THE EFFECT OF OBESITY ON UGT ENZYME EXPRESSION, ACTIVITY
AND MODELED HEPATIC CLEARANCE BY GLUCURONIDATION

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

MOLECULAR BIOSCIENCES AND BIOENGINEERING

DECEMBER 2013

BY STEFFEN GERD OESER

THESIS COMMITTEE:

ABBY C. COLLIER, CHAIRPERSON
JON-PAUL BINGHAM
ROBERT RICHMOND
ALEXANDER J. STOKES

KEYWORDS: UGT, OBESITY, UDP-GLUCURONOSYLTRANSFERASE,
PHARMACOKINETICS, DETOXIFICATION
GENERAL ABSTRACT

The uridine 5'-diphospho-glucuronosyltransferases (UGTs) are, arguably, the most important pathway of conjugative metabolism. Despite this, little is known about how they may be impacted by demographic variables such as obesity or sex. Since obesity is at epidemic proportions worldwide, this has important implications for drug and chemical toxicity. We hypothesized that since obesity causes a number of changes to both the phenotype and function of the liver, that these changes might also impair UGT enzymes. Using biochemical activity assays, mass spectrometry and protein immunoblot, we show that with increasing obesity there is reduced activity for isoforms UGT1A, 1A4, and 1A9. When all obese individuals are classified as “overweight and/or obese” and compared to normal weight individuals (utilizing body mass index categories established by the Centers for Disease Control), we see that isoforms 1A1, 1A6, 1A9 and 2B7 have significantly lower activity for the overweight group. When physiologically-based pharmacokinetic modeling was used, the total liver clearance for the substrates used was significantly lower for UGT1A, 1A9, and 1A4/trifluoperazine (an a-typical antipsychotic drug). Categorized clearance was significantly lower in the obese group for isoforms 1A6 and 2B7. Sex differences exist that are isoform specific. Activities of UGT1A1, 1A6 and 1A9 were significantly decreased with increasing BMI in males, but not females, which showed general 1A to be significantly decreased in the obese group. Finally, an unintended but beneficial outcome from this study was to demonstrate excellent concordance between mass spectrometry and protein levels for UGT1A1, 1A6, 1A9 and 2B7 in these adult samples. In addition, mass spectrometry shows very good concordance
with fluorescence activity for UGT1A1 and 1A9 in adults, demonstrating the utility of fluorescence-based approaches. In summary, the activities of UGT enzymes are decreased by obesity and there may be sex differences within this result.
DEDICATION

I dedicate this thesis to my mother Sharon Dale Oeser; as proud as I know I make her, she also makes me. To my father Gerd (Jerry) Herbert Oeser, a more interesting combination of creativity and logic was never seen, until they had me. To my sister, Christiana, I admire and envy you and your family to no end. I miss you all, every day.
ACKNOWLEDGMENTS

I would like to acknowledge my advisor, Dr. Abby C. Collier, for guidance, advice, feedback, and the opportunity to further my education; my committee members, Drs. Jon-Paul Bingham, Robert Richmond, and Alexander Stokes for advice on my project, results, and positive feedback; my lab mates who have helped me acclimate to the new lab, especially Luc Rougee, Brittany Sato, and Shogo Miyagi; my family who I have missed tremendously, and finally my good friend and colleague Dr. Yvonne Baumer who supplies endless advice, entertainment, and distraction both inside and outside of work.
TABLE OF CONTENTS
GENERAL ABSTRACT ..................................................... III
DEDICATION ................................................................ V
LIST OF FIGURES .......................................................... IX
LIST OF ABBREVIATIONS ................................................. X
PAPERS ARISING FROM THESIS ................................. XIII

CHAPTER 1: GENERAL INTRODUCTION .................. 1
1.1 GENERAL INTRODUCTION ................................. 2
1.1.1 BACKGROUND ..................................................... 2
1.1.2 UGT OVERVIEW .................................................. 3
1.1.3 OBESITY AND BMI ............................................. 4
1.1.4 RELEVANCE ....................................................... 5
1.2 DETAILED INTRODUCTION ............................. 6
1.2.1 OBESITY AND NHANES ................................. 6
1.2.2 HEALTH DISPARITIES FROM OBESITY .......... 7
1.2.3 BODY FAT COMPOSITION AND BMI ............. 10
1.2.4 UGT DYNAMICS IN THE LIVER ......................... 11

CHAPTER 2: MATERIALS AND METHODS .......... 18
2.1 MICROSOME EXTRACTION ................................. 19
2.2 LIVER SAMPLES ..................................................... 19
2.3 UGT WESTERN BLOTTING PROTOCOL ............. 19
2.3.1 WESTERN BLOT FILM TRANSFER .................. 20
2.3.2 WESTERN BLOT ANALYSIS ............................. 20
2.4 FLUORESCENT ACTIVITY ASSAYS : ................. 21
2.4.1 1A1 GLUCURONIDATION ASSAY USING BILIRUBIN 21
2.4.2 UGT1A4 GLUCURONIDATION ASSAY USING TRIFLUOPERAZINE 22
2.4.3 GENERAL 1A GLUCURONIDATION ASSAY USING 4MU 22
2.4.4 1A9 GLUCURONIDATION ASSAY USING 4MU WITH NFA 23
2.5 XENOTECH MASS SPECTROMETRY ASSAY .... 24
2.6 ANALYSIS METHODS ............................................. 25
2.6.1 PHARMACOKINETIC ANALYSIS ...................... 25
2.6.2 STATISTICAL ANALYSIS .................................. 28

CHAPTER 3: RESULTS .................................................. 30
3.1 RELATIVE PROTEIN EXPRESSION, WESTERN BLOTING 31
3.1.1 WESTERN BLOT SAMPLE LAYOUTS ................ 31
3.1.2 WESTERN BLOT REPRESENTATIVE PLOTS ....... 32
3.1.2.1 UGT 1A1 WESTERN BLOT ......................... 32
3.1.2.2 UGT 1A4 WESTERN BLOT ......................... 33
3.1.2.3 UGT 1A6 WESTERN BLOT ......................... 34
3.1.2.4 UGT 1A9 WESTERN BLOT ......................... 35
LIST OF FIGURES

FIGURE 1 - UNITED STATES MALE (LEFT) AND FEMALE (RIGHT) OBESITY TRENDS OVER TIME ........................................... 7
FIGURE 2 - CAUSE, INFLUENCES AND EFFECTS OF OBESITY ......................................................................................... 8
FIGURE 3 - ILLUSTRATIONS OF BODY FAT MEASUREMENTS ................................................................................................ 9
FIGURE 4 - ILLUSTRATION OF CHANGES TO LIVER IN COMPOSITION FROM NORMAL TO FATTY ...................... 11
FIGURE 5 - ILLUSTRATION SHOWING PHASE I AND PHASE II PROCESSES ................................................................. 13
FIGURE 6 - METHODS OF GLUCURONIDATION OF VARIOUS SUBSTRATES ........................................................................ 14
FIGURE 7 - DENDROGRAM SHOWING HOMOLOGY OF UGT ISOFORMS ................................................................................ 15
FIGURE 8 - THEORIZED ANCHORING OF UGT INTO ENDOPLASMIC RETICULUM MEMBRANE ........................................ 16
FIGURE 9 - UGT ISOFORM 1A1 WESTERN BLOT .................................................................................................................. 32
FIGURE 10 - UGT ISOFORM 1A4 WESTERN BLOT .................................................................................................................. 33
FIGURE 11 - UGT ISOFORM 1A6 WESTERN BLOT .................................................................................................................. 34
FIGURE 12 - UGT ISOFORM 1A9 WESTERN BLOT .................................................................................................................. 35
FIGURE 13 - UGT ISOFORM 2B7 WESTERN BLOT .................................................................................................................. 36
FIGURE 14 – PROTEIN EXPRESSION SCATTERPLOTS FOR ISOFORMS 1A1 AND 1A4 ......................................................... 39
FIGURE 15 – PROTEIN EXPRESSION SCATTERPLOTS FOR ISOFORMS 1A6 AND 1A9 ......................................................... 41
FIGURE 16 - PROTEIN EXPRESSION SCATTERPLOTS FOR ISOFORM 2B7 ........................................................................ 43
FIGURE 17 - CATEGORIZED EXPRESSION DATA FOR ALL UGT ISOFORMS EXAMINED .................................................. 45
FIGURE 18 - FLUORESCENCE-BASED ACTIVITY SCATTERPLOTS UGT ISOFORMS 1A AND 1A1 .............................. 48
FIGURE 19 - FLUORESCENCE-BASED ACTIVITY SCATTERPLOTS FOR ISOFORMS 1A9 ................................................. 50
FIGURE 20 - CATEGORIZED FLUORESCENCE-BASED ACTIVITY DATA FOR ALL ISOFORMS EXAMINED .................. 51
FIGURE 21 - ACTIVITY SCATTERPLOTS AS MEASURED BY MASS SPECTROMETRY FOR ISOFORMS 1A1 AND UGT .......... 54
FIGURE 22 - ACTIVITY SCATTERPLOTS AS MEASURED BY MASS SPEC FOR UGT ISOFORMS 1A4 AND 1A6 ...... 56
FIGURE 23 - ACTIVITY SCATTERPLOTS AS MEASURED BY MASS SPECTROMETRY FOR UGT ISOFORMS 1A9 AND 2B7 .......... 58
FIGURE 24 - CATEGORIZED ACTIVITY DATA UTILIZING MASS SPEC FOR GENERAL UGT ISOFORMS .......................... 60
FIGURE 25 - MASS SPECTROMETRY BASED ACTIVITY MEASUREMENTS VS. RELATIVE PROTEIN DENSITY .......... 63
FIGURE 26 - FLUORESCENCE-BASED ACTIVITY MEASUREMENTS VS. RELATIVE PROTEIN DENSITY ......................... 65
FIGURE 27 - MASS SPECTROMETRY-BASED ACTIVITY MEASUREMENTS VS. FLUORESCENCE-BASED ACTIVITY .......... 66
FIGURE 28 - WELL-STIRRED MODELED HEPATIC CLEARANCE FOR ALL SAMPLES VS. BODY MASS INDEX ........... 69
FIGURE 29 - FEMALE WELL-STIRRED MODELED HEPATIC CLEARANCE VS. BMI ......................................................... 71
FIGURE 30 - MALE WELL-STIRRED MODELED HEPATIC CLEARANCE VS. BMI .............................................................. 72
FIGURE 31 - MODELED HEPATIC WELL-STIRRED (WS) CLEARANCE MEASUREMENTS VS. BMI CATEGORY ..... 74
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>4MUG</td>
<td>4-methyl umbelliferone glucuronide</td>
</tr>
<tr>
<td>4MU</td>
<td>4-methylumbelliferone sodium salt</td>
</tr>
<tr>
<td>NADPH</td>
<td>alpha-nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BPA</td>
<td>bisphenol A</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>body surface area</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>c</td>
<td>concentration</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>CDC</td>
<td>centers for disease control</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ε</td>
<td>extinction coefficient</td>
</tr>
<tr>
<td>fu</td>
<td>fraction unbound</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>HLM</td>
<td>human liver microsome</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>MgCl</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>m</td>
<td>meter</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µL</td>
<td>microliter</td>
</tr>
<tr>
<td>µM</td>
<td>micromoles per liter</td>
</tr>
<tr>
<td>mGST</td>
<td>microsomal glutathione-S-transferase</td>
</tr>
<tr>
<td>MPPGL</td>
<td>microsomal protein per gram liver</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimoles per liter</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>Code</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>UGT1A7</td>
<td>UDP-glucuronosyltransferase 1A7 isoform</td>
</tr>
<tr>
<td>UGT2A</td>
<td>UDP-glucuronosyltransferase 2A subfamily</td>
</tr>
<tr>
<td>UGT2B</td>
<td>UDP-glucuronosyltransferase 2B subfamily</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>UDP-glucuronosyltransferase 2B7 isoform</td>
</tr>
<tr>
<td>UGT3A</td>
<td>UDP-glucuronosyltransferase 3A subfamily</td>
</tr>
<tr>
<td>UGTIA1</td>
<td>UDP-glucuronosyltransferase IA1 isoform</td>
</tr>
<tr>
<td>UGTIA</td>
<td>UDP-glucuronosyltransferase IA subfamily</td>
</tr>
<tr>
<td>UGTIA3</td>
<td>UDP-glucuronosyltransferase IA3 isoform</td>
</tr>
<tr>
<td>UGTIA6</td>
<td>UDP-glucuronosyltransferase IA6 isoform</td>
</tr>
<tr>
<td>UGTIA8</td>
<td>UDP-glucuronosyltransferase IA8 isoform</td>
</tr>
<tr>
<td>UGTIA9</td>
<td>UDP-glucuronosyltransferase IA9 isoform</td>
</tr>
<tr>
<td>UGTIA4</td>
<td>UDP-glucuronosyltransferase IA4 isoform</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferase super-family</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>λ</td>
<td>wavelength</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
</tbody>
</table>
PAPERS ARISING FROM THESIS

Papers in preparation

Oeser, S.G., Rougée, L.R. and Collier, A.C., Obesity negatively impacts UGT isoform activity and hepatic clearance. *In preparation.*
CHAPTER 1: GENERAL INTRODUCTION
1.1 GENERAL INTRODUCTION

1.1.1 BACKGROUND
In the mid 1800’s the first experiments were conducted that began to show how ingested compounds (now typically called xenobiotics) were transformed into chemicals, which were then ultimately eliminated from the human body (Murphy 2001). Over the next 100 years a continuous flow of information collected on metabolic research resulted in the publication of “Detoxification Mechanisms” by Richard Williams, which delineated drug metabolism as a unique branch of scientific discovery (Williams 1959). Since then, the field of drug metabolism has continually evolved to incorporate varying types of xenobiotic transformation, the separation of phase I and phase II metabolism processes and the mechanisms by which metabolism is regulated. Initially the Cytochrome P450’s, a family of enzymes responsible for a large portion of phase I metabolism discovered in the 1960s (Omura and Sato 1964), became the main focus for drug metabolism and held the spotlight for over 30 years. More recently, Uridine 5’-diphospho-glucuronosyltransferases (UGTs) and other Phase II enzymes have been focused on in research as they are responsible for the ultimate step in detoxification of a large number of the top 200 prescribed drugs as well as other environmental chemicals (Williams, Hyland et al. 2004).

The UGTs are a family of membrane bound enzymes located in the endoplasmic reticulum that are approximately 550 amino acids long. They were first widely associated with the detoxification of bilirubin, a yellowish breakdown product from red blood cells REF. Bilirubin is responsible for the yellow color associated with bruises,
urine, and jaundice. Deficient conjugation with bilirubin leads to some severe diseases such as Crigler Najar and Gilberts syndrome. Additionally, UGTs are critical for detoxifying a number of environmental compounds including bisphenol A (BPA) and benzo[α]pyrene (from cooked meats) (Mazur, Kenneke et al. 2010). While the liver is the primary site of glucuronidation, intestinal UGTs may affect oral bioavailability of several drugs and dietary supplements, and may be responsible for chemoprevention in this tissue (Tukey and Strassburg 2000). If we are able to identify a link between obesity and dysregulation of UGT activity, it would give us further insight into the ways in which obesity can affect the health and detoxification of a person, and possible avenues of pursuit for treatment.

1.1.2 UGT OVERVIEW
The uridine 5'-diphospho-glucuronosyl transferases (UGTs) are responsible for the inactivation of a broad spectrum of prescribed drugs, chemicals and endobiotics (Williams, Hyland et al. 2004). These enzymes catalyze the addition of glucuronic acid to lipophilic substrates in order to increase their polarity and facilitate excretion. Currently, more than 40% of clinically used drugs are subjected to glucuronidation (Wells, Mackenzie et al. 2004). In addition to clinical drugs, UGT enzymes assist in protection against environmental toxins, carcinogens, and dietary toxins as well as regulating numerous endogenous molecules, including bilirubin, steroid hormones and bile acids (Guillemette 2003). The UGT superfamily is split into the UGT1, UGT2, and UGT3 gene families, which are further broken down into the UGT1A, UGT2A, UGT2B, and UGT3A subfamily of enzymes (Meech and Mackenzie 2010). Due to their involvement in many
detoxification processes, the UGT1A and UGT2B subfamilies are of significant interest in drug metabolism research. The UGT2A family are only found in the nasal epithelia (Jedlitschky, Cassidy et al. 1999) and are involved in olfaction, while the UGT3A isoforms were only recently discovered are of unknown function. Hence, the UGT2A and 3A isoforms will not be considered further here.

Although a number of organs participate in detoxifying the body, the majority of detoxification occurs in the liver. This critical organ has the ability to regenerate both its mass and functionality after physical injury, conditions such as non-alcoholic fatty liver disease, associated with obesity, can cause progressive deterioration of the liver and its ability to function properly (Kirovski, Schacherer et al. 2010; Gandhi 2012).

1.1.3 OBESITY AND BMI
According to the Centers for Disease Control, as of 2010, ~36% of U.S. adults over the age of 20 are considered obese as calculated by a Body Mass Index (BMI) greater than 30 kg/m² (Flegal, Carroll et al. 2012; Ogden, Carroll et al. 2012). In addition to multiple adverse health effects, obesity results in morphological and physiological liver changes such as: hepatomegaly, inflammation, steatosis, reduced high-density lipoprotein levels, and insulin resistance (Kirovski, Schacherer et al. 2010; Walsky, Bauman et al. 2012).

Since the greatest abundance of UGTs is found in the liver, these alterations have the potential to impact the expression and activities of these enzymes (Guillemette 2003). Recently, Xu et al. discovered that the expression and activity of UGT isoforms were dysregulated in the livers of obese mice and mice with fasting-induced steatosis (Xu,
Kulkarni et al. 2012). Alterations in glucuronidation rates can have extensive consequences; deficient glucuronidation can result in the accumulation and toxicity of chemicals, while greater elimination can inappropriately remove necessary compounds (Wells, Mackenzie et al. 2004). Toxicity is of great concern in the development of clinical drugs, the efficacy of which depends greatly on the characteristics of the drug (acidity, polarity, size, etc.), as well as the inherent variability of the enzymes and transporters within the patient. Predicting an individual’s capacity to clear a drug requires detailed quantitative models (Wells, Mackenzie et al. 2004).

1.1.4 RELEVANCE
If UGTs are not able to detoxify the body due to impaired production from obesity, we must examine where and how these changes are occurring in order to repair UGT activity or find an alternate route of detoxification. This is particularly relevant to the increased requirement for altered drug dosing in obese patients. At present, there are few rationally based clinical guidelines for altered dosing in the obese, as dosing schedules have come into being through trial and error by experiences clinicians. In this study, we have investigated the impact of obesity, as well as the potential for sex differences to affect the expression and activity of major drug metabolizing UGT isoforms.
1.2 DETAILED INTRODUCTION

1.2.1 OBESITY AND NHANES
The National Center for Health Statistics (NCHS) in conjunction with the Centers for Disease Control and Prevention (CDC) utilizes a National Health and Nutrition Examination Survey (NHANES), initiated in 1960, to measure and track the health and nutritional status of adults and children in the United States (Flegal, Carroll et al. 2012). The CDC.gov website describes that the NHANES survey examines a nationally representative sample of about 5,000 persons each year and since 1999 has become a continuous program which has a changing focus on a variety of health and nutrition measurements to meet emerging needs. The NHANES interview includes demographic, socioeconomic, dietary, and health-related questions while the examination component consists of medical, dental, and physiological measurements, as well as laboratory tests administered by medical personnel. The data from this survey is used to establish prevalence rates of chronic conditions in the population as well as examine risk factors for certain diseases or conditions. While the survey studies many different diseases, medical conditions, and health indicators, one particularly useful set of information are the data related to obesity, which has become and increasing epidemic. Utilizing the NHANES, the CDC estimates that as of 2010, 35.7% of U.S. adults over the age of 20 are considered obese as calculated by a Body Mass Index (BMI) greater than 30 kg/m² (Flegal, Carroll et al. 2012; Ogden, Carroll et al. 2012). While the percentages of overweight male and female adults has stayed relatively stable over the last 20 years, obese and morbidly obese adults have significantly increased during the same period of time (Figure 1).
1.2.2 HEALTH DISPARITIES FROM OBESITY

Obesity is the excess accumulation of fat, which often times results in a number of adverse health effects, such as diabetes, heart disease, hypertension, and cancer all of which ultimately result in lowered life expectancy which makes it the leading preventable cause of death worldwide (Bray 2004). The primary causes of obesity are excessive food intake and decreased physical activity. Secondary causes can be genetic, medical, or psychiatric, although these causes are far fewer in abundance than the primary cause. In his publication, Bray et al. states that the effects of obesity result from two factors: the increased mass of adipose tissue and the increased secretion of pathogenetic products from enlarged fat cells (Figure 2) (Bray 2004).
social stigma, sleep apnea, and osteoarthritis, while the latter contributes to metabolic factors like insulin-resistance, proinflammatory state, procoagulant state, increased risk of cardiovascular disease and hypertension, as well as increased risk of cancer all of which results in an increased risk of shortened life expectancy (Bray 2004).

1.2.3 MEASUREMENTS OF BODY FAT COMPOSITION

Measuring this excess body fat can be accomplished by any number of methods (Figure 3) (Perry 2010). Skin fold calipers can be used to measure subcutaneous fat amounts from various locations around the body. These measurements should be performed by skilled measurers and are accurate if done so, but can be time consuming. Calipers also have a limited range of opening, so measuring extremely obese individuals may not be
possible if the skin folds are too large. Bioelectric impedance measurements involve running electric current through the body (typically through the feet) to measure resistance caused by fat in the body. While quick and convenient, this measurement is often inaccurate and not reproducible. Differences in hydration levels can cause problems with the accuracy of the readings as well. Hydrostatic weighing involves being weighed while submerged under water (after exhaling all air in lungs) to determine overall density and determine body fat composition relative to bone and muscle. Up until a few years ago, this measurement was the gold standard of analysis given its high accuracy and reproducibility. Unfortunately it is inconvenient, time consuming and expensive to perform. Current dual-energy X-ray absorptiometry (DEXA) scans have become the newest gold standard measurement for body fat composition. It is extremely reproducible and accurate. Results of the scan allow total body mineral, lean mass, and fat tissue mass of an individual. Unfortunately it is expensive and likely the machine is only found in medical centers. The most common measuring technique is based on anthropometric measurements that rely on physical exterior body measurements such as height, weight, or circumference measurements to estimate body fat composition. This measurement only requires a tape measurer and a scale. Its easy to administer and cheap, but questionable accuracy due to it being a correlative estimate and depending on the numbers of body areas that are measured (Wang, Deurenberg et al. 1998).

Figure 3 - Illustrations of body fat measurements from left to right (bioelectric impedance, skin fold calipers, DEXA scan, dunk tank, and anthropomorphic).
1.2.4 BODY FAT COMPOSITION AND BMI

Body Mass Index (BMI) the most commonly used method for measuring body fat composition is based on anthropometric measurements. It takes height and weight in order to estimate body fat composition on a scale with units of kg/m$^2$. Since height and weight are commonly taken during doctor visits and for pathology samples, this measurement is most commonly used as an indicator of body fat composition.

Unfortunately this measurement does not work well across various races or for highly muscular individuals (Wang, Deurenberg et al. 1998). A recent publication indicates that BMI, when compared to DEXA scans, was inaccurate up to 1/3 of the time (Kennedy, Shea et al. 2009), correcting BMI based on this data would make 64% of American women obese. A drawback to using BMI is that the cutoffs used can vary over time and between populations as it is based on the relationship between body weight and disease/death. Another drawback to using BMI as an indicator of body fat composition is that the health disparities associated with obesity increase if a majority of body fat is localized around the abdomen (Berentzen, Angquist et al. 2012). Even with these drawbacks, BMI is still used most frequently because of the ease at obtaining it and frequency of utilization. It is presumed that abdominal fat mass is more highly associated with negative health outcomes because the abdominal fat tends to incorporate into the organs in and around the stomach area. Such accumulation can lead to dysregulation of organ function, as is common in the liver in obese individuals (Marchesini, Moscatiello et al. 2008). Changes to the liver from obesity can include increased size (hepatomegaly), inflammation, increased fat deposits (steatosis), reduced HDL levels, and insulin resistance (Figure 4) (MFMER 1998-2013). Since the liver is a major site of
detoxification, protein synthesis, digestion biochemicals production, glycogen storage, decomposition of red blood cells, and hormone production it is imperative that any changes to the liver from obesity be examined for root cause. While the liver has the capability of performing compensatory growth from as little at 25% in an attempt to maintain function, it is not capable of complete regeneration, which is an important distinction. The liver can grow in size in order to compensate for loss from injury, but diseases such as hepatitis, cirrhosis, fatty liver, and cancer, are throughout the liver and so cannot be overcome if disease is not treated.

1.2.5 UGT DYNAMICS IN THE LIVER
Life has evolved a complex system for detoxifying the body from interactions that occurs within an organism, endobiotics, and the outside world, xenobiotics, on a daily basis. Presently the word xenobiotics is used typically to describe any number of drugs, pollutants, pesticides, and If natural or artificial substances that may be ingested or

Figure 4 - illustration of changes to liver in composition from normal (upper left) to fatty (upper right) as well as size in normal (lower left)
acquired from interaction with the environment. The body must work quickly to remove these chemicals from the body to minimize any injury to the system from toxicity. To combat these toxins, the body has an arsenal of metabolic enzymes that aid in deactivating, then excreting these toxins as well as breaking down internal/endogenous substances that must be removed to prevent toxicity. It typically does this by initially activating them through various methods such as hydrolysis, hydration, oxidation, or reduction, before conjugating the newly activated compound with glucuronic or sulphuric acid, or glutathione and then excreting it in bile or urine. This process has been divided into two processes termed, phase I and phase II metabolism. The enzymes involved in phase I metabolism typically act by adding or exposing a functional group such as –OH, -NH$_2$, or –SH, which usually inactivates the xenobiotic and tags it for conversion into metabolites that can either be either directly excreted or become water soluble conjugates (Figure 5) (Anderson 2005). The most well known phase I enzyme class are the cyochrome P450s (CYP450s) that act on well-known chemicals like acetaminophen, ethanol, caffeine, progesterone, and naproxen to name a few. Phase II enzymes are commonly conjugation enzymes that form a covalent linkage to the drug/xenobiotic and glucuronic acid, sulfate, glutathione, amino acids, or acetate. Once conjugated, these drugs become largely inactivated and polar which allows them to be easily excreted in
bile or urine. Uridine 5’-diphospho-glucuronosyltransferases (UDP-glucuronosyltransferases or UGTs for short) are the most well known class of the phase II enzymes, which work by glucuronidation, the addition of the glycosyl group from a UTP-sugar (UDPGA) to the foreign substrate/chemical like nicotine, ibuprofin, warfarin and acetaminophen as well (Figure 6) (Jancova, Anzenbacher et al. 2010). Currently more than 40% of clinically used drugs are subject to glucuronidation and so it is an important class of enzymes to examine (Court 2010). Most of these enzymes are produced in large
quantities in the liver, a key organ for detoxifying the body, although other organs also participate in metabolism.

1.2.6 UGT AND DETOXIFICATION

A large majority of research on xenobiotic metabolism has focused on the CYP450s, which are fairly well characterized to date (although more information is continually being added about this class of enzymes). UGT characterization is increasingly a growing field of interest in pharmacology and xenobiotic metabolism largely due to the fact that they are responsible for aiding in the detoxification of a large number of the top 200 drugs (Meech and Mackenzie 2010). It is well known that this detoxification assists in the protection against environmental toxins, carcinogens, dietary toxins and participates in the homeostasis of numerous endogenous molecules, including bilirubin, steroid hormones and biliary acids (Guillemette 2003).

Figure 6 - Methods of glucuronidation of various substrates showing the sugar donor (UDPGA) and conjugated products.
The UGT family of enzymes is split into the UGT1, UGT2, and UGT3, which are further broken down into the isoforms UGT1a, UGT2a, UGT2b, and UGT3a (Figure 7) (Meech and Mackenzie 2010; Rowland, Miners et al. 2013). Generally the UGTs are about 530 amino acids (AAs) in length, with approximately 25 AAs being attributed to a signaling protein which acts to direct the enzyme to the endoplasmic reticulum and is subsequently cleaved off. The 1A isoform, employing alternatively spliced variants, is responsible for a total of 9 variants (labeled 1A1, 1A3, etc) while the UGT2 family is split up into 2A and 2B. The newly discovered UGT3 family is still in the process of being characterized, but appears as though UGT3A has isoforms 1 and 2. While UGTs are present across multiple species (based on sequence homology) the isoforms are not always structurally
identical. The details of the production and anchoring of the various isoforms is still under investigation, but the current hypothesis is that the carboxyl group extends out into the cytosol, with a helix domain anchoring that portion through the membrane of the endoplasmic reticulum into the lumen (Figure 8)(Rouleau, Collin et al. 2013).

Figure 8 - Theorized anchoring of UGT into endoplasmic reticulum membrane.
With the importance that UGTs play in detoxifying the body, it is important to continue to characterize any sources that may impair or induce action of these enzymes. Since these enzymes are primarily produced in the liver and obesity is known to modify the liver both in form and function, it is necessary to determine whether obesity may impact the production and function of these critical enzymes.
CHAPTER 2: MATERIALS AND METHODS
2.1 Microsome Extraction
Homogenization buffer was created by combining 2mM phenylmethanesulfonyl fluoride (dissolved in DMSO) to 0.1 M Tris-HCl buffer with 5mM MgCl₂ at pH 7. This was added 1:4 (w:v) ratio to tissue and homogenized before centrifugation at 10,000g for 20 minutes at 4°C. S9 postmitochondrial fraction was transferred to an ultracentrifuge tube and spun at 100,000g for 20 minutes at 4°C for 1 hour. This supernatant was transferred to a new microcentrifuge tube and stored at -80°C (Collier, Tingle et al. 2000).

2.2 Liver Samples
Human liver microsomes (HLM) were sourced from Xenotech, LLC (Lenexa, KS) (n = 23) and Invitrogen Inc. (San Diego, CA) (n = 13). Microsomes were re-suspended in 0.1 M Tris buffer with 5 mM MgCl₂ pH 7.4, and protein content was assayed by the bicinchoninic acid method using bovine serum albumin as a standard (Smith, Krohn et al. 1985).

2.3 UGT Western Blotting Protocol
Protein expression was assessed using SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. Primary antibodies for each isoform were generously gifted by Professor Michael Coughtrie from the University of Dundee (Scotland, UK). Briefly, 4 µg of individual adult microsome samples and pooled (n = 200) adult liver microsome positive control were resolved on a 10% SDS-PAGE gel under reducing conditions, transferred to polyvinylidene difluoride membranes with semi-dry transfer (Biorad, Hercules, CA) then blocked in Tris-HCl buffered saline pH 9.0 (TBS) with 0.1% v/v
Tween 20 (TBST) and 2% (w/v) fish skin gelatin overnight at 4 °C. Subsequently, membranes were probed with primary antibodies (1:8,000) diluted in TBST with 2% fish skin blocker at room temperature (RT) for three hours. Membranes were washed four times for ten minutes each with TBST and subsequently incubated with secondary donkey-anti-sheep IgG-horseradish peroxidase antibody (Donkey anti Sheep HRP from Jackson Labs p/n 713-035-003) (1:40,000) diluted in TBST for 1 h at RT. Membranes were again washed four times for ten minutes and developed using chemiluminescent detection ECL+ (Perkin Elmer NEL105001EA). (Coughtrie, Burchell et al. 1987)

2.3.1 Western Blot Film Transfer
Generally with the above western blot protocol, to undertake transfer to film, the exposure time was between 15 seconds to 1 min, with membranes imaged using Blue Devil Film (p/ n #30-101). Exposure was as minimized to avoid possible oversaturation, these were then developed (Konica SRX-101A) then digitized using a scanner (Canon Canoscan 9000F 32-bit).

2.3.2 Western Blot Analysis
Resulting blot intensities were extracted using ImageJ software (version 1.45s). Briefly, image was inverted, then equal sized boxes were used to extract intensities of each band. The intensity of the sample bands from each blot were divided by the intensity from the pooled sample to perform interblot normalization. Prism Software (version 5.0b) was then used to perform statistical calculations and create graphs.
2.4 Fluorescent Activity Assays:

2.4.1 1A1 Glucuronidation Assay Using Bilirubin

Background: This assay measured UGT1A1 activity by quantifying the amount of bilirubin glucuronide, however it slightly underreports the activity since it does not take into account the difference between mono-glucuronides and di-glucuronides.

Method: Diazo-reagent mixture was prepared by combining: 0.1 mL of ethyl anthranilate (0.66 mmol) suspended in 10 mL of 0.15M HCl then treated with 0.3 mL of NaNO₂ (5 mg/ml) and incubated at room temperature for 5 mins. Subsequently it was treated with 0.1 mL of ammonium sulphamate (10 mg/mL) and incubated at room temperature for 3 mins before use.

Microsomes in Tri-HCl buffer for a final concentration of 0.5 mg/mL were added to 50 ug/mg protein alamethicin in DMSO, 125 µM bilirubin in 0.1 M NaOH with 1 mM EDTA, 0.4 M Tris-HCl buffer with 5 mM MgCl₂ at pH 7.7 and UDPGA in Tris-HCl buffer at a final concentration of 5 mM. This was immediately incubated in dark at 37°C in water bath for 15 mins then and equal volumes of glycine-HCl buffer (pH 2.7) were added to stop reaction. After placing on ice 1/2 volume of ethyl anthranilate diazo-reagent was added before incubating at room temperature for 30 minutes. Reaction was stopped by adding 1/3 volume of freshly prepared ascorbic acid solution (10%) then iced for a few minutes before equal volumes of 2-pentanone:n-butyl acetate (17:3, v/v) were added before freezing overnight. After freezing solid, solution was thawed, vortexed for another 10 seconds and centrifuged for 2500g for 10 minutes. Lastly, the mixture was frozen again and absorbance of the top (unfrozen) phase was measured at 530 nm in a clear 96 well plate that was resistant to organic solvents.
Concentrations were calculated using $A = \varepsilon \times c \times l$, where $A$ is absorbance, $\varepsilon$ is the molar extinction coefficient ($44.4 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$ at 530 nm), $c$ is concentration and $l$ is path length of the sample. (Doostdar, Grant et al. 1993), (Heirwegh, Fevery et al. 1974), (Viollon-Abadie, Lassere et al. 1999), (Viollon-Abadie, Bigot-Lasserre et al. 2000)

2.4.2 UGT1A4 Glucuronidation Assay Using Trifluoperazine

**Background:** This assay measured UGT1A4 activity with substrate depletion of trifluoperazine (TFP) detected by fluorescence, which was quenched by formation of glucuronide.

**Method:** Microplate was placed on ice and the following were combined: Microsomal protein (0.1 mg/mL), alamethicin (50 µg/mg protein at 1 mg/mL in DMSO), hecogenin, if desired (200 µM in ethanol), TFP (100 µM), and 50 mM Tris-HCl pH 7.5 which contained 10 mM MgCl$_2$. This mixture was incubated at 37°C in microplate reader for 5 minutes before UDPGA (5 mM) was added which initiated the reaction. Emission was read at $\lambda = 475$ nm with excitation of $\lambda = 310$ nm every minute for 60 mins. (Uchaipichat, Mackenzie et al. 2006; Miyagi and Collier 2007)

2.4.3 General 1A Glucuronidation Assay Using 4MU

**Background:** This assay measured general UGT activity. Isoforms known to metabolize 4MU include UGT1A1, 1A3, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17. Isoforms of the UGT1A family have higher capacity and lower affinity for the substrate.
**Method:** In a microplate on ice the following were added: Alamethicin dissolved in DMSO to final concentration of 0.5 µg/mg protein (final concentration of DMSO did not exceed 1% v/v), microsomal sample protein (20 mg/mL in Tri-HCl buffer for a final concentration of 0.5 mg/mL), tris buffer pH 7.4 contained 5 mM MgCl₂ and 4MU (final concentration of 100 µM) and 0.05 mg/ug protein alamethicin. The reaction mixture was incubated at 37°C in microplate reader for 2 min before UDPGA was added to final concentration 5 mM which initiated the reaction. Fluorescence was read immediately at \( \lambda_{ex} = 355 \text{ nm}, \lambda_{em} = 460 \text{ nm} \) every minute for 15 min. (Collier, Tingle et al. 2000; Miners, Bowalgaha et al. 2011)

**2.4.4 1A9 Glucuronidation Assay Using 4MU with NFA**

To the general 1A glucuronidation assay (above) also added niflumic acid inhibitor (2.5 µM final concentration) which inhibited activity of all isoforms except UGT1A9.

Incubated this reaction mixture at 37°C in microplate reader for 2 min before UDPGA was added to a final concentration 5 mM which initiated the reaction. Fluorescence was read immediately at \( \lambda_{ex} = 355 \text{ nm}, \lambda_{em} = 460 \text{ nm} \) every minute for 15 min. (Collier, Tingle et al. 2000; Miners, Bowalgaha et al. 2011)
2.5 Xenotech Mass Spectrometry Assay
UDP-Glucuronosyltransferase Characterization (supplied by Xenotech)

Reaction volumes were typically 200µL and consisted of a final concentration of 0.5 mM CHAPS, 50 µg/mg protein alamethicin, 100mM Tris HCl, ph 8.0 at RT, 1mM EDTA, 10mM MgCl2, 0.1mM saccharic acid 1,4-lactone, and 8mM UDPGA. The reactions were stopped with 2% v/v formic acid in acetonitrile for UGT isoforms 1A1, 1A4, 1A6, 1A9 and 1% v/v perchloric acid for isoform 2B7. Protein, substrates and incubation times for the isoforms were as follows: 1A1 [50 µg/mL microsome, beta-estradiol, 10 min], 1A4 [250µg/mL microsome, 0.025mM trifluoperazine, 5 min], 1A6 [10µg/mL microsome, 0.5mM naphthol, 10 min], 1A9 [50µg/mL microsome, 0.05mM propofol, 10 min], 2B7 [50µg/mL microsome, 1mM morphine, 10 min]. Manual incubations were typically conducted in glass culture tubes. Zero-time incubations served as blanks and zero-time incubations spiked with metabolite standard (typically samples ranged between 0.025 to 5 µM) served as the metabolite standards. Microsomes (diluted to x20 the final incubation concentration) were pre-incubated in the presence of an activator. Three basic microsomal activation conditions could have been used: native (no activator), CHAPS or alamethicin. Each activator was prepared in 20 mM EDTA, pH 7.4. The microsomal activation was typically performed by adding equal volume of the protein and activator solution (50% microsomes, 50% activator/EDTA; v/v) and was preincubated for 15-25 min, on ice, prior to incubation with the substrate.

Liver microsomes were incubated at 37 ± 1°C in mixtures that contained Tris-HCl (100 mM), EDTA (1.0 mM), MgCl2 (10 mM), D-saccharic acid 1,4-lactone (100 µM),
UDPGA (8.0 mM) and substrate at the final concentrations indicated. Reactions were started by the addition of the cofactor, UDPGA, and were usually stopped after zero to 10 minutes by the addition of 175 µL of Stop Reagent. Precipitated protein was removed by centrifugation (400-2500 x g for 5-15 min at 5-25°C). A portion of the supernatant fraction was analyzed by LC-MS/MS.

2.6 ANALYSIS METHODS

2.6.1 Pharmacokinetic Analysis

**Obesity**

Individual classification of obesity was determined using BMI, calculated as body weight in kg divided by the square of the height in meters. Individuals were classified into categories based on BMI (Table 1) (NIH 1998).

**Pharmacokinetics, Scaling and Statistical Analyses**

Using in vitro enzyme kinetic results, hepatic drug clearance was modeled using both the well-stirred (Equation 1) and parallel tube (Equation 2) models:

\[
(Eq. 1) \quad Cl_{hepatic} (L/hr) = \frac{Q_{hepatic} \times f_u \times Cl_{int(whole Liver)}}{Q_{hepatic} + f_u \times Cl_{int(whole Liver)}}
\]

\[
(Eq. 2) \quad Cl_{hepatic}(L/hr) = Q_{hepatic} \times \left( \frac{-Cl_{int(whole Liver)} \times f_u}{Q_{hepatic}} \right) \times 1 - e^{-Cl_{int(whole Liver)} \times f_u / Q_{hepatic}}
\]
In these equations, $Cl_{hepatic}$ is the modeled hepatic clearance, $Q_{hepatic}$ is hepatic blood flow, and $f_u$ is the fraction of the drug unbound. Since microsomes were used for incubations, the unbound fraction in microsomes ($f_{u,mic}$) was used to adjust for non-specific binding. Experimental values reported in the literature were used wherever possible (Table 2). In the absence of such values, $f_{u,mic}$ was determined experimentally by loading 0.1 mg/mL of protein, 5 µg/mL alamethicin, 0.5 mM naphthol with 10 µg/mL of XT200 (Xenotech XTreme 200 Pool of 100 male and 100 female HLMs, P/N H2630) microsomal protein in 100 mM Tris-HCL, and shaking for 1 h in a micro-equilibrium dialyzer (Hoefer, Inc. P/N SP-74162501D) before quantifying unbound protein that crossed the membrane.

Experimental intrinsic clearances from microsomes ($CL_{int(microsomes)} = \frac{V_{max}}{K_m}$) was scaled up to whole liver hepatic intrinsic clearance ($Cl_{int(whole\ liver)}$) by using values of microsomal protein per gram of liver (MPPGL) and liver weight (LW) (Equation 3).

Literature values for $K_m$ were used to calculate intrinsic clearance (Table 3).

$\text{(Eq. 3)} \ Cl_{int(whole\ liver)} = Cl_{int(microsomes)}(L/min/mg) \times MPPGL(mg/g) \times LW (g)$

**Allometric Model**

For comparison, an allometric model was used. Since the MPPGL value was unknown, the standard variable of 40 mg/g was used (Barter, Chowdry et al. 2008). A standard liver weight of 1500 g and an average hepatic blood flow of 90 L/hr for an adult were assumed for allometry.
In Vitro to In Vivo Extrapolation (IVIVE) Model

A previously described IVIVE model was used (Howgate, Rowland Yeo et al. 2006; Ghobadi, Johnson et al. 2011) using equations that take into account individual variability in system parameters and obesity. The following equations were used:

Since body surface area (BSA) correlates with organ sizes and blood flow better than height and weight alone, a BSA value for each individual was calculated using the Du Bois and Du Bois equation (Equation 4) (Du Bois and Du Bois 1916):

(Eq. 4) $BSA(m^2) = \text{weight}(kg)^{0.425} \times \text{height}(cm)^{0.725} \times 0.007184$

Based on in vivo subject data from published models of liver size, liver volume (LV) has been related to BSA (Equation 5) (Johnson, Tucker et al. 2005):

(Eq. 5) $LV(L) = \left( \sqrt{BSA} \times 0.72 + 0.171 \right)^3$

However, this equation only agrees with a BMI $\leq 40 \text{ kg/m}^2$, so a scaling factor of 1.25 was used to compensate for individuals with a BMI greater than 40 kg/m$^2$ (Ghobadi, Johnson et al. 2011).

To convert $LV$ to liver weight ($LW$), $LV$ was multiplied by liver density, 1,080 g/L (Equation 6) (Heinemann, Wischhusen et al. 1999):

(Eq. 6) $LW(g) = LV(L) \times \text{density}(g/L)$

Based on age in years, individual MPPGLs were calculated (Equation 7) (Barter, Chowdry et al. 2008):

(Eq. 7) $MPPGL(\text{mg/g}) = 10^{(1.407 + 0.0158 \times \text{age} - 0.00038 \times \text{age}^2 + 0.000024 \times \text{age}^3)}$
The relationship between cardiac output (CO) and BSA was used to determine individual $Q_{hepatic}$ values. The cardiac index as a function of age in adults (ages > 20) (Equation 8) was converted to CO by multiplying by individual BSA (Equation 9) (Howgate, Rowland Yeo et al. 2006):

\[(Eq. 8) \text{Cardiac index}(L/min/m^2) = 3 + (0.01 \times (age(\text{years}) - 20))\]

\[(Eq. 9) \text{Cardiac output}(L/h) = \text{cardiac index}(L/min/m^2) \times 60(\text{min/hr}) \times BSA(m^2)\]

By multiplying CO with the percentage of blood flow to the liver, cardiac output was converted to $Q_{hepatic}$. Current evidence does not suggest a significant difference in $Q_{hepatic}$ between normal weight and obese individuals, so percent of blood flow to the liver was modified to account for relative increases through BSA (Ghobadi, Johnson et al. 2011). Similar adjustments were also made to account for differences in $Q_{hepatic}$ contribution between sex (male vs. female), as well as BMI classification (healthy vs. overweight).

2.6.2 Statistical Analysis
Analysis was performed using GraphPad Prism 5 (La Jolla, CA). All data for BMI was treated as continuous data for correlation analysis using the Pearson’s test for linear correlation when the data was found to be normally distributed and Spearman’s test for monotonic correlation when the data was not normally distributed. Normality of the data was tested using the D’Agostino-Pearson omnibus normality test.

The data for activity and clearance was then categorized based on BMI category (Table 1) into normal weight (NW) and overweight (OW) groups. The groups were compared by an unpaired two-tailed t-test (with Welch’s correction when variances were statistically
different as determined by F-test). Data was then separated by sex and analyzed again as described above. Finally, an unpaired two-tailed t-test was used to determine any overall differences between males and females.

Table 1. BMI category ranges used for analysis

<table>
<thead>
<tr>
<th>Category</th>
<th>BMI Range (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal BMI (NW)</td>
<td>18.5 – 24.9</td>
</tr>
<tr>
<td>Overweight/Above Normal BMI (OW)</td>
<td>&gt; 25.0</td>
</tr>
</tbody>
</table>

Due to the small sample size, in addition to significant results (p ≤ 0.05), trends approaching significance (p ≤ 0.1) are discussed.

Table 2. Characteristics of samples examined.

<table>
<thead>
<tr>
<th></th>
<th>HLM Female</th>
<th>HLM Male</th>
<th>MS Female</th>
<th>MS Male</th>
<th>*MS 4MU Female</th>
<th>*MS 4MU Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>14</td>
<td>22</td>
<td>25</td>
<td>31</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td>Normal</td>
<td>3</td>
<td>6</td>
<td>4</td>
<td>9</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Overweight</td>
<td>3</td>
<td>8</td>
<td>6</td>
<td>12</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Obese</td>
<td>6</td>
<td>7</td>
<td>13</td>
<td>9</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Morbidly Obese</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Average BMI NW</td>
<td>23 ± 3.76</td>
<td>22 ± 6</td>
<td>22 ± 5</td>
<td>22 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average BMI OW</td>
<td>33 ± 27.89</td>
<td>32 ± 29</td>
<td>32 ± 21</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*MS data for 4MU assay was performed on a different set of samples than those used for all other MS assays
CHAPTER 3: RESULTS
3.1 Relative Protein Expression, Western Blotting

3.1.1 Western Blot Sample Layouts
All samples were assayed on mini-blot setups (Figures 9-13) with each blot (labeled A, B, and D) holding 14 samples, and blot C holding 13 samples. Each blot contained a ladder that was visible on the PVDF membrane, but does not image onto the film. Ladder was transferred manually by marking the film after development and was solely used to confirm approximate size of protein (approximately 57kDa). Two of the blots (A and C) contained a specific recombinant protein that reacts to the UGT isoform being analyzed, while blots B and D contained recombinant protein specific to another isoform being analyzed (as listed in the figure legends of Figures 9-13); this assisted in checking for specificity. For isoforms 1A9 and 2B7, the specific was loaded on B and D. For each isoform a total of 3 replicates per sample were run.
3.1.2 Western Blot Representative Plots

3.1.2.1 UGT 1A1 Western Blot

Figure 9 - UGT isoform 1A1 western blot showing blots A-D and sample lanes numbered 1-14. Lanes 1 in blots A and C show a specific recombinant UGT protein at 57kDa, while lanes 1 in blot B and D contain a recombinant protein for UGT 1A4. Lane 3 on all blots show a pooled sample for interblot normalization. Lanes 4 and above contain human liver microsome (HLM) samples.

UGT 1A1 shows good specific protein expression for all samples examined with bands near 57kDa consistent with the reported protein size (Guillemette 2003) for this particular isoform (57.1kDa). Lane 3 on all plots contains a pool of 200 HLMs which were used for interblot normalization. Blots A and C contained a 1A1 positive recombinant control in lane 1 which expressed at the desired protein size. A negative recombinant control for isoform 1A4 was included in lane 1 on blots B and D and did not show expression.
3.1.2.2 UGT 1A4 Western Blot

Figure 10 - UGT isoform 1A4 western blot showing blots A-D and sample lanes numbered 1-14. Lanes 1 in blots A and C show a specific recombinant UGT protein at 57kDa, while lanes 1 in blot B and D contain a recombinant protein for UGT 1A4. Lane 3 on all blots show a pooled sample for interblot normalization. Lanes 4 and above contain HLM samples.

UGT 1A3 shows good specific protein expression for all samples examined with bands near 57kDa consistent with the reported protein size (Guillemette 2003) for this particular isoform (57kDa). Lane 3 on all plots contains a pool of 200 HLMs which were used for interblot normalization. Blots A and C contained a 1A4 positive recombinant control in lane 1 which expressed at the desired protein size. A negative recombinant control for isoform 1A1 was included in lane 1 on blots B and D and did not show expression.
3.1.2.3 UGT 1A6 Western Blot

Figure 11 - UGT isoform 1A6 western blot showing blots A-D and sample lanes numbered 1-14. Lanes 1 in blots A and C show a specific recombinant UGT protein at 57kDa, while lanes 1 in blot B and D contain a recombinant protein for UGT 1A4. Lane 3 on all blots show a pooled sample for interblot normalization. Lanes 4 and above contain HLM samples.

UGT 1A6 shows good specific protein expression for all samples examined with bands near 57kDa consistent with the reported protein size (Guillemette 2003) for this particular isoform (57.9kDa). Two additional nonspecific bands appear, but are separated enough from the band of interest not to cause interference with analysis. Lane 3 on all plots contains a pool of 200 HLMs which were used for interblot normalization. Blots A and C contained a 1A6 positive recombinant control in lane 1 which expressed at the desired protein size. A negative recombinant control for isoform 1A9 was included in lane 1 on blots B and D and did not show expression.
3.1.2.4 UGT 1A9 Western Blot

Figure 12 - UGT isoform 1A9 western blot showing blots A-D and sample lanes numbered 1-14. Lanes 1 in blots B and D show a specific recombinant UGT protein at 57kDa, while lanes 1 in blot A and C contain a recombinant protein for UGT 1A6. Lane 3 on all blots show a pooled sample for interblot normalization. Lanes 4 and above contain HLM samples.

UGT 1A9 shows good specific protein expression for all samples examined with bands near 57kDa consistent with the reported protein size (Guillemette 2003) for this particular isoform (57.4kDa). There is minor nonspecific signal, but it is separated enough from the band of interest not to interfere with analysis. Lane 3 on all plots contains a pool of 200 HLMs which were used for interblot normalization. Blots B and D contained a 1A9 positive recombinant control in lane 1 which expressed at the desired protein size. A negative recombinant control for isoform 1A6 was included in lane 1 on blots A and C and did not show expression.
3.1.2.5 UGT 2B7 Western Blot

Figure 13 - UGT isoform 2B7 western blot showing blots A-D and sample lanes numbered 1-14. Lanes 1 in blots B and D show a specific recombinant UGT protein at 57kDa, while lanes 1 in blot A and C contain a recombinant protein for UGT 1A1. Lane 3 on all blots show a pooled sample for interblot normalization. Lanes 4 and above contain HLM samples.

UGT 2B7 shows good specific protein expression for all samples examined with bands near 57kDa consistent with the reported protein size (Guillemette 2003) for this particular isoform (57kDa). Lane 3 on all plots contains a pool of 200 HLMs which were used for interblot normalization. Blots B and D contained a 1A1 positive recombinant control in lane 1 which expressed at the desired protein size. A negative recombinant control for isoform 1A6 was included in lane 1 on blots A and C and did not show expression.
<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Relative Protein Density</th>
<th>Sex</th>
<th>BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xenotech HLM0489</td>
<td>0.93 0.95 0.89 0.88 0.93</td>
<td>Male</td>
<td>21.2</td>
</tr>
<tr>
<td>Xenotech HLM0459</td>
<td>0.93 0.97 0.84 0.97 1.02</td>
<td>Female</td>
<td>22.1</td>
</tr>
<tr>
<td>CellzDirect Hu8043</td>
<td>0.87 1.09 0.87 0.87 0.92</td>
<td>Female</td>
<td>22.3</td>
</tr>
<tr>
<td>Xenotech HLM0438</td>
<td>0.84 1.01 0.98 0.91 0.94</td>
<td>Male</td>
<td>22.8</td>
</tr>
<tr>
<td>CellzDirect Hu8062</td>
<td>0.97 1.03 0.82 0.87 0.91</td>
<td>Male</td>
<td>23</td>
</tr>
<tr>
<td>Xenotech HLM0430</td>
<td>1.11 1.05 0.98 0.99 0.91</td>
<td>Male</td>
<td>23.5</td>
</tr>
<tr>
<td>Xenotech HLM0458</td>
<td>1.1 0.97 0.98 1.03 0.8</td>
<td>Male</td>
<td>23.8</td>
</tr>
<tr>
<td>CellzDirect Hu8032</td>
<td>1.11 0.93 0.95 0.93 0.98</td>
<td>Female</td>
<td>24.9</td>
</tr>
<tr>
<td>Xenotech HLM0452</td>
<td>0.82 0.94 0.92 1.01 1.03</td>
<td>Male</td>
<td>25</td>
</tr>
<tr>
<td>CellzDirect Hu8053</td>
<td>1.01 1.09 0.98 1.07 0.95</td>
<td>Male</td>
<td>25.7</td>
</tr>
<tr>
<td>Xenotech HLM0483</td>
<td>0.73 0.98 0.87 0.89 0.94</td>
<td>Male</td>
<td>26.2</td>
</tr>
<tr>
<td>Xenotech HLM0434</td>
<td>0.9 1 0.88 0.98 0.96</td>
<td>Male</td>
<td>26.4</td>
</tr>
<tr>
<td>Xenotech HLM0453</td>
<td>1 0.93 0.98 1.06 0.93</td>
<td>Female</td>
<td>26.4</td>
</tr>
<tr>
<td>CellzDirect Hu8059</td>
<td>1.09 1.03 0.96 1 0.83</td>
<td>Male</td>
<td>26.6</td>
</tr>
<tr>
<td>Xenotech HLM0442</td>
<td>0.8 0.93 1 0.9 0.92</td>
<td>Male</td>
<td>27.2</td>
</tr>
<tr>
<td>Xenotech HLM0315</td>
<td>1.21 1.08 1.02 1.07 1.12</td>
<td>Male</td>
<td>27.5</td>
</tr>
<tr>
<td>CellzDirect Hu8064</td>
<td>1.07 0.9 0.95 0.95 0.92</td>
<td>Female</td>
<td>28.4</td>
</tr>
<tr>
<td>Xenotech HLM0431</td>
<td>0.9 0.94 0.87 0.93 0.97</td>
<td>Male</td>
<td>29.5</td>
</tr>
<tr>
<td>CellzDirect Hu8077</td>
<td>0.97 0.97 0.84 0.94 0.95</td>
<td>Male</td>
<td>29.5</td>
</tr>
<tr>
<td>CellzDirect Hu8034</td>
<td>0.73 0.97 0.76 0.86 0.91</td>
<td>Male</td>
<td>29.5</td>
</tr>
<tr>
<td>CellzDirect Hu8057</td>
<td>0.95 0.94 0.87 0.91 1.09</td>
<td>Male</td>
<td>31.2</td>
</tr>
<tr>
<td>Xenotech HLM0393</td>
<td>1.03 1.05 1.07 1.12 1.15</td>
<td>Female</td>
<td>31.3</td>
</tr>
<tr>
<td>Xenotech HLM0429</td>
<td>0.94 0.93 0.96 0.93 1</td>
<td>Male</td>
<td>31.6</td>
</tr>
<tr>
<td>CellzDirect Hu8071</td>
<td>0.86 0.99 0.87 0.87 0.95</td>
<td>Male</td>
<td>34.4</td>
</tr>
<tr>
<td>Xenotech HLM0439</td>
<td>0.93 1 0.89 0.96 0.93</td>
<td>Female</td>
<td>34.4</td>
</tr>
<tr>
<td>Xenotech HLM0258</td>
<td>0.77 0.92 0.87 0.84 1.01</td>
<td>Male</td>
<td>35.1</td>
</tr>
<tr>
<td>Xenotech HLM0445</td>
<td>1.03 0.97 0.85 0.99 0.94</td>
<td>Female</td>
<td>35.9</td>
</tr>
<tr>
<td>CellzDirect Hu8055</td>
<td>0.97 0.95 1.04 1.03 1.02</td>
<td>Female</td>
<td>36.4</td>
</tr>
<tr>
<td>CellzDirect Hu8040</td>
<td>1.09 0.96 1.05 1.05 1.05</td>
<td>Female</td>
<td>36.5</td>
</tr>
<tr>
<td>Xenotech HLM0441</td>
<td>0.92 1.05 0.85 0.91 0.93</td>
<td>Male</td>
<td>36.6</td>
</tr>
<tr>
<td>Xenotech HLM0455</td>
<td>1.05 1.03 0.96 1.05 0.93</td>
<td>Female</td>
<td>37.8</td>
</tr>
<tr>
<td>CellzDirect Hu8058</td>
<td>1.01 0.88 0.93 1 1.04</td>
<td>Male</td>
<td>38.7</td>
</tr>
<tr>
<td>Xenotech HLM0348</td>
<td>1.09 0.98 0.92 0.99 0.92</td>
<td>Female</td>
<td>39.3</td>
</tr>
<tr>
<td>Xenotech HLM0428</td>
<td>0.87 0.99 0.98 0.93 1.11</td>
<td>Female</td>
<td>40.2</td>
</tr>
<tr>
<td>Xenotech HLM0422</td>
<td>0.82 1.01 0.93 0.95 1.03</td>
<td>Male</td>
<td>41.1</td>
</tr>
<tr>
<td>Xenotech HLM0447</td>
<td>1.01 1.01 0.74 0.77 0.93</td>
<td>Female</td>
<td>53.9</td>
</tr>
<tr>
<td>CellzDirect Female NW Pooled</td>
<td>0.96 0.96 0.91 0.93 0.93</td>
<td>Female</td>
<td>21</td>
</tr>
<tr>
<td>CellzDirect Male NW Pooled</td>
<td>0.9 0.94 0.89 0.92 0.9</td>
<td>Male</td>
<td>21</td>
</tr>
<tr>
<td>CellzDirect NW Pooled</td>
<td>0.93 0.96 0.9 0.91 0.88</td>
<td>n.a.</td>
<td>21</td>
</tr>
<tr>
<td>CellzDirect OW Pooled</td>
<td>0.9 0.94 0.92 0.93 0.93</td>
<td>n.a.</td>
<td>25</td>
</tr>
<tr>
<td>CellzDirect Obese Pooled</td>
<td>0.94 0.94 0.9 0.92 0.98</td>
<td>n.a.</td>
<td>31</td>
</tr>
<tr>
<td>CellzDirect Obese II Pooled</td>
<td>0.88 0.89 0.91 0.96 0.88</td>
<td>n.a.</td>
<td>41</td>
</tr>
</tbody>
</table>
Table 3 – Summary of relative protein density for all UGT isoforms examined. Data is given as relative protein density in ratio format as a ratio of sample density to pooled sample density. Data was then analyzed for statistical significance comparing relative protein density to body mass index (BMI) in kg/m$^2$. 
3.1.3 Analysis of Western Blots

3.1.3.1 Protein Expression, Continuous Linear Data

*UGT1A1 and 1A4 relative protein density vs. BMI*

**UGT1A1 and 1A4 relative protein density vs. BMI**

![1A1 BMI vs RPD (all)](image1)

![1A4 BMI vs RPD (all)](image2)

![1A1 BMI vs RPD (female)](image3)

![1A4 BMI vs RPD (female)](image4)

![1A1 BMI vs RPD (male)](image5)

![1A4 BMI vs RPD (male)](image6)

Figure 14 – Protein expression scatterplots utilizing continuous linear data for isoforms 1A1 (left) and 1A4 (right). Plots show data of relative protein density (RPD) vs body mass index (BMI) for all samples (black points), only female samples (red), and only male samples (blue). Number of samples (n), Pearsons r correlation.
(r) and statistical significance (p) is shown on each plot, which is an average of 3 experiments.

Isoforms 1A1 and 1A4 do not show significant correlations for the group of samples examined in any of the stratified conditions. When separating data out by males and females data was still not significant. Sample size was relatively small considering that UGTs are known to be highly variable between individuals.
UGT1A6 and 1A9 relative protein density vs. BMI

Figure 15 – Protein expression scatterplots utilizing continuous linear data for isoforms 1A6 (left) and 1A9 (right). Plots show data of RPD vs BMI for all samples (black points), only female samples (red), and only male samples (blue). Number of samples (n), Pearson's r correlation (r) and statistical significance (p) is shown on each plot, which is an average of 3 experiments.
Isoforms 1A6 and 1A9 do not show significant correlations for the group of samples examined in any of the stratified conditions. When separating data out by males (Figure 15 bottom) and females (Figure 15 middle) data was still not significant.
Figure 16 - Protein expression scatterplots utilizing continuous linear data for isoform. Plots show data of RPD vs BMI for all samples (black points), only female samples (red), and only male samples (blue). Number of samples (n), Pearson’s r.
correlation (r) and statistical significance (p) is shown on each plot, which is an average of 3 experiments.

Isoform 2B7 does not show significant correlation (p=0.15) when all samples are examined as a whole (Figure 16A). When separating data out by sex, male samples (Figure 16C) showed a mild significant positive correlation (p-value of 0.047), between relative protein expression and BMI; as BMI increased, so did relative protein expression. While the significance is not very strong (p-value < 0.05), it shows that for isoform 2B7 more protein may be produced when individuals have a higher BMI (Figure 16C).
3.1.3.2 Protein Expression, Categorized Data

Figure 17 - Categorized expression data for all UGT isoforms examined via western blot was grouped according to normal weight (NW) and over weight (OW) for all samples (black), female samples (F) in red, and male (M) in blue. Error bars are standard deviations (SD). Numbers at the bottom of bars indicate total number of averaged samples for given data point.
Relative protein expression data was averaged based on BMI categories established by the CDC utilizing the NHANES health evaluation survey (Table 1). It was hoped that by grouping the data into discrete categories that extra power would be afforded to the t-test in order to call significance in certain cases. Unfortunately by binning the data, it left some of the categories with a very few number of samples. Female normal weight samples only had 3 samples (Figure 17, F-NW) that were being grouped together, which did not show significance for any of the isoforms examined (Figure 17, red columns). For all samples (Figure 17 black), isoforms 1A1 and 1A4 (Figure 17, top) had lower average expression for the overweight group when compared to the normal weight group, the reverse was true for the rest of the isoforms 1A6, 1A9 (Figure 17, middle) and 2B7 (Figure 17, bottom) which showed higher average expression in the overweight group. For the female samples (red) all isoforms except 1A4 (Figure 17 upper-right) showed higher average expression in the overweight group compared to the normal weight group. When examining the male samples (blue), all isoforms showed decreasing expression in the overweight group compared to the normal group except for isoform 2B7 which showed a higher expression in the overweight group (Figure 17, bottom).

**3.1.3.3 Protein Expression Summary**

Treating BMI as a continuous variable, UGT2B7 relative protein expression correlated in a significant positive fashion with BMI for only male samples (p = 0.047) (Figure 10C).

When samples were grouped into BMI categories, no significant differences were observed in relative protein expression of UGT isoforms between normal weight (NW)
and over weight (OW). When separated by sex, only UGT1A1 approached significance (p = 0.057) with males having lower overall levels of protein expression than females (graph not shown). UGT Fluorescence-Based Activity Measurements

**Fluorescence Activity Measurements Troubleshooting and Protocol Modifications**

UGT1A1

Protocol calls for assay to be done in glass tubes. Protocol was modified to be performed in deepwell plate Axygen p/n P-DW-20CSIND. Initial experiments were performed to test the reactivity of the plates to the organic solvent used in the experiment. None was visible. Sealing film could be used to seal the plates, but do not allow the organic solvent to come in contact with it, or it will dissolve the adhesive and contaminate sample. Silicone sealing mat was used for much of the process, although it tended to react poorly to freezing step and so it was necessary to switch to sealing film at that stage. Overnight -20°C incubation was used for the last step to ensure that the aqueous layer froze sufficiently for organic layer removal. Optical read plates must be used because they are non-reactive to the organic phase.
3.2 Activity Data via Fluorescence-based assays

3.2.1 Fluorescent Activity Data, Continuous Linear Scatterplots

General UGT and UGT 1A1 Activity

Figure 18 – Fluorescence-based activity scatterplots utilizing continuous linear data for isoform 1A1 (right) and general UGT assay (left). Plots show enzyme activity vs body mass index (BMI) for all samples (black), only female samples (red), and only male samples (blue). Number of samples (n), Pearsons r correlation (r) and
statistical significance (p) is shown on each plot, which is an average of 3 experiments.

Isoform 1A1 (Figures 18D, E, F) and general UGT (Figures 18A, B, C) did not show any significant correlations for the samples examined (p-values were higher than 0.05).

While the comparisons of activity to BMI tended to have negative slopes even when stratifying by sex (Figures 18B, C, E, F), the relatively low number of samples for these comparisons may have inhibited confidence for these comparisons.

UGT 1A4 and 1A9 Activity
UGT 1A4 and 1A9 Activity

Figure 19 - Fluorescence-based activity scatterplots utilizing continuous linear data for isoform 1A4 (left) and 1A9 (left). Plots show enzyme activity vs BMI for all samples (black), only female samples (red), and only male samples (blue). Number of samples (n), Pearsons r correlation (r) and statistical significance (p) is shown on each plot, which is an average of 3 experiments.
Isoforms 1A4 and 1A9 did not show any significant correlations for the samples examined (Figure 19A-F). While the comparisons of activity to BMI tended to have negative slopes even when stratifying by sex (Figure 19B, C, E, F), the relatively low number of samples for these comparisons may have inhibited confidence for these comparisons.

### 3.2.2 Fluorescent Activity Data, Categorized

![Graphs showing fluorescent activity data for UGT 1A and isoforms]

**Figure 20** - Categorized fluorescence-based activity data for general UGT (plot A) and isoforms 1A1 (plot B), 1A4 (plot C), and 1A9 (plot D). Grouped according to normal weight (NW) and over weight (OW) for all samples (black), female samples (F) in red, and male (M) in blue. Error bars are standard deviations (SD). Numbers at the bottom of bars indicate total number of averaged samples for given data point.
Fluorescent activity data was averaged based on BMI categories established by the CDC utilizing the NHANES health evaluation survey (Table 1). Constraining the data left some of the categories with a very few number of samples; Female (red) normal weight samples only had 3 samples that were being grouped together (Figure 20, F-NW), which did not show significance for any of the isoforms examined (Figure 20A-D). None of the comparisons resulted in a statistically significant difference and so trends will be discussed below. For all samples (black), isoform 1A1 (Figure 20B) had lower average activity for the overweight group when compared to the normal weight group, the reverse was true for the 1A9 (Figure 20D) and general UGT (Figure 20A) which showed higher average expression in the overweight group. For the female samples (red) 1A9 (figure 20D) and general UGT (Figure 20A) showed higher average expression in the overweight group when compared to the normal weight group, while the reverse was true for 1A1 (Figure 20B). When examining the male samples (blue), general UGT (Figure 20A) showed no discernable difference between the normal and overweight groups while 1A1 (Figure 20B) and 1A9 (Figure 20D) showed a decrease and increase in the overweight group respectively. Isoform 1A4 was not examined in detail as the results from the experiment do not appear to show any discernable difference in any of the groups examined.

3.2.3 Fluorescence-based activity summary
While no statistically significant findings appeared, when examining linear continuous data for fluorescence-based activity vs. BMI, there appeared to be a consistent negative trend that showed decreasing activity with increasing BMI (Figures 18 and 19). This did not, however, always corroborate with the categorized data (Figure 20), which showed
increasing activity in some of the overweight groups (Figures 20A and D). This discrepancy could be due to a large number of factors, but high intrinsic interpersonal variability of UGT activity and relatively low sample numbers likely contribute to this variation and lack of significance.
3.3 Activity Measurements via Mass Spectrometry
3.3.1 Continuous Linear Scatterplots
Activity Data via Mass Spec, Continuous Linear Scatterplots UGT1A and 1A1

Figure 21 - Activity scatterplots as measured by mass spectrometry (mass spec) utilizing continuous linear data for UGT isoform 1A1 (right) and general UGT (left). Plots show enzyme activity vs body mass index (BMI) for all samples (black), only female samples (red), and only male samples (blue). Number of samples (n),
Pearson's r correlation (r) and statistical significance (p) is shown on each plot, which is an average of 3 experiments.

The female samples in 1A1 (Figure 21E) showed a positive trend between mass spec activity and BMI, but all other trends were negative for isoform 1A1 and general UGT (Figures 21A-D, F). Only when looking at all samples for general UGT was there a significant negative linear correlation between activity and BMI (p=0.028) (Figure 21A); as BMI increased, activity decreased. In the male samples for 1A1 (Figure 21F), there was an approaching significance (p=0.068) for 1A1 which also showed a negative correlation between activity and BMI.
Activity Data via Mass Spec, Continuous Linear Scatterplots UGT1A4 and 1A6

Figure 22 - Activity scatterplots as measured by mass spec utilizing continuous linear data for UGT isoforms 1A4 (left) and 1A6 (right). Plots show enzyme activity vs BMI for all samples (black), only female samples (red), and only male samples (blue). Number of samples (n), Pearsons r correlation (r) and statistical significance (p) is shown on each plot, which is an average of 3 experiments.
When analyzing data for UGT isoforms 1A4 (Figure 22, left) and 1A6 (Figure 22, right), a consistent negative trend between BMI and mass spec activity is seen (Figures 22A-F). Most of these comparisons were not statistically significant (p\geq 0.05). Investigation of all of the samples for isoform 1A4 (Figure 22A) shows that there was a statistically significant negative correlation (p=0.02) between BMI and activity showing a decrease in activity with an increase in BMI. A similar trend was seen for 1A6 (Figure 22D), however the correlation was only approaching statistical significance (p=0.054).
Activity Data via Mass Spec, Continuous Linear Scatterplots UGT1A9 and 2B7

Figure 23 - Activity scatterplots as measured by mass spectrometry (mass spec) utilizing continuous linear data for UGT isoforms 1A9 (left) and 2B7 (right). Plots show enzyme activity vs BMI for all samples (black), only female samples (red), and only male samples (blue). Number of samples (n), Pearson’s r correlation (r) and statistical significance (p) is shown on each plot, which is an average of 3 experiments.
By inspecting all plots (Figure 23A-F), a negative trend between BMI and mass spec activity is seen. Most of these comparisons did not result in statistical significance (as defined by a cutoff of $p \geq 0.05$). All samples for isoform 1A9 (Figure 23A), show a statistically significant negative correlation ($p=0.039$) between BMI and activity illustrating a significant decrease in activity with increasing BMI. This is also true when looking just at the male samples (Figure 23C) that show a significant negative correlation ($p=0.048$) between activity and BMI. For isoform 2B7 there is an approaching significant negative correlation between activity and BMI for all samples (Figure 23D) and also for just the male samples (Figure 23F) ($p=0.081$ and 0.079 respectively).
3.3.2 Activity Data via Mass Spec, Categorized

Figure 24 - Categorized activity data utilizing mass spec for general UGT (plot A) isoforms 1A1 (plot B), 1A4 (plot C), 1A6 (plot D), 1A9 (plot E), 2B7 (plot F), grouped according to normal weight (NW) and over weight (OW) for all samples (black), female samples (F) in red, and male (M) in blue with standard deviation error bars. Numbers at the bottom of bars indicate total number of averaged samples for given data point. A (*) represents a p-value ≤0.05, while (**) is P≤0.01.
Mass spec activity data was averaged based on BMI categories established by the CDC utilizing the NHANES health evaluation survey (Table 1). Examination of all samples for isoforms 1A1, 1A6, 1A9, and 2B7 (Figures 24 B, D, E and F respectively), show significantly (p≤0.05) lower activity in overweight samples compared to normal weight samples while the remaining isoform 1A4 (Figure 24C) and general UGT (Figure 24A) show approaching significance (p-values of 0.057 and 0.075 respectively). Female (red) samples for general UGT (Figure 24A) show a similar significant decrease in activity for the overweight samples (p≤0.05), while in male (blue) samples significance (p≤0.01) was seen only in 1A1 (Figure 24B) and 1A6 (p≤0.05) (Figure 24D). Approaching significance was seen, showing lower mass spec activity in overweight female samples for 1A6 (Figure 24D) with a p-value of 0.096. In males lower activity was seen in the overweight samples that approached significance for 1A9 (Figure 24E) and 2B7 (Figure 24F) with p-values of 0.07 and 0.086 respectively.

3.3.3 Enzyme Activity Summary
When BMI was treated as a continuous variable, the activity of general UGT, UGT1A4 and UGT1A9, had a statistically significant negative correlation; as BMI increased, activity decreased (Figures 16, 17, 18 respectively). Isoforms UGT1A6 and UGT2B7 approached significance (p = 0.054 and 0.081 respectively) demonstrating the same negative correlation (Figures 17 and 18).

When activity was stratified by sex for continuous BMI, no significant correlations were observed in females. Conversely, in males, UGT1A9 was observed to have a significant
negative correlation, while the UGT1A1 and UGT2B7 isoforms approached significance (p = 0.068 and 0.079 respectively) (Figures 16 and 18).

After grouping samples into categories, OW individuals were found to have significantly lower activity compared to the NW individuals for the UGT1A1, 1A6, 1A9, and 2B7 isoforms (Figure 19), with UGT1A4 and general UGT1A approaching significance (p = 0.057 and 0.075 respectively) (Figure 19). When stratified by sex, OW females had significantly lower activity compared to NW females for general UGT1A, while UGT1A6 approached significance (p = 0.096) (Figure 19). In males, UGT1A1 and UGT1A6 activity was significantly lower in the OW compared to NW individuals, while the same trend approached significance for UGT1A9 and UGT2B7 (p = 0.070 and 0.086 respectively) (Figure 19). No significant overall sex differences between males and females were observed.
3.4 Assay Correlations

3.4.1 Protein Expression vs. Mass Spectrometry Activity

Figure 25 – Mass spectrometry based activity measurements vs. relative protein density (RPD) expression measurements for UGT 1A1 (plot A), 1A4 (plot B), 1A6 (plot C), 1A9 (plot D), and 2B7 (plot E). Number of samples (n), Pearsons r correlation (r) and statistical significance (p) is shown on each plot, which is an average of 3 experiments.
Intersecting samples between the western blot and mass spec activity assay were correlated on a continuous linear scale and correlations were determined using Prism software. Isoforms 1A1, 1A6, 1A9 and 2B7 (Figures 25A, C, D and E respectively) showed a statistically significant ($p \leq 0.05$) correlation between these two assays. This is encouraging, and possibly even surprising for two reasons: first is that there are relatively few numbers of samples being compared ($n=14$), second is that UGTs are known to be subject to post-translational modification that can reduce their activity after the protein is expressed. This can suggest a number of things: perhaps that post-translational modification is not as influential as may have been theorized (Ishii, Nurrochmad et al. 2010), that for this particular set of samples the effect of modification did not result in a large change in activity, or that perhaps when averaging data over a large number of samples that the effect of modification is balanced out.
3.4.2 Fluorescence Activity vs. Protein Expression

Figure 26 – Fluorescence-based activity measurements vs. relative protein density (RPD) expression measurements for UGT 1A1 (plot A), 1A4 (plot B), and 1A9 (plot C). Number of samples (n), Pearsons r correlation (r) and statistical significance (p) is shown on each plot, which is an average of 3 experiments.

The same set of samples was run in-house utilizing a western blot expression assay and a fluorescence-based activity assay. The high number of samples (n=43) shows strong correlation between these two assays for isoforms 1A1 (Figure 21A) and 1A9 (Figure 21C). This is encouraging as it shows that, while utilizing separate assays we can get similar results for the expression and/or activity of these UGT isoforms for the assays utilized. While they do show good correlation, they are in fact measuring separate things. A direct substitution of these two assays is not possible, but perhaps we can examine the
poorly correlating samples to determine what may be causing a change between the transcription and translation.

3.4.3 Mass Spectrometry Activity vs. Fluorescence Activity

![Figure 27 - Mass spectrometry-based activity measurements vs. fluorescence-based activity measurements for UGT 1A1 (plot A), 1A4 (plot B), and 1A9 (plot C). RF = relative fluorescence. Number of samples (n), Pearson's r correlation (r) and statistical significance (p) is shown on each plot, which is an average of 3 experiments.

The mass spec-based and fluorescent-based assays are both measuring activity. Mass spec typically does this by running a glucuronidation reaction and then, through mass spec, examines the amount of glucuronidated product that is produced before factoring in amount of protein utilized and reaction time to get activity. This is similar to the 1A1 (Figure 27A) assay in which the glucuronidated product is isolated and examined for fluorescence. For UGTs 1A4 (Figure 27B) and 1A9 (Figure 27C) fluorescence-based
assays, fluorescence depletion is measured; the glucuronidation removes the fluorescence of the substrate and so the reduction in fluorescence is converted into the activity of the microsomal enzyme. The significant correlation of the 1A1 (Figure 27A) and 1A9 (Figure 27C) assays is an indication that a cheaper, and easier fluorescence-based assay can be used as a substitute for mass spec which requires costly machinery, but is thought to be more sensitive. This would be especially useful if perhaps the equipment is not available or large numbers of samples are being measured. Isoform 1A1 has especially significant correlation (p<0.0001), while 1A9 has less significant correlation (p=0.038). This may be due to the fact that for isoform 1A9 there is a two-experiment protocol that may increase the noise of the data. First, a general UGT assay is run, then the same assay is run and NFA is added, which inhibits all 1A isoforms except for 1A9. Through subtraction then the activity of 1A9 can be resolved. In a final note, while a general UGT assay was used both for mass spec and for the fluorescent-based assay the intersection of samples was extremely small (n=3) as the data supplied by Xenotech on the 1A assay was on a completely different set of samples than was used for the western blot and fluorescent activity assay which is why a correlation was not made.

3.4.4 Assay Correlation Summary
While examinations of UGTs can be costly and/or time consuming, we show that in at least two isoforms there are consistent and reliable methods available on three platforms (MS activity, fluorescent activity, and relative protein density on western blot) that offer similar results; showing very good correlations for isoforms 1A1 and 1A9 (Figures 25-27). In addition to these two isoforms, MS and western blot also showed good
correlation for UGT 1A6 and 2B7 (Figures 25C and E). This illustrates that while there are many potential substrates for these two isoforms, the ones outlined in this paper are both maximally, or in the very least equivalently, acted upon by their respective UGTs offering alternative methods of examination for the same UGT isoform. Additionally, since fluorescent approaches are generally less expensive and require less technical expertise, the accuracy and precision of fluorescence-based platforms has been demonstrated with good correlation to the gold standard mass spectrometry assay.

The good correlation between the assays may assist us in interpretation of the results. While we did see some consistent trends in the data for the fluorescence-based activity measurements and the protein expression data, significance was a problem in resolving whether in fact the trends were true. We presumed that this was due to a relatively low number of samples being examined. Enzymes are known to have high amounts of inter-individual variation that can be caused by factors including polymorphisms and concurrent exposure to other drugs, and so the limited sample size may have reduced our ability to observe significant differences due to this variability (Wells, Mackenzie et al. 2004; Izukawa, Nakajima et al. 2009). With the additional number of samples being examined in the MS data (n=56 vs. the 47 in the expression and fluorescent assays), we saw many more significant negative correlations between activity and BMI. Since the MS assay also correlated well with the expression assay in a number of cases, we may be able to infer that expression is also negatively impacted.
3.5 Physiologically-Based Pharmacokinetic Models

3.5.1 Mass Spectrometry-Based, Well-Stirred Clearance

Figure 28 – Well-stirred modeled hepatic clearance for all samples vs. body mass index (BMI) for general UGT (plot A), 1A1 (plot B), 1A4 (plot C), 1A6 (plot D), 1A9 (plot E), and 2B7 (plot F). The correlation (r) and p-value (p) are listed in addition to the number of samples (n) for an average of 3 experiments.
The modeled hepatic clearance for all samples shows a general decrease of clearance with increasing BMI, which is shown to be statistically significant (p<0.05) for general UGT (Figure 28A), as well as isoforms 1A4 (Figure 28C) and 1A9 (Figure 28E). Additionally, isoforms 1A6 and 2B7 (Figures 28D and F respectively) show approaching significance of p=0.054 and 0.081 respectively.
3.5.2 Mass Spectrometry-Based Well-Stirred Female Clearance

Figure 29 – Female well-stirred modeled hepatic clearance vs. BMI for general UGT (plot A), 1A1 (plot B), 1A4 (plot C), 1A6 (plot D), 1A9 (plot E), and 2B7 (plot F). The correlation (r) and p-value (p) are listed in addition to the number of samples (n) for an average of 3 experiments.

When stratified by sex, no significant correlations were seen in female samples (Figure 29). Modeled well-stirred clearance for UGT 1A1 (Figure 29B) had a positive trend
showing an increase in clearance with increasing BMI, while all other isoforms (Figures 29A, C-F) showed decreasing modeled hepatic clearance with increasing BMI.

3.5.3 Mass Spectrometry-Based Well-Stirred Male Clearance

Figure 30 - Male well-stirred modeled hepatic clearance vs. BMI for general UGT (plot A), 1A1 (plot B), 1A4 (plot C), 1A6 (plot D), 1A9 (plot E), and 2B7 (plot F). The
correlation (r) and p-value (p) are listed in addition to the number of samples (n) for an average of 3 experiments.

In males, a significant negative correlation was found between continuous BMI and clearance for the UGT1A9 isoform (Figure 30E) in the well-stirred models (p=0.047 and 0.054 respectively). The same negative correlation approached significance for isoforms UGT1A1 and UGT2B7 (p = 0.068 and 0.079 respectively) in both the well-stirred and parallel tube models (Figures 30A and F). In general, there were few differences seen between the parallel tubes and well-stirred mixing models of hepatic clearance. For simplicity, only the well-stirred model data was supplied, and parallel tubes model data was discussed when there were deviations between the two models (as was just discussed for the male samples in Figures 30A, E, and F).
3.5.4 Mass Spectrometry Categorized Well-Stirred Clearance

Figure 31 - Modeled hepatic well-stirred (WS) clearance measurements vs. BMI category for normal BMI (NW) vs. above normal BMI (OW) for all samples in black, female samples (F) in red, and male samples (M) in blue with standard deviation error bars. General UGT (plot A), as well as UGT isoforms 1A1 (plot B),
1A4 (plot C), 1A6 (plot D), 1A9 (plot E), and 2B7 (plot F) were examined. Numbers at the bottom of bars indicate total number of averaged samples for given data point. A (*) represents a p-value ≤0.05, while (**) is P≤0.01).

After grouping samples into weight categories, the OW category was observed to have lower overall clearance, compared to the NW category, through the UGT1A6 and UGT2B7 isoforms in both the parallel tube (p = 0.0006 and 0.043 respectively) and well-stirred (p = 0.004 and 0.042 respectively) models, while the same trend approached significant for the UGT1A4, UGT1A9, and general UGT1A isoforms (p = 0.070, 0.086, and 0.075 respectively) for the well-stirred model only. The same trend was discovered with the parallel tube model, with the exception that the UGT1A9 isoform no longer approached significance (p = 0.148, Figure 31).

When stratified by sex, general UGT1A clearance was statistically lower in OW compared to NW female individuals; this was true for both the parallel tube (p = 0.047) and well-stirred (p = 0.047) models. In the male population, UGT1A1 and UGT1A6 showed statistically lower overall clearance in the OW samples for both the parallel tube (p = 0.011 and 0.006 respectively) and well-stirred (p = 0.014 and 0.008 respectively) models, while UGT2B7 approached significance with a p-value f 0.087 for both the parallel tube and well-stirred models (Figure 31)

When comparing overall clearance between males to females, no significant differences were recorded.
3.5.5 Clearance Summary
When examining the clearance data from the IVIVE model, only one significant difference was observed for comparisons made for either the total sample population or between sexes in either the well-stirred or parallel tube equations. Females had significantly lower overall clearance through UGT1A4 for both the well-stirred (p = 0.029) and parallel tubes model (p = 0.027) when compared to males.

Conversely using allometric scaling numerous significant findings were reported. A statistically significant negative correlation between continuous BMI and modeled hepatic clearance was found for UGT1A4, UGT1A9 and general UGT in both the well-stirred (Figures 28C, B and A respectively) and parallel tube models (p = 0.017, 0.039 and 0.027 respectively). The same negative correlation approached significance for UGT1A6 and UGT2B7 in both models (p = 0.054 and 0.081 respectively, same values for both models) (Figure 28).
CHAPTER 4: SUMMARY AND CONCLUSION
4.1 UGT Activity
This thesis reports for the first time the influence of obesity on the activity and expression of major UGT drug metabolizing isoforms in the human liver. Though there were no significant changes in protein expression, the activity of the enzymes generally decreased with increasing BMI for all of the isoforms investigated. These findings have significant bearing since these isoforms are involved in a variety of clinical drug and metabolic pathways including those related to cancer treatment, psychiatric conditions, and organ transplantation (Desai, Innocenti et al. 2003; Bernard and Guillemette 2004; Sandson, Armstrong et al. 2005). Our findings suggest that obese individuals may have a reduced ability to detoxify compounds through these isoform-specific pathways, potentially adding drug toxicity to the already extensive list of negative effects directly resulting from obesity itself (Desai, Innocenti et al. 2003; Bernard and Guillemette 2004; Sandson, Armstrong et al. 2005; Kramer, Sagartz et al. 2007).

A majority of metabolic research involving cytochrome P450s (CYP450s) has typically show that no consistent generalizations can be made regarding obesity and enzyme activity. Recent findings from Brill et al. (Brill, Diepstraten et al. 2012), however, describe that drug activity is dysregulated in obese patients, but varies greatly depending on the specific metabolic or elimination pathway involved; some showing an increase, while others show a decrease, or no change whatsoever. In Brill et al.’s review, some substrates showed improved modeling when an obesity factor was used as a covariate for clearance, while others required no such compensation. In opposition to the CYP data, our study showed a consistent and significant negative correlation between activity and
BMI, with all isoforms decreasing activity with increased BMI. Since obese adults frequently experience polypharmacy, more so than normal weight adults, insufficient modeling of multiple drug interactions poses a risk to the patient due to potential toxic buildup over time from deficient metabolism of drugs from the system of these individuals (Kit, Ogden et al. 2012). Obesity influences many physiological aspects that may result in changes to metabolism and subsequently to elimination of these drugs. While the influence of obesity on tubular reabsorption is unknown, recent findings by Brill et al. have suggested an increase in liver blood flow, glomerular filtration and tubular secretion in obese patients; however, these particular findings were determined using only a few high extraction-ratio drugs, and differences in body weights between patient groups was limited (Brill, Diepstraten et al. 2012). These factors are not directly involved in our *in vitro* experiments, but it is important to keep these concepts in mind when discussing potential relationships to *in vivo* data. Additionally, most *in vivo* studies tend to examine specific drugs and their whole body biotransformation, which includes multiple isoforms and pathways and may be subject to other drugs that have been coadministered.

In this study, focus is on individual UGT isoforms to determine the impact obesity would have on a single aspect of the metabolic process for the substrate drug, activity (Court 2010). Moreover, there are examples of clinical drugs that have single metabolic pathways including SN38 (UGT1A1), trifluoperazine (UGT1A4), and propofol (UGT1A9). Thus, these data may be of clinical relevance for these drugs (Court 2010).
Unfortunately the number of studies looking at UGT-metabolized drugs and obesity is small, but most show a significant increase in UGT biotransformation in obese compared to non-obese subjects (Court 2010). These findings are not necessarily in direct opposition to the data we present here, because most of the published studies rely on clinical blood measurements instead of the microsomal experiments performed here. While we clearly showed decreases in enzyme activities, the concurrent pharmacokinetic modeling demonstrated that clearances trended lower but did not significantly decrease. This implies that complementary pathways of metabolism or transport (such as the sulfotransferases or transport proteins) may compensate for the decrease in direct glucuronidation. Additionally, there may be many confounding factors that are present in vivo which do not apply to this very specific study examining just how the liver, by way of in vitro data, responds to specific substrates. This thorough examination of in vitro expression and activity data can be used to ensure that modeling parameters for drugs metabolism and elimination include obesity as a factor that must be accounted for.

4.2 Activity and Sex
There are many factors, both genetic and environmental, that account for the large variation in activity of UGTs, but very limited data on sex differences, exist. This is especially true when considering obesity as a cofounding factor which provides no published information whatsoever. Publications relating to genetic differences, in the form of polymorphisms, appear to be most prevalent in discussions of UGT variation. These publications show isoforms such as 1A1, 1A4, and 2B7 to have inhibited glucuronidation abilities with a particular polymorphism and induced glucuronidation for
1A6, 1A9 and 2B7 with other polymorphisms (Court 2010). The relevance of these differences in glucuronidation varies depending on the prevalence of the substrate that these isoforms act on. In the case of 1A9 and 2B7, which glucuronidate propofol and morphine respectively, changes in the activity of these enzymes add to the difficulty of dosing for these powerful anesthetic and pain relief drugs which typically require constant monitoring to avoid overdosing. Environmental factors such as drinking and smoking are commonly recorded in patient histories and so publication data exists which illustrate that higher 1A4 and 1A6 activity exists in smokers than in nonsmokers (Court 2010) while 2B7 glucuronidation is unchanged (Fleischmann, Remmer et al. 1986). A positive drinking history is also reported to be responsible for higher glucuronidation activity in all UGT1A isoforms examined in this study. Clearly, genetic (in the form of polymorphisms), and environmental factors can modify UGT activity, but in general, limited studies exist addressing sex differences in humans for UGTs. Two recent studies, however, have found sex to be linked to differences in UGT activity, with females being at higher risk for clinically relevant adverse drug reactions (Anderson 2008; Soldin, Chung et al. 2011). Anderson et al. (2008) state that evidence points to females having lower UGT activity than males, which may be responsible for causing females to have higher incidence of drug-induced liver toxicity, gastrointestinal adverse events due to NSAIDs, and allergic skin rashes. In accordance with this it has been discussed that physiological, hormonal, and genetic differences between males and females may affect the prevalence, incidence and severity of responses to therapy (Soldin, Chung et al 2011). As an example, body fat content, which tends to be higher in women, is described to be
responsible for sex-related pharmacokinetic differences in the distribution of diazepam and may have similar effects on certain drugs such as the hyperlipidemic drug fenofibrate (glucuronidated by UGT 1A1, 1A3, 1A9 and 2B7) which do show higher glucuronidation rates in females (Liu, Vincent-Viry et al. 1991).

In contrast to most of the existing data, our study found that activity for UGT isoforms 1A1, 1A6 and 1A9 was significantly decreased with increasing BMI in males, but not in females. This may simply show that males were less susceptible to the negative affects of obesity on activity than females rather than showing specifically that males and females had overall discrepant glucuronidation activities. In apparent contradiction to most of the male trends, it was general 1A that was significantly lower in overweight females, but not in overweight males, in our study. It is interesting to see that individual UGT1A isoforms were lower in overweight males, but the general 1A activity was lower in overweight females. Since we saw trends that indicated a decrease in every case (except for female 1A1) with increasing BMI, our significance in males and not in females may simply have been a result of insufficient power. Males had twice as many samples in the normal weight group than females had which may have driven the t-test statistically. In agreement with this being a power issue is the fact that when all samples were looked at as a whole (males and females combined), there tended to be more total significant negative correlations discovered. In the statistically significant overweight male correlations, reduced activity of these isoforms may impair the ability of overweight individuals to effectively detoxify their bodies leading to buildup of potentially toxic
endogenous molecules and exogenous chemicals. These particular isoforms are known to be involved in the elimination of the anticonvulsant lamotrigine (1A4), nonsteroidal anti-inflammatory drugs (1A6), and mycophenolic acid, a standard immunosuppressive drug (1A9) (Bernard and Guillemette 2004). Obesity may be an important factor in dosing of these types of drugs if activity is impaired in these individuals. While our study indicates that UGT inhibition due to obesity is more common in males than in females, the limited sample size may reduce our ability to observe significant differences in females due to the high interindividual variability of UGTs (Wells, Mackenzie et al. 2004; Izukawa, Nakajima et al. 2009).

4.3 Assay Correlation
Evidence for the correlation between activity and expression of UGT isoforms is sparse with only a few studies showing direct comparisons between activity and expression data (Ouzzine, Pillot et al. 1994; Haberkorn, Heydel et al. 2002). Ouzzine et al. demonstrated good correlation between UGT1A6 activity and expression, while Haberkorn et al did the same utilizing the family 1 UGTs in rats being fed retinol. Our study demonstrates a strong correlation exists between activity and relative protein expression for four of the UGT isoforms (1A1, 1A6, 1A9, 2B7). This was encouraging given the relatively small number of samples in common for the mass spec-based activity vs. expression comparisons. In the case of 1A1 and 1A9 fluorescent activity vs. expression that had a larger number of samples, the significance of correlation was extremely high. These results are very interesting given that UGTs are known to have post-translational modifications that can modify their activity after production (Ishii, Nurrochmad et al.)
2010). Even so, the amount of protein for these four isoforms appears to be directly related to the amount of activity towards the substrates used in the adults in our sample. This may illustrate that genetic drivers are more important than environmental influences. Alternatively effects such as post-translational modification may not have a large influence on the activity of the isoforms examined, or that specific environmental influences that vary person-to-person may be averaged out over a large number of samples. Even with the good correlation between expression and activity, we recommended that these assays are not interchangeable. In much the same way that BMI and body fat composition correlate, but are not exactly looking at the same thing, these two assays may correlate but are not examining the same thing. This data shows that, on a large scale, expression and activity may not be subject to the effects of post-translational modification.

For enzyme activity, it is known that determination of enzyme activity is dependent on the substrate, and given that, these correlations may not exist with alternate substrates. In response to this we have the two activity assays that are compared to each other. Isoform 1A1 utilizes bilirubin for the fluorescent assay while the mass spec assay used estradiol. The correlation for these two assays that only contained 17 samples had a p-value <0.0001. While the same substrates were not being glucuronidated, they were likely either maximally, or in the worst-case scenario, similarly glucuronidated by UGT1A1. This also shows, in part, that correct experimental design was used as we were in the $V_{max}$ portion of the incubations. In a more interesting substrate-dependent scenario, UGT
1A9 had a correlation p-value of 0.038 which itself isn’t fascinating, except that the two activity assays not only utilized different substrates, but also that the fluorescent assay used the subtraction of two assays (one with an inhibitor and one without). Correlation of these activity assays is important as it shows that while different substrates are likely glucuronidated at different levels, as long as they are maximally glucuronidated by a particular isoform, that either one could be used as a measurement of enzyme activity.

Additionally, we demonstrated that the fluorescent assays for UGT1A1 and 1A9 have extremely high concordance between mass spec and fluorescence approaches. Fluorescent assays are much more accessible given that they require a plate reader and not a mass spectrometry machine. Additionally, training to run the reader is exponentially faster and less expensive than training to competently use a mass spec. Hence the cost difference between the two is many orders of magnitude. It is, therefore; of great interest that we demonstrate that the accuracy and precision of fluorescence-based assays for UGT1A1 and UGT1A9 are comparable.

4.4 Physiologically-Based Pharmacokinetic Models
Drug development and dosing is a process that requires an extensive understanding of absorption, distribution, metabolism, and elimination (ADME) characteristics of a drug. Physiologically based pharmacokinetic models provide a method to account for an individual’s phenotypic characteristics in order to better model the ADME of a drug within the subject. There are, however, limitations to these models, especially with regard to UGTs, which have not been as extensively characterized as cytochrome P450s
(CYP450s). The equation used to calculate mg protein per gram of liver (MPPGL) has been shown to be accurate experimentally for CYP450s, but not expressly for UGTs. If UGT metabolism deviates substantially from the established model for CYP450s, the use of these models for UGT clearance may not be appropriate. When employing the IVIVE model, we find no significant difference between normal weight and obese individuals in UGT clearance. If these results are accurate, then the in vitro to in vivo extrapolation (IVIVE) model may be well suited to account for clearance differences due to obesity. However, reservations to this interpretation exist due to the inherent interindividual variations seen in UGTs. It is possible that our limited sample size and/or appropriateness of the model itself may mask actual differences in clearance between normal and obese individuals.

4.5 Study Limitations
While BMI correlates to body fat and obesity, it is not a direct measure of these factors. Deficiencies in the BMI model show that it does not correlate well to body fat content for muscular individuals (Lele 2007) and varies between populations and ethnic groups (Misra, Wasir et al. 2005). Since height and weight are commonly collected for health screenings and pathology reports, BMI while not perfect, is a convenient metric to estimate obesity. Regardless of convenience, a height to waist ratio is superior to waist circumference and BMI alone for detecting cardiometabolic risk factors in both sexes across multiple ethnic groups (Ashwell, Gunn et al. 2012). Therefore, the incorporation of this metric (were it to exist for our samples) may alter our findings.
4.6 Conclusion
With the obese population of the world steadily increasing in both size and numbers, this disease may impact a larger component of the population. Many clinical trials only include mildly overweight to moderately obese patients and may not adequately reflect the impact of obesity, especially in morbidly obese individuals, on drug activity and clearance (Brill, Diepstraten et al. 2012). Our study begins to provide evidence that obesity plays a significant role in altering the activity, and possibly expression, of drugs processed through glucuronidation. This study also shows that these alterations from obesity may be different in males and females, another important characteristic to consider. Moreover, the obese population may require additional attention for drug development to ensure proper establishment of dosing regimens. With continued refinements to drug dose modeling, especially for obese and morbidly obese individuals, it may be possible to improve drug administration to the individual and reduce both the frequency and severity of adverse side effects in this population.

4.7 Future Studies
With evidence of UGT activity impairment and possible changes to protein expression, determination of cause and further effects are advisable. With additional information on the substrates used for activity measurements and complex modeling equations it would be possible to model the hepatic clearance of these isoforms for each individual in the study. While changes in expression and activity in obese individuals is a concern, modeling of hepatic clearance will aid us in assessing whether these changes result in
modifications to the clearance abilities of these individuals. Modeled hepatic clearance can tell us whether toxicity from drugs should be a concern for those with higher BMIs.

Recent studies have shown that methylation states of promoter regions play an important role in the functioning of UGT isoforms (Gagnon, Bernard et al. 2006). While current methylation studies show that it is highly influential in tumor cells and cell type differentiation, no studies have directly looked at methylation in relation to obese individuals that also show characteristic liver changes, sometimes attributable to a disease state. There have been studies showing a so-called “metabolic memory” by which certain metabolic disease states are predisposed in people by early exposure to certain conditions (Takizawa, Mizutani et al. 2013). We believe that this may happen in liver cells, exposed to conditions brought on by systematic changes that occur in obesity. It is proposed that a methylation study of fatty livers versus normal livers and/or high BMI versus normal BMI livers can be performed and differences in UGT methylation interrogated.

Current research seems to indicate that fat loading of hepatic cells lowers activity of several CYP enzymes, this research has not yet been performed on UGTs. By inducing steatosis and then examining the mRNA levels of these samples we can determine the affect of mRNA levels in hepatic cells. Alternatively we may interrogate the changes induced on nuclear transcription factors such as Peroxisome Proliferator-Activated Receptors (PPARs) which are important in lipid metabolism and homeostasis, or Hepatic Nuclear Factors (HNFs) which are obligate for UGT expression in the liver. If PPAR
function is disrupted or deficient, it could lead to an overabundance of intracellular FFAs. In conjunction with increased FFAs, there would also be increased ROS leading to oxidative stress.

4.8 Final Conclusion
The key contributions of this study are the discovery that UGT activity, but not protein expression is inhibited by obesity. Preliminary pharmacokinetic modeling indicates that for at least one isoform (UGT1A4) this also significantly affects clearance of the drug Trifluoperazine. Since protein levels did not diminish we contend that post-translational modifications and or allosteric interactions on the enzyme protein may be responsible for the decrease in enzyme activity. The major secondary contribution is the confirmation that for UGT1A1 and 1A9 (at least) fluorescence assays are as accurate and precise as mass spectrometry. This is a practical outcome that can save researchers time and money. In future, we hope to use more sophisticated modeling approaches to define the amount of clearance that is inhibited by obesity and guide clinical dosing appropriately by setting guidelines for drug dosing and environmental exposure, specific to obese people.
REFERENCES


Williams, R. T. (1959). Detoxication mechanisms; the metabolism and detoxication of drugs, toxic substances, and other organic compounds. New York,, Wiley.

BIOSAFETY AND ETHICAL CONSIDERATIONS

Biosafety Institutional Approval
The PI, Dr. Abby Collier, possesses all the appropriate Institutional approvals from The University of Hawaii Environmental Health and Safety office (BSP 2 and 3). Along with Dr. Collier, Steffen Oeser has requested and received exempt approval from the University of Hawaii IRB to perform this research.

Human Subjects Research
Microsomal samples are provided by Xenotech, which will have already de-identified the data before supplying it. We will have access to height, weight, BMI, sex and race, but no personally identifying information. Liver samples to be used for the methylation study will be de-identified and obtained from the Hawaii Organ and Tissue Bank in which the recipients have previously authorized samples to be used for research and all donors are deceased. Demographic data will be gained from Hawaii Organ and Tissue Bank staff, extracted from de-identified files. Patient population #1 is a set of deceased donors that have consented to their organs being archived in a biorepository for research. We will be using donated human livers. We will be using 24 adult livers of mixed age, sex and ethnicity. Second population is commercially supplied (Xenotech, Lenexa, KS) human liver cells and data (n=400) from non-surviving donors. These will be received from Xenotech deidentified with only demographic data (age, sex, height, Weight, ethnicity, cause of death). The PhD student (Steffen Oeser) has received exempt category 4 IRB approval for this research with Dr. Collier as his named PI from the University of Hawaii Committee on Human Studies (CHS #21144). Dr. Collier completed NIH
training for the protection of human subjects in 2003 and has current CITI certification (latest date 5/13/12). The student (Steffen Oeser) completed CITI training on 3/14/13. The risks to the subjects are extremely minimal since we will use de-identified tissues. The knowledge gained through this study will show the impact of obesity on UGT isoforms.

DSMP: Not applicable

Inclusion of Women and Minorities

Women are not the main subjects of this research but they are equally considered in that they make up an almost equal portion (57 female samples, 66 male samples) of our pool of examined biological information for all characterizations. We will be examining sex differences in our data and comparing both expression and activity rates of UGTs. This will allow us to determine whether trends are similar between the sexes for all isoforms examined.

Minorities are included in this project as we have tissues from native Asians, and African Americans, Hispanics, as well as Caucasians. Since we do not plan to look at ethnicities specifically, no single ethnic group will be specifically excluded or included for analysis. No generalizations based on race will be made, as we will not consider racial differences for any particular analysis in this set of studies.

Inclusion of Children

Children are not subjects of this research, only adult tissues are used.

Animal Research

Not applicable, this proposal includes human tissues, cells and cell lines.