geometric mean for the respective group and individual values are shown as aligned dots plot.

Figure 1.5: Comparison of transporter expression between in-house measurements and Groen et al. 2018 and Wang et al. 2016. Min to max reported with mean shown as the middle point.

MANUSCRIPT 2

PRESENCE OF DRUG METABOLIZING ENZYMES INDUCERS IN COMMERCIALLY AVAILABLE HUMAN LIVER SAMPLES RESULTS IN ALTERED METABOLIC PROFILES

Prepared for *Drug Metabolism and Disposition*

Benjamin J. Barlock 1, Rohitash Jamwal 1, Ali Mahta 2, Fatemeh Akhlaghi 1

- 1 Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, RI, 02881, USA
- 2 Department of Neurology, Brown University, Providence, RI

2.1 Abstract

Background & Aims: Human liver tissue utilized in drug metabolism (DM) studies is retrieved from brain-dead individuals receiving an array of therapeutic agents during hospitalization. The anticonvulsant agent phenytoin, a potent cytochrome P450 (CYP) 3A4 inducer, is among such agents that potentially can impact the expression and activity of DM enzymes. Here we identify and highlight the need to screen human liver tissues for the presence of phenytoin.

Methods: One-hundred and six samples from an in-house repository of human livers were screened for the presence of phenytoin and 1,702 other compounds utilizing AB SCIEX forensic library and a TOF-instrument. Phenytoin concentrations were then quantified in phenytoin positive tissue using LC-MS/MS. Activity levels for CYP3A4 were collected in-house as well as provided by the vendor. Expression of CYP proteins were analyzed by an untargeted proteomic analysis. mRNA levels for all CYPs and certain nuclear transcription factors were also measured. Non-parametric independent sample tests were run in SPSS and comparisons with a p-value <0.05 were considered significant.

Results: Phenytoin was present in 34 of the 106 human livers (32%) and the functional activity and protein level of CYP3A4 were significantly higher in these samples.

Conclusions: Caution should be taken when performing studies with commercially available liver sample and microsomes prepared thereof. The use of ambiguous protein expression values due to induction from phenytoin may decrease the accuracy of PBPK models developed using such data. Whenever possible, we suggest that liver

tissue, primary hepatocytes and liver microsomes should be screened for phenytoin to prevent ambiguous results due to the induction of DMEs.

2.2 Introduction

Livers from human donors contain valuable information regarding drug metabolism and how it is affected by disease and other exogenous and endogenous factors. When performing drug metabolism studies using human livers, it is imperative to know what medications are still remaining in the system as many drugs on the market are either inducers or inhibitors of drug metabolizing enzymes¹. The FDA classifies drugs based on their potential to inhibit or induce as potent, moderate or weak and lists examples while an exhaustive list can be found in the University of Washington Metabolism and Transport Drug Interaction Database^{2,3}. These drugs can significantly impact and change the efficiency of drug metabolizing enzymes (DMEs), specifically the hepatic cytochrome P450 (CYP) family of proteins⁴. By using mass spectrometry, it is possible to screen these livers for thousands of medications and their metabolites that may alter the metabolic profile.

Phenytoin is a highly effective anticonvulsant and one of the most commonly prescribed antiepileptic drugs (AED) in clinical practice. Seizure is a common complication of patients with a wide variety of brain pathologies including spontaneous intracerebral hemorrhage, brain tumors and patients with traumatic brain injury (TBI). Phenytoin is commonly used in TBI patients who develop seizure. It is also recommended as either primary agent or alternative AED to Levetiracetam for seizure prophylaxis in this population⁵. Post traumatic epilepsy can occur in about 5-7% of patients with TBI² and prophylactic use of AEDs can prevent early post traumatic epilepsy. In addition to phenytoin, a plethora of medications are given when attempting to save a patient's life and once a patient is pronounced brain dead prior to

organ procurement. These drugs include but are not limited to sedatives, analgesics including opioids, vasopressors, paralytics, etc⁶. Therefore, it can be expected that tissue obtained from such organ donors may contain many of these drugs.

In this study, we describe the screening of commercially available human liver samples for the presence of ~2,000 drugs and metabolites with emphasis on CYP3A inducers. Additionally, we describe the effects seen in the protein and mRNA expression as well as the activity levels of CYP3A4 due to the presence of phenytoin. Finally, we demonstrate how the use of CYP3A4 values obtained from contaminated samples can cause significant alterations in drug profiles obtained when creating a PBPK model.

2.3 Materials and Methods

2.3.1 Chemicals and reagents

Protein preparation kit, TPCK-treated trypsin, trypsin digested β -galactosidase, and MS tuning solution were purchased from SCIEX (Framingham, MA). Acquity UPLC Peptide BEH C18 column and VanGuard pre-columns were obtained from Waters Corp. (Waltham, MA). Dithiothreitol (DTT) was purchased from Roche Diagnostics (Indianapolis, IN). MS grade acetonitrile, methanol and formic acid were procured from ThermoFisher (Waltham, MA). RNeasy Mini Kit was purchased from Qiagen Inc. (Valencia, CA). Phenytoin and phenytoin-d10 were purchased from Sigma Aldrich (St. Louis, MO). All other reagents and solvents used in this study were of analytical grade.

2.3.2 Human Liver Bank

Frozen human liver samples from brain dead donors were purchased from Sekisui XenoTech LLC, Kansas City, KS. All the livers are from organ donors involved in automobile accidents and therefore are IRB-exempt. The detailed demographics of the donors is given in **Table 2.1** including cause of death.

2.3.3 Forensic sample preparation

Human liver tissue (~50 mg) was homogenized using a bead homogenizer in 8 M urea containing 10 mM DTT and 50 mM TEAB (tri-ethyl ammonium bicarbonate) as described elsewhere (manuscript in preparation). For the qualitative drug screening analysis, 400 µl of ice-cold extraction solution (acetonitrile containing 1% formic acid and 7.5 ng THC-d3 as an internal standard) was added to 100 µl of homogenate sample. Samples were then vortexed and sonicated.

Precipitated proteins were pelleted by spinning at 10,000 *g* for 5 min at 10°C. Supernatant was collected in clean glass tubes and was dried using a SpeedVac at room temperature (Savant SPD2010, ThermoFisher, Waltham, MA). Dried samples were reconstituted in equal mixture of methanol and water containing 0.1% formic acid. The samples were vortexed and sonicated for 30 sec and transferred to a centrifuge tube. Subsequently, samples were spun at 10,000 rpm for 5 min at 10°C and 10 µL of resulting supernatant was injected into LC-MS/MS.

2.3.4 Screening for drugs

Qualitative drug screening was performed in positive ionization mode on a SCIEX 5600 TripleTOF mass spectrometer equipped with a DuoSpray ion source (AB Sciex, Concord, Canada) coupled to an Acquity UHPLC HClass system (Waters Corp.,

Milford, MA, USA). Drugs were separated on a Phenomenex Kinetex Phenyl-Hexyl (4.6 x 50 mm, 2.6 μ m, 100 Å). Samples were kept at 10°C in the autosampler. The analytical column was kept at 50°C and injection volume was 10 μ l. The chromatographic separation was run for 25 minutes at 500 μ l/min using mobile phase A (99.9% 25 mM ammonium formate, 0.1% formic acid) and mobile phase B (99.9% methanol, 0.1% formic acid). The solvent composition of the linear gradient is as follows; 95% A at 0 min: 80% A at 1 min: 25% A at 14 min: 10% A from 16 – 19 min: 50% A at 22 min: and 95% A from 24 – 25 min. Analyst TF 1.7 was used to acquire the mass spectrometry data. The SWATH-MS experiments were performed over a m/z range of 100-700 with overlapping SWATH window widths of 20 Da resulting in 28 windows. Declustering potential was set to 80 and a collision energy of 35 V with a spread of 15 V was used for ions with a +1 charge state. Source specific parameters are as follows; gas 1 (GS1): 55 psi, gas 2 (GS2): 60 psi, curtain gas (CUR): 25 psi, source temperature (TEM): 500°C, ionspray voltage floating (ISVF): 5500 V.

The raw data files from SWATH-MS experiments were run through the drug screening library for identification of drugs in the liver homogenate. The Forensic HR-MS/MS Spectral Library (v2.1) was used to mine the data on MasterView v1.1 (SCIEX, Framingham, MA).

2.3.5 Quantification of phenytoin by LC-MS/MS

For quantification of phenytoin, an MRM based targeted method was used. Phenytoin was extracted from homogenate by adding 200 μ l of ice-cold extraction solution (acetonitrile containing 1% formic acid and 7.5 ng THC-d3). Samples were

spun down at 10,000 *g* for 5 min at 10°C and the supernatant was dried using a SpeedVac at room temperature. Samples were then reconstituted in methanol. The samples were vortexed and sonicated for 30 sec and transferred to a centrifuge tube. Subsequently, samples were spun at 10000 *g* for 5 min at 10°C and 5 µL of the resulting supernatant was analyzed on a Xevo TQ MS (Waters Corp., Waltham, MA).

An Acquity UPLC[®] system coupled to a Xevo[®] TQ MS detector was used for quantification of 1-phthalazinone (Waters Corp, Milford, MA, USA). Separation of analytes was achieved over a run-time of 2.5 min on Acquity UPLC BEH C18 column (2.1 x 50 mm, 130Å, 1.7 µm) attached to Acquity UPLC BEH C18 VanGuard pre-column (2.1 x 5 mm, 130Å). The flow rate was 0.5 mL/min and 5 µL of samples was injected. The analytical column and autosampler were kept at 45°C and 10°C, respectively. The separation of analytes was achieved over a 2.5 min linear gradient elution method using 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The gradient conditions were as followed: 2% B to 98% B (0.5-1 min), 98% B (1-1.5 min), 98% B to 2% B at 2.25 min and maintained at 2% B for rest of the run-time. MassLynx[™] (V 4.1) and TargetLynx[™] (V 4.1) were used to control the system and integration of the analyte peaks, respectively. The ratio of analyte to internal standard was used to quantify the phenytoin level in homogenate samples. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode and quantification was performed in positive electrospray ionization (ESI). The source desolvation temperature was 650°C and desolvation gas flow rate was 1000 L/h. Mass/charge of precursor and product ions for phenytoin and phenytoin-d10 (internal

standard) are given in **Table 2.2**. Measured phenytoin concentration was converted to $\mu\text{g/ml}$ based on total liver volume equations⁷.

2.3.6 Proteomic and mRNA studies

Preparation of microsomes, CYP3A4 functional activity, protein and mRNA levels were obtained from the liver bank determined in our previous studies^{8,9}. Protein expression levels were achieved by running data dependent acquisition files through MaxQuant (ver 1.5.2.10) where the “Total Protein Approach” was then applied in order to convert intensity values to a picomole per mg of microsomal protein scale¹⁰. Methods can be found in the supplementary information.

2.3.7 PBPK modeling

SimCYP population-based simulator (ver 17, Certara LP, Sheffield, UK) was used to create a physiologically base pharmacokinetic (PBPK) model. CYP3A4 protein and enzyme kinetic parameters for midazolam were manually changed to reflect with and without phenytoin containing liver samples. The Caucasian population (Sim-NEurcaucasian) was used and 10 virtual trials with 1000 subjects per trial were run. Male and female proportions were equal, and ages were 20-65 years old. In vitro V_{max} and K_m values were obtained from Jamwal et al. and protein expression was measured in house and previously published^{8,9}. All other parameters were kept as default and estimated plasma concentrations of phenytoin were used for analysis.

2.3.8 Statistical analysis

Independent student's t-test was used to determine the difference between groups containing phenytoin and groups that were clean of phenytoin. Statistical values of

$p < 0.05$ were considered significant for the analysis. SPSS 24 (IBM Corp., Armonk, NY) and Prism 6.0 (GraphPad Inc., La Jolla, CA) were used to perform statistical tests and create graphs.

2.4 Results

2.4.1 Drugs found in forensic library

Drugs were considered a match if the MS/MS fragmentation pattern matched the reference library with less than 10 ppm error. Across the 106 human liver samples, 75 different drugs were found in at least one or more samples. Phenytoin was found in 34 of the 106 samples (32%). No correlation was found between phenytoin and cause of death. Other abundant drugs found were methylprednisolone, dexamethasone, ofloxacin, diphenhydramine and amiodarone. A list of the other drugs found can be seen in **Table 2.3**. THC-d3 was spiked into all samples as an internal control and was found in all samples. Blanks only contained THC-d3 and a pooled human liver microsome sample contained methylprednisolone, phenytoin and THC-d3.

2.4.2 Phenytoin concentration

Measured phenytoin levels ranged between 1 and 21 ug per g of liver tissue. The equation $\text{Liver Volume} = 0.722 * \text{BSA}^{1.176}$ was used to convert values to $\mu\text{g/ml}^7$. Values ranged between 1.7 and 16.8 $\mu\text{g/ml}$. Normal therapeutic levels of phenytoin are between 10 and 20 ug/ml^{11} . No correlation was found between phenytoin concentration and mRNA, activity or protein expression. Due to the nature of the samples, length of phenytoin therapy and time of administration is unknown.

2.4.3 Effect of phenytoin on CYP450 expression and activity

CYP3A4 protein expression was found to be significantly higher in samples containing phenytoin. Liver samples containing phenytoin had levels of 74.84 ± 16.18 pmol/mg of protein while liver samples without phenytoin expressed 34.9 ± 7.08 pmol/mg of protein ($p=0.0003$). Similar results were seen in CYP3A5 ($p=0.032$) and CYP2B6 ($p=0.004$). CYP2A6 showed a similar trend but was not significant. The expression of the remaining CYPs showed little to no significant change. These data can be seen in **Figure 2.1** and **Table 2.3**.

The mRNA expression of CYP2A6 ($p=0.0002$), 2B6 ($p<0.0001$), 2C8 ($p=0.0001$), 2C9 ($p=0.0033$) and 3A4 ($p<0.0001$) in samples containing phenytoin were found to be significantly increased when compared those that were found to be phenytoin free. Relative CYP3A4 mRNA expression was 2.348 ± 1.904 and 0.45 ± 0.71 in livers with phenytoin compared to those without phenytoin, respectively. CYP2A6, CYP2B6 and CYP2C9 were all found to be slightly significantly increased as well ($p<0.05$). These data can be seen in **Figure 2.2** and **Table 2.4**.

CYP3A4/5 mediated testosterone hydroxylation was found to be significantly higher in samples containing phenytoin when performed in house and by the vendor. In house Vmax for livers containing phenytoin was found to be 268.9 ± 92.86 pmol/min/mg protein compared to 158.8 ± 54.57 pmol/min/mg protein for livers that were absent of phenytoin ($p<0.0001$). Similar trends were seen in values measured by the vendor at 3136 ± 652.43 pmol/min/mg protein and 1460 ± 191.1 pmol/min/mg protein for livers containing phenytoin and those without, respectively. Activity levels for midazolam hydroxylation by CYP3A4/5 ($p=0.0008$) and CYP2B6 ($p<0.0001$)

were also found to be significantly increased when phenytoin was present. CYP2A6 was found to be slightly significantly increased ($p=0.012$) and CYP2C9 was found to be significantly decreased ($p=0.011$). The activity of the other CYP enzymes showed no significant difference. These data can be seen in figure **Figure 2.3** and **Table 2.5**.

2.4.4 PBPK model

SimCyp (ver 17, Certara LP, Sheffield, UK) was used to model the elimination of midazolam in healthy patients with parameters changed to reflect the differences of patients on phenytoin. An IV bolus dose of 5 mg midazolam was given over 30 s. For the no phenytoin control group, the normal population was used with 100 patients and 10 runs. For the phenytoin containing group, values for V_{max} , K_m and CYP3A4 were changed from 158.8 pmol/min/mg protein, 2.19 μ M and 34.9 pmol/mg protein to 268.9 pmol/min/mg protein, 2.08 μ M and 74.84 pmol/mg protein, respectively, to reflect the values collected in our lab. It was found that the AUC, C_{max} and T_{max} changed from 1304 ng/ml.h, 126.8 ng/ml and 0.12 h to 753.8 ng/ml.h, 120.7 ng/ml and 0.12 h respectively. AUC showed a significant decrease ($p<0.0001$) and changed by about 50%. This data can be shown in **Figure 2.4**.

2.5 Discussion

Initially the goal of our study was purely based on curiosity of what medications were found in the liver samples being used for proteomic and enzymatic studies. Once the samples were run through the screening library it was discovered that such a large percentage contained phenytoin. At that point it was decided to

explore the effect of phenytoin through its absolute quantification and its effect on protein, mRNA and enzymatic activity.

In this study we found 75 unique drugs present in 106 human liver samples. Four or more drugs were found in 50% of all samples with one sample containing as many as 11 medications or metabolites. Phenytoin was found in one third of all samples. Due to its abundance in samples and CYP3A4 induction characteristics, phenytoin was chosen for further quantification¹³. There was no correlation between any of the abundant drugs and cause of death. Phenytoin was expected to be seen in a larger proportion of samples obtained from patients who passed away due to head trauma, but it was not statistically significant¹⁴. Phenytoin is widely used either as first line or an alternative agent to levetiracetam for seizure prophylaxis in patients with TBI and is typically given for the first 7 days after trauma¹⁵.

In 2014, there were 288,000 hospitalizations due to TBI with 56,800 cases leading to death in the United States¹⁶. The average length of stay for a TBI hospitalization is about 7 days in patients who require ICU admission and 3.2 days for those who did not require the ICU¹⁷. As it takes between 7 and 10 days for phenytoin to reach steady state (10-20µg/ml), it can be assumed that most patients, following the recommended 7-day dosing for seizure prophylaxis, will have reached or be close to steady state levels prior to declaration of brain death or organ procurement¹⁸. Full induction effects can be expected to be the EC50 for phenytoin has been found to be 23µM or 5.8µg/ml¹⁹. Since the therapeutic range of phenytoin is 10-20µg/ml, it can be assumed that patients receiving phenytoin were above the EC50 for the duration of therapy²⁰. In the situation of seizure prophylaxis for traumatic brain injury steady state

is normally reached quicker. In some clinical practices, a loading dose of 15 to 20 mg phenytoin equivalents (PE) per kg of IV fosphenytoin, a prodrug of phenytoin, is given at 150 PE/min^{21,22}. Fosphenytoin has a half-life of 15 min so it can be used to rapidly increase the concentrations of phenytoin and therefore shorten the time it takes to achieve steady state²¹, bringing the plasma concentration to around 3 µg/ml before phenytoin therapy is started. This also avoids the untoward cardiovascular effects and injection site pain that are associated with rapid infusion of phenytoin (greater than 50 mg/min)²¹. It can take up to 2 weeks to see full CYP3A4 induction as a result of phenytoin, although effects of CYP3A4 induction can be seen as quickly as 48 hours after the start of phenytoin therapy^{23,24}. There was no correlation between the concentration of phenytoin and the expression levels of CYP3A4 mRNA or protein. This could be explained by the nonlinear pharmacokinetics of phenytoin²⁵. Due to the nature of the samples, it is not possible to determine if patients has been taking phenytoin prior as opposed to during hospitalization course to achieve more of an insight²⁶.

Induction of CYP3A4 is a result of phenytoin binding to pregnane X receptor (PXR) leading to co-binding with retinoid X receptor (RXR) and the eventual binding to DNA. This results in increased production of mRNA for CYP3A4 and in turn, an increased expression of protein. PXR binding is also responsible for the regulation of CYP3A5, 2B6, 2C8, 2C9, 2C19 and 1A2^{27,28}. Similar effects can be attributed to the modulation of constitutive androstane receptor (CAR) which works in the same way as RXR but has a smaller ligand binding domain. CAR is also responsible for the modulation of CYP1A2, 2B6, 2C9 and 3A4^{27,29}.

An increase in a CYP3A4 leads to an increased metabolic rate or activity of the enzyme and thus an increased clearance and decreased half-life of any given CYP3A4 substrate. For CYP3A4 to return to normal levels, it must be catabolized by the body. It was found by Fleishaker et al., that when healthy subjects received 200 mg of phenytoin every 8 hours, the CYP3A4 mediated metabolite of cortisol, 6 β -hydroxycortisol (6 β -OHC), significantly increased with the first effects being noticed at 48 hours²³. The normal half-life for CYP3A4 is about 3 days, meaning that once an inducer like phenytoin is removed from the body, the enzyme remains in an induced state for up to 2 weeks, assuming it takes 5 half-lives to return to baseline³⁰. Once a donor has been declared brain dead and their family members authorize the organ retrieval process, it can take 24-36 h before the donor is in the operating room, ready for organ procurement³¹. This means that there is not time for the enzyme to return to a non-induced state if the patient was on phenytoin.

No major inhibitors of metabolism were found in our study. An inhibitor, unlike an inducer, binds to the protein or enzyme directly. This can be either a reversible binding or irreversible (mechanism-based) and can either be in the active binding site or at an allosteric location. Reversible inhibitors return to a normal level much faster than irreversible as it will take until new enzyme is created in order to return to normal function³². It was expected that if any inhibitors were found in the liver samples, their effect would not affect protein or mRNA expression unless there was irreversible binding. Additionally, activity was not expected to be affected as the inhibitor would have been lost during ultracentrifugation during the preparation of microsomes.

Human liver microsomes (HLM) are commonly used and provide valuable support to in vitro drug metabolism studies. HLMs primarily comprise of membrane vesicles from hepatocyte endoplasmic reticulum and contain membrane bound phase I (CYPs) and phase II enzymes (UGTs)³³. It is expected that over the course of the preparation of microsomes, drugs would be removed during any ultracentrifugation steps. The purchased pooled liver microsome sample that we tested was shown to contain phenytoin. The presence of an inducer indicates that donors used in the pool should be expected to have an altered CYP protein expression. This can confound the true functional activity of CYP enzymes. Our research suggests that commercially obtained HLM should be screened for common CYP inducers or prepared in house with the removal of samples containing CYP inducers.

When using liver samples containing drugs of induction, results obtained and used in PBPK models can cause altered results. We found that using values obtained from phenytoin containing livers, the AUC for midazolam was decreased by about 50% due to the induction of CYP3A4. This can cause a challenge when creating disease populations where true effects may be masked or exaggerated due to the presence of a DME altering drug such as phenytoin.

In summary, our research suggests that commercially available human liver samples contain numerous xenobiotics from the time of death of the organ donors. It appears that there are multiple inducers of metabolism remaining in the liver samples and the presence of the potent CYP3A4 inducer, phenytoin, resulted in increased protein expression, mRNA expression and activity levels of CYP3A4, as well as slight induction in protein and mRNA expression for numerous other CYPs.

This is a concern when developing PBPK models for translation to humans. As we have shown, using values obtained from samples that were free of phenytoin result in a significantly different profile when compared to samples that contained phenytoin. This is especially important when creating models for special populations, such as disease models, where drug metabolizing enzymes are possibly dysregulated in the first place. The addition of phenytoin and other metabolism altering drugs to an already perturbed metabolic profile could lead to an over or under estimation of PK parameters when attempting to translate to the clinic.

2.6 References

1. Wilkinson GR. Drug metabolism and variability among patients in drug response. *N Engl J Med*. 2005;352(21):2211-2221.
2. FDA. Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers. 2017.
3. Hachad H, Ragueneau-Majlessi I, Levy RH. A useful tool for drug interaction evaluation: the University of Washington Metabolism and Transport Drug Interaction Database. *Hum Genomics*. 2010;5(1):61-72.
4. Lin JH, Lu AY. Inhibition and induction of cytochrome P450 and the clinical implications. *Clin Pharmacokinet*. 1998;35(5):361-390.
5. Wilson CD, Burks JD, Rodgers RB, Evans RM, Bakare AA, Safavi-Abbasi S. Early and Late Posttraumatic Epilepsy in the Setting of Traumatic Brain Injury: A Meta-analysis and Review of Antiepileptic Management. *World Neurosurg*. 2018;110:e901-e906.
6. Korte C, Garber JL, Descourouez JL, Richards KR, Hardinger K. Pharmacists' guide to the management of organ donors after brain death. *Am J Health Syst Pharm*. 2016;73(22):1829-1839.

7. Johnson TN, Tucker GT, Tanner MS, Rostami-Hodjegan A. Changes in liver volume from birth to adulthood: a meta-analysis. *Liver Transpl.* 2005;11(12):1481-1493.
8. Jamwal R, Barlock BJ, Adusumalli S, Ogasawara K, Simons BL, Akhlaghi F. Multiplex and Label-Free Relative Quantification Approach for Studying Protein Abundance of Drug Metabolizing Enzymes in Human Liver Microsomes Using SWATH-MS. *J Proteome Res.* 2017;16(11):4134-4143.
9. Jamwal R, de la Monte SM, Ogasawara K, Adusumalli S, Barlock BB, Akhlaghi F. Nonalcoholic Fatty Liver Disease and Diabetes Are Associated with Decreased CYP3A4 Protein Expression and Activity in Human Liver. *Mol Pharm.* 2018;15(7):2621-2632.
10. Wisniewski JR, Rakus D. Multi-enzyme digestion FASP and the 'Total Protein Approach'-based absolute quantification of the Escherichia coli proteome. *J Proteomics.* 2014;109:322-331.
11. Pfizer. Dilantin (phenytoin). *US Food and Drug Administration Website.*
12. Adusumalli S, Jamwal R, Obach RS, Ryder TF, Leggio L, Akhlaghi F. Role of Molybdenum-Containing Enzymes in the Biotransformation of the Novel Ghrelin Receptor Inverse Agonist PF-5190457: A Reverse Translational Bed-to-Bench Approach. *Drug Metab Dispos.* 2019;47(8):874-882.
13. Luo G, Cunningham M, Kim S, et al. CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug Metab Dispos.* 2002;30(7):795-804.
14. Alimardani S, Sadrai S, Masoumi HT, et al. Pharmacokinetic Behavior of Phenytoin in Head Trauma and Cerebrovascular Accident Patients in an Iranian Population. *J Res Pharm Pract.* 2017;6(4):217-222.
15. Torbic H, Forni AA, Anger KE, Degrado JR, Greenwood BC. Use of antiepileptics for seizure prophylaxis after traumatic brain injury. *Am J Health Syst Pharm.* 2013;70(9):759-766.
16. Taylor CA, Bell JM, Breiding MJ, Xu L. Traumatic Brain Injury-Related Emergency Department Visits, Hospitalizations, and Deaths - United States, 2007 and 2013. *MMWR Surveill Summ.* 2017;66(9):1-16.

17. Levant S, Chari K, DeFrances C. National Hospital Care Survey Demonstration Projects: Traumatic Brain Injury. *Natl Health Stat Report*. 2016(97):1-16.
18. Bolt J, Gorman SK. Precision, bias, and clinical utility of the Sheiner-Tozer equation to guide phenytoin dosing in critically ill adults. *J Clin Pharmacol*. 2013;53(4):451-455.
19. Vermet H, Raoust N, Ngo R, et al. Evaluation of Normalization Methods To Predict CYP3A4 Induction in Six Fully Characterized Cryopreserved Human Hepatocyte Preparations and HepaRG Cells. *Drug Metab Dispos*. 2016;44(1):50-60.
20. Turnbull DM, Rawlins MD, Weightman D, Chadwick DW. "Therapeutic" serum concentration of phenytoin: the influence of seizure type. *J Neurol Neurosurg Psychiatry*. 1984;47(3):231-234.
21. Pfizer. Cerebyx (fosphenytoin sodium).
22. Division of Acute Care Surgery Clinical Practice Policies, Guidelines, and Algorithms: Post-Traumatic Seizure Prophylaxis in Patients with Traumatic Brain Injury Clinical Practice Guideline. 2017.
23. Fleishaker JC, Pearson LK, Peters GR. Phenytoin causes a rapid increase in 6 beta-hydroxycortisol urinary excretion in humans--a putative measure of CYP3A induction. *J Pharm Sci*. 1995;84(3):292-294.
24. Zhu M, Kaul S, Nandy P, Grasela DM, Pfister M. Model-based approach to characterize efavirenz autoinduction and concurrent enzyme induction with carbamazepine. *Antimicrob Agents Chemother*. 2009;53(6):2346-2353.
25. Martin E, Tozer TN, Sheiner LB, Riegelman S. The clinical pharmacokinetics of phenytoin. *J Pharmacokinet Biopharm*. 1977;5(6):579-596.
26. Thorn CF, Whirl-Carrillo M, Leeder JS, Klein TE, Altman RB. PharmGKB summary: phenytoin pathway. *Pharmacogenet Genomics*. 2012;22(6):466-470.
27. Brodie MJ, Mintzer S, Pack AM, Gidal BE, Vecht CJ, Schmidt D. Enzyme induction with antiepileptic drugs: cause for concern? *Epilepsia*. 2013;54(1):11-27.

28. Wolf KK, Wood SG, Hunt JA, et al. Role of the nuclear receptor pregnane X receptor in acetaminophen hepatotoxicity. *Drug Metab Dispos.* 2005;33(12):1827-1836.
29. Lee CH, Ito Y, Yanagiba Y, et al. Pyrene-induced CYP1A2 and SULT1A1 may be regulated by CAR and not by AhR. *Toxicology.* 2007;238(2-3):147-156.
30. Yang J, Liao M, Shou M, et al. Cytochrome p450 turnover: regulation of synthesis and degradation, methods for determining rates, and implications for the prediction of drug interactions. *Curr Drug Metab.* 2008;9(5):384-394.
31. Wojda TR, Stawicki SP, Yandle KP, et al. Keys to successful organ procurement: An experience-based review of clinical practices at a high-performing health-care organization. *Int J Crit Illn Inj Sci.* 2017;7(2):91-100.
32. Zhou S, Yung Chan S, Cher Goh B, et al. Mechanism-based inhibition of cytochrome P450 3A4 by therapeutic drugs. *Clin Pharmacokinet.* 2005;44(3):279-304.
33. Zhang H, Gao N, Tian X, et al. Content and activity of human liver microsomal protein and prediction of individual hepatic clearance in vivo. *Sci Rep.* 2015;5:17671.

2.7 Tables

	Phenytoin Free	Phenytoin Containing
N	72	33
Male	35	21
Female	38	12
Height (cm)	169 ± 11	170 ± 10
BMI (kg/m²)	34 ± 12	29 ± 8
Age (years)	52 ± 13	50 ± 11
Ethnicity (C, AA, H)	63, 8, 2	27, 2, 3
Cause of death		
Anoxia	40	7
Cerebrovascular aneurysm	26	15
Head trauma	6	10
Myocardial Infarction	1	0
Intracerebral Hemorrhage	0	1
Smoking status		
Non-smoker	34	13
Smoker	39	19

Table 2.1 Demographic data for the phenytoin containing and phenytoin free livers. BMI: Body mass index, C: Caucasian, AA: African American, H: Hispanic

Analyte specific mass spectrometry parameters						
Analyte	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Dwell time (s)	Cone voltage (V)	Collision voltage (V)	Retention time (min)
Phenytoin	253.0958	104.1512	0.050	20	35	1.3
		182.1704	0.050	20	20	1.3
		225.1000	0.050	20	10	1.3
Phenytoin D ₁₀	263.1596	109.1476	0.050	20	35	1.3
		192.2356	0.050	20	20	1.3
		235.2691	0.050	20	10	1.3

Table 2.2. Analyte specific mass spectrometry parameters of phenytoin and phenytoin D₁₀

Citalopram n=8	Fluconazole n=6	Laudanosine n=4
Clindamycin n=8	Propoxyphene n=6	Midazolam n=3
Metoprolol n=8	Amitriptyline n=5	Paroxetine n=3
Sertraline n=8	Lorazepam n=5	Promethazine n=3
Cyclobenzaprine n=7	Verapamil n=5	Protriptyline n=3
Diazepam n=6	Trimipramine n=4	Quetiapine n=3
Ephedrine n=6	Diltiazem n=4	Trimethoprim n=3

Table 2.3 List of drugs found in 3 or more human liver samples

Picomole of protein per milligram of microsomal protein ¹				
Enzyme	No Phenytoin	Phenytoin	Phenytoin ratio	p-value
CYP1A2	10.06 (7.368-13.73)	8.26 (4.604-14.82)	0.82	0.541
CYP2A6	23.17 (16.46-32.6)	38.83 (23.35-64.56)	1.68	0.074
CYP2B6	1.149 (0.831-1.589)	3.361 (1.832-6.166)	2.93	0.0041
CYP2C8	21.25 (17.1-26.4)	23.74 (15.14-37.23)	1.12	0.137
CYP2C9	46.11 (40.65-52.29)	41.11 (33.24-50.84)	0.89	0.692
CYP3A4	34.9 (26.88-45.32)	74.84 (48.16-116.3)	2.14	0.0003
CYP3A5	4.64 (3.205-6.722)	8.768 (5.418-14.19)	1.89	0.032

Table 2.4 Effect of phenytoin on protein expression of various CYP450 proteins. Geometric mean and 95% confidence interval of protein expression in pmol per mg of microsomal protein for CYPs in phenytoin containing livers and non-phenytoin containing livers. Mann Whitney U test was used to determine significance.

Relative mRNA Expression ¹				
Enzyme	No Phenytoin	Phenytoin	Phenytoin ratio	p-value
CYP1A2	1.017 (0.653-1.582)	0.915 (0.491-1.706)	0.86	0.748
CYP2A6	0.706 (0.414-1.206)	3.397 (1.639-7.041)	4.81	0.0002
CYP2B6	0.4011 (0.257-0.625)	3.26 (1.658-6.408)	8.13	<0.0001
CYP2C8	0.689 (0.459-1.034)	2.211 (1.35-3.62)	3.21	0.0001
CYP2C9	0.8016 (0.517-1.244)	2.432 (1.487-3.976)	3.03	0.0033
CYP3A4	0.452 (0.2933-0.696)	2.348 (1.159-4.757)	5.19	<0.0001
CYP3A5	1.008 (0.7196-1.412)	1.218 (0.847-1.754)	1.2	0.275

Table 2.5 Effect of phenytoin on mRNA expression of various CYP450 proteins. Geometric mean and 95% confidence interval of mRNA expression for CYPs in phenytoin containing livers and non-phenytoin containing livers. Mann Whitney U test was used to determine significance.

Enzyme Activity Level ¹				
Enzyme	No Phenytoin	Phenytoin	Phenytoin ratio	p-value
CYP1A2 (Phenacetin O-alkylation)	327.2 (268.5-398.8)	343.5 (233.5-505.4)	1.04	0.986
CYP2A6 (Coumarin 7-hydroxylation)	480.2 (356.2-647.3)	777.4 (447.5-1350)	1.62	0.012
CYP2B6 (Bupropion hydroxylation)	181.2 (136.4-240.6)	632.1 (433.2-922.4)	3.49	<0.0001
CYP2C8 (Amodiaquine N-dealkylation)	1576 (1292-1922)	1960 (1404-2738)	1.24	0.273
CYP2C9 (Diclofenac 4-hydroxylation)	2279 (2069-2509)	1682 (1368-2068)	0.74	0.011
CYP3A4 (Testosterone 6 β -hydroxylation)	1430 (1141-1792)	3136 (2078-4734)	2.19	<0.0001
CYP3A5 (Midazolam 1-hydroxylation)	278.1 (221.3-349.4)	539.7 (353.1-824.8)	1.94	0.0008

Table 2.6 Effect of phenytoin on enzymatic activity of various CYP450 proteins. Geometric mean and 95% confidence interval of functional activity for CYPs in phenytoin containing livers and non-phenytoin containing livers. Mann Whitney U test was used to determine significance.

2.8 Figures

Figure 2.1 Effect of phenytoin on protein expression of various CYP450 proteins. Protein expression in pmol per mg of microsomal protein of CYP1A2, 2A6, 2B6, 2C8, 2C9, 3A4 and 3A5 in livers containing phenytoin compared to livers not containing phenytoin. Mann Whitney U test was performed and geometric mean with 95% confidence interval are shown.

Figure 2.2 Effect of phenytoin on mRNA expression of various CYP450 proteins. mRNA expression of CYP1A2, 2A6, 2B6, 2C8, 2C9, 3A4 and 3A5 in livers containing phenytoin compared to livers not containing phenytoin. Mann Whitney U test was performed and geometric mean with 95% confidence interval are shown.

Figure 2.3 Effect of phenytoin on enzymatic activity of various CYP450 proteins. Activity levels of CYP1A2, 2A6, 2B6, 2C8, 2C9, 3A4 and 3A5 in livers containing phenytoin compared to livers not containing phenytoin. The following compound metabolism was used to determine functional activity from the vendor: CYP1A2: Phenacetin O-alkylation; CYP2A6: Coumarin 7-hydroxylation; CYP2B6: Bupropion hydroxylation; CYP2C8: Amodiaquine N-dealkylation; CYP2C9; Diocefance 4-hydroxylation; CYP3A4/5: Testosterone 6 β -hydroxylation and Midazolam 1-hydroxylation Mann Whitney U test was performed and geometric mean with 95% confidence interval are shown.

Figure 2.4 Effect of phenytoin on PB/PK modeling simulation of IV midazolam dose. SimCyp used to create a phenytoin containing and phenytoin free population. Midazolam plasma concentration simulated using in both states.

MANUSCRIPT 3

NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD) AND DIABETES – EFFECT ON THE PROTEIN ABUNDANCE AND ACTIVITY OF HUMAN HEPATIC DRUG METABOLIZING ENZYMES AND TRANSPORTERS

Prepared for submission to Liver International

Benjamin J Barlock¹, Rohitash Jamwal¹, Suzanne M. de la Monte², Fatemeh

Akhlaghi¹

¹ Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, RI, 02881, USA

² Departments of Medicine, Pathology, Neurology, and Neurosurgery, Rhode Island Hospital and the Warren Alpert Medical School of Brown University, Providence, RI 02903, USA.

3.1 Abstract

Background & Aims. Differential basal expression of drug metabolizing enzymes (DMEs) and transporters in disease states can contribute to significant differences in systemic exposure of xenobiotics. To gain insight into the alterations of these proteins during non-alcoholic fatty liver disease (NAFLD) and diabetes, we investigated the protein abundance and activity in 106 human liver samples.

Methods. In-solution trypsin digestion of proteins in whole tissue lysate was carried out using pressure-cycling technology. Data were acquired in data-dependent acquisition (DDA) and sequential windowed acquisition of all theoretical ion mass spectra (SWATH-MS) modes on a time of flight (TOF) mass spectrometer, and absolute protein levels were determined using total protein approach. Functional activity data from the tissue vendor were used to evaluate the effect of disease on cytochrome P450 (CYP450) activity and further correlated with protein abundance.

Results. It was found that NAFLD altered CYP20A1, CYP2E1, CYP3A4, CYP2J2, CYP4F2, UGT1A3, UGT1A4, UGT2A3, SULT1A1, SULT1C2, MDR1, BSEP and MRP3 protein expression. Diabetes was found to significantly alter the protein expression of CYP4F2, CYP4F3, CYP4F11, SULT2A1, SULT1A1, UGT1A3, UGT2B4, OATP1B3 and OATP2B1. It is important to point out that when livers containing the anticonvulsant, phenytoin, were excluded from the analysis, the differences seen in CYP3A4 were no longer significant.

Conclusions: NAFLD and diabetes alter the protein expression of some hepatic drug disposition proteins.

3.2 Introduction

The human liver, facilitated by several drug metabolizing enzymes (DMEs) and transporters, is the primary organ responsible for the elimination of xenobiotics and endogenous compounds. Nonalcoholic fatty liver disease (NAFLD), a chronic liver condition is characterized by the presence of >5% of macrovascular steatosis in individuals without high alcohol intake (<20 g/day for women, <30 g/day for men) and the spectrum of the disease ranges from the benign fatty liver to severe nonalcoholic steatohepatitis (NASH).¹ NAFLD is projected to be associated with significant economic (~\$103 billion) and clinical burden in the US.² The prevalence of NAFLD in the USA was found to be 30% with 10.3% of patients with advanced fibrosis.³ A recent meta-analytic report estimated higher prevalence in the Middle East and South America with a global NAFLD prevalence of 25.24% (95% CI 22.1-28.7).⁴ The disease is commonly associated with hepatic fat accumulation, insulin resistance, obesity, and cardiovascular diseases.⁵ Advanced stages of NAFLD are associated with hepatocyte ballooning, lobular inflammation and/or fibrosis along with steatosis.⁶ Recent studies have shown a significant dysregulation of hepatic DMEs in S9 fractions from cirrhotic liver samples.⁷ However, the information on the expression of other DMEs in NAFLD is limited, and wherever available, lacks consensus.⁸ The current literature suggests alterations in hepatic cytochrome P450 (CYP) expression, activity or both, however, the directionality of change lacks agreement.⁹⁻¹²

Diabetes and NAFLD tend to go hand in hand. In patients with type II diabetes, the prevalence of NAFLD ranges between 40% and 70%.¹³ The risk factors for diabetes are very similar to those of NAFLD as aforementioned.¹⁴ Diabetes has

traditionally been proven to show downregulation in CYP3A4, the enzyme responsible for metabolism of 50% of marketed drugs.¹⁵⁻¹⁷ It is important to study both of these diseases in tandem as the overlap in, not only risk factors, but effect on drug disposition can exacerbate the dysfunction of important enzymes and transporters.

The last decade has seen a rise in the use of mass spectrometry based techniques for quantification of the protein expression.^{18,19} Label-free quantification (LFQ) has emerged recently as an attractive approach for comparative analysis of protein expression across different samples.²⁰ Accurate and robust quantification with LFQ approaches is complex, and different strategies for extracting quantitative data have been developed.²⁰ Despite being the gold standard, targeted methods of protein quantification are costly and need significant time for optimization of mass spectrometer conditions. A comprehensive cost comparison of different mass spectrometry-based techniques reported significant cost savings with label-free based quantitative proteomics.²¹ Recently, members of the proteomics community have come together in order to attempt to create a standard set of rules to follow when doing proteomic analysis in order to help reduce the variability that can be seen between methodology and different groups.²²

Traditionally, the expression and activity of DMEs have been measured in a human liver microsomal fraction. However, the quantification of DMEs in subcellular fractions usually suffers from batch-to-batch variability in recovery and enrichment of proteins.²³ Therefore, we developed a simple whole tissue lysate method for simultaneous quantification of hepatic drug metabolism enzymes in human liver using

LC-MS/MS. The method was applied to study the effect of NAFLD and diabetes on the expression of clinically important drug metabolizing enzymes.

3.3 Materials and Methods

3.3.1 Chemicals and reagents

TPCK-treated trypsin, trypsin digested β -galactosidase and mass spectrometer tuning solution were purchased from SCIEX, Framingham, MA. Acquity UPLC Peptide BEH C18 analytical column and VanGuard pre-columns were procured from Waters Corp. (Waltham, MA). 1,4-Dithiothreitol (DTT) was obtained from Roche Diagnostics (Indianapolis, IN). Bovine serum albumin, sodium deoxycholate and iodoacetamide (IAA) were procured from Sigma Aldrich (St. Louis, MO). MS grade acetonitrile and formic acid were purchased from ThermoFisher (Waltham, MA).

3.3.2 Human liver and homogenate preparation

An overview of the study workflow is given in figure 1. Frozen human liver samples from brain dead donors were purchased from Sekisui XenoTech LLC (Kansas City, KS). The detailed demographics of the donors are given in **Table 3.1**. Livers were graded by a histopathologist as previously described and were categorized as control, NAFL or NASH ¹⁶. Approximately 50 mg liver tissue was weighed and homogenized in 1000 μ l of homogenization buffer (8 M urea, 50 mM Triethylammonium bicarbonate, 10 mM DTT v/v) using a bead homogenizer (Omni Bead Ruptor, Kennesaw GA) ²⁴. Samples were then spun at 1,000 g for 5 min and the supernatant was collected. The total protein concentration of the resulting sample was determined using Pierce BCA protein assay kit (ThermoFisher Scientific, Waltham, MA). Lipid peroxidation and total cholesterol were measured as described

previously.²⁴ Triacylglycerides (TAG) were measured using a colorimetric assay kit as per manufacturer's protocol (Cayman Chemicals, Ann Arbor, MI).

3.3.3 *In-solution trypsin digestion*

Protein digestion was conducted as described previously with few adaptations^{24,25}. Samples (~250 µg protein) were spiked with 2 µg bovine serum albumin (BSA) and denatured with 25 µL DTT (100 mM) at 35°C for 30 min in a shaking water bath (120 rpm). After denaturation, samples were alkylated in the dark with 25 µL IAA (200 mM) for 30 min at room temperature. Samples were then concentrated using the cold water, methanol and chloroform (1:2:1) precipitation method (centrifugation at 10000 rpm, 5 min at 10°C). The protein pellet was washed with ice-cold methanol and suspended in 100 µL of 50 mM ammonium bicarbonate (pH ~8) containing 3% w/v sodium deoxycholate (DOC). Further, TPCK-treated trypsin (12.5 µg) was added to samples at a ratio of 1:20 (trypsin: protein) and samples were transferred into digestion tubes (PCT MicroTubes, Pressure Biosciences Inc., Easton, MA). The barocycler was run at 35°C, for 75 cycles with 60-sec pressure-cycle (50-sec high pressure, 10-sec ambient pressure, 25 kpsi). Subsequently, 10 µg trypsin was again added to each sample and digestion was repeated as mentioned above.

Further, to 110 µL of digested peptides sample, 10 µL of acetonitrile/water (1:1 v/v, containing 5% formic acid) was added to precipitate DOC. Samples were spun (10,000 rpm for 5 min at 10°C) to remove the precipitate and 100 µL supernatant was collected. Subsequently, 25 µL of the digested peptide sample was injected on the analytical column and were analyzed using LC-MS/MS method described below.

3.3.4 *LC-MS/MS analysis*

Data-dependent analysis (DDA) was performed in positive ionization mode using a DuoSpray™ ion source on a Sciex 5600 TripleTOF™ mass spectrometer (AB Sciex, Concord, Canada) equipped with an Acquity UPLC HClass system (Waters Corp., Milford, MA, USA). Gas 1 (GS1), gas 2 (GS2) and curtain gas (CUR) were maintained at 55, 60 and 25 psi, respectively. Ion spray voltage floating (ISVF) was kept at 5500 V while the source temperature (TEM) was 450°C. Declustering potential (DP), collision energy (CE) and collision energy spread (CES) were set at 100, 10 and 15 respectively. During the survey scan, all the ions between a mass range of m/z 300-1250 and exceeding 25 cps were used for MS/MS analysis. Former target ions were excluded for 8 sec and the mass tolerance for TOF-MS was 50 mDa with a 100 milliseconds accumulation time. For product scan, data were acquired from 100 to 1250 m/z with an accumulation time of 75 milliseconds with a total cycle time of 3.5 sec. Product ion analysis was done under dynamic accumulation and rolling collision energy. The SWATH-MS acquisition parameters were the same except TOF masses were collected from m/z 300 to 1500 and SWATH data were acquired (m/z 400-1100) over 70 SWATH windows with a size of 10 Da each.

Chromatographic separation was achieved over 180 min gradient method at 100 $\mu\text{L}/\text{min}$ on an Acquity UPLC Peptide BEH C18 (2.1 X 150 mm, 300 Å, 1.7 μm) preceded by an Acquity VanGuard pre-column (2.1 X 5 mm, 300 Å, 1.7 μm). Mobile phase A was water containing 0.1% formic acid and mobile phase B was acetonitrile containing 0.1% formic acid. Gradient conditions used were 98% A from 0 to 5 min, 98% to 70% A from 5 to 155 min, 70% to 50% A from 155 to 160 min, 50% to 5% A from 160 to 170 min, 5% to 98% A held from 170 to 175 min. The gradient was held

at initial conditions from 175 min until the end of the run to equilibrate the column before the start of next run. The flow was diverted to waste for the first 8 minutes and last 20 minutes of the acquisition. Autosampler was maintained at 10°C, and the column was kept at 50°C. Trypsin-digested β -galactosidase peptides were injected to monitor TOF detector mass calibration.

3.3.5 Data processing

The absolute level of proteins was determined from DDA and DIA data using “Total Protein Approach”.²⁶ Homogenate samples were analyzed as previously described using Spectronaut (Biognosys, Schlieren, Switzerland). The search was performed using Pulsar algorithm for identification of peptides and proteins from DDA and DIA data. BGS Factory settings were used for the search which included specific trypsin/P digestion with a minimum of 7 amino acids, maximum of 52 amino acids and 2 missed cleavages. Fixed modifications included carbamidomethyl (C) and variable modifications included acetyl (protein N-terminus) and oxidation (M). All other options were kept as the BGS factory settings as well. Raw data files and search results are available at Japan Proteome Standard Repository (*jPOSTrepo* JPST000372, *ProteomeXchange* PXD008593), a publicly available data repository for proteomics data²⁷.

The spectral library was then used to analyze DIA data in a DIA analysis in Spectronaut. Samples were first converted to HTRMS files using the Spectronaut Converter (Biognosys, Schlieren, Switzerland). All settings were run as the BGS factory settings except “Used Biognosys’ iRT kit” was unselected and “PTM localization” was unselected. All other settings were left the same. Raw intensity after

normalization was exported and bovine serum albumin (BSA) was monitored for variability.

Raw normalized intensity from Spectronaut was further converted to picomoles of protein per gram of liver tissue (pmol/g liver) use the total protein approach (TPA) and protein yield (protein per gram of liver or PPGL) ²⁶.

$$\text{Protein} \left(\frac{\text{pmol}}{\text{mg protein}} \right) = \frac{\text{Protein intensity}}{\text{Total intensity} \times \text{MW (g/mol)}} \times 10^9$$

$$\text{Protein} \left(\frac{\text{pmol}}{\text{g liver}} \right) = \text{Protein} \left(\frac{\text{pmol}}{\text{mg protein}} \right) \times \text{protein per gram of liver (mg)}$$

3.3.6 Statistical analysis

Natural log was taken of all values for statistical tests. Student's t-test was used to compare the differences for two groups and a one-way ANOVA followed by Bonferroni multiple comparison correction three or more groups. The vendor provided functional activity was used for correlation analysis with protein concentration. The details of the assay's conditions are described elsewhere.²⁴ Spearman-correlation analysis was performed between the functional activity and protein levels. $P < 0.05$ was considered significant throughout the analysis. Prism 6 (GraphPad Inc., La Jolla, CA) and SPSS 26 (IBM Corp., Armonk, NY) were used for graphing and statistical testing, respectively.

3.4 Results

NAFLD liver samples exhibited significantly elevated levels of malondialdehyde and cholesterol (**Table 3.1**). The livers from NAFL and NASH

donors were larger in weight than the control group. The total protein yield from samples with NAFLD was marginally lower as compared to the control but was determined to be non-significant. Donors with NAFLD also showed a trend of increased body weight and body mass index.

3.4.1 NAFLD and CYP450 protein expression

The protein expression of CYP1A2 was significantly lower in NAFLD samples ($p<0.05$). In addition, there was a significant decrease in CYP3A4 in NAFL and NASH condition in comparison to control ($p<0.05$) (figure 2). Additionally, CYP2E1 and CYP2A6 were found to be downregulated in NAFL conditions but not NASH conditions ($p<0.05$). We observed no difference in the protein levels of CYP2D6, CYP2C8, and CYP2C9 among different study groups. A trend of lower expression was observed for CYP2B6; however, the reduction was not significantly different from control samples. These data can be seen in **Table 3.2**, **Figure 3.2** and **Figure 3.3**.

3.4.2 Diabetes and CYP450 protein expression

The only CYP to show any significant difference was CYP3A5, which was downregulated in diabetes. CYP3A4 showed downregulation as well but was not found to be statistically significant. There was no significant difference found in the remainder of the CYPs. These data can be seen in **Table 3.5**, **Figure 3.7** and **Figure 3.8**.

3.4.3 NAFLD and CYP450 enzymatic activity

Like protein abundance, CYP1A2 phenacetin O-dealkylation activity was significantly lower in NAFL and NASH (figure 3). CYP3A4 mediated testosterone

hydroxylation was moderately decreased in NAFLD group. CYP3A4 was tested in samples containing CYP3A5*3/*3 as it is the nonfunctioning form of CYP3A5. The activity of other CYP450 enzymes remained mostly similar between control and disease state.

3.4.4 Diabetes and CYP450 enzymatic activity

None of the CYP450 enzymes showed significant alteration due to diabetes. CYP3A4 was again tested in samples containing CYP3A5*3/*3.

3.4.5 Correlation of CYP450 enzymatic activity and protein expression

Non-parametric Spearman correlation analysis was performed between the determined protein levels (expressed as pmol/mg protein) and vendor provided enzyme activity (**Figure 3.1**). A significant correlation with varying strength was observed for all the 9 CYP450 enzymes. CYP1A2, 2A6, 2B6, 2D6, and 3A4 exhibited strong correlation ($r > 0.7$) while it was moderate ($r = 0.5-0.7$) for CYP2C8, 2C9 and 2E1. Interestingly, the association between CYP2C19 protein and activity was weakest ($r = 0.395$) among all the proteins.

3.4.6 NAFLD and phase II drug metabolizing enzymes

UGT1A3 was found to be significantly downregulated in NASH ($p < 0.05$) and downregulated in NAFL but not significant. The rest of the UGTs were found to have no significant differences (figure 5). None of the SULT enzymes identified showed any significant difference in either group. Data can be observed (**Table 3.3**, **Figure 3.4** and **Figure 3.5**).

3.4.7 Diabetes and phase II drug metabolizing enzymes

SULT2A1 was found to be downregulated in diabetic livers ($p<0.05$) while SULT1A1 was found to be very close to significant ($p=0.60$). SULT1B1, SULT1C2 and SULT1A2 remained unchanged between the three groups. UGT1A3 was found to be downregulated in diabetes ($p<0.05$) and UGT2B4 had a non-significant downregulation. The rest of the UGTs remained unchanged (**Table 3.6**, **Figure 3.9** and **Figure 3.10**).

3.4.8 NAFLD and drug transporters

Bile salt export pump (BSEP) was seen to be downregulated in NASH conditions ($p<0.05$). Multidrug resistance-associated protein 3 (MRP2) showed downregulation across the disease but did not end up being statistically significant. MDR1 was found to be significantly upregulated in NASH ($p<0.05$) (**Table 3.4** and **Figure 3.6**).

3.4.9 Diabetes and drug transporters

None of the efflux transporters showed any significant difference across the disease. OATP1B3 was found to have significant downregulation ($p<0.01$) in diabetes while OATP1B1 showed a trend of upregulation but was not significant. OATP2B1 was also found to be significantly downregulated but not significant. OCT3 showed significant downregulation ($p<0.05$) while OCT1, OAT2 and OAT7 all remained the same across control and diabetic samples (**Table 3.7** and **Figure 3.11**).

3.4.10 Effect of phenytoin

When livers containing phenytoin were removed from the data set, CYP3A4 was no longer found to be significantly downregulated in either the NAFL or NASH groups. Values returned to similar levels found in the control samples. The same

thing was seen in CYP3A4 regarding diabetes. This can be seen in **Table 3.8** and **Figure 3.12**.

3.5 Discussion

NAFLD and diabetes are often accompanied by other comorbidities that may confound the observations and could partially explain the wide discrepancy in the literature. Therefore, the results and discussion of this work are mainly restricted to the effect of NAFLD and diabetes specifically on the expression of DMEs without any focus on underlying conditions.

Studies in human liver microsomes have shown both downregulation of CYP3A4 activity with the progression of NAFLD and severity of steatosis while other studies show that there is no significant difference occurring.^{9,10} Similar reports of downregulation have been previously reported in microsomes from diabetic liver samples.¹⁵ A recent study from our group found almost a 2-fold decrease of CYP3A4 protein expression and activity in liver microsomes from NAFLD donors as well as donors who had both NAFLD and diabetes.¹⁶ Studies in microsomal fractions from diabetic livers found significant downregulation of CYP3A4 protein and activity but not mRNA.¹⁵ One confounder that could explain the discrepancy that is currently being reported is the presence of an inducer of CYP3A4, like phenytoin. As we have previously reported, phenytoin significantly increases CYP3A4 protein, mRNA and activity and could lead to masking the true effect of NAFLD. Our data suggest that initially progression of NAFLD significantly lowers the protein and activity of CYP3A4, but once the livers containing phenytoin were removed, the CYP3A4 related levels returned to the healthy baseline (**Table 3.8** and **Figure 3.12**).

This study found significant downregulation of CYP1A2 in both the NAFL and NASH livers. The same downregulation of CYP1A2 protein and activity has been reported in human liver microsomes and hepatocytes from fatty liver grafts.^{9,28} It has also been found that CYP1A2 is decreased by about 50% in mice with obstructive sleep apnea (OSA).²⁹ OSA has been found to occur in 25%-30% of obese patients as well as having an association with NAFLD.³⁰ Patients with NAFLD and OSA could explain a portion downregulation that is seen in CYP1A2 in this study.

CYP2E1 mediated biotransformation reactions in the liver generate a significant amount of reactive oxygen species which further promotes the oxidative stress in NAFLD.³¹ Groups have reported that CYP2E1 is induced in diabetes where we have shown no effect either in protein expression or enzymatic activity.^{32,33} Groups have also shown similar results to ours in the suppression of CYP2E1 in NAFL and NASH.^{9,34,35} CYP2E1 mRNA and protein expression were found to be lower in another study in livers from NAFLD patients whereas activity was unaltered.⁹

CYP2C8 protein expression was downregulated in NASH-associated liver biopsy while CYP2C9 levels were unchanged compared to control.³⁶ Other studies in human tissue observed no differences in the expression of either isoform.³⁷ No studies have shown the effect of diabetes on CYP2C8 or CYP2C9 protein expression or activity. CYP2C8 activity was similar in different stages of NAFLD and control livers whereas CYP2C9 activity was reported to be higher.⁹ A recent study in a 3-dimensional hepatocyte culture model of NAFLD, showed CYP3A4 activity was found to be upregulated while CYP2C9 decreased.³⁸ No studies could be found showing any dysregulation in CYP2C8 or CYP2C9 in regards to diabetes as our data

also show. CYP2C18 and CYP2C19 were determined to have no meaningful difference in NAFLD or diabetes and there is no reported literature reporting any affect either.

CYP2B6 is one of most polymorphic enzymes, which accounts for significant variability in its expression and activity.³⁹ Similar to CYP2B6, CYP2D6 is another highly polymorphic enzyme responsible with varying phenotypes and degree of metabolism of its substrates.⁴⁰ Fisher et al. observed elevated CYP2B6 mRNA without any difference in protein and activity during the progression of NAFLD.⁹ Current literature reports no significant alterations in CYP2D6 expression and activity in human liver.⁹ We found no significant alteration in CYP2B6 or CYP2D6 protein expression in our NAFLD or diabetic liver samples but both cases showed a trend toward downregulation of CYP2B6 protein expression but not enzymatic function.

CYP20A1 was found to remain the same in NAFL when compared to healthy but downregulated in NASH ($p < 0.05$). There is not much literature reporting the function of CYP20A1 in regard to metabolism in the liver and it has the designation of being an orphan CYP.⁴¹ Additional orphan CYPs, CYP4F11, CYP4F22 and CYP4V2 were found in the dataset but showed no significant difference in either stage of the disease. CYP4F11 was also found to have no difference in NASH patients in a study performed by Kakehasi et al.³⁶ CYP20A1 and CYP4F11 were shown to be significantly downregulated in diabetes and CYP4V2 showed the same trend with no significance but like with NAFLD, there is no current literature on the effect of diabetes.

Three CYPs, CYP2J2, CYP4A11 and CYP4F12, were all identified to be related to fatty acid metabolism⁴¹. We found that CYP2J2 was reduced in the NASH group but the NAFL group remained similar to the healthy group although the reduction was not significant. CYP2J2 has also been found to be responsible for the metabolism of arachidonic acid into epoxyeicosatrienoic acids, which have a cardioprotective effect.⁴² The downward trend of CYP2J2 and the loss of these cardioprotective fatty acids may exacerbate the cardiovascular affects associated with NAFLD. Both CYP4A11 and CYP4F12 showed no difference across NAFLD or diabetes and CYP2J2 showed no difference in diabetes as well. This conflicts with gene expression data that show overexpression of CYP4A11 in human NAFLD livers.^{43,44}

CYP27A1, CYP51A1, CYP8B1 and CYP7B1 are all related to sterol metabolism⁴¹. CYP8B1 mRNA was found to be downregulated in NASH samples while CYP7B1 mRNA has found to be increased in NASH livers.⁴⁵ Our data suggest that there is no significant difference in the protein expression of CYP8B1 but CYP8B1 trended downwards in NASH conditions but was not statistically significant. Both enzymes are known to be involved with the conversion of cholesterol into bile acids. There are no literature reports on the effect of NAFLD or diabetes on CYP51A1 or CYP27A1 in humans. The downregulation of CYP27A1 and CYP7B1 could be a factor to the increased cholesterol levels noted during the progression of NAFLD.

CYP4F2 saw trends of downregulation in NAFL and both CYP4F2 and CYP4F3 saw similar trends in diabetes but none were significant. These enzymes are involved with eicosanoid metabolism and along with CYP2J2, CYP4A11 and

CYP4F11 are involved with the metabolism of arachidonic acid and other polyunsaturated fatty acids.^{41,42,46,47}

While the effect of NAFL on CYP450 enzymes has been extensively studied, only limited information on the regulation of UGT enzymes is available in human.⁴⁸ Hardwick and colleagues identified significant alterations in mRNA of different UGT isoforms, but the acetaminophen glucuronidation activity was unchanged.⁴⁸ UGT2B17 and UGT2B7 were identified to have similar protein levels between control and NASH biopsy samples.³⁶ Yalcin et al. also found that SULT1A1 protein expression and activity were significantly decreased in steatosis and diabetes.⁴⁹ Others have reported downregulation of human SULT1A2 in NAFLD.^{50,51} Similarly, we found decreased SULT1A1 protein decreased in NAFL and NASH but it was not significant.⁴⁸ SULT1A2 showed non-significant decreases in NAFL and was significantly downregulated in diabetes ($p < 0.05$). Multiple UGTs have been shown to glucuronidate fatty acids like arachidonic and linoleic acid. Specifically, in the UGT1A family, UGT1A1, UGT1A3, UGT1A4 and UGT1A9 are seen create many of the glucuronide metabolites.⁵² Our data show that there is significant downregulation in UGT1A3 in NASH and diabetes that diabetes may exacerbate the effect of NAFLD by decreasing the rate at which certain fatty acids can be cleared from the body. A similar trend was seen in UGT1A4, but it was not significant. Similar to reports for CYP450 enzymes, regulation of UGTs and SULTs is as heterogeneous; however, there is a scarcity of data for the latter.

In addition to DMEs, xenobiotic transporters play a large role in the elimination of a drug and are subject to dysregulation due to NAFLD and diabetes.

There is limited literature available assessing the effect of diabetes on human liver transporters. The majority of papers are presenting data on BCRP, which was not quantified in our study.⁵³ In regard to NAFLD, our results show that there is a significant downregulation in BSEP in the NASH group. It has been found that there is bile acid dysregulation in NAFLD patients and samples, which could be attributed to the downregulation of BSEP and the buildup of bile acids in the liver.⁵⁴ It has also been shown that mRNA of BSEP significantly decreases as NAFLD progresses in both male and female populations.⁵⁵ We found MDR1 or P-gp to be significantly upregulated in NASH. This matches data published by Hardwick et al. This could be a result of NRF2 induction due to oxidative stress conditions associated with NAFLD.⁵⁶ In regards to MRP3 where we see no significant difference, groups have shown induction in NAFL and NASH designations while other groups show increased protein in only NASH samples.⁵⁵⁻⁵⁷ OATP1B3 was significantly upregulated in diabetes but not NAFLD and the other OATP family proteins both saw non-significant differences in diabetes and NAFLD. The 8 other clinically relevant transporters that were measured in our samples showed little to no difference across the NAFLD and diabetes grouping. It has been reported that the majority of basolateral efflux transporters are upregulated in NASH while uptake transporters are downregulated.⁵⁶ Recently, numerous transporters were shown to be changing mainly from steatosis to NASH livers. There was a significant decrease found in OATP1B1, OATP1B3, OATP2B1, OAT2 and NTCP while OAT7, MDR1 and MRP3 showed significant increase.⁵⁷ As NAFLD is a multifaceted disease with numerous variables, it is important to look at each of the individual variables to determine their impact. This is

difficult, though, as it is generally not possible to procure samples that are specific enough in their pathology to perform a valid analysis. Another component that can cause variability in reported values is the method of expressing the transporter units. Some groups report as pmol/mg of membrane protein while other factor protein extraction into the equation and use pmol/g of liver. As the extraction of protein can differ from liver to liver based on the amount of fat or fibrotic tissue present, this can add additional differences to final protein expression values.

One potential explanation for such a wide discrepancy in literature can be attributed to the interindividual variability, and sample size used to derive the inferences. The availability of human liver tissue for studying the hepatic drug metabolizing enzymes remains a challenge for researchers due to ethical consideration and the availability of tissue with adequate clinical information. Additionally, the methods of reporting expression, especially with protein, can differ from group to group. The differences that can occur expressing in terms of mg of membrane protein, mg of total protein or g of liver can cause high variability. Misuse of classification systems and incorrect characterization of study samples may have also lead to different conclusions.⁵⁸ The sampling of human liver tissue is critical in the identification of the perturbations in the expression of proteins. Histopathological differences were found in liver biopsies from the right and left lobes of bariatric patients.⁵⁹ Our knowledge of the drugs that the donors were taking at the time of death is limited only to the information passed on by the vendor. These possible medications have been shown by our group to significantly alter the metabolic profile of CYP3A4 and could, therefore, be suppressing the true effect of a disease state. Furthermore,

there were no data available on the duration of hospitalization of donors before the organs were harvested.

To summarize, our research shows that CYP1A2 and BSEP are highly affected by NAFLD. CYP3A4 initially appears to be affected but once phenytoin is accounted for, these differences become non-significant. The remainder of significantly altered CYPs seem to be mainly related to fatty acid synthesis and sterol metabolism. Apart from CYP1A2 and CYP3A4, the majority of CYPs related to drug metabolism seem to be unaffected by NAFLD. A lack of adverse clinical reports also indicates that most drugs appear to be well-tolerated by these patients despite the differences in the expression of drug metabolism enzymes. Additionally, glucuronidation by UGTs seems to be affected by both NAFLD and diabetes leading to a possible exacerbated effect, especially related to the formation of bile acids. It appears that majority of enzymes that are affected by NAFLD are related to the fatty acid metabolic or bile acid synthesis pathways rather than the drug metabolic pathway. Multiple factors in NAFLD and diabetes may affect the expression and activity of DMEs, and a comprehensive study needs to be conducted to answer some of the questions around the discrepancies observed in the literature.

3.6 References

1. Vuppalanchi R, Chalasani N. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: Selected practical issues in their evaluation and management. *Hepatology*. 2009;49(1):306-317.
2. Younossi ZM, Blissett D, Blissett R, et al. The economic and clinical burden of nonalcoholic fatty liver disease in the United States and Europe. *Hepatology*. 2016;64(5):1577-1586.
3. Le MH, Devaki P, Ha NB, et al. Prevalence of non-alcoholic fatty liver disease and risk factors for advanced fibrosis and mortality in the United States. *PLoS One*. 2017;12(3):e0173499.
4. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*. 2016;64(1):73-84.
5. Brunt EM, Tiniakos DG. Histopathology of nonalcoholic fatty liver disease. *World J Gastroenterol*. 2010;16(42):5286-5296.
6. Younossi Z, Henry L. Contribution of Alcoholic and Nonalcoholic Fatty Liver Disease to the Burden of Liver-Related Morbidity and Mortality. *Gastroenterology*. 2016;150(8):1778-1785.
7. Prasad B, Johnson K, Xiao G, et al. Protein Abundance of Phase I and II Drug Metabolizing Enzymes in Alcoholic and Hepatitis C Cirrhosis Livers. *The FASEB Journal*. 2017;31(1 Supplement):668.617.
8. Cobbina E, Akhlaghi F. Non-alcoholic fatty liver disease (NAFLD) - pathogenesis, classification, and effect on drug metabolizing enzymes and transporters. *Drug Metab Rev*. 2017;49(2):197-211.
9. Fisher CD, Lickteig AJ, Augustine LM, et al. Hepatic cytochrome P450 enzyme alterations in humans with progressive stages of nonalcoholic fatty liver disease. *Drug Metab Dispos*. 2009;37(10):2087-2094.
10. Kolwankar D, Vuppalanchi R, Ethell B, et al. Association between nonalcoholic hepatic steatosis and hepatic cytochrome P-450 3A activity. *Clin Gastroenterol Hepatol*. 2007;5(3):388-393.
11. Niemela O, Parkkila S, Juvonen RO, Viitala K, Gelboin HV, Pasanen M. Cytochromes P450 2A6, 2E1, and 3A and production of protein-aldehyde adducts in the liver of patients with alcoholic and non-alcoholic liver diseases. *J Hepatol*. 2000;33(6):893-901.
12. Woolsey SJ, Mansell SE, Kim RB, Tirona RG, Beaton MD. CYP3A Activity and Expression in Nonalcoholic Fatty Liver Disease. *Drug Metab Dispos*. 2015;43(10):1484-1490.
13. Anstee QM, McPherson S, Day CP. How big a problem is non-alcoholic fatty liver disease? *BMJ*. 2011;343:d3897.
14. European Association for the Study of the L, European Association for the Study of D, European Association for the Study of O. EASL-EASD-EASO

- Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol.* 2016;64(6):1388-1402.
15. Dostalek M, Court MH, Yan B, Akhlaghi F. Significantly reduced cytochrome P450 3A4 expression and activity in liver from humans with diabetes mellitus. *Br J Pharmacol.* 2011;163(5):937-947.
 16. Jamwal R, de la Monte SM, Ogasawara K, Adusumalli S, Barlock BB, Akhlaghi F. Nonalcoholic Fatty Liver Disease and Diabetes Are Associated with Decreased CYP3A4 Protein Expression and Activity in Human Liver. *Mol Pharm.* 2018;15(7):2621-2632.
 17. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther.* 2013;138(1):103-141.
 18. Fallon JK, Smith PC, Xia CQ, Kim MS. Quantification of Four Efflux Drug Transporters in Liver and Kidney Across Species Using Targeted Quantitative Proteomics by Isotope Dilution NanoLC-MS/MS. *Pharm Res.* 2016;33(9):2280-2288.
 19. Prasad B, Unadkat JD. Optimized approaches for quantification of drug transporters in tissues and cells by MRM proteomics. *AAPS J.* 2014;16(4):634-648.
 20. Wong JW, Cagney G. An overview of label-free quantitation methods in proteomics by mass spectrometry. *Methods Mol Biol.* 2010;604:273-283.
 21. Al Feteisi H, Achour B, Barber J, Rostami-Hodjegan A. Choice of LC-MS methods for the absolute quantification of drug-metabolizing enzymes and transporters in human tissue: a comparative cost analysis. *AAPS J.* 2015;17(2):438-446.
 22. Prasad B, Achour B, Artursson P, et al. Toward a Consensus on Applying Quantitative Liquid Chromatography-Tandem Mass Spectrometry Proteomics in Translational Pharmacology Research: A White Paper. *Clin Pharmacol Ther.* 2019;106(3):525-543.
 23. Xu M, Saxena N, Vrana M, et al. A Targeted LC-MS/MS Proteomics-Based Strategy to Characterize In Vitro Models Used in Drug Metabolism and Transport Studies. *Anal Chem.* 2018.
 24. Jamwal R, Barlock BJ, Adusumalli S, Ogasawara K, Simons BL, Akhlaghi F. Multiplex and Label-Free Relative Quantification Approach for Studying Protein Abundance of Drug Metabolizing Enzymes in Human Liver Microsomes Using SWATH-MS. *J Proteome Res.* 2017;16(11):4134-4143.
 25. Prasad B, Evers R, Gupta A, et al. Interindividual variability in hepatic organic anion-transporting polypeptides and P-glycoprotein (ABCB1) protein expression: quantification by liquid chromatography tandem mass spectroscopy and influence of genotype, age, and sex. *Drug Metab Dispos.* 2014;42(1):78-88.
 26. Wisniewski JR, Rakus D. Multi-enzyme digestion FASP and the 'Total Protein Approach'-based absolute quantification of the Escherichia coli proteome. *J Proteomics.* 2014;109:322-331.
 27. Okuda S, Watanabe Y, Moriya Y, et al. jPOSTrepo: an international standard data repository for proteomes. *Nucleic Acids Res.* 2017;45(D1):D1107-D1111.

28. Donato MT, Lahoz A, Jimenez N, et al. Potential impact of steatosis on cytochrome P450 enzymes of human hepatocytes isolated from fatty liver grafts. *Drug Metab Dispos.* 2006;34(9):1556-1562.
29. Zhang XB, Zeng YM, Chen XY, Zhang YX, Ding JZ, Xue C. Decreased expression of hepatic cytochrome P450 1A2 (CYP1A2) in a chronic intermittent hypoxia mouse model. *J Thorac Dis.* 2018;10(2):825-834.
30. Ahmed MH, Byrne CD. Obstructive sleep apnea syndrome and fatty liver: association or causal link? *World J Gastroenterol.* 2010;16(34):4243-4252.
31. Aubert J, Begriche K, Knockaert L, Robin MA, Fromenty B. Increased expression of cytochrome P450 2E1 in nonalcoholic fatty liver disease: mechanisms and pathophysiological role. *Clin Res Hepatol Gastroenterol.* 2011;35(10):630-637.
32. Wang Z, Hall SD, Maya JF, Li L, Asghar A, Gorski JC. Diabetes mellitus increases the in vivo activity of cytochrome P450 2E1 in humans. *Br J Clin Pharmacol.* 2003;55(1):77-85.
33. Yap CG, Zaini A, Othman I. Targeted CYP2E1 quantification and its correlation to currently acceptable clinical biochemical indices. *J Biol Res (Thessalon).* 2016;23:15.
34. Leung TM, Nieto N. CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease. *J Hepatol.* 2013;58(2):395-398.
35. Nakamuta M, Kohjima M, Morizono S, et al. Evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease. *Int J Mol Med.* 2005;16(4):631-635.
36. Kakehashi A, Stefanov VE, Ishii N, et al. Proteome Characteristics of Non-Alcoholic Steatohepatitis Liver Tissue and Associated Hepatocellular Carcinomas. *Int J Mol Sci.* 2017;18(2).
37. Chtioui H, Semela D, Ledermann M, Zimmermann A, Dufour JF. Expression and activity of the cytochrome P450 2E1 in patients with nonalcoholic steatosis and steatohepatitis. *Liver Int.* 2007;27(6):764-771.
38. Kostrzewski T, Cornforth T, Snow SA, et al. Three-dimensional perfused human in vitro model of non-alcoholic fatty liver disease. *World J Gastroenterol.* 2017;23(2):204-215.
39. Zanger UM, Klein K. Pharmacogenetics of cytochrome P450 2B6 (CYP2B6): advances on polymorphisms, mechanisms, and clinical relevance. *Front Genet.* 2013;4:24.
40. Zhou SF. Polymorphism of human cytochrome P450 2D6 and its clinical significance: Part I. *Clin Pharmacokinet.* 2009;48(11):689-723.
41. Guengerich FP, Cheng Q. Orphans in the human cytochrome P450 superfamily: approaches to discovering functions and relevance in pharmacology. *Pharmacol Rev.* 2011;63(3):684-699.
42. Solanki M, Pointon A, Jones B, Herbert K. Cytochrome P450 2J2: Potential Role in Drug Metabolism and Cardiotoxicity. *Drug Metab Dispos.* 2018;46(8):1053-1065.
43. Kohjima M, Enjoji M, Higuchi N, et al. Re-evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease. *Int J Mol Med.* 2007;20(3):351-358.

44. Nakamuta M, Kohjima M, Higuchi N, et al. The significance of differences in fatty acid metabolism between obese and non-obese patients with non-alcoholic fatty liver disease. *Int J Mol Med*. 2008;22(5):663-667.
45. Lake AD, Novak P, Shipkova P, et al. Decreased hepatotoxic bile acid composition and altered synthesis in progressive human nonalcoholic fatty liver disease. *Toxicol Appl Pharmacol*. 2013;268(2):132-140.
46. Powell PK, Wolf I, Jin R, Lasker JM. Metabolism of arachidonic acid to 20-hydroxy-5,8,11, 14-eicosatetraenoic acid by P450 enzymes in human liver: involvement of CYP4F2 and CYP4A11. *J Pharmacol Exp Ther*. 1998;285(3):1327-1336.
47. Hardwick JP. Cytochrome P450 omega hydroxylase (CYP4) function in fatty acid metabolism and metabolic diseases. *Biochem Pharmacol*. 2008;75(12):2263-2275.
48. Hardwick RN, Ferreira DW, More VR, et al. Altered UDP-glucuronosyltransferase and sulfotransferase expression and function during progressive stages of human nonalcoholic fatty liver disease. *Drug Metab Dispos*. 2013;41(3):554-561.
49. Yalcin EB, More V, Neira KL, et al. Downregulation of sulfotransferase expression and activity in diseased human livers. *Drug Metab Dispos*. 2013;41(9):1642-1650.
50. Stepanova M, Hossain N, Afendy A, et al. Hepatic gene expression of Caucasian and African-American patients with obesity-related non-alcoholic fatty liver disease. *Obes Surg*. 2010;20(5):640-650.
51. Younossi ZM, Baranova A, Ziegler K, et al. A genomic and proteomic study of the spectrum of nonalcoholic fatty liver disease. *Hepatology*. 2005;42(3):665-674.
52. Turgeon D, Chouinard S, Belanger P, et al. Glucuronidation of arachidonic and linoleic acid metabolites by human UDP-glucuronosyltransferases. *J Lipid Res*. 2003;44(6):1182-1191.
53. Chen F, Li DY, Zhang B, et al. Alterations of drug-metabolizing enzymes and transporters under diabetic conditions: what is the potential clinical significance? *Drug Metab Rev*. 2018;50(3):369-397.
54. Kalhan SC, Guo L, Edmison J, et al. Plasma metabolomic profile in nonalcoholic fatty liver disease. *Metabolism*. 2011;60(3):404-413.
55. Okushin K, Tsutsumi T, Enooku K, et al. The intrahepatic expression levels of bile acid transporters are inversely correlated with the histological progression of nonalcoholic fatty liver disease. *J Gastroenterol*. 2016;51(8):808-818.
56. Hardwick RN, Fisher CD, Canet MJ, Scheffer GL, Cherrington NJ. Variations in ATP-binding cassette transporter regulation during the progression of human nonalcoholic fatty liver disease. *Drug Metab Dispos*. 2011;39(12):2395-2402.
57. Vildhede A, Kimoto E, Pelis RM, Rodrigues AD, Varma MVS. Quantitative Proteomics and Mechanistic Modeling of Transporter-mediated Disposition in Non-Alcoholic Fatty Liver Disease. *Clin Pharmacol Ther*. 2019.
58. Brunt EM, Kleiner DE, Behling C, et al. Misuse of scoring systems. *Hepatology*. 2011;54(1):369-370; author reply 370-361.

59. Merriman RB, Ferrell LD, Patti MG, et al. Correlation of paired liver biopsies in morbidly obese patients with suspected nonalcoholic fatty liver disease. *Hepatology*. 2006;44(4):874-880.

3.7 Tables

	Control	NAFL	NASH
N	42	34	30
Age (y)	46.5 (41.9 - 51.6)	52.8 (49.5 - 56.4)	50.8 (47.3 - 54.5)
Female	16	19	15
Male	26	15	15
Caucasian	33	20	28
Afro-American	8	1	1
Hispanic	1	3	1
Body weight (kg)	83.5 (76.2 - 91.5)	89.8 (80.6 - 100.1)	92.7 (86 - 99.9)
BMI (kg/m²)	29.3 (26.5 - 32.3)	31.2 (28 - 34.8)	32.2 (29.9 - 34.7)
Liver weight (g)	1482.4 (1374.9 - 1598.3)	1798.5 (1603.2 - 2017.6) *	1839 (1678.4 - 2014.9) **
Malondialdehyde (nmol/mg protein)	0.6 (0.5 - 0.7)	0.9 (0.8 - 1.2) **	1.2 (1 - 1.6) **
Cholesterol (µg/g liver)	14.2 (12.4 - 16.2)	18.7 (16 - 21.9) **	20.8 (17.9 - 24.1) **
Triacyl glycerides (mg/g liver)	12.18 (10.01 - 14.82)	15.8 (12.01 - 20.79)	14.44 (12.1 - 17.23)
PPGL (mg/g)	83.1 (77.4 - 89.3)	78.9 (71.8 - 86.8)	79.8 (72.1 - 88.3)
Non-diabetic	21	19	13
Diabetic	21	15	17

Table 3.1 Demographic characteristics of control, NAFL and NASH groups.

BMI: Body-mass index; PPGL: Protein per gram liver. Adjusted p-value from Bonferroni comparison correction. * and ** represent p-value <0.05 and <0.01, respectively.

Picomole of protein per gram of liver					
Enzyme	Healthy	NAFL	NASH	Healthy vs NAFL ¹	Health vs NASH ¹
CYP1A2	303.0 (179.5-421.3)	116.0 (66.41-202.5)	128.4 (73.99-222.8)	0.013	0.040
CYP20A1	49.46 (40.66-60.17)	41.36 (33.25-51.44)	33.67 (25.46-44.52)	0.607	0.039
CYP27A1	264.9 (189.0-371.2)	300.0 (200.3-449.3)	262.4 (164.2-419.5)	1	0.880
CYP2A6	618.8 (413.2-926.6)	315.5 (183.4-542.9)	432.1 (294.9-633.1)	0.041	0.488
CYP2B6	102.9 (65.61-161.5)	46.57 (26.62-81.48)	62.91 (39.87-99.28)	0.051	0.429
CYP2C8	350.2 (227.8-538.3)	205.8 (128.3-330.0)	309.6 (198.7-482.6)	0.129	1
CYP2C9	908.2 (641.2-1287)	567.5 (365.6-880.8)	629.4 (366.4-1081)	0.285	0.604
CYP2C18	25.19 (19.50-32.53)	23.62 (16.77-33.26)	27.74 (19.67-39.14)	1	1
CYP2C19	32.93 (23.89-45.38)	29.39 (17.98-48.06)	42.36 (27.45-65.39)	1	1
CYP2D6	148.9 (106.0-209.1)	105.5 (63.13-176.2)	109.1 (68.39-174.0)	0.674	0.863
CYP2E1	853.5 (661.7-1102)	420.2 (287.7-613.7)	557.4 (337.4-920.9)	0.012	0.253
CYP2J2	43.60 (34.66-54.83)	40.14 (29.48-54.66)	30.38 (22.20-41.59)	1	0.165
CYP3A4	447.6 (300.4-667.0)	230.3 (139.3-380.7)	254.7 (169.8-381.9)	0.019	0.040
CYP3A5	56.63 (35.54-90.23)	32.55 (22.79-46.48)	39.60 (25.42-61.71)	0.200	0.758
CYP3A7	18.34 (10.53-31.95)	14.92 (8.564-26.00)	7.846 (4.338-14.19)	1	0.103
CYP4A11	403.0 (300.2-540.9)	367.0 (267.6-503.4)	287.8 (169.5-488.8)	1	0.460
CYP4A22	58.75 (40.66-84.89)	64.75 (49.38-84.90)	61.47 (42.09-89.76)	1	1
CYP4F2	194.2 (138.0-273.4)	118.7 (76.00-185.5)	207.7 (143.9-299.7)	0.083	0.131
CYP4F3	231.6 (174.4-307.5)	223.1 (161.7-307.9)	260.8 (178.6-380.8)	1	1
CYP4F11	125.3 (98.80-159.0)	106.9 (79.94-143.1)	108.1 (80.63-145.0)	1	1
CYP4F12	19.35 (16.38-22.85)	17.40 (13.24-22.87)	16.61 (12.45-22.16)	1	0.966
CYP4V2	42.52 (34.55-52.33)	41.87 (30.83-56.88)	36.76 (24.20-55.84)	1	1

CYP51A1	48.28 (33.96-68.65)	47.30 (34.44-64.95)	47.22 (31.01-71.92)	1	1
CYP7B1	17.96 (13.42-24.03)	18.26 (13.98-23.85)	11.40 (7.499-17.33)	1	0.131
CYP8B1	254.3 (162.9-397.0)	368.8 (307.2-442.8)	322.3 (234.8-443.2)	0.360	1

Table 3.2 Effect of NAFLD on protein expression of various CYP450 proteins.
Geometric mean and 95% confidence interval of protein expression in pmol per g of liver tissue. ¹P-value Bonferroni multiple comparison correction after ANOVA

Picomole of protein per gram of liver					
Enzyme	Healthy	NAFL	NASH	Healthy vs NAFL ¹	Healthy vs NASH ¹
SULT1A1	883.8 (624.3-1251)	579.1 (380.2-882.2)	539.3 (298.9-973.2)	0.394	0.277
SULT1A2	70.74 (46.28-108.1)	63.55 (39.66-101.9)	89.15 (58.59-135.6)	1	1
SULT1A3	21.92 (16.69-28.78)	21.03 (13.82-31.99)	23.93 (18.05-31.71)	1	1
SULT1B1	134.9 (98.96-184.0)	131.0 (95.42-179.9)	142.1 (102.4-197.4)	1	1
SULT1C2	85.01 (51.50-140.3)	164.2 (86.99-310.0)	116.5 (62.93-215.6)	0.227	1
SULT2A1	1548 (1046-2291)	1099 (703.7-1715)	1717 (1146-2573)	0.348	1
UGT1A1	147.8 (104.4-209.2)	139.8 (93.14-209.9)	629.4 (366.4-1081)	1	1
UGT1A3	161.3 (127.0-204.8)	109.2 (76.83-155.2)	117.7 (72.53-190.9)	0.194	0.013
UGT1A4	294.6 (212.8-407.8)	169.6 (106.2-270.9)	172.9 (108.6-275.4)	0.151	0.203
UGT1A6	170.2 (121.5-238.3)	158.6 (104.8-239.9)	240.2 (165.9-347.8)	1	0.781
UGT1A9	592.2 (422.5-830.1)	531.2 (336.4-838.9)	477.1 (268.1-848.7)	1	1
UGT2A3	50.91 (40.70-63.66)	59.72 (44.03-81.00)	62.59 (43.31-90.46)	1	0.809
UGT2B4	559.0 (435.1-718.2)	463.1 (353.2-607.4)	395.2 (270.6-577.2)	1	0.314
UGT2B7	1359 (906.8-2037)	1137 (732.0-1765)	1185 (705.3-1992)	1	1
UGT2B10	648.5 (493.1-852.8)	382.7 (243.3-602.0)	414.0 (255.1-671.6)	0.144	0.306
UGT2B15	203.4 (142.7-290.1)	251.9 (170.1-373.0)	276.8 (175.5-436.5)	1	0.744
UGT2B17	896.5 (607.6-1323)	951.9 (598.2-1514)	1104 (715.9-1703)	1	1
UGT3A1	17.81 (12.65-25.07)	12.01 (7.297-19.77)	16.80 (8.865-31.83)	0.811	1

Table 3.3 Effect of NAFLD on protein expression of various phase II metabolic proteins. Geometric mean and 95% confidence interval of protein expression in pmol per g of liver tissue. ¹ P-value Bonferroni multiple comparison correction after ANOVA

Picomole of protein per gram of liver					
Enzyme	Healthy	NAFL	NASH	Healthy vs NAFL ¹	Healthy vs NASH ¹
MDR1	25.89 (21.31-31.45)	24.51 (17.77-33.80)	39.41 (32.34-48.03)	1	0.043
MDR3	26.68 (21.41-33.25)	19.52 (16.22-23.50)	24.96 (17.18-36.28)	1	1
BSEP	22.87 (19.80-26.42)	27.83 (22.08-35.07)	18.20 (15.42-21.47)	0.345	0.020
MRP2	14.13 (11.16-17.90)	13.09 (10.07-17.01)	14.33 (10.70-19.18)	1	1
MRP3	11.79 (9.726-14.29)	10.09 (7.703-13.21)	8.260 (5.822-11.72)	1	0.191
MRP6	29.04 (23.21-36.34)	27.42 (20.78-36.17)	30.27 (23.00-39.85)	1	1
OCT1	79.57 (53.77-117.8)	102.9 (73.75-143.6)	101.8 (64.15-161.5)	0.980	1
OCT3	18.66 (14.79-23.54)	21.97 (17.62-27.38)	22.40 (17.01-29.50)	0.975	0.863
OAT2	91.46 (67.19-124.5)	73.03 (49.36-108.1)	76.32 (51.08-114.0)	1	1
OAT7	53.62 (40.52-70.97)	55.09 (39.56-76.72)	61.27 (40.34-93.06)	1	1
OATP1B1	68.27 (56.50-82.49)	65.58 (50.28-85.54)	54.19 (38.13-77.01)	1	0.608
OATP1B3	67.65 (53.50-85.54)	49.27 (33.64-72.17)	56.15 (37.82-83.35)	0.451	1
OATP2B1	28.16 (21.79-36.39)	26.87 (19.43-37.16)	30.97 (23.38-41.01)	1	1

Table 3.4 Effect of NAFLD on protein expression of various transporter proteins. Geometric mean and 95% confidence interval of protein expression in pmol per g of liver tissue. ¹P-value Bonferroni multiple comparison correction after ANOVA

Picomole of protein per gram of liver			
Enzyme	Non-Diabetic	Diabetic	Student's t test p-value ¹
CYP1A2	198.9 (133.6-296.1)	139.5 (92.22-211.0)	0.219
CYP20A1	45.52 (38.67-53.59)	36.73 (29.84-45.20)	0.124
CYP27A1	292.1 (216.3-394.6)	258.5 (191.9-348.1)	0.599
CYP2A6	483.8 (339.4-689.7)	388.6 (268.6-562.1)	0.506
CYP2B6	85.44 (60.41-120.9)	55.89 (36.62-85.27)	0.126
CYP2C8	348.8 (252.4-482.1)	241.1 (165.9-350.3)	0.197
CYP2C9	737.0 (530.4-1024)	652.1 (461.1-922.2)	0.649
CYP2C18	28.01 (23.3-33.57)	22.69 (17.18-29.97)	0.226
CYP2C19	37.96 (28.46-50.63)	32.49 (23.51-44.91)	0.614
CYP2D6	143.9 (105.5-196.2)	110.5 (78.25-156.1)	0.272
CYP2E1	606.7 (445.9-825.7)	533.8 (397.9-716.2)	0.583
CYP2J2	35.36 (27.78-45.01)	39.05 (31.31-48.69)	0.501
CYP3A4	406.8 (296.4-558.1)	253.4 (178.8-359.2)	0.078
CYP3A5	59.14 (40.73-85.88)	34.26 (24.71-47.51)	0.029
CYP3A7	13.28 (8.262-21.36)	14.30 (9.225-22.16)	0.825
CYP4A11	361.7 (272.1-480.8)	341.1 (257.9-451.1)	0.871
CYP4A22	58.24 (44.36-76.48)	65.22 (50.35-84.49)	0.482
CYP4F2	196.2 (144.3-266.6)	150.1 (110.8-203.4)	0.313
CYP4F3	262.8 (209.2-330.1)	200.8 (152.2-264.9)	0.135
CYP4F11	117.3 (91.74-150.0)	99.43 (81.06-122.0)	0.326
CYP4F12	18.14 (15.09-21.81)	18.25 (15.13-22.03)	0.909
CYP4V2	43.38 (35.73-52.67)	39.78 (20.50-51.88)	0.663

CYP51A1	44.49 (33.81-58.52)	52.05 (39.48-68.83)	0.422
CYP7B1	16.80 (13.06-21.61)	15.25 (11.90-19.55)	0.607
CYP8B1	277.0 (204.5-375.1)	293.8 (218.8-394.5)	0.807

Table 3.5 Effect of diabetes on protein expression of various CYP450 proteins.
Geometric mean and 95% confidence interval of protein expression in pmol per g of liver tissue. ¹ P-value from student's t-test.

Enzyme	Non-Diabetic	Diabetic	Student's t-test p-value ¹
SULT1A1	879.0 (628.9-1228)	544.8 (384.2-772.6)	0.060
SULT1A2	88.78 (63.25-124.6)	65.63 (47.07-91.51)	0.207
SULT1A3	21.70 (16.68-28.24)	21.82 (17.13-27.80)	0.975
SULT1B1	117.5 (86.44-159.8)	134.6 (107.8-168.0)	0.483
SULT1C2	99.84 (67.53-147.6)	115.4 (69.51-191.7)	0.643
SULT2A1	1986 (1540-2561)	1091 (762.8-1562)	0.012
UGT1A1	148.7 (110.3-200.3)	127.0 (93.85-191.7)	0.463
UGT1A3	149.5 (121.7-183.6)	99.13 (76.83-155.2)	0.019
UGT1A4	247.8 (183.6-334.5)	196.8 (140.8-275.5)	0.300
UGT1A6	178.2 (134.0-237.1)	189.8 (142.7-252.4)	0.600
UGT1A9	567.5 (419.9-767.1)	503.3 (349.0-725.9)	0.585
UGT2A3	57.90 (47.24-70.96)	54.30 (42.54-69.31)	0.621
UGT2B4	542.4 (424.2-693.6)	403.7 (327.5-497.6)	0.065
UGT2B7	1414 (1006-1989)	1088 (784.4-1508)	0.261
UGT2B10	494.2 (356.6-684.9)	438.5 (322.5-596.2)	0.598
UGT2B15	263.8 (202.5-343.7)	227.0 (161.4-319.3)	0.456
UGT2B17	1212 (894.3-1644)	857.0 (605.0-1214)	0.130
UGT3A1	13.22 (9.019-19.36)	15.18 (10.80-21.34)	0.652

Table 3.6 Effect of diabetes on protein expression of various phase II metabolic proteins. Geometric mean and 95% confidence interval of protein expression in pmol per g of liver tissue. ¹P-value from student's t-test.

Enzyme	Non-Diabetic	Diabetic	Student's t test p-value ¹
MDR1	27.78 (23.69-32.58)	28.10 (22.47-35.14)	0.935
MDR3	24.56 (20.06-30.06)	27.69 (22.49-34.09)	0.146
BSEP	21.48 (18.56-24.87)	18.71 (16.34-21.41)	0.830
MRP2	14.13 (11.86-17.99)	12.55 (10.31-15.27)	0.285
MRP3	9.583 (7.761-11.83)	10.45 (8.618-12.68)	0.591
MRP6	29.75 (24.19-36.60)	28.31 (23.86-33.59)	0.763
OCT1	96.76 (71.16-131.6)	84.12 (61.19-115.6)	0.513
OCT3	23.75 (20.76-27.16)	17.90 (14.39-22.26)	0.028
OAT2	80.62 (60.32-107.8)	78.90 (60.79-102.4)	0.853
OAT7	47.62 (36.42-62.27)	64.08 (50.79-80.85)	0.106
OATP1B1	55.40 (44.02-69.72)	70.07 (59.13-83.04)	0.098
OATP1B3	70.50 (57.46-86.49)	44.08 (33.02-58.85)	0.010
OATP2B1	31.61 (25.56-39.11)	24.65 (19.56-31.07)	0.125

Table 3.7 Effect of diabetes on protein expression of various transporter proteins. Geometric mean and 95% confidence interval of protein expression in pmol per g of liver tissue. ¹ P-value from student's t-test.

Effect of phenytoin on CYP3A4 in NAFLD and diabetes				
Enzyme	Healthy	NAFL	NASH	ANOVA p-value
Phenytoin Free	N=29	N=26	N=17	
All Livers	N=42	N=34	N=30	
Protein (Phenytoin Free)	399.2 (244.9-650.8)	277.7 (152.8-504.7)	250.4 (159.5-393.1)	0.304
Protein (All Livers)	447.6 (300.4-667.0)	230.3 (139.3-380.7)	254.7 (169.8-381.9)	0.009
Activity (Phenytoin Free)	1867 (1384-2519)	1071 (688.1-1668)	1214 (902.2-2272)	0.099
Activity (All Livers)	2544 (3379-1915)	1412 (919.6-2168)	1377 (1035-2186)	0.042

	Non-Diabetic	Diabetic	Student's t-test p-value
Phenytoin Free	N=35	N=31	
All Livers	N=53	N=43	
Protein (Phenytoin Free Livers)	398.9 (268.8-591.9)	230.1 (147.7-358.4)	0.276
Protein (All Livers)	406.8 (296.4-558.1)	253.4 (178.8-359.2)	0.063
Activity (Phenytoin Free Livers)	1496 (1029-2173)	1256 (1002-1828)	0.300
Activity (All Livers)	1972 (1448-2688)	1549 (1240-2155)	0.096

Table 3.8 Effect of phenytoin on protein expression and enzymatic activity of CYP3A4. Geometric mean and 95% confidence interval of protein expression in pmol per g of liver tissue. P-value from ANOVA for NAFLD samples. P-value from P-value from student's t-test for diabetic samples.

3.8 Figures

Figure 3.1 Correlation of protein expression and functional activity of CYP enzymes. r represents non-parametric Spearman correlation coefficient.

Figure 3.2 Effect of NAFLD on protein expression of various CYP450 proteins. Graphs represent Tukey box plots with median (horizontal line); + represent mean; P-value from one-way ANOVA run on ln transformed data with Bonferroni correction. * and ** represent p-value <0.05 and <0.01, respectively.

Figure 3.3 Effect of NAFLD on protein expression of various CYP450 proteins. Graphs represent Tukey box plots with median (horizontal line); + represent mean; P-value from one-way ANOVA run on ln transformed data with Bonferroni correction. * and ** represent p-value <0.05 and <0.01, respectively.

Figure 3.4 Effect of NAFLD on protein expression of various SULT proteins. Graphs represent Tukey box plots with median (horizontal line); + represent mean; P-value from one-way ANOVA run on ln transformed data with Bonferroni correction. * and ** represent p-value <0.05 and <0.01, respectively.

Figure 3.5 Effect of NAFLD on protein expression of various UGT proteins. Graphs represent Tukey box plots with median (horizontal line); + represent mean; P-value from one-way ANOVA run on ln transformed data with Bonferroni correction. * and ** represent p-value <0.05 and <0.01, respectively.

Figure 3.6 Effect of NAFLD on protein expression of various drug transporter proteins. Graphs represent Tukey box plots with median (horizontal line); + represent mean; P-value from one-way ANOVA run on ln transformed data with Bonferroni correction. * and ** represent p-value <0.05 and <0.01, respectively.

Figure 3.7 Effect of diabetes on protein expression of various CYP proteins. Graphs represent Tukey box plots with median (horizontal line); + represent mean; P-value from independent Student's t-test run on ln transformed data. * and ** represent p-value <0.05 and <0.01, respectively.

Figure 3.8 Effect of diabetes on protein expression of various CYP450 proteins. Graphs represent Tukey box plots with median (horizontal line); + represent mean; P-value from independent Student's t-test run on ln transformed data. * and ** represent p-value <0.05 and <0.01, respectively.

Figure 3.9 Effect of diabetes on protein expression of various UGT proteins. Graphs represent Tukey box plots with median (horizontal line); + represent mean; P-value from independent Student's t-test run on ln transformed data. * and ** represent p-value <0.05 and <0.01, respectively.

Figure 3.10 Effect of diabetes on protein expression of various SULT proteins. Graphs represent Tukey box plots with median (horizontal line); + represent mean; P-value from independent Student's t-test run on ln transformed data. * and ** represent p-value <0.05 and <0.01, respectively.

Figure 3.11 Effect of diabetes on protein expression of various drug transporter proteins. Graphs represent Tukey box plots with median (horizontal line); + represent mean; P-value from independent Student's t-test run on ln transformed data. * and ** represent p-value <0.05 and <0.01, respectively.

Figure 3.12 Effect of phenytoin on CYP3A4 protein expression and enzymatic metabolism of testosterone in regard to NAFLD and diabetes. Graphs represent Tukey box plots with median (horizontal line); + represent mean; P-value from independent Student's t-test (diabetes) and one-way ANOVA with Bonferroni correction (NAFLD). * and ** represent p-value <0.05 and <0.01, respectively.

MANUSCRIPT 4

EVALUATION OF THE AMERICAN LIFESTYLE INDUCED OBESITY SYNDROME (ALIOS) MOUSE AS A MODEL FOR HUMAN DRUG METABOLIZING ENYMES UTILIZING SWATH-MS PROTEOMICS

Prepared for submission to Liver International

Benjamin J. Barlock, Nick DaSilva, Rohitash Jamwal, Angela L. Slitt, Fatemeh

Akhlaghi

University of Rhode Island, College of Pharmacy, Department of Biomedical and
Pharmaceutical Sciences, Kingston, RI 02881

4.1 Abstract

Background & Aims. Non-alcoholic fatty liver disease (NAFLD) is a highly prevalent disease in our modern society. It is crucial to develop an accurate mouse model that is representative of the disease. The American Lifestyle Induced Obesity Syndrome (ALIOS) model aims to cause mice to develop NAFLD through diet and lifestyle management simulating western culture. Determining the proteomic changes occurring to the drug metabolizing profile is critical to assess if the model accurately translates to humans.

Methods. Pressure-cycling technology aided trypsin digestion was carried out on the whole tissue lysate from human and mouse livers. Both a data-dependent and data-independent acquisition method was used to acquire data on a time of flight (TOF) mass spectrometer. Absolute protein quantification was determined and fold change analysis in each species was performed between the healthy and disease state.

Results. It was found that about 75% of cytochrome P450s (CYPs) enzymes related to xenobiotic metabolism showed changes that were comparable between mouse and humans. Phase II metabolic enzymes were accurate 50% of the time and transporter differences were accurate in the two proteins that were measured in mouse liver. The enzymes that did not properly match up were mainly proteins that had poor orthology between the species.

Conclusions: The ALIOS model looks to be a reasonable representation of the majority of drug metabolism related CYPs.

4.2 Introduction

Nonalcoholic fatty liver disease (NAFLD) is a highly prevalent disease in modern society. It has been found that 30% of the population in the US has some degree of NAFLD, with 10.3% of patients exhibiting advanced fibrosis resulting in an approximate \$103 billion economic burden within US^{1,2}. NAFLD is characterized by the presence of >5% macrovascular steatosis within the liver in people with low to no alcohol intake (<20g/day for females, <30g/day for males)³. Both healthy and diseased livers have ranging levels of hepatocyte ballooning and lobular inflammation however NAFLD does not truly develop until steatosis occurs. NAFLD can range from benign fatty liver to nonalcoholic steatohepatitis (NASH) and can eventually lead to hepatocellular carcinoma. While the benign stages of the disease are reversible, once a liver becomes fibrotic, the damage cannot be repaired with current therapeutic interventions⁴. Other disease states including obesity, insulin resistance, cardiovascular diseases, and lipotoxicity are all generally associated with NAFLD as well⁵.

As the physiological characteristics of the liver change as the disease progresses, it is important to determine how the disease affects the function of drug metabolizing enzymes and transporters concentrated within the liver. Drug transporters are responsible for the passage of drugs and drug metabolites from the blood into the cell and vice versa⁶. They, along with drug metabolizing enzymes are responsible for the elimination of drugs from the body under normal circumstances.

Mouse models are an important starting point for the study of diseases including NAFLD. When looking at models, mice have multiple advantages when compared to

in vitro cell models. Mouse models are better able to translate to humans as the complexity of a whole animal can more accurately portray human disease state than a cell culture model. Diet, genetics, and lifestyle are all able to be modified in order to create the most accurate model. It is unrealistic, though, to assume that one animal model will represent all aspects of a disease. Therefore, it is important to realize that numerous models may be needed to understand different disease aspects. Additionally, species differences can lead to decreased accuracy of these models due to differences and variations in numerous genes that are expressed in humans and mice.⁷ Though there are some genetic differences, the genomes of mice and human are strikingly homologous leading to the assumption that mice are a good representative model.

Some other factors that need to be taken into consideration when comparing a mouse model to a human is their difference in size, metabolic rate and lifespan.⁸ Regarding specific NAFLD models, not only the genome and proteome have to accurately reflect the changes that are occurring in humans but the histological changes that are seen must be consistent too. Steatosis, lobular inflammation, hepatocyte ballooning and fibrosis must all develop as they are all involved in the classification and designation of scoring a liver to determine the severity of NAFLD.⁹ There have been numerous cases of drugs that show great potential in the treatment of NAFLD in preclinical mouse studies but completely fail once in the clinic.¹⁰

New tools are being developed to assist with the validation and exploration of the mouse proteome in relation to human. Mass spectrometry-based protein quantification methods have begun to rise as a gold standard over the past decade. While targeted quantification methods remain the current gold standard, label-free quantification

(LFQ) methods are becoming ever more popular due to the cost effectiveness and reduction of time required to prepare and analyze samples. Although less reproducible as targeted methods, LFQ methods are proving their worth when employed correctly. Tools like this are allowing a deep dive into the proteomes of different mouse models to help determine if the proteome is seeing the same changes in NAFLD that are being noted in humans. This can provide validation when translating to humans and the clinic and will help with the development of new drugs to treat NAFLD faster and for less money.

4.3 Materials and methods

4.3.1 Chemicals and reagents

Acquity UPLC Peptide BEH C18 analytical column and VanGuard pre-columns we purchased from Waters Corp. (Waltham, MA). MS tuning solution, TPCK-treated trypsin and trypsin digested β -galactosidase were procured from SCIEX (Framingham, MA). Bovine serum albumin (BSA), sodium deoxycholate (DOC) and iodoacetamide (IAA) were purchased from Sigma Aldrich (St. Louis, MO). 1,4-Dithiothreitol (DTT) was obtained from Roche Diagnostics (Indianapolis, IN). MS grade acetonitrile and formic acid were purchased from ThermoFisher (Waltham, MA).

4.3.2 Human liver bank

Frozen human liver samples were purchased from Sekisui XenoTech LLC (Kansas City, KS). All liver samples were procured from patients involved in automobile accidents, therefore are IRB-exempt. All livers came from brain dead

donors ranging from 21 to 64 years old. A list of the detailed demographic data is shown in **Table 1**. Human livers were graded by a histopathologist as previously described and further classified as a control, NAFL or NASH liver¹¹. Mouse livers were similarly graded and scored by a board certified veterinary pathologist using the method described by Kleiner et al¹².

4.3.3 Animal Treatment and Study Design

C57BL/6 (n=16) aged 4 weeks were purchased from Jackson Laboratories (Bar Harbor, ME USA) and housed with at least 2 mice per cage under 12-hour light/day cycles. Mice were acclimated for 1 week prior to having bedding pooled and redistributed weekly for two weeks. Mice were fed either a low-fat diet (LFD)(12% kcal from fat)(n=8) or a high-fat diet (HFD)(45% kcal from fat)(n=8). Bedding was pooled weekly within treatment groups, mixed and redistributed within the treatment group to account for a microbial cohousing effect¹³⁻¹⁵. Water consumption, food intake and body weight were monitored weekly. At the end of 18 weeks, 16 mice were sacrificed and whole blood was collected using cardiac puncture. Whole blood was centrifuged and immediately separated into two aliquots for future analysis. Mice were sacrificed by isoflurane and decapitation following blood collection.

4.3.4 Mouse Diet Selection

The ALIOS (American Lifestyle Induced Obesity Syndrome) diet models a western diet while also pairing with sedentary behavior and sugar rich drinking water. The diet consisted of hydrogenated vegetable oil (trans and saturated fats) and cholesterol (0.2% w/w) in addition to water containing high sugar content (42 g/L: 55% w/v fructose, 45% w/v glucose)(TD.120330). Diets were prepared by Envigo-

Teklad (Madison, WI USA) and chosen based on previously cited studies that show the development of NASH phenotypes including liver neutral lipid accumulation, inflammation and necrosis within 16 weeks. The dietary inclusion of cholesterol and high fructose/glucose in drinking water also has been shown to further promote the NASH phenotype¹⁶⁻¹⁸. A custom, grain free, phytoestrogen free diet (TD.2020) was used as the low-fat diet control.

4.3.5 Tissue preparation

One ml of homogenization buffer (8 M urea, 50 mM triethylammonium bicarbonate, 10 mM DTT v/v) was added to approximately 50 mg liver tissue and homogenized using a bead homogenizer (Omni Bead Ruptor, Kennesaw, GA)¹⁹. Samples were spun down at 1,000 x g for 5 min and the supernatant was collected. A Pierce BCA protein assay kit (ThermoFisher Scientific, Waltham, MA) was used to estimate protein concentration. Lipid peroxidation and total cholesterol were measured in human livers as previously described¹⁹. A colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI) was used to measure triacylglycerides (TAG) according to the manufacturer's protocol.

4.3.6 In-solution trypsin digestion

Protein digestion was performed with slight modifications to the method described by Jamwal et al.²⁰. Samples (250 µg protein) were spiked with 2 ng BSA and denatured with a DTT at a final concentration of 20 mM at 95°C for 15 minutes in a water bath shaking at 100 rpm. Samples were then alkylated using IAA (33mM) for 30 minutes at room temperature in the dark. Following alkylation, samples were concentrated using ice-cold methanol, chloroform and water (2:1:1) to precipitate the

proteins. Samples were spun at 10,000 x g for 5 minutes. The pellet was collected and washed with ice-cold methanol. The remaining pellet was resuspended in 125 μ l of 50 mM ammonium bicarbonate (pH 7.8) containing 3% w/v DOC. TPCK-treated trypsin was additionally added to the sample at a ratio of 1:20 (trypsin:protein). Samples were transferred to PCT MicroTubes (Pressure Biosciences Inc., Easton, MA) and the tubes were then placed in the NEP-Barocycler (Pressure Biosciences Inc., Easton, MA) run at 35°C for 75 cycles (50 sec at 25 kpsi, 10 sec at ambient pressure). Subsequently, the same amount of trypsin was added again, and the samples were placed back in the barocycler for the same cycle. After digestion, trypsin was quenched, and DOC was precipitated by the addition of formic acid (5% in acetonitrile) at a final concentration of 0.1%. Samples were spun at 10000 x g for 5 min at 10°C and the supernatant was collected for LC-MS/MS analysis. Samples were injected at a volume of 25 μ L and analyzed using the LC-MS/MS method below.

4.3.7 LC-MS/MS analysis

Mass spectrometry was performed as described previously with slight modifications¹⁹. Data was collected on a SCIEX 5600 TripleTOF® mass spectrometer using a DuoSpray™ ion source (SCIEX, Concord, Canada) coupled to an Acquity UHPLC HClass system (Waters Corp., Milford, MA). An Acquity UPLC Peptide BEH C18 (2.1 X 150 mm², 300 Å, 1.7 μ m) with an Acquity VanGuard pre-column (2.1 X 5 mm², 300 Å, 1.7 μ m) was used for sample separation. The column was kept at 50°C and the autosampler at 10°C. A linear gradient run at 100 μ L/min for 180-min was used to achieve separation. Mobile phase A consisted of 99.9% acetonitrile and 0.1% formic acid, and mobile phase B consisted of 99.9% water and

0.1% formic acid. The gradient was composed of 98% A from 0 to 5 min, 98% to 70% A from 5 to 155 min, 70% to 50% A from 155 to 160 min and 50% to 5% from 160 to 170 min. Mobile phase A was held at 5% from 170 to 175 min and the column returned to starting conditions at 175 min and was held until the end of the run-in order to equilibrate the column prior to the next sample. Trypsin-digested β -galactosidase peptides was used for mass calibration and monitoring of the QTOF detector every 4 samples.

4.3.8 Mass spectrometer acquisition parameters

Positive ionization mode was used for both data-independent (SWATH – Sequential Windowed Acquisition of all Theoretical Ions) and data-dependent acquisition. The specific parameters were as follows: gas 1 55 psi, gas 2 60 psi, curtain gas 25 psi, temperature 450°C, ion spray voltage floating 5500 V, declustering potential 100, collision energy 10 and collision energy spread 15.

For the data dependent acquisition, a maximum of 50 candidate ions were monitored each survey scan. These ions had a charge state from 2 to 4. All ions that fell between m/z 300-1250 and that were more intense than 25 cps were selected for MS/MS analysis. Dynamic accumulation and a rolling collision energy based on each ion's m/z were used. A mass tolerance of 50 mDa was set during the initial survey scan of 0.75 sec. The total cycle time was 3.50 sec.

The parameters used for SWATH-MS acquisition were the same as above except for the following: temperature 400°C and TOF masses were collected from m/z 300-1500. Seventy SWATH windows per cycles were collected over m/z 400-1100 with each window size being m/z 10. The total cycle time was 3.95 sec.

4.3.9 Data processing

Data-dependent acquisition (DDA) samples were searched using Spectronaut (Biognosys, Schlieren, Switzerland) against reviewed Swiss-Prot identifiers (October 2016). The Pulsar algorithm was used for identification of peptides and proteins from both DDA and DIA data. BGS factory settings were used for the search. Tryptic peptides were searched for with a minimum length of 7 amino acids and a maximum of 52 amino acids with a maximum of 2 missed cleavages. All other options were kept as the BGS factory settings. The spectral library generated from the DDA files was used to then analyze the DIA files in Spectronaut. All settings in Spectronaut were kept as the BGS factory settings except “PTM localization” and “used Biognosys iRT kit” were unselected. Normalized intensity values were exported and converted using excel.

4.3.10 Data normalization

The normalized output from Spectronaut were converted to picomoles per gram of liver (pmol/g liver) by first using the Total Protein Approach (TPA) followed by protein yield (protein per gram of liver or PPGL)²¹. The following equations demonstrate how values were converted to their final form:

$$\text{Protein} \left(\frac{\text{pmol}}{\text{mg protein}} \right) = \frac{\text{Protein intensity}}{\text{Total intensity} \times \text{MW (g/mol)}} \times 10^6$$

$$\begin{aligned} &\text{Absolute Protein} \left(\frac{\text{pmol}}{\text{g liver}} \right) \\ &= \text{Absolute Protein} \left(\frac{\text{pmol}}{\text{mg protein}} \right) \times \text{protein per gram of liver (mg)} \end{aligned}$$

The natural log of the final values was taken, and statistical analysis was performed.

4.3.11 Statistical analysis

Student's t-test was used to determine the difference between NASH and healthy groups. Statistical values of $p < 0.05$ were considered significant for the analysis. SPSS 24 (IBM Corp., Armonk, NY) and Prism 6.0 (GraphPad Inc., La Jolla, CA) were used to perform statistical tests and create graphs.

4.4 Results

A list of human proteins analyzed, and their orthologous mouse gene can be found in **Table 4.1**.

4.4.1 Human liver histology

Criteria for the non NAFLD group of human livers included no level of steatosis but some degree of lobular inflammation and hepatocyte ballooning and fibrosis. For these samples specifically, only one sample had a degree of hepatocyte ballooning, while about 50% had either low grade inflammation and/or fibrosis. In order to allow this model to apply to the greatest population size possible, diabetes status was disregarded. For the NASH group, steatosis levels ranged from 5% to >67%. All samples had some degree of inflammation and hepatocyte ballooning while most samples had low to mid-grade fibrosis. A full table of demographics can be seen in **Table 4.2**.

4.4.2 Mouse liver histology

Mice in the healthy group were found to have no steatosis at week 18. Both necrosis and inflammation were seen to be from 0-10% and no biliary hyperplasia was seen. In the high fat diet group, lipid accumulation was seen to be >40% in all mice.

Inflammation was seen to range from 0-25% and necrosis remained the same at 0-10%. Biliary hyperplasia increased from 0% to between 0 and 10%. ALIOS mice additionally became less sensitive to glucose and insulin resistance compared to the healthy mice indicating some level of diabetes. A full table of demographics can be seen in **Table 4.3**.

4.4.3 Total protein extracted

Total protein extracted per gram of liver tissue was calculated in both species and used to convert the units of protein expression. Between the high-fat and low-fat mouse groups, no significant protein extraction differences were seen (154.4 ± 5.92 vs 162.4 ± 11.95 mg/g liver) whereas total liver weight was increased by about 140% in the high fat group (3.052 ± 0.22 g vs 1.27 ± 0.02 g). Similar trends were seen in the human group. The healthy group was able to extract 85.35 ± 3.0 mg/g liver where the NASH group extracted 83.08 ± 4.7 mg/g liver. Total liver weight was also found to be significantly increased from the healthy group to the NASH group (1.528 ± 60 g vs 1901 ± 97 g).

4.4.4 Effect of NASH on human drug metabolizing enzymes and transporters

CYP1A2 ($p < 0.01$) and CYP2J2 ($p < 0.01$) were all found to be significantly downregulated in NASH samples compared to healthy samples. CYP2C8 and CYP4A11 were also both found to be downregulated but were not significant. UGT1A4 ($p < 0.05$) and BSEP ($p < 0.05$) were also both found to be significantly decreased in the NASH group. Interestingly, UGT2A3 was found to be significantly upregulated in the NASH group ($p < 0.05$). The remainder of proteins remained similar to the healthy group (**Table 4.4**). CYP3A4 levels were analyzed in samples that were

free of phenytoin. Prior to the phenytoin sample removal, CYP3A4 showed significant decrease ($p<0.01$).

4.4.5 Effect of NASH on mouse drug metabolizing enzymes and transporters

Cyp1a2 ($p<0.01$), Cyp2c29 ($p<0.001$), Cyp2c37 ($p<0.001$), Cyp2j5 ($p<0.05$), Cyp7b1 ($p<0.05$) and Cyp8b1 ($p<0.05$) were all found to be significantly downregulated in the mice with NASH. Cyp4a01 ($p<0.05$) was found to be upregulated in the NASH group. Additionally, Ugt2a3 ($p<0.05$) and Bsep ($p<0.05$) were both found to be significantly decreased in the NASH samples compared to healthy. The remainder of the proteins remained similar to baseline (**Table 4.5**).

4.4.6 Comparison of species effect

The majority of drug disposition proteins analyzed showed similar trends when comparing the mouse to the human liver samples. UGT2A3, CYP4A11 and CYP4A22 showed opposite trends in humans than mice. CYP2C8 and CYP2C9 showed much greater and significant downregulation in mice than in human. The remaining proteins showed the similar trends when comparing NASH to healthy samples in both species (**Table 4.6** and **Figures 1-3**).

4.5 Discussion

Mouse models are an important step in pre-clinical research. These models can provide valuable information to assist with the validation that a drug has the expected efficacy and safety profile that is necessary to move on to human studies. Not only are mouse models important for new drug entities, they are also important in understanding how the metabolic profile of a disease population will change. NAFLD

is an ever-expanding field that new mouse models are being developed and validated for.

There are numerous models of NAFLD that have been developed over the years that incorporate genetics, diet and lifestyle or some combination of all three. Some examples of these include the high fat/high cholesterol/high sugar diet (HF-HC-HS), *ob/ob* mice, a methionine/choline deficient (MCD) diet, the Diet Induced Animal Model of Non-Alcoholic Fatty Liver Disease (DIAMOND) and the ALIOS models. While all these models lead to different characteristics of NASH, each one has their pitfalls as it is with all animal models. The MCD diet is a nutrient deficiency model where mice are fed high sucrose and low-fat food that is missing methionine and choline. While this diet is able to achieve a NASH histology, the mice do not gain weight or become insulin resistant²². Although the HF-HC-HS diet accurately achieves all changes normally seen in NASH, it does not accurately represent a western culture diet as the cholesterol content is significantly higher in the diet used for the model²³.

The DIAMOND model uses a specific genetic mix between the 129S1/SvImJ and the C57BL/6J strains of mice combined with a high fat diet and water with high fructose/glucose in order to accurately develop NASH²⁴. These results took 4 years to obtain, though, and the results were not due to purely diet, but due to a genetic factor as well as both parent strains did not have as pronounced NASH histology²⁴. Another model that does not have a dietary component is the *ob/ob* mouse model where there is a loss of function mutation resulting in the gene that encodes for leptin causing it to not work properly. As leptin acts to maintain homeostasis by reducing food intake, this leads to severely obese mice. These mice show the traditional signs of being

diabetic and can develop benign hepatic steatosis but do not progress into the NASH territory^{25,26}. Finally, another diet like DIAMOND is the ALIOS model. This uses a realistic, high fat diet and water with high sugar content in order to simulate a western, fast food diet along with a sedentary lifestyle. These mice result in the NASH phenotype after 16 weeks and show all components except for hepatocyte ballooning^{16,27}.

The ALIOS diet was chosen for this study as it is comparable to common western diets and had no genetic factor introduced. The diet included a consumption of sugar, cholesterol and saturated fats that is similar to eat fast food while also adding sugar water to simulate soda consumption²⁸⁻³¹. Additionally, the mice had a sedentary lifestyle to help stimulate weight gain due to lack of exercise. This was done by directly placing food in the cage, rather than in a top mounted feeder. Glucose and insulin intolerance were noticed at 12 and 15 weeks, respectively. This means the mice had reached diabetic conditions prior to sacrifice and their NASH diagnosis. It has been shown that after 12 weeks on a supplemented cholesterol and 45% kcal diet weight gain and insulin resistance develop but the addition of fructose is needed in order to develop simple steatosis, NASH and even fibrosis¹⁶⁻¹⁸. Mells et al. found that at 16 weeks, mild to moderate NASH had developed in aged mice³².

The majority of these models, mainly *ob/ob* and MCD, have been explored to determine their effect on the mRNA, protein and enzymatic activity levels of drug metabolizing enzymes expressed in the liver³³. These results have not been consistent as it is when studying human liver enzymes in NASH as well. The ALIOS model currently has no literature reports on the effect on DME expression. It is additionally

important to verify that the metabolic profile of the liver proteome remains consistent between the mouse model and the human proteome that it is trying to mimic.

The ALIOS model shows good representation of the CYPs related to drug metabolism. Both models show downregulation of CYP1A2 which has been reported previously in human liver microsomes and hepatocytes^{34,35}. The mouse model showed significant downregulation in both CYP2C8 and CYP2C9 where the human samples showed non-significant downregulation. One literature report showed some downregulation of CYP2C8 in NASH while no change in CYP2C9 while others report no change in either enzyme^{36,37}. The CYP2C family consists of 4 genes in humans while there are 15 functional genes in mice. It is difficult to define orthologous genes from human to mouse due to these differences as well as the sequence variation in each³⁸. It has been found that for both CYP2C8 and CYP2C9, the top 4 orthologous mouse genes in sequence homology are Cyp2c29, Cyp2c37, Cyp2c38 and Cyp2c39 and they all match both human genes between 68 and 78%³⁹. These difficulties could explain the discrepancies that are being noted between the human and mouse models. CYP27A1, CYP2D6 and CYP2E1 all showed no change in either model which is consistent with literature except for CYP2E1 which has been reported to be downregulated^{35,40,41}. CYP2J2 showed downregulation in both species. This enzyme is related to fatty acid synthesis but there is no current literature stating if there is any change due to NASH. CYP4A11 is also involved with fatty acid metabolism as has also been reported to be upregulated by NASH in mice⁴². While we show that CYP4A11 is not changing in NASH, it has been previously reported that the CYP4A11 gene is significantly increased in NAFLD livers^{43,44}. This fluctuation in

results between the species could be due to the lack of orthologous CYP4A family proteins. CYP4A11 and CYP4A22 are closely related in humans with other forms being seen in mice that make extrapolation and comparison difficult⁴⁵. UGT1A6 and UGT2A3 are responsible for the glucuronidation of numerous drugs, lipids and fat-soluble vitamins^{46,47}. As humans have 1 copy of UGT1A6 while mice have 2 copies of, *Ugt1a6a* and *Ugt1a6b*, this could explain the discrepancy between species in the protein expression⁴⁸. There are no reports as to the change of UGT2A3 in a mouse or human model. The discrepancy seen between species could be due to the fact that the UGT2 family enzymes have 70% sequence homology making it difficult to elucidate orthologs⁴⁶.

In human livers, in addition to what was visible in the mouse model, we have seen progression of NAFLD lead to significant downregulation in CYP1A2, CYP20A1, CYP2E1, CYP4F2, and CYP2J2. CYP3A4 initially shows downregulation, but this can be attributed to the presence of the potent inducer, phenytoin, being contained in some of these liver samples. We have also seen changes in phase II metabolic enzymes including SULT1A1, SULT1C2, UGT1A3, UGT1A4, UGT2A3 and transporters such as MDR1, BSEP and MRP3.

For this study, mice in the control group showed no signs of diabetes or high NAS pathology. The healthy livers had no levels of steatosis while having low levels of lobular inflammation and necrosis as can normally be found in human livers that are not considered to have NAFLD⁴⁹. By week 18 in the high fat diet group, mice were seen to have developed symptoms of diabetes such as insulin resistance and glucose intolerance. The mouse livers at this point were seen to have high levels of

steatosis with all mice having >40% lipid accumulation while inflammation and necrosis remained around similar levels. In order to properly match this model to human data, our healthy group consisted of non-diabetic donors who had been scored and determined to have no level of NAFLD. Donors with or without diabetes and diagnosed NASH were used to match with the high fat diet mouse group. All human fibrosis levels were considered, as fibrosis was not tested for in the mouse samples. Interestingly, even though the total liver weight was significantly different between groups in both species, the total protein yield remained similar between the groups. This could be due to the changes in pathology throughout the liver. The section that the pathologists examined may have had altered fat levels when compared to the section that was used for protein measurement. It would be expected that protein yield would decrease as fat content increased yet there is no strong correlation seen.

While other mouse models of NASH have reported combined effect of diet and genetics on drug-metabolizing enzymes, no literature is currently available on how these enzymes are changing in the ALIOS mouse model. Based on the current study, the ALIOS diet model exhibits reasonable representation of changes in some of the major phase I and phase II drug metabolizing enzymes as compared to human livers from individuals with NASH. Importantly, CYPs that have orthologous mouse genes such as CYP3A4 and CYP1A2 look similar in regard to disease progression in both mice and human. Additionally, BSEP, an important bile acid transporter, seems to be dysregulated in both models. The further development of mouse models is extremely important in the advancement of drug and disease research and the ALIOS model looks to be an accurate representation of major drug metabolism routes.

4.6 References

1. Le MH, Devaki P, Ha NB, et al. Prevalence of non-alcoholic fatty liver disease and risk factors for advanced fibrosis and mortality in the United States. *PLoS One*. 2017;12(3):e0173499.
2. Younossi ZM, Blissett D, Blissett R, et al. The economic and clinical burden of nonalcoholic fatty liver disease in the United States and Europe. *Hepatology*. 2016;64(5):1577-1586.
3. Vuppalanchi R, Chalasani N. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: Selected practical issues in their evaluation and management. *Hepatology*. 2009;49(1):306-317.
4. Younossi Z, Henry L. Contribution of Alcoholic and Nonalcoholic Fatty Liver Disease to the Burden of Liver-Related Morbidity and Mortality. *Gastroenterology*. 2016;150(8):1778-1785.
5. Brunt EM, Tiniakos DG. Histopathology of nonalcoholic fatty liver disease. *World J Gastroenterol*. 2010;16(42):5286-5296.
6. Rausch-Derra LC, Hartley DP, Meier PJ, Klaassen CD. Differential effects of microsomal enzyme-inducing chemicals on the hepatic expression of rat organic anion transporters, OATP1 and OATP2. *Hepatology*. 2001;33(6):1469-1478.
7. Pound P, Ritskes-Hoitinga M. Is it possible to overcome issues of external validity in preclinical animal research? Why most animal models are bound to fail. *J Transl Med*. 2018;16(1):304.
8. Perlman RL. Mouse models of human disease: An evolutionary perspective. *Evol Med Public Health*. 2016;2016(1):170-176.

9. Takahashi Y, Soejima Y, Fukusato T. Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World J Gastroenterol*. 2012;18(19):2300-2308.
10. Ratzliff V, Bedossa P, Francque SM, et al. Lack of efficacy of an inhibitor of PDE4 in phase 1 and 2 trials of patients with nonalcoholic steatohepatitis. *Clin Gastroenterol Hepatol*. 2014;12(10):1724-1730 e1725.
11. Jamwal R, de la Monte SM, Ogasawara K, Adusumalli S, Barlock BB, Akhlaghi F. Nonalcoholic Fatty Liver Disease and Diabetes Are Associated with Decreased CYP3A4 Protein Expression and Activity in Human Liver. *Mol Pharm*. 2018;15(7):2621-2632.
12. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005;41(6):1313-1321.
13. Anhe FF, Varin TV, Le Barz M, et al. Gut Microbiota Dysbiosis in Obesity-Linked Metabolic Diseases and Prebiotic Potential of Polyphenol-Rich Extracts. *Curr Obes Rep*. 2015;4(4):389-400.
14. Anhe FF, Roy D, Pilon G, et al. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. population in the gut microbiota of mice. *Gut*. 2015;64(6):872-883.
15. Miyoshi J, Leone V, Nobutani K, et al. Minimizing confounders and increasing data quality in murine models for studies of the gut microbiome. *PeerJ*. 2018;6:e5166.
16. Tetri LH, Basaranoglu M, Brunt EM, Yerian LM, Neuschwander-Tetri BA. Severe NAFLD with hepatic necroinflammatory changes in mice fed trans fats and a high-fructose corn syrup equivalent. *Am J Physiol Gastrointest Liver Physiol*. 2008;295(5):G987-995.

17. Ito M, Suzuki J, Tsujioka S, et al. Longitudinal analysis of murine steatohepatitis model induced by chronic exposure to high-fat diet. *Hepatol Res.* 2007;37(1):50-57.
18. Ferrere G, Leroux A, Wrzosek L, et al. Activation of Kupffer Cells Is Associated with a Specific Dysbiosis Induced by Fructose or High Fat Diet in Mice. *PLoS One.* 2016;11(1):e0146177.
19. Jamwal R, Barlock BJ, Adusumalli S, Ogasawara K, Simons BL, Akhlaghi F. Multiplex and Label-Free Relative Quantification Approach for Studying Protein Abundance of Drug Metabolizing Enzymes in Human Liver Microsomes Using SWATH-MS. *J Proteome Res.* 2017;16(11):4134-4143.
20. Jamwal R, Barlock BJ, Adusumalli S, Ogasawara K, Simons BL, Akhlaghi F. Multiplex and Label-Free Relative Quantification Approach for Studying Protein Abundance of Drug Metabolizing Enzymes in Human Liver Microsomes Using SWATH-MS. *J Proteome Res.* 2017.
21. Wisniewski JR, Rakus D. Multi-enzyme digestion FASP and the 'Total Protein Approach'-based absolute quantification of the Escherichia coli proteome. *J Proteomics.* 2014;109:322-331.
22. Rinella ME, Green RM. The methionine-choline deficient dietary model of steatohepatitis does not exhibit insulin resistance. *J Hepatol.* 2004;40(1):47-51.
23. Cole BK, Feaver RE, Wamhoff BR, Dash A. Non-alcoholic fatty liver disease (NAFLD) models in drug discovery. *Expert Opin Drug Discov.* 2018;13(2):193-205.
24. Asgharpour A, Cazanave SC, Pacana T, et al. A diet-induced animal model of non-alcoholic fatty liver disease and hepatocellular cancer. *J Hepatol.* 2016;65(3):579-588.

25. Wang B, Chandrasekera PC, Pippin JJ. Leptin- and leptin receptor-deficient rodent models: relevance for human type 2 diabetes. *Curr Diabetes Rev.* 2014;10(2):131-145.
26. Kelesidis T, Kelesidis I, Chou S, Mantzoros CS. Narrative review: the role of leptin in human physiology: emerging clinical applications. *Ann Intern Med.* 2010;152(2):93-100.
27. Dowman JK, Hopkins LJ, Reynolds GM, et al. Development of hepatocellular carcinoma in a murine model of nonalcoholic steatohepatitis induced by use of a high-fat/fructose diet and sedentary lifestyle. *Am J Pathol.* 2014;184(5):1550-1561.
28. In: *Health, United States, 2011: With Special Feature on Socioeconomic Status and Health.* Hyattsville (MD)2012.
29. Volynets V, Louis S, Pretz D, et al. Intestinal Barrier Function and the Gut Microbiome Are Differentially Affected in Mice Fed a Western-Style Diet or Drinking Water Supplemented with Fructose. *J Nutr.* 2017;147(5):770-780.
30. Savard C, Tartaglione EV, Kuver R, et al. Synergistic interaction of dietary cholesterol and dietary fat in inducing experimental steatohepatitis. *Hepatology.* 2013;57(1):81-92.
31. Panasevich MR, Meers GM, Linden MA, et al. High-fat, high-fructose, high-cholesterol feeding causes severe NASH and cecal microbiota dysbiosis in juvenile Ossabaw swine. *Am J Physiol Endocrinol Metab.* 2018;314(1):E78-E92.
32. Mells JE, Fu PP, Kumar P, Smith T, Karpen SJ, Anania FA. Saturated fat and cholesterol are critical to inducing murine metabolic syndrome with robust nonalcoholic steatohepatitis. *J Nutr Biochem.* 2015;26(3):285-292.
33. Merrell MD, Cherrington NJ. Drug metabolism alterations in nonalcoholic fatty liver disease. *Drug Metab Rev.* 2011;43(3):317-334.

34. Donato MT, Lahoz A, Jimenez N, et al. Potential impact of steatosis on cytochrome P450 enzymes of human hepatocytes isolated from fatty liver grafts. *Drug Metab Dispos.* 2006;34(9):1556-1562.
35. Fisher CD, Lickteig AJ, Augustine LM, et al. Hepatic cytochrome P450 enzyme alterations in humans with progressive stages of nonalcoholic fatty liver disease. *Drug Metab Dispos.* 2009;37(10):2087-2094.
36. Kakehashi A, Stefanov VE, Ishii N, et al. Proteome Characteristics of Non-Alcoholic Steatohepatitis Liver Tissue and Associated Hepatocellular Carcinomas. *Int J Mol Sci.* 2017;18(2).
37. Chtioui H, Semela D, Ledermann M, Zimmermann A, Dufour JF. Expression and activity of the cytochrome P450 2E1 in patients with nonalcoholic steatosis and steatohepatitis. *Liver Int.* 2007;27(6):764-771.
38. Scheer N, Kapelyukh Y, Chatham L, Rode A, Buechel S, Wolf CR. Generation and characterization of novel cytochrome P450 Cyp2c gene cluster knockout and CYP2C9 humanized mouse lines. *Mol Pharmacol.* 2012;82(6):1022-1029.
39. Pan ST, Xue D, Li ZL, et al. Computational Identification of the Paralogs and Orthologs of Human Cytochrome P450 Superfamily and the Implication in Drug Discovery. *Int J Mol Sci.* 2016;17(7).
40. Leung TM, Nieto N. CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease. *J Hepatol.* 2013;58(2):395-398.
41. Nakamuta M, Kohjima M, Morizono S, et al. Evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease. *Int J Mol Med.* 2005;16(4):631-635.
42. Owada Y, Tamura T, Tanoi T, et al. Novel non-alcoholic steatohepatitis model with histopathological and insulin-resistant features. *Pathol Int.* 2018;68(1):12-22.

43. Kohjima M, Enjoji M, Higuchi N, et al. Re-evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease. *Int J Mol Med*. 2007;20(3):351-358.
44. Nakamuta M, Kohjima M, Higuchi N, et al. The significance of differences in fatty acid metabolism between obese and non-obese patients with non-alcoholic fatty liver disease. *Int J Mol Med*. 2008;22(5):663-667.
45. Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, Nebert DW. Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics*. 2004;14(1):1-18.
46. Mackenzie PI, Bock KW, Burchell B, et al. Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily. *Pharmacogenet Genomics*. 2005;15(10):677-685.
47. Turgeon D, Chouinard S, Belanger P, et al. Glucuronidation of arachidonic and linoleic acid metabolites by human UDP-glucuronosyltransferases. *J Lipid Res*. 2003;44(6):1182-1191.
48. Shiratani H, Katoh M, Nakajima M, Yokoi T. Species differences in UDP-glucuronosyltransferase activities in mice and rats. *Drug Metab Dispos*. 2008;36(9):1745-1752.
49. Bedossa P, Poitou C, Veyrie N, et al. Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. *Hepatology*. 2012;56(5):1751-1759.

4.7 Tables

Human gene	Mouse Gene
BSEP	Bsep
CYP1A2	Cyp1a2
CYP27A1	Cyp27a1
CYP2C8	Cyp2c29?
CYP2C9	Cyp2c37?
CYP2D6	Cyp2d9
CYP2E1	Cyp2e1
CYP2J2	Cyp2j5
CYP3A4	Cyp3a11
CYP3A5	Cyp3a13
CYP4A11	Cyp4a10
CYP4A22	Cyp4a12a
CYP7B1	Cyp7b1
CYP8B1	Cyp8b1
OATP1B1	Slco1b2
SULT1A1	Sult1a1
UGT1A1	Ugt1a1
UGT1A4	Ugt1a5
UGT1A6	Ugt1a6a Ugt1a6b
UGT2A3	Ugt2a3
UGT2B17	Ugt2b17

Table 4.1. Comparison of human DME gene names and their orthologous gene in mice.

	Human Control	Human NASH
N	42	30
Age (y)	46.5 (41.9 - 51.6)	50.8 (47.3 - 54.5)
Female	16	15
Male	26	15
Caucasian	33	28
African American	8	1
Hispanic	1	1
Body weight (kg)	83.5 (76.2 - 91.5)	92.7 (86 - 99.9)
BMI (kg/m²)	29.3 (26.5 - 32.3)	32.2 (29.9 - 34.7)
Liver weight (g)	1482.4 (1374.9 - 1598.3)	1839 (1678.4 - 2014.9) **
Malondialdehyde (nmol/mg protein)	0.6 (0.5 - 0.7)	1.2 (1 - 1.6) **
Cholesterol (µg/g liver)	14.2 (12.4 - 16.2)	20.8 (17.9 - 24.1) **
Triacyl glycerides (mg/g liver)	12.18 (10.01 - 14.82)	14.44 (12.1 - 17.23)
PPGL (mg/g)	83.1 (77.4 - 89.3)	79.8 (72.1 - 88.3)
Non-diabetic	21	13
Diabetic	21	17
Steatosis (0,1,2,3)	0 – 100%	0 – 0%
	1 – 0%	1 – 23.3%
	2 – 0%	2 – 33.3%
	3 – 0%	3 – 43.3%
Lobular Inflammation (0,1,2,3)	0 – 38%	0 – 0
	1 – 52%	1 – 77%
	2 – 10%	2 – 17%
	3 – 0	3 – 6%
Hepatocyte Ballooning (0,1,2,3)	0 – 98%	0 – 0
	1 – 2%	1 – 73%
	2 – 0	2 – 27%
Fibrosis (0,1,2,3)	0 – 50%	0 – 33.3%
	1 – 40%	1 – 53.3%
	2 – 7%	2 – 10%
	3 – 3%	3 – 3.3%

Table 4.2. Demographic characteristics of control and NASH groups. BMI: Body-mass index; PPGL: Protein per gram liver. P-value from non-parametric Mann Whitney U test. * and ** represent p-value <0.05 and <0.01, respectively.

	Mouse LFD	Mouse HFD
N	5	4
Age (weeks)	18	18
Body weight (kg)	35.55 ± 0.79	42.28 ± 0.81
Liver weight (g)	3.27 ± 0.02	3.05 ± 0.22**
PPGL (mg/g)	162.4 ± 11.95	154.4 ± 5.92
Steatosis (0,1,2,3,4,5)	0 – 100%	0 – 0%
	1 – 0%	1 – 0%
	2 – 0%	2 – 0%
	3 – 0%	3 – 0%
	4 – 0%	4 – 71.5%
	5 – 0%	5 – 28.5%
Necrosis (0,1,2,3,4,5,)	0 – 37.5%	0 – 71.5%
	1 – 62.5%	1 – 28.5%
	2 – 0%	2 – 0%
	3 – 0%	3 – 0%
	4 – 0%	4 – 0%
	5 – 0%	5 – 0%
Inflammation (0,1,2,3,4,5)	0 – 25%	0 – 28.5%
	1 – 75%	1 – 57.2%
	2 – 0%	2 – 14.5%
	3 – 0%	3 – 0%
	4 – 0%	4 – 0%
	5 – 0%	5 – 0%

Table 4.3 Demographic characteristics of mouse low-fat diet (LFD) and high-fat diet (HFD) groups. PPGL: Protein per gram liver. P-value from student's t-test. ** represents p-value <0.01.

Enzyme	Healthy	NASH	Fractional Difference	Student's t-test p-value
BSEP	22.87 (19.80-26.42)	18.20* (15.42-21.47)	-0.213	0.039
CYP1A2	275.0 (179.5-421.3)	128.4* (73.99-222.8)	-0.485	0.027
CYP27A1	264.9 (189.0-371.2)	262.4 (164.2-419.5)	0.166	0.978
CYP2C8	350.2 (227.8-538.3)	309.6 (198.7-482.6)	-0.177	0.215
CYP2C9	908.2 (641.2-1287)	629.4 (366.4-1081)	-0.171	0.091
CYP2D6	148.9 (106.0-209.1)	109.1 (68.39-174.0)	-0.167	0.265
CYP2E1	853.8 (661.7-1102)	557.4 (337.4-920.9)	-0.084	0.099
CYP2J2	43.60 (34.66-54.83)	30.38 (22.20-41.59)	-0.287	0.057
CYP3A4	434.1 (282.3-667.6)	285.3 (199.6-407.7)	-0.325	0.132
CYP3A5	56.63 (35.54-90.23)	39.60 (25.42-61.71)	-0.458	0.280
CYP4A11	403.0 (300.2-540.9)	287.8 (169.5-488.8)	-0.147	0.231
CYP4A22	58.75 (40.66-84.89)	61.47 (42.09-89.76)	-0.0488	0.866
CYP7B1	17.96 (13.42-24.03)	11.40 (7.499-17.33)	-0.257	0.065
CYP8B1	254.3 (162.9-397.0)	322.3 (234.3-443.2)	-0.025	0.421
OATP1B1	68.27 (56.60-82.49)	54.19 (38.13-77.01)	-0.041	0.210
SULT1A1	883.8 (624.3-1251)	539.3 (298.9-973.2)	-0.14	0.124
UGT1A1	147.8 (104.4-209.2)	117.7 (72.53-190.9)	-0.072	0.427
UGT1A4	294.6 (212.8-407.8)	172.9 (108.6-275.4)	-0.287	0.053
UGT1A6	170.2 (121.5-238.5)	240.2 (165.9-347.8)	0.441	0.171
UGT2A3	50.91 (40.70-63.66)	62.59 (43.31-90.46)	0.309	0.307
UGT2B17	869.5 (607.6-1323)	1104 (715.9-1703)	0.140	0.475

Table 4.4 Effect of NASH on protein expression of various human DMES and transporter proteins. Geometric mean and 95% confidence interval of protein expression in pmol per g of liver tissue. P-value from independent Student's t-test on ln transformed data. * represents p-value <0.05.

Enzyme	Healthy	NASH	Fractional Difference	Student's t-test p-value
BSEP	47.96 (40.68-56.55)	35.07* (26.97-45.60)	-0.266	0.015
CYP1A2	271.7 (203.7-362.6)	118.2** (58.69-238.1)	-0.544	0.008
CYP27A1	885 (700.5-1120)	771.6 (652.5-912.3)	-0.137	0.236
CYP2C8	1381 (1065-1790)	363.0*** (126.6-403.7)	-0.721	<0.001
CYP2C9	627.7 (508.0-775.6)	226.1*** (126.6-403.7)	-0.626	<0.001
CYP2D6	1141 (930.7-1399)	921.6 (597.4-1422)	-0.179	0.186
CYP2E1	2258 (1757-2903)	2368 (2068-2711)	0.034	0.677
CYP2J2	236.2 (176.1-316.7)	147.4* (86.41-251.5)	-0.363	0.042
CYP3A4	644.7 (454.8-913.9)	624.7 (475.5-820.7)	-0.052	0.851
CYP3A5	155.5 (122.2-197.7)	117.1 (70.78-193.6)	-0.229	0.139
CYP4A11	531.4 (434.1-650.4)	741.8* (517.7-1063)	0.409	0.036
CYP4A22	334.8 (279.4-401.1)	399.3 (260.0-613.1)	0.213	0.247
CYP7B1	166.4 (110.2-251.2)	78.82 (15.18-409.4)	-0.472	0.072
CYP8B1	260.2 (195.9-345.5)	168.2* (102.5-275.8)	-0.344	0.045
OATP1B1	289.5 (213.8-392.0)	257.0 (191.6-344.8)	-0.122	0.448
SULT1A1	1759 (1352-2289)	1425 (1154-1760)	-0.199	0.129
UGT1A1	629.7 (528.5-750.3)	649.1 (499.6-843.4)	0.033	0.774
UGT1A4	207.3 (164.1-261.8)	184.9 (131.6-259.8)	-0.106	0.422
UGT1A6	3314 (2759-3981)	3252 (3056-3461)	-0.026	0.813
UGT2A3	930.9 (749.2-1157)	711.0* (576.2-877.3)	-0.241	0.038
UGT2B17	3371 (2618-4340)	2907 (2623-3221)	-0.150	0.209

Table 4.5. Effect of NASH on protein expression of various mouse orthologs of DMEs and transporter proteins. Geometric mean and 95% confidence interval of protein expression in pmol per g of liver tissue. P-value from independent Student's t-test on ln transformed data. * represents p-value <0.05.

Comparison of ratio differences			
Enzyme	Human	Mouse	Human/Mouse trend
BSEP	-0.213	-0.266	↓↓/↓↓
CYP1A2	-0.485	-0.544	↓↓/↓↓
CYP27A1	0.166	-0.137	↔/↔
CYP2C8	-0.177	-0.721	↔/↓↓
CYP2C9	-0.171	-0.626	↔/↓↓
CYP2D6	-0.167	-0.179	↔/↔
CYP2E1	-0.084	0.034	↔/↔
CYP2J2	-0.287	-0.363	↓/↓↓
CYP3A4	-0.325	-0.052	↔/↔
CYP3A5	-0.458	-0.229	↔/↔
CYP4A11	-0.147	0.409	↔/↑↑
CYP4A22	-0.0488	0.213	↔/↑
CYP7B1	-0.257	-0.472	↓/↓
CYP8B1	-0.025	-0.344	↔/↓↓
OATP1B1	-0.041	-0.122	↔/↔
SULT1A1	-0.14	-0.199	↓/↓
UGT1A1	-0.072	0.033	↔/↔
UGT1A4	-0.287	-0.106	↓/↓
UGT1A6	0.441	-0.026	↑/↔
UGT2A3	0.309	-0.241	↑/↓↓
UGT2B17	0.140	-0.150	↑/↓

Table 4.6. Comparison of alteration to DMEs and transporters in mouse and human livers. Values presented are fractional differences between healthy and NASH samples in both species. ↓↓ and ↑↑ represent $p < 0.05$, ↓ and ↑ represent a non-significant trend and ↔ represents no change.

4.8 Figures

Figure 4.1 Effect of NASH on protein expression of various CYP450 proteins in human and mice. Graphs represent ratio of NASH mean protein expression to healthy mean protein expression. Student's t-test was used to test for significance between NASH and healthy groups in each species. *, ** and *** represent p-value <0.05, <0.01 and <0.001, respectively

Figure 4.2. Effect of NASH on protein expression of various CYP450 proteins in human and mice. Graphs represent ratio of NASH mean protein expression to healthy mean protein expression. Student's t-test was used to test for significance between NASH and healthy groups in each species. *, ** and *** represent p-value <0.05, <0.01 and <0.001, respectively

Figure 4.3 Effect of NASH on protein expression of various UGT and drug transporter proteins in human and mice. Graphs represent ratio of NASH mean protein expression to healthy mean protein expression. Student's t-test was used to test for significance between NASH and healthy groups in each species. *, ** and *** represent p-value <0.05, <0.01 and <0.001, respectively

CONCLUSION

This work demonstrates the alterations found in drug disposition proteins in cases of both NAFLD and diabetes. This first part of this dissertation put the methodology in place to accurately analyze the effect of these diseases. Drug transporters are low abundant membrane proteins and therefore traditionally hard to accurately measure. The methodology developed allowed for the quantification of 13 clinically relevant drug transporters that accurately matched literature values. This method was able to additionally be applied to the measurement of phase I and II DMEs. Additionally, the first part showed how enzyme inducing medications, such as phenytoin, remain in the liver after it has been donated to science. As a result, certain medications can significantly alter a metabolic profile as it was seen with the induction of CYP3A4.

With the knowledge gained in part I of this manuscript, part II was then able to accurately measure DMEs and transporters and assess the validity of an American diet-based mouse model. It was seen that numerous phase I and II metabolic enzymes were significantly changing across both NAFLD and diabetes and in some cases, the effect was most likely being exacerbated by both diseases. This is of large concern as patients with NAFLD and diabetes generally have numerous other health conditions that they are being treated for. This indicates that they would be taking numerous medications that have the potential to be altered by the noted effect on the enzymes.

These results translated nicely to the mouse model which showed that most drug metabolizing CYPs were altered in the same direction across species. Although

some of the phase II metabolic enzymes were seen to be differentially changing, this can be explained by the differences in the mouse genome and the difficulty in the measurement of certain proteins. This mouse model is able to represent the realistic changes occurring due to the NAFLD being induced by diet and lifestyle and not genetics. There are no mouse models that will accurately predict all factors of a given disease, but the ALIOS model looks to accurately represent the phase I drug disposition profile of a human with NAFLD and/or diabetes.