

EFFECT OF OBESITY ON HEPATIC DRUG METABOLISM

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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September 2013

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Acknowledgements

First and foremost I would like to thank my advisor Dr. L'Aurelle Johnson for her scientific guidance and support over the years of my graduate training. It was a great pleasure to work with her. Her constant motivation over the past few years gave me the encouragement to carry on with my graduate work even when things were not on brighter side. I also wish to express my sincere appreciation and gratitude to Dr. Rory Remmel for his guidance. The guidance that I received from both Dr. Remmel and Dr. Johnson has contributed tremendously to my scientific and personal life.

I wish to thank Dr. Robert Straka for his constructive criticism and suggestions on my thesis work. His feedback ensured that I was always aware of translational utility of my research work. Also, I would like to thank Dr. Lisa Peterson for her guidance and valuable scientific feedback during the course of my research work.

I would like to acknowledge the help of our collaborators Dr. Butterick and Dr. Kotz at the V.A. Medical centre and Dr. Rector, Dr. Perfield and Laura at the University of Missouri. Also, I would like to acknowledge the help extended by Jim Fisher at the CPAS lab.

I would like to thank all the members of ECP administrative staff for their support and making sure that I navigated through the different rules and regulations of graduate school smoothly. Dede, Carol Ann and Erin have been great people to interact with in the department.

I would like to thank Dr. Mike Sinz and Bristol-Myers Squibb for training me in the technique of hepatocyte isolations.

Also, a special thanks to all my lab mates Harrison, Aaron, Jin, Darcy, Anja, Lucy, Gina and Ryan for giving me all the support in the lab.

A special thanks to all my friends Raj, Suresh, Akshanth, Azher, and Jia who have helped me a lot throughout my graduate student life. Without them the journey would not have been fun.

I would also thank Dr. Iyer and Dr. Coutinho my professors in India. Their blessings continue to guide and motivate me.

Lastly and the most important my family Tai, Shrikant, Arunima, Aai, Baba, and Neha who are a source of strength, care and love and without whom nothing is possible.

DEDICATION

This thesis is dedicated to my God and

His manifestations *MY PARENTS*

And

To my loving and caring wife *NEHA*

Abstract

Obesity has increased markedly over the last few decades and is now a major public health crisis in the U.S. affecting over 1/3 of the US population. Optimization of dosing in obese individuals is a challenge due to the lack of knowledge regarding changes in the pharmacokinetics (PK) of therapeutic agents in obese individuals. Thus the aim of this thesis was to determine the effect of obesity on drug metabolism and evaluate methods that could potentially predict changes in pharmacokinetics in the obese population.

The impact of obesity on drug metabolism in children has not been determined and our clinical study (Chapter 2) was the first of its kind to examine the effect of childhood obesity on CYP1A2, CYP2D6, CYP3A4, xanthine oxidase, and NAT2 activity using caffeine and dextromethorphan as probe drugs. Our results conclusively indicate that obesity results in an elevation of xanthine oxidase and NAT2 enzyme activities in obese children as compared to lean children, whereas there was no difference in CYP1A2, CYP2D6 and CYP3A4 activity between obese and lean children. This study provides a potential mechanism of altered 6-mercaptopurine exposure in the obese pediatric cancer population.

While clinical studies would provide the most optimum method to predict clearance of therapeutic agents in humans, studies have reported that clearance can also be predicted using animal data. In Chapter 3, we examined mouse, rat and porcine model of obesity in order to determine the utility of these animal models to predict PK in obese humans and to identify a model that would best reflect the human obesity mediated changes in drug metabolism. The study indicated species dependent differences in CL_{int} of various drugs that were due to, either changes in expression of drug metabolizing enzymes or

changes in enzyme substrate affinity. The study demonstrated that obesity can alter enzyme activity in a species and model dependent manner. Furthermore this study identified that the rat High Fat Diet animal model of obesity is the best representation of the obesity mediated alterations in humans.

In Chapter 4, in collaboration with Drs. Scott Rector and Jim Perfield, University of Missouri, Columbia, we demonstrated obesity mediated alterations of drug metabolism enzyme activity can be prevented by sterculic oil dietary supplementation. These effects are mediated through signal transduction pathways which regulate CAR and PXR transcription factors. These results establish that obesity mediated changes are biochemically dependent and not weight dependent.

In Chapter 5, we developed a proof of concept that would help generate biochemically obese hepatocytes. In absence of hepatocytes from obese individuals, these hepatocytes can be used as a tool to predict obesity mediated changes in drug clearance. Our studies indicate that individually, leptin, resistin, IL-6 and TNF- α can modulate expression of various DMEs in a concentration dependent and isoform specific manner. This study demonstrates that the obesity microenvironment is important in obesity mediated changes in drug metabolism. Additional studies would help establish a more robust method to generate and validate these obese hepatocytes.

In summary, the work in this thesis has helped identify the drug metabolism enzymes that are altered in the obese children, the utility of using animal models of obesity as tools to study the impact of obesity on pharmacokinetics/pharmacodynamics, proven that it is possible to reverse obesity mediated changes in drug metabolism and

developed an in vitro model that may be used to predict changes in drug disposition in the obese population. These findings are important for to better develop dosing strategies in obese humans with concomitant disease.

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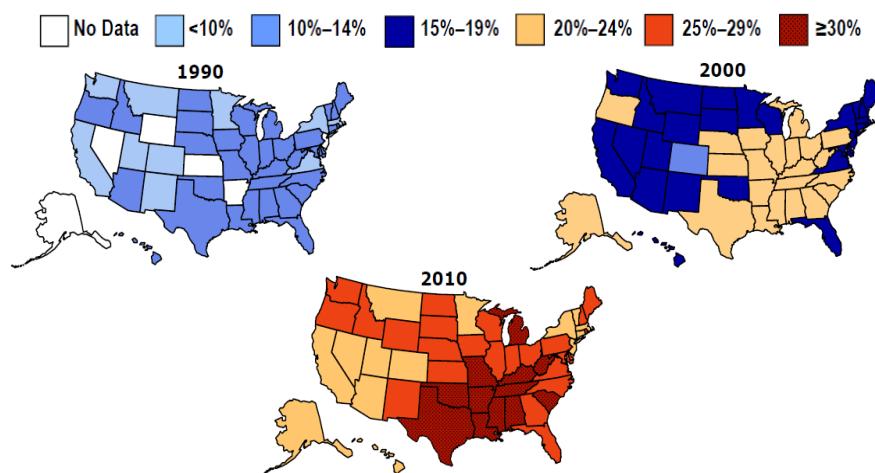
Chapter I

Literature Review

1.1 Obesity

1.1.1. Prevalence and causes of obesity: Obesity is a growing epidemic and a major health crisis in United States of America. The rates of obesity have nearly doubled over the last two decades with nearly 35 % of adults and 18 % of children being classified as obese as of 2010, whereas in 1990s the prevalence of obesity in US was nearly 15 % in adults.(1) This increase in prevalence of obesity has been attributed to complex interaction between genetic, cultural, social, and environmental factors. However, environmental factors are considered to be a major reason for the increased rates of obesity in US population. By analysis of four consecutive studies conducted by National Health and Nutrition Examination Survey (NHANES), Kant et al. reported that there is a parallel between increased consumption of high density foods and growing prevalence of obesity. Also, socio-economic status has been associated with obesity, since there is an increased consumption of high fat diet food, rather than fruits and vegetables, by individuals in lower income groups.(2)

Fig 1.1. Percentage of obese adults in US (Source: CDC)



These prevalence rates are based on the clinical definition of obesity that is, in adults, defined in terms of body mass index (BMI). BMI is a ratio of the weight (kg) to the height (m^2) and based on BMI, individuals are either classified as normal weight, overweight, obese and morbidly obese (**Table 1.1**).

Table 1.1: Classification of obesity status.

BMI	Obesity
> 40	Morbidly Obese
30 - 39.9	Obese
25 - 29.9	Overweight
19 - 24.9	Lean

In the pediatric population obesity is defined in terms of the percentile; with children at or above the 95th percentile for height and weight being classified as obese, as compared to children of same sex and age.(1)

The growing frequency of obesity is considered to be a serious health and financial crisis, since obesity is associated with severe medical complications and increased financial burden on healthcare system.(3) Based on Centre for Disease Control (CDC) reports, it was estimated that medical costs associated with obesity have been as high as \$ 147 billion in the year 2008.(1)

Also, studies have shown that BMI is associated with increased incidence of various diseases like diabetes, cardiovascular diseases, cancer etc. For example, Barker et al. have reported that the prevalence of hypertension was nearly 41.9 % in men and 37.8 %

in women with BMI > 30.(4) Andersen et al have shown that BMI at age 7 is associated with risk of coronary heart disease at the age of 25, with each unit increase in BMI being associated with 10 % increase in odds of CHD event when adjusted for birth weight. (5) In addition to these life threatening diseases obesity has been associated with other debilitating diseases such as osteoporosis, arthritis etc. that can alter the quality of life. One of the major causes of the diseases associated with obesity is changes in the metabolic profile in obese individuals. The observed alterations in the plasma levels of various biochemicals is due to changes in the content and size of adipose tissue.(3)

1.1.1 Adipose Tissue

In humans, adipose tissue occurs in two types: a. white adipose tissue (WAT) and b. brown adipose tissue.

Brown adipose tissue is found mainly in human neonates and is important for the regulation of body temperature through non-shivering thermogenesis.

White adipose tissue is the most abundant adipose tissue in humans and is the main site of energy storage. WAT is composed of many cell types with adipocytes being the most abundant. In addition to adipocytes, adipose tissue also contains pre-adipocytes (adipocytes that have not yet been loaded with lipids), endothelial cells, fibroblasts, leukocytes and, most importantly, macrophages. The number of macrophages present in WAT is directly correlated with adiposity and with adipocyte size in both human subjects and mice, with no significant differences present between subcutaneous and visceral WAT. Recently, it has been recognized that the white adipocytes are major secretory

cells responsible for secretion of various fatty acids as well other lipids such as prostanoids (synthesized by WAT) and retinol and cholesterol (not synthesized, but stored). Also adipose tissue is responsible for the secretion of various protein moieties similar to the cytokines known as adipokines. These adipokines mainly, leptin, resistin, and serpin E1, form a major portion of the adipose ‘secretome’ (Fig 1.2).(6)

Studies have shown that the adipose tissue content in a lean individual is nearly 20 % of the total body weight, while in an obese individual the adipose tissue is much higher and is nearly half of the total body weight. Since the adipose tissue is involved in the secretion of various adipocytokines, it has been reported that the levels of these adipocytokines are higher in obese children as well as adults as compared to the lean controls (Table 1.2).(7)(8)

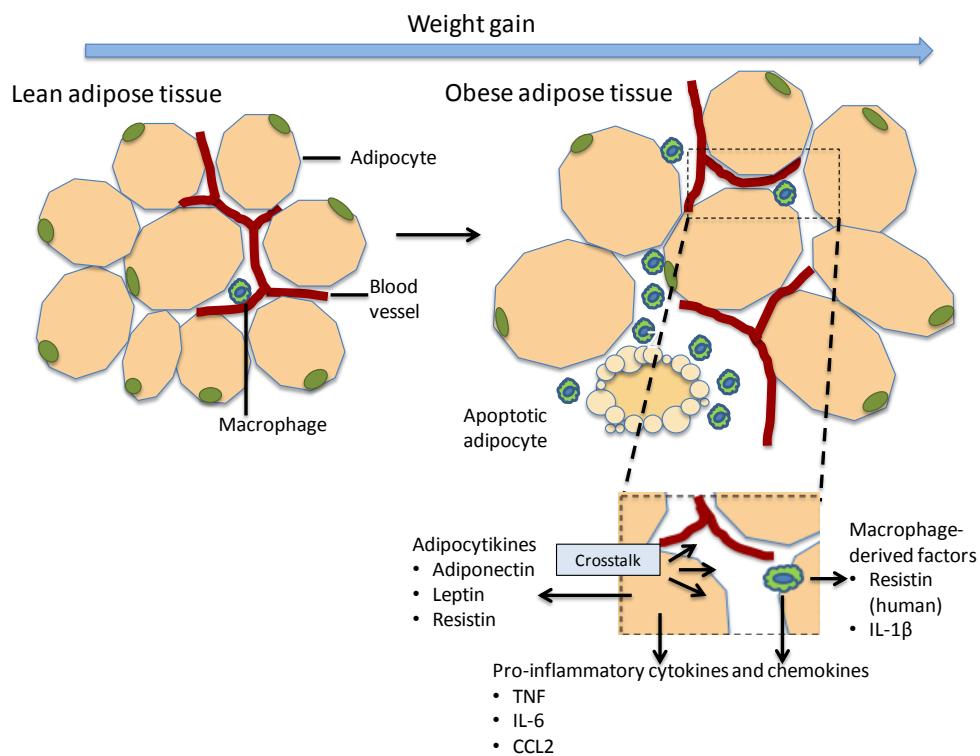
Table 1.2: Plasma levels of adipokines and cytokines in obese individuals.

Variable	Pediatric		Adult	
	Control (n = 63)	Obese (n = 63)	Control (n = 16)	Obese (n = 9)
Leptin (ng/ml)	7.9 ± 5.1	19.9 * ± 7.4	7 ± 3	17 ± 2 *
IL - 1 (pg/ml)	3.6 ± 1	33 * ± 8.9		
IL - 2 (U/L)	0.9 ± 0.1	0.4 * ± 0.1		
IL - 6 (pg/ml)	13.1 ± 3.9	45.2 * ± 11.8	1.2 ± 0.3	2.8 ± 0.5 *
TNF - α (pg/ml)	3.9 ± 1	9.2 * ± 2.3	8 ± 1	9.2 ± 1.4
Resistin (ng/mL)	12.8 ± 8.3	24.6 * ± 12.9	6.3 ± 0.8	7.8 ± 0.5 *
Adiponectin (μg/mL)	13.3 ± 1.8	8.6 * ± 0.8	11 ± 1.8	8.8 ± 1.3

* p-value < 0.05

The adipokines adiponectin, leptin, resistin and visfatin provide an important link between obesity, inflammation and insulin resistance. Leptin and resistin are the most important adipokines that are secreted by the adipose tissue. Also, the adipose tissue is involved in the increased secretion of inflammatory cytokines like interleukin 6 (IL-6), IL-1, tumor necrosis factor (TNF- α) and monocyte chemo-attractant protein (MCP). MCP is known to recruit macrophages to the adipose leading to increased secretion of TNF- α . Thus, obesity is a state of chronic inflammation associated with increased levels of circulating cytokines, increased synthesis of acute phase reactant proteins like C-reactive protein (CRP) and the activation of subsequent inflammatory pathways (Fig 1.2).(7)

Fig 1.2: Secretion of adipokines and cytokines by adipose tissue.



The activation of inflammatory pathways along with changes in the fatty acid content that are associated with obesity are known to cause changes in the expression and activity of drug metabolizing enzymes.(9) These changes in the activity of DMEs are known to modulate the clearance of therapeutic agents that are commonly administered to obese individuals for the treatment of various comorbidities associated with obesity.(6)

1.2 Effect of Obesity on Hepatic Drug Metabolism: Animal and Human Studies

Over the past two decades various in vitro (microsomal) and in vivo studies (animal models of obesity or obese humans) have been conducted in order to identify the effect of obesity on drug metabolism.(9)(10)(11) However, most of these studies have been reported in various animal models of obesity that are commonly used as mimics of human obese phenotype in preclinical setting. The following section gives a brief overview of the various animal models that have been used to date.

1.2.1 Animal models of Obesity:

Animal models of obesity can be subdivided into two distinct types: a. Genetic models of obesity. and b. Diet-induced models of obesity (12)

The animal models of obesity are ideally expected to mimic human obesity in terms of the causes of the disorder (termed as construct validity). Another important aspect is that these animal models should respond to pharmacological treatments in a manner that is similar to humans (termed as predictive validity).

Despite being phylogenetically unrelated to humans, rats and mice are the predominant models that are used for understanding, preventing and curing human obesity. Interestingly, these animals show neurological changes that are similar to those in humans. Previous studies have established that human and rodent neurotransmitters and peptides that regulate food intake and energy homeostasis produce effects that are similar in both species.(12)

a. Genetic models of obesity:

Genetic models of obesity tend to acquire the obese phenotype due to the occurrence of single gene mutation that results in increased food intake leading to obesity. The most widely used animal models of obesity include the *ob/ob* mouse, *db/db* mouse and the Zucker *fa/fa* rat. These animals are overweight within several weeks of birth and eventually become 1.5 to 3 heavier than their lean control. After 6 months, nearly 50% of their body weight is due to the presence of fat deposits.

Due to the single gene mutation the *ob/ob* mice lack leptin, while the *db/db* mice and Zucker *fa/fa* rats lack the leptin receptor, but have marked elevations in circulating leptin.(13) The absence of leptin receptor or leptin (satiety hormone) leads to the higher intake of food leading to obesity. All three models display hyperphagia, hyperinsulinaemia and hyperlipidaemia but differ in the degree of hyperglycemia. The *db/db* mice are hyperglycemic and are also used as a model of diabetes, whereas *ob/ob* mice display mild to moderate hyperglycemia and Zucker *fa/fa* rats are not hyperglycaemic but develop insulin-resistance.(14) However, despite the similar phenotype, one of the major concerns associated with use of *ob/ob* mouse model of

obesity, is the lack of leptin in these mice which is contrast to the significantly higher leptin levels associated with human obesity.(8)

b. Diet-induced models of obesity:

The diet-induced model of obesity (DIO) or high fat diet (HFD) model of obesity involves providing rats or mice free access to high fat diets over a period of 12-16 weeks. These animals over time exhibit increased weight gain along with an increase in body fat. Apart from changes in the body weight these animals exhibit insulin resistance, glucose intolerance, elevated leptin levels and a mild dyslipidaemia as compared with rats fed a standard diet with lower fat content. Importantly, such changes in DIO mice and rats mimic the changes observed in obese patients. An important aspect with the use of DIO models is that the changes seen in DIO animals are remarkably consistent with those seen in obese patients in the clinic. However, a study involving identification of changes in the expression of various genes validated the DIO rat as a model of human obesity.(15) Apart from the rodent model of obesity, recent studies by Sturek et al. have characterized a novel porcine model of obesity. Ossabaw pigs serve as an excellent model for investigating metabolic syndrome, progression to type II diabetes and coronary heart disease. When fed HFD the Ossabaw pigs develop obesity, insulin resistance, dyslipidemia, and hypertension in a manner similar to that observed in human obesity.(16)

Despite the development of various animal models of obesity over the past few decades, no single model is applicable to all the research associated with obesity. However, it is

important to consider that certain models have greater utility as compared to others. In order to study the effect of obesity on drug metabolism it is not only important to consider the validity of obesity phenotype, but also compare the homology and regulation of oxidative and conjugative enzymes involved in drug disposition.(17, 18) Recent studies have shown that though the rodent models are predictors of drug metabolism in humans, the porcine CYPs share a much greater homology with humans and may serve as better predictor. (19) The following section describes the different aspects of drug metabolism and provides a brief description of changes associated with obesity in DMEs in different animal models of obesity.

1.2.2. Hepatic Drug Metabolism

The liver is the major organ involved in metabolism of xenobiotics; however other organs like the intestine, kidneys, lungs etc. are also known to be involved in xenobiotics metabolism. The major enzymes that are involved in the biotransformation process are mainly the Cytochrome P450s (CYPs) and the Uridine diphosphate glucuronosyltransferases (UGTs), with minor contribution from other oxidative and conjugative enzymes. The primary role of these enzymes in biotransformation involves conversion of xenobiotics to a more polar form that can be easily excreted via the kidneys. Thus, the expressions of these enzymes play an important role in clearance of xenobiotics from the body (20).

In humans 18 families of CYPs, including 43 subfamilies have been identified, with the CYP 1, 2 and 3 family accounting for nearly 75 % of total hepatic CYP content (Fig

1.3.). These heme-containing enzymes located in the endoplasmic reticulum of the cells are known to oxidize a variety of substrates. Of importance to human drug metabolism are CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4/5 isoforms, of which CYP3A is the most abundant and important human hepatic CYP subfamily accounting for metabolism of nearly 50 % of therapeutic agents (Fig 1.1). (20) Based on sequence identity, human UGTs are classified into four families; UGT1, UGT2, UGT3 and UGT8. UGT1 and UGT2 families are the most important in terms of drug metabolism. The human UGT1 gene contains 9 distinct functional first exons UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10 and shared set of four exons, designated 2–5. Alternate splicing of the distinct first exons results in formation of nine functional transcripts, which result in synthesis of proteins with common carboxy-acid terminal but different amino acid terminal. The UGT1 locus (encoding UGT1A2p, UGT1A11p, UGT1A12p, and UGT1A13p) contains mutations leading to formation of inactive protein. The members of the UGT2 family are encoded by separate genes comprising six exons and hence have different protein sequence amongst members of the same subfamily. The UGT2 family is further sub-divided in to two distinct sub-families 1.UGT2A (2A1, 2A2 and 2A3) and 2. UGT2B (2B4, 2B7, 2B10, 2B11, 2B15, 2B17, and 2B28), which contain three and seven members, respectively. Like UGT1A family, there are five UGT2B pseudogenes (UGT2B24P, UGT2B25P, UGT2B26P, UGT2B27P and UGT2B28P) that have been identified. Of the various UGT1A and UGT2B family members, the following hepatic isoforms: UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15 play an important role in drug and xenobiotic

metabolism (Table 1.3) (21, 22)

Fig 1.3: Relative hepatic content of various CYP isoforms in humans (in %). (20)

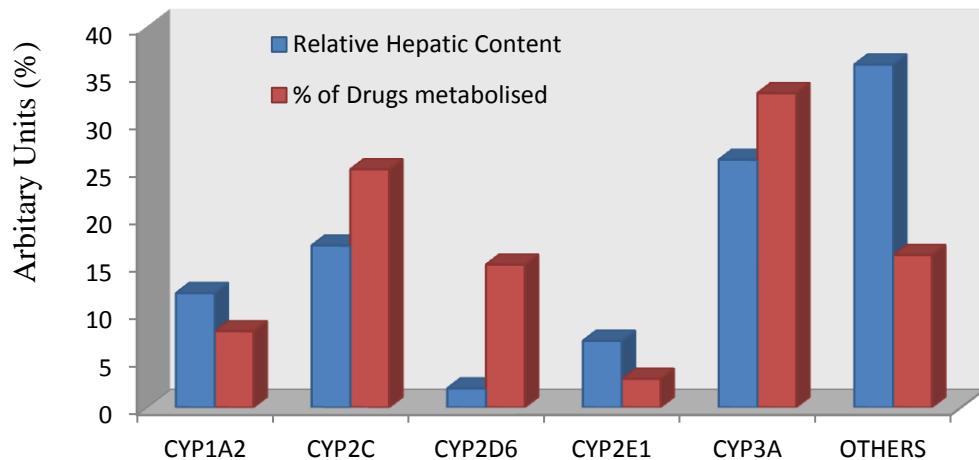


Table 1.3: Substrates, inhibitors and inducers of CYPs and UGTs in humans.

Enzyme	Substrate	Inhibitor	Inducer
1A2	theophylline, caffeine	fluvoxamine	omeprazole, β-naphthoflavone
2B6	efavirenz	Ticlopidine, thiotapec	phenobarbital
2C8	repaglinide, rosiglitazone	gemfibrozil	rifampin
2C9	warfarin, tolbutamide	fluconazole, amiodarone	rifampin
2C19	omeprazole, esoprazole, lansoprazole,	omeprazole,	rifampin
2D6	desipramine, dextromethorphan,	paroxetine, quinidine, fluoxetine	none identified
2E1	chlorzoxazone	disulfiram	ethanol
3A4/3A5	midazolam, triazolam	atazanavir, itraconazole,	rifampin, carbamazepine
UGT1A1	bilirubin, ezetimibe estradiol	atazanavir, indinavir	Rifampicin,
UGT1A3	alizarin, apigenin, buprenorphine,	25-trihydroxyvitamin D(3)	ponasterone A

Enzyme	Substrate	Inhibitor	Inducer
UGT1A4	chlorpromazine, clozapine, doxepin	hecogenin	phenobarbital, phenytoin
UGT1A6	atenolol, labetolol, propranolol	Oltipraz, TCDD	tbutylhydroquinone
UGT1A7	estriol, 2-oh-estradiol, 4- oh-estrone	magnolol	TCDD
UGT1A9	mycophenolic acid, r- oxazepam, paracetamol, propofol,	niflumic acid	thiazolinediones
UGT2B7	flurbiprofen, morphine, zidovudine.	ketoconazole, valproic acid	barbiturates
UGT2B15	lorazepam, s-oxazepam	flunitrazepam	

Combined together, CYPs and UGTs are responsible for the hepatic clearance of more than 90% of drugs from the body. Apart from these enzymes there are other oxidative enzymes like aldehyde oxidase (AO), xanthine oxidase (XO) and conjugative enzymes like sulphotransferases, N-acetyltransferases and glutathione S-transferase that are known to metabolize selective marketed drugs. Hence it is important to understand and predict the changes in activity of these enzymes in human obesity.

Various pre-clinical models of obesity have been used to understand the impact of obesity on drug metabolism. However, the use of mouse, rat and porcine model of obesity is associated with marked species differences in the content and activity of various isoforms of CYPs and UGTs.

Species differences in Drug Metabolism:

Species differences in drug metabolizing enzymes have been identified and reported for most of enzymes. However, of importance are those differences that are mainly in the CYPs and UGTs since these enzymes are mainly responsible for the metabolism of therapeutic agents. Shimada et al. and Stevens et al. have reported differences in the total P450 content in liver microsomes isolated from mouse, rat, pig, monkey and humans (**Table 1.4**). (17)(23)

Table 1.4: Hepatic P450 content in different species.

Species	P450 Content pmol/mg protein
Human	307 ± 160
Monkey	1030 ± 106
Pig (Female)	488 ± 83
Rat	673 ± 50
Mouse	719 + 41

Apart from the differences in the total P450 content there are also differences in the expression and homology of various CYP isoforms in these animal models as compared to humans. The following table gives a list of various human CYPs involved in drug metabolism along with their respective orthologs in mice, rats and pigs.(24)

Table 1.5: List of CYPs in humans along with their preclinical species orthologs.

Family	Subfamily	Human	Mouse	Rat	Porcine
CYP1	A	1A1, 1A2	1A1, 1A2	1A1, 1A2	CYP1A2
	B	1B1	1B1	1B1	1B1
CYP2	A	2A6, 2A7, 2A13	2A4, 2A5, 2A12, 2A22	2A1, 2A2, 2A3	2A19
	B	2B6, 2B7	2B9, 2B10	2B1, 2B2, 2B3	2B22
	C	2C8, 2C9, 2C18, 2C19	2C29, 2C37,	2C6, 2C7*, 2C11*, 2C12*, 2C13*, 2C22, 2C23	2C33, 2C42, 2C49
	D	2D6, 2D7, 2D8	2D9, 2D10, 2D11, 2D12, 2D22	2D1, 2D2, 2D3, 2D4, 2D18	2D21, 2D25
	E	2E1	2E1	2E1	2E1
3	A	3A4, 3A5, 3A7, 3A43	3A11, 3A13, 3A16, 3A25,	3A1/3A23, 3A2*, 3A9*, 3A18*, 3A62	3A29, 3A39, 3A46

* Gender difference in expression

The differences in protein sequence and expression in these animal models have been reported to alter substrate specificity and rate of metabolism of various substrates that have been studied using human liver microsomes or recombinant human CYPs.(25)(26)

The following table lists the differences/similarities in substrate specificity in various animal models commonly used to assess drug metabolism in preclinical setting(19, 24)

Table 1.6: Species comparison of various CYP isoforms, substrates, inhibitors and inducers.

CYP		Human	Rat	Mouse	Porcine
1A	CYP	1A1/2	1A1/2	1A1/2	1A1/2
	Inducer	OM, BNF, 3-MC	BNF, 3-MC, PB	PB	BNF, 3-MC,
	Substrate	Phenacetin, ER	Phenacetin, ER	Phenacetin ER, MR	ER
2A	CYP	2A6	Absent	Absent	2A19
	Inducer	PB, RIF		PB	PB
	Substrate	Coumarin	N/A	N/A	Coumarin
2B	CYP	2B6/7	2B1/2	2B9/10/13/19	2B
	Inducer	PB	PB	PB	PB, Rifampicin
	Substrate	S-mephenytoin, Bupropion, BR, PR	BR, PR, testosterone	BR, PR	PR, mephenytoin
2C	CYP	2C8/9/18/19	2C11, CYP2C6	2C29	2C29
	Inducer	RIF (2C9, 2C19), PB (2C19)	Dex	Phenytoin	Rifampicin, PB
	Substrate	Paclitaxal (2C8), Tolbutamide, Diclofenac (2C9), S-Mephenytoin (hydroxylation, 2C19)	Diclofenac, Testosterone (16α- and 2α-hydroxide)	Tolbutamide	Diclofenac,
2D	CYP	2D6	2D1/2	2D9–13	2D25
	Inducer	No known inducer			
2E	Substrate	Dextromethorphan	Dextromethorphan	Buforolol	Tolterodine
	CYP	2E1	2E1	2E1	2E1
	Inducer	Isoniazid, PB, RIF	Ethanol, Acetonepyrazine	Ethanol	Ethanol, Isoniazid
3A	Substrate	Chlorzoxazone	Chlorzoxazone, p-NP	Chlorzoxazone p-NP	Chlorzoxazone
	CYP	3A4/5	3A1/2	3A11	3A29
	Inducer	RIF	DEX, PB	PB	DEX, Rifampicin
4A	Substrate	Testosterone, Midazolam, Nifedipine	Testosterone, Midazolam	Testosterone, Nifedipine	Nifedipine, Testosterone
	CYP	4A9/11	4A1/2/3/8	4A10/12/14	4A21
	Inducer	Clofibrate	Clofibrate (4A1)		Clofibrate
	Substrate	Lauric acid	Lauric acid	Lauric acid	Lauric acid

Apart from differences in the CYPs, there are reported differences in various UGTs in animal models. However, the data regarding the substrate specificity and relative activity of these enzymes is not available since recombinant enzymes in all animal species have not been cloned and expressed.

The intrinsic clearance of various commonly used human UGT probes has been determined in mouse and rat liver microsomes and these results (Table 1.7) indicate clear differences in extent of hepatic glucuronidation among different species. These differences in glucuronidation rates can be associated to differences in expression and homology of the UGTs catalyzing these reactions.(18, 23)

Table 1.7: Intrinsic clearance of various UGT substrates.

Substrate	Mouse	Rat	Humans
	CL _{int} (μl/min/mg protein)		
Estradiol	179	193	40
4-MU	1975	1146	1245
4-NP	341	213	314
Propofol	62	14	14
MPA	78	17	250
Morphine	46	27	2

4-MU: 4-methylumbelliferaone; 4-NP: 4-nitrophenol; MPA: mycophenolic acid

One of the major differences in rodents and humans, with respect to UGT expression, is that in mice Ugt1a4 is a pseudogene while in rats Ugt1a4 and Ugt1a9 are both pseudogenes. However, in humans, both UGT1A4 and UGT1A9 are important UGTs that are known to glucuronidate variety of therapeutic agents (Table 1.3). For other UGT1A

sub-families, there is poor homology between rodent and human UGTs. The mouse and rat Ugt1a6 has the highest homology (70%) with human UGT1A6, while other subfamilies have less than 70 % homology. Interestingly, in absence of Ugt1a9 in rats, rUgt1a8 shares nearly 68% homology with human UGT1A9.(21) However, due to lack of specific studies using recombinant rat and mouse Ugts it is difficult to identify the functional relevance of the similarities/differences in UGTs in different animal species. Since disease states are known to modulate the clearance of various therapeutic agents, the choice of animal model to study the impact of particular disease state on drug metabolism should take into account the differences in regulation and catalytic activity of DMEs among different species.(27)

1.2.3 Effect of obesity on drug metabolism: A review

Obesity is one such disease state that is known to regulate the expression and activity of these drug metabolizing enzymes. Over the last two decades numerous studies have reported alterations in pharmacokinetics of specific therapeutic agents in obese as compared to lean. For example Caraco et al. reported decreased clearance of anti-epileptic drug carbamazepine (CYP3A4 substrate) in obese patients as compared to lean controls (19.8 ± 1.2 ml/min vs 23.0 ± 1.3 mL/min).(28) Similarly other investigators have shown increased clearance of lorazepam, chlorzoxazone in obese individuals.(9) Since, however, for drugs with low to moderate clearance, the concentration at steady state is a function of the intrinsic clearance of that drug, it is important to determine the activity in obese individuals using specific probe drugs. Recent studies in animal models of obesity and clinical studies involving obese patients have demonstrated that there are clinically

significant changes in the activity of important drug metabolizing enzymes in the obese phenotype. These changes have been reported in most of the members of CYP family like CYP1A, CYP2A, CYP2B, CYP2C, CYP2E, CYP3A and CYP4A either in animal models, clinical studies or using post-mortem samples from obese individuals.(10, 29, 30)(31)

CYP1A2: This isoform constitutes nearly 12 % of total hepatic CYP content and is responsible for metabolism of nearly 15 % of the marketed drugs. Variety of substrates are metabolized via this enzyme and includes various analgesics, antiarrhythmics, anticancer agents, antidepressants etc. Decreased CYP1A2 expression and activity in animal models of obesity is one of the consistent observations that have been reported by various groups. Interestingly, though Suh et al. using obese Zucker rats reported decreased mRNA levels of CYP1A2 at 12 weeks, they observed a significant increase in mRNA expression of CYP1A2 at 6 weeks, compared to their respective lean controls.(32) Similarly, results in the genetic and diet induced obesity mouse models are in agreement with the reported changes in rat models of obesity. Yoshinari et al. and Kirpitch et al. have reported decreased mRNA levels of CYP1A2 in db/db and high fat diet mouse models of obesity respectively.(33, 34) Studies using hepatocytes isolated from obese individuals or using post mortem liver samples have also observed decreased mRNA levels and activity of CYP1A2.(31) However, most of the clinical studies, including our study in obese pediatric children have reported no changes in clearance of drugs that are specifically metabolized by CYP1A2, except one study by Jusko et al. which reported increased clearance of theophylline in obese individuals as compared to lean.(35)

CYP2A: It is one of least important DME since it constitutes only 4 % of total hepatic CYP content and is responsible for metabolism of only 3 % of the marketed agents. Considering its limited role in metabolizing therapeutic agents only few studies have been reported in animal models of obesity as well as in obese humans. In animal studies, Weltman et al. reported decreased activity in HFD mouse model, while Watson et al. have reported increased activity in ob/ob mouse model.(10, 29) Similarly, in humans Fisher et al. have reported increased activity in livers obtained from obese humans while Rubio et al. have reported decreased activity.(31,36) However, in each of the reported studies the extent/mechanism of obesity was different which could have led to the conflicting results observed in those studies.

CYP2B: CYP2B6 accounts for 6% of total CYPs in the liver and has been shown to metabolize nearly 10% of therapeutic agents. Though this P450 is known to metabolize some important drugs like efavirenz, and other tricyclic antidepressants, its regulation in obesity is not well understood. Studies in ob/ob mice have shown that the mRNA levels of Cyp2b10 (the mouse ortholog of human CYP2B6), are lower in male obese mice while they are higher in female obese mice.(11) Studies by Watson et al. have shown that activity of Cyp2b is higher in ob/ob mice as compared to lean control.(10) Even, studies using human liver samples have shown that there is either an increase or decrease in the mRNA levels of CYP2B6 in obese as compared to lean controls.(31) However, studies have shown that in presence of inflammatory mediators like TNF- α , IL-6 the expression and activity of CYP2B6 is lower due to increased proteosomal degradation.(37)

CYP2C: The human CYP2C family consists of multiple isoforms viz CYP2C8, CYP2C9 and CYP2C19 that accounts for nearly 18 % of total hepatic CYP content and is known

to metabolize approximately 25 % of the marketed drugs. Some of the commonly used therapeutics agents that are metabolized via this pathway include anticoagulants, antidiabetics, proton pump inhibitors etc. CYP2Cs are involved in clinically significant drug-drug interactions due to their role in metabolizing a wide variety of drugs. Despite the clinical importance of CYP2C few studies have explored the effect of obesity on expression and activity of CYP2Cs, both, in animal models as well as in humans. Studies in db/db mouse model have reported no change in Cyp2c29 protein even though the mRNA levels of this enzyme were found to be elevated.(33) In the rat model of obesity studies have shown that mRNA expression and activity of CYP2C11 is decreased in the obese animals as compared to the lean control.(38) In contrast to the uniform decrease seen in rats, studies using human liver have shown no changes in CYP2C9 activity. Interestingly, the clearance of ibuprofen, a CYP2C9 substrate, has been reported to be higher in obese individuals as compared to lean, while clearance of phenytoin was not found to be significantly different between both the groups.(9) Similarly, studies using post mortem human livers obtained from obese have shown decreased protein levels and activity of CYP2C19, while pharmacokinetic studies have shown increased clearance of diazepam (CYP2C19 substrate) in obese as compared to lean.(27) No studies in rats and mice have explored the effect of obesity on CYP2C19 substrates.

CYP2D6: It is an enzyme that constitutes nearly 2-8 % of the total hepatic CYP content but is responsible for the metabolism of nearly 25 % of therapeutic agents, including various anti-depressants, anti-HIV, anti-arrhythmics etc. Few studies in rodent models of obesity have explored changes in respective CYP2D activities. Also, considering the genetic polymorphisms associated with CYP2D6 conducting adequately powered clinical

studies that evaluate the effect of obesity are difficult and have not been reported. However, a study by Donato et al. has shown that fatty acids are associated with decreased CYP2D6 activity, while study by Fisher et al. has shown that the CYP2D6 activity is unchanged in human livers.(39)(31) Despite these findings, additional studies are needed to determine the magnitude of change and the mechanism of these changes in CYP2D6 expression and activity.

CYP2E1: It is responsible for metabolism of nearly 5 % of marketed drugs and constitutes nearly 7 % of total hepatic content. However, despite its lower expression and limited involvement in metabolism of therapeutic agents it is one of the most studied enzymes in obesity since it is highly conserved across species. Also, it is the first enzyme that was reported to be increased in patients with fatty liver.(9) Most of the studies in obese humans indicate increased activity of this enzyme as measured by clearance of probe substrate chlorzoxazone. Similarly studies in genetic and diet induced rat models also indicate increased activity of CYP2E1. An exception was a study by Zhang et al. which reported decreased CYP2E1 activity in obese Zucker rats. (40) While studies in rat and humans shown similar changes in CYP2E1 activity in obese phenotype, most of the studies using the genetic mouse models have reported either decreased activity and expression of CYP2E1 or no changes in the CYP2E1 activity.(11) (41) However, studies using the high fat diet model of obesity have shown that the CYP2E1 activity is higher in the obese mice as compared to the lean mice.(42) Thus, the studies reported in preclinical high fat diet models of obesity have reported changes in CYP2E1 activity which is in agreement with the changes reported in humans. O'Shea et al. reported increased oral clearance of chlorzoxazone in obese (6.23 ± 1.7 mL/min/kg) as compared to lean ($4.15 \pm$

0.81 mL/min/kg) volunteers, while Miller et al. also observed increased clearance of enflurane (substrate of CYP2E1) in obese as compared to lean.(43,44) In contrast some researchers have reported no differences in activity of CYP2E1. Interestingly, studies in patients undergoing bariatric surgery or diet restriction in order to reduce weight, have shown decrease in CYP2E1 activity following intervention.(45)

CYP3A: This is one of most important human hepatic enzyme that constitutes nearly 26 % of total hepatic CYP content and is responsible for metabolism of greater than 50 % of therapeutic agents. There are multiple isoforms of this enzyme that are expressed at either the neonatal stage (CYP3A7) or during the adult life (CYP3A4 and CYP3A5). There is a great variability in the hepatic and intestinal expression of CYP3A subfamily of enzymes. Considering, its major role in metabolism of multiple substrates it has been implicated in various drug-drug interactions and is also a focus of various studies reported by researchers in obese preclinical models of obesity. Studies in obese high fat diet and Zucker rat model of obesity have consistently reported decreased expression and activity of CYP3A1/23. Suh et al. have reported temporal changes in expression of CYP3A with higher expression of CYP3A in 6 weeks old Zucker rats as compared to lean control. However, in 12 week old Zucker rats the authors reported decreased expression of CYP3A as compared to the lean control.(32) Similarly, clinical studies in humans have reported decreased clearance of probe drugs, midazolam, alprazolam and triazolam in obese individuals as compared to lean control. In a study by Abernethy et al. the clearance of triazolam was found to be significantly lower in obese (340 ± 44 mL/min) as compared to lean individuals (531 ± 38 mL/min).(27) Also, studies by Caraco et al. have reported increased clearance of carbamazepine in obese individuals following weight loss

(31.6 ± 5 mL/min vs 20.4 ± 1.8 mL/min).(28) However, this decreased clearance of probe substrates has been reported to be substrate specific, with no change in clearance of other probes like midazolam and cyclosporine. Interestingly, recent studies by Fisher et al. using postmortem liver samples from obese also reported no change in either the expression or activity of CYP3A4.(31) In general, the data reported using mouse model of obesity is much more conflicting, with some groups reporting no changes in Cyp3a activity in either ob/ob or db/db mouse model of obesity,(10, 33, 41) while some groups based on studies in high fat diet model of obesity have reported decreased or increased activity of Cyp3a.(34)(46) Though there has been extensive effort to identify effect of obesity on CYP3A there is clearly a need to conduct well powered clinical studies that would not only quantify the changes in CYP3A activity using multiple probe drug approach, but also associate these changes with a particular biochemical signature that could be predictive of the changes expected in obese individuals.

Other oxidative enzymes: Xanthine oxidase (XO) is an enzyme that is responsible for the metabolism of purine drugs mainly 6-mercaptopurine, allopurinol (also a suicide inhibitor) and endogenous substrates like hypoxanthine and xanthine. Various studies in rodent models of obesity and clinical studies have reported that the levels of xanthine oxidase are higher in the obese as compared to the lean controls. A study by our group in pediatric population has shown that xanthine oxidase activity, as assessed by metabolism of caffeine, was 30 % higher in the obese group.(47) This obesity mediated increased activity is a cause of concern since xanthine oxidase catalytic cycle is known to produce reactive oxygen species which is a risk factor for cardiovascular diseases. Aldehyde

oxidase (AO) another oxidative enzyme that has recently been shown to be involved in metabolism of various preclinical drug candidates as well as some therapeutic agents has been found to be upregulated in the high fat mouse model of obesity.(38) However, for AO no clinical studies have been reported in obese individuals.

Apart from the oxidative enzymes various studies have reported changes in the expression and activity of various conjugative enzymes like UGTs, NATs, Glutathione S-transferases (GSTs).

UDP glucuronosyltransferases: Conjugation via glucuronide formation is one of the major pathways of drug metabolism catalyzed by multiple intestinal, hepatic and renal UGTs. Apart from metabolizing xenobiotics UGTs play an important role in conjugating endogenous substrates like bilirubin, steroid hormones, bile acids, eicosanoids, and neurotransmitters. Polymorphisms resulting in loss of UGT function have been known to cause Gilberts syndrome, Crigler-Najjer syndrome and various cancers. Considering its importance in metabolism and association with various disease states, studies have been conducted in animal models and obese humans to explore the effect of obesity on expression and activity of various isoforms of UGTs.(22)

In ob/ob mouse model of obesity Watson et al. reported no changes in overall activity of UGTs (assessed by glucuronidation of 4-methyumbellifereone, a general UGT probe).(10) However recent studies by Xu et al. have reported increased mRNA expression of Ugt1a1, 1a6, 1a9, 2a3, 3a1 and 3a2 in ob/ob mice. Also, the glucuronidation of acetaminophen was found to be nearly 47 % higher in ob/ob mice as compared to C57BL/6J mice (lean control). (30) Interestingly, in high fat diet mouse model of obesity,

a study by Koide et al. reported no changes in expression and activity of Ugt1a1, Ugt2b, while a study by Kirpich et al. reported no changes in mRNA expression of Ugt1a9 and Ugt2b1.(34, 46) In rat, genetic and diet model of obesity the results are much more consistent with Kim et al. reporting nearly 60 % decreased expression of Ugt1a1, Ugt1a6 and Ugt2b1 in obese Zucker rats, while Osabe et al. reported similar decrease in mRNA expression of Ugt1a1, 1a6, 1a7 and 2b1 in the diet induced rat model of obesity.(38) Surprisingly, very few studies have been reported that determine the changes in glucuronidation capacity of obese individuals. Using acetaminophen metabolism as marker of UGT1A6, UGT1A9 and UGT2B15 researchers have shown that the clearance of acetaminophen via glucuronidation pathway is higher in obese as compared to lean. Similarly researchers have reported increased clearance of oxazepam (UGT1A9, UGT2B7 and UGT2B15 substrate) and lorazepam (general UGT substrate) in obese as compared to lean.(9)

Sulfotransferases: Sulfotransferases, like UGTs, are involved in the metabolism of both endogenous substrates and xenobiotics. However, unlike UGTs, the important drug metabolizing SULTs are cytosolic and belong to the SULT1 and SULT2 families. SULTs are the major detoxification enzymes in the developing liver however, in the adult liver they are responsible for the conjugation of less than 25 % therapeutic agents that are eliminated via conjugation. Some of the common substrates of SULTs include acetaminophen, albuterol, terbutaline and hormonal contraceptives.

In ob/ob mouse model, there were gender dependent changes in Sult expression with increased Sult2a1/2 expression in males with no change in females.(11) However, Koide

et al. reported decreased Sult2a1 activity in high fat diet mouse model of obesity. In the same study, the researchers observed no changes in the expression of Sult1a1. (46) In Zucker rat model, investigators have reported no changes in activity, while in humans there is a decreased expression of SULT1A1 in liver of obese patients.(48)

Glutathione S-transferase: Glutathione S-transferases are a group of enzymes that catalyze the transfer of glutathione to electrophilic carbon of nonpolar xenobiotics. The important mammalian GSTs are cytosolic and based on their amino acid sequence are divided into seven classes. Various studies have shown that genetic polymorphisms and disease states modulate the expression and activity of GSTs. Various studies in animal models of obesity as well as clinical studies have reported changes in expression and/or activity of GSTs. These changes are isoforms specific and animal model dependent.

Table 1.8: Changes in total GST.

Species	Model of obesity	mRNA	Protein	Activity	Reference
Mouse	Ob/Ob mice (12 weeks)	ND	ND	No change	Watson et al.
	Ob/Ob mice (16 weeks)	ND	ND	Decreased	Barnett et al.
	Ob/Ob mice (16 weeks)	ND	ND	Decreased	Roe et al.
Rat	Zucker rat at (20 weeks)	ND	No change	ND	Chaudhary et al.
	Zucker rat at (14 weeks)	Decreased	ND	ND	Kim et al.

Species	Model of obesity	mRNA	Protein	Activity	Reference
Human	Progression to NAFLD	ND	Increased (tGSTA)	ND	Hardwick et al.
		ND	Decreased (tGSTM)	ND	
		ND	Increased (tGSTP)	ND	
		ND	ND	Decreased (tGST)	

ND: Not determined; tGST: total GST; A: Alpha; M: Mu; P: Pi

Other conjugative enzymes: N-acetyl transferase is an enzyme responsible for acetylation of drugs like isoniazid. In our clinical study in obese pediatric patients, we observed genotype dependent increased NAT activity in obese group as compared to the lean group.(47)

1.2.4 Mechanism of obesity mediated changes in DMEs:

It is a well documented observation that several drug metabolizing enzymes are either induced or downregulated by variety of endobiotics and xenobiotics including therapeutic agents as well as biochemicals altered during disease states. The regulation of DMEs occurs at either the transcriptional level or at a post-translational level. Ligand activated nuclear receptors are known to transcriptionally induce the expression of various DMEs via binding to regulatory elements in the promoter region of the target genes. Ligands that either bind to these receptors or cause degradation of them can modulate the expression of target DMEs. Several nuclear receptors along with their target genes have been identified and are listed in Table 1.9(20, 49, 50)

Table 1.9: List of transcriptional regulators along with their target genes.

Transcription factor (Full Form)	Ligands		Target Gene	
	Endogenous	Xenobiotics	CYPs	UGTs
AhR Aryl-hydrocarbon receptor	Bilirubin, tryptophan	polycyclic and halogenated aromatic amines	1A1, 1A2,1B1	1A1, 1A6
CAR Constitutive androstane receptor	androstanol, DHEA	phenobarbital, CITCO	2B6, 2C9, 2C19, 3A4/5	1A1, 2B4
SXR/PXR Pregnane X receptor	pregnenolone, estrogens, DHEA	rifampicin, phenobarbital, dexamethasone	1A, 1B, 2A, 3B6, 2C, 3A4/5/7	1A1,1A3, 1A4,1A9
VDR Vitamin D receptor	1,25-dihydroxyvitamin D3	Doxercalciferol, paricalcitol	2B6, 2C9, 3A4	1A3,1A4
FXR Farsenoid X receptor	Bile acids, unsaturated fatty acids	Ivermectin	3A4	2B4, 2B7
PPAR α Peroxisome proliferator-activated receptor	Fatty acids	Fibrates	1A,2A, 2C, 2E, 4A	1A1,1A9, 2B4
Nrf2**	Antioxidant pathway	Sources of oxidative stress	1A2	1A1,1A3

**NFE2L2: Nuclear factor (erythroid-derived 2)-like 2

The modulation in levels of these transcription factors along with induction in protein degradation pathways has been implicated to play a role in altering the levels of target DMEs. The following mechanisms have been reported as potential mediators of reported changes in DMEs in obesity.

Transcriptional Regulation of Drug Metabolizing Enzymes:

Obesity is a state of chronic inflammation and mechanistic studies that have been reported for various cytokines provide an insight into the mechanism of regulation of oxidative and conjugative enzymes in obese condition.(37)

Multiple *in vitro* and *in vivo* studies have shown that inflammatory stimuli (associated with inflammation, infection and obesity) or administration of inflammatory cytokines, is responsible for downregulation of CYP mRNA. The reduction in CYP mRNA has been associated with concomitant decrease in CYP protein and activity. However, very few studies provide regulatory pathways that might be responsible for the observed changes. Delaporte et al. demonstrated using nuclear run-on assay, that following induction of proinflammatory pathways in rats, there was decreased transcription of CYP1A1 and CYP1A2 genes.(51) However, later studies by Cheng et al. demonstrated that though primary mechanism of downregulation is decreased transcriptional activity, increased rates of mRNA degradation would also play an important role in decreasing the mRNA levels following cytokine treatment.(11) Tinel et al. demonstrated that downregulation of CYP2C11 and CYP3A by IL-2 in rat hepatocytes was related to induction of oncogene transcriptional factor *c-myc*, these observations were confirmed by adding inhibitors of *c-myc* which resulted in blockade of IL-2 mediated downregulation of CYP2C11 and CYP3A.(52) Iber et al. demonstrated that cytokine activated transcription factor NF-κB, bound to a low affinity negative κB response element on promoter region of CYP2C11 and CYP3A leading to decreased transcription.(53) However, the most detailed study involving CYP3A4, regarding molecular mechanisms was conducted by Jover et al. who demonstrated using human hepatocytes that cytokines caused induction of mRNA

expression of transcription factor CCAAT-enhancer binding protein β (C/EBP β), which subsequently forms C/EBP β -LIP (Liver-enriched transcriptional inhibitory protein). This C/EBP β -LIP competes with constitutively expressed C/EBP β -LAP (Liver-enriched transcriptional activating protein) leading to transcriptional repression of CYP3A4 mRNA expression.(54)

Proinflammatory cytokines have also been shown to downregulate the expression of nuclear receptors CAR and PXR. Thus, cytokines like IL-6 do not decrease the transcriptional activity of CAR and PXR but suppress expression of their target genes by decreasing the expression of these NRs. However, the transcriptional activity of CAR and PXR might be decreased, since studies have also found decreased expression of RXR, which is an obligatory heterodimer partner of CAR and PXR and necessary for high affinity binding of these NRs to promoter region of target genes. Apart from cytokines, polyunsaturated fatty acids (which are higher in obese) have also found to decrease the expression of CAR and to a lesser extent PXR.(31, 33, 55) Fatty acids are known to activate PPARs and HNF4 α .(56) While PPARs are known to induce the expression of CYP4A and various UGT isoforms, HNF4 α has been reported to regulate CYP2A6, 2B6, 2C9, 2C19 and CYP3A4/5. Also, HNF4 α is known to regulate the expression of CAR and PXR thus indirectly controlling expression of various CYPs and UGTs.(57)

Studies have shown that obesity is associated with oxidative stress, and cells respond to this stress by upregulating various antioxidant genes. The antioxidant response is controlled by a specific transcription factor called Nrf2. Under normal conditions, Nrf2 forms a complex with Keap1 and undergoes proteosomal degradation. Under oxidative stress, the Nrf2 is released from the Keap1 complex and translocates to the nucleus where

it promotes the transcription of target genes like GSTs, UGTs, SULTs etc.(57) Studies by Garg et al. and Lamsa et al. have shown that under oxidative stress CYP1A is negatively regulated while CYP2A is induced.(58, 59) Apart from these reported mechanisms, there are various other unproven hypothesis involving changes in levels of glucagon, epinephrine, bile acids that can explain some of the changes in mRNA observed in obesity.

Clearly, studies have been done that demonstrate the importance of transcriptional regulation in causing alterations in the expression of DMEs. However, apart from transcriptional regulation, various studies have shown that there are changes at the posttranslational level which would influence the amount of active protein and hence the phenotype.

Posttranslational Mechanism of Obesity mediated changes in DMEs

Studies using rat hepatocytes have reported decreased protein levels following treatment with various cytokines that are not correlated with the changes in mRNA expression of the protein. Since the decline in the protein content could not be explained by pre-transcriptional change, the researchers proposed the contribution of posttranslational modifications to the observed changes in protein levels. Induction of nitric oxide synthase (NOS) and xanthine oxidase (XO) has been correlated with decrease in CYP protein content. Several studies have shown that induction of NOS in presence of cytokines results in formation of nitric oxide which is known to bind to the heme moiety of the CYP enzymes resulting in lower activity and acceleration of protein degradation by nitrosylation of the heme.(60) Addition of inhibitors of NOS resulted in reversal of these

effects. Carlson et al. demonstrated that addition of cytokines to primary rat hepatocyte culture resulted in decreased CYP2B levels ($33\% \pm 9\%$ of control), and addition of NOS inhibitor L-NMA significantly prevented the cytokine mediated decrease in CYP content.(61) Apart from NOS, induction of xanthine oxidase activity has been reported in presence of various cytokines. The increased xanthine oxidase activity has been associated with increased generation of superoxide radicals which may contribute to degradation of CYP protein. Moochhala et al. reported decreased CYP3A content and an increased activity of XO in rat hepatocytes treated with interferon. The effects of IFN were attenuated on addition of allopurinol a known XO inhibitor.(62) Studies have shown that XO is not necessary mechanism for DME downregulation, however, in presence of redundant mechanisms it might contribute to the downregulation of various DMEs.(63) Despite various mechanisms that have been used to explain the changes in DME expression, the observed changes are varied and dependent upon the model, extent and cause of obesity. Also, while clinical and animal studies have been reported that show obesity mediated changes in drug metabolism and hence clearance, few have sought to predict the changes in clearance of therapeutic agents in obese individuals. Thus, the aim of the current thesis was to determine the effect of obesity on drug metabolism and to explore various methods/tools that are necessary to predict clearance in obese individuals. This would help achieve the long term goal of predicting accurate dosing in obese individuals as well as predicting drug – drug interactions and bioactivation potential of therapeutic agents in this population.

1.3 Research Objectives:

1. Determine the effect of obesity in obese pediatric population:

Using a probe drug approach, the activity of drug metabolizing enzymes was estimated in obese pediatric population. Caffeine and dextromethorphan were used to assess the changes in enzyme activity of CYP1A2, NAT2, Xanthine oxidase, CYP3A and CYP2D6 in obese children as compared to lean controls.

2. Comparison of changes in drug metabolism in animal models of obesity:

The purpose of this study was to evaluate the changes in drug metabolism enzyme activity in rodent and non-rodent models of obesity with a two-fold purpose of:

- i) Identifying the feasibility of using these animal models of obesity as predictors of changes in clearance in obese individuals using the allometric scaling approach and
- ii) Identifying an animal model that best mimics the changes seen in obese humans.

3. Determine the effect of anti-obesity agent on drug metabolism in rat animal model of obesity:

The objective of this study was determine whether changes in plasma levels of various biochemicals following anti-obesity intervention in obese animals would result in reversal of obesity mediated changes in drug metabolism.

4. Evaluate the effect of biochemicals on DME activity and development of biochemically obese hepatocytes to predict changes in hepatic clearance in obese individuals:

The aim of this study was to test the feasibility of generating biochemically obese hepatocytes, which could be used to predict the changes in hepatic clearance of

therapeutic agents in obese individuals. Also, such hepatocytes would provide a better insight into the bioactivation and drug-drug interaction potential of various therapeutic agents in obese individuals.

Chapter II

Effect of Obesity on Drug Metabolism in Pediatric Population

This Chapter has been published in Br. J. Clin. Pharmacol. 2011 Jul;72(1):109-15.

Additional study results have been added at the end of chapter.

2.1. Introduction:

The effect of obesity on activity of conjugative and oxidative enzymes involved in drug metabolism has been studied in adults to some extent. Specifically obese adults exhibited a 35%, 38% and 36% decrease in clearance of CYP3A4/5 substrates carbamazepine, methylprednisolone and triazolam, respectively (1-4). However activities of other CYP isoforms (e.g. CYP2C19, CYP2E1 and CYP2D6) were increased as compared to non-obese adults.(5-8) Studies of conjugative enzymes using lorazepam and oxazepam as probes, demonstrated that obese adults also have an enhanced capacity for biotransformation via the glucuronide conjugation pathway and that these changes were proportional to total body weight (TBW).(9)

In contrast to the current knowledge of the effect of obesity on drug metabolism in adults, little is known about the impact of obesity on drug metabolism in children. With the prevalence of pediatric obesity approaching 30%, (10-13) extensive knowledge of how each enzyme is affected is needed to begin to understand the impact of obesity on the pharmacokinetics of therapeutic agents in the pediatric population. Caffeine phenotyping in children is a successful non-invasive tool for the measurement of oxidative and conjugative drug metabolism enzymes cytochrome P450 1A2 (CYP1A2), xanthine oxidase (XO), and N-acetyltransferase 2 (NAT2) Scheme 1. Specifically this probe has been utilized in pediatric populations to determine the effects of disease on oxidative and conjugative capacities in these individuals.(14-16) The current study evaluated the effect of obesity on drug metabolism in children using caffeine to assess CYP 1A2, NAT2, and XO activity.

2.2. Methods:

2.2.1. Study Design: The study was approved by the Institutional Review Board (IRB 0610M94328) and was conducted at the Clinical and Translational Sciences Institute of the University of Minnesota. All subjects 7 years of age and older signed an informed assent, whereas all parents/ guardians signed an informed consent which was also approved by the IRB. The participants were included based on the following criteria: 1) Pre-pubertal children between ages 6-10 yrs., 2) Children with BMI less than 85th percentile (control) or greater than 95th percentile (obese) for age and gender (<http://www.cdc.gov/obesity/childhood/defining.html>), 3) Children not allergic to caffeine and 4) Children currently not on any medication that interferes with drug metabolism. Obese volunteers were recruited from the University of Minnesota Weight Management Clinic; whereas lean subjects were recruited randomly.

Study volunteers were asked to refrain, for 48 hrs prior to administration of the caffeine, from consuming any substances that would alter basal phase I and phase II enzyme activity. Baseline saliva, blood and overnight urine samples were collected prior to the start of study. Following administration of caffeine (11.5 mg as 4oz Coca Cola®) urine samples were collected in two hour increments over 8 hours.(15) Whole body dual-energy X-ray absorptiometry (DEXA), scans were performed on each study subject to assess total body fat composition.

2.2.2. Quantification of caffeine metabolites: Concentrations of caffeine and its metabolites were determined using LC/MS as previously described by Weimann et al.(17) Briefly caffeine and metabolites were separated on a Phenomenex fusion reverse

phase (50mm x 3.0mm, 3.5um) column at 30°C. The separation was performed by gradient elution starting at 5% acetonitrile increasing to 30% acetonitrile over 4 minutes at flow rate of 0.3 ml/min. Electrospray ionization was performed in the positive ion mode to detect caffeine and in the negative mode to detect the 17U (1,7-dimethyluric acid), 1X (1-methylxanthine), AFMU (5-acetylamino-6-formylamino-3-methyluracil), and 1U (1-methyluric acid) metabolites. The $([M + H]^+/[M - H]^-)$ ions were selected by the first mass filter for all analytes. The coefficient of variation was less than 7.5% for all metabolites at all concentration levels. The accuracy was between 90-105%. Calibration curves for caffeine and metabolites were linear in the range of 2.5 – 2500 ng/ml. The lower limit of quantification for caffeine, 17U, 1X, 1U, and AFMU metabolite was 2.5 ng/ml.

2.2.3. Assessment of drug metabolizing enzyme activity: Based on previously reported studies, the effect of obesity on CYP1A2, XO and NAT2 enzyme activities were evaluated using urinary metabolic ratio (MR) of metabolites of caffeine. CYP1A2, NAT2 and XO activity was assessed using equation 1, 2, 3 respectively. (18) (19) (20) For all the enzymes of interest, the MR was defined as concentration of metabolite to concentration of drug, thus a higher MR ratio is associated with increased enzyme activity.

Equation 1:

$$CYP1A2 = \frac{AFMU + 1U + 1X}{17U}$$

Equation 2:

$$\text{NAT2} = \frac{\text{AFMU}}{\text{AFMU} + 1\text{U} + 1\text{X}}$$

Equation 3:

$$XO = \frac{1\text{U}}{(1\text{U} + 1\text{X})}$$

2.2.4. DNA isolation and genotype determination: Genomic DNA was isolated from peripheral blood lymphocytes using standard techniques.(21) DNA quantitation was carried out by measuring the absorbance at 260 nm.

NAT2 and CYP1A2 single nucleotide polymorphisms (SNPs) were determined by TaqMan from Applied Biosystems (Foster City, CA). The PCR and probe primers were designed by Applied Biosystems Inc for the following NAT2 polymorphisms; rs1208, rs1801279, rs1801280, rs1799929, rs 1799930, and rs1041983; and CYP1A2 polymorphisms rs762551, rs12720461, rs2069514, rs2069526, rs2470890, and rs28399424. All reactions and analysis were conducted in a 96-well plate format. The reaction components for each genotyping reaction were as follows: 1 μ L of DNA (20 ng/ μ L), 12.5 μ L of TaqMan master mix (Applied Biosystems, Branchburg, NJ), 0.625 μ L of primer/probe mix (Applied Biosystems, Foster City, CA) and water up to a total volume of 25 μ L. The thermocycler conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 92°C–15s and 60°C–1 min. The reaction was then analyzed on an Applied Biosystems PRISM model 7500 sequence detection system and software.

2.2.5. Obesity biomarker determination: Plasma obesity biomarkers were determined using Human Obesity MultiAnalyte Profiling Base Kit purchase from R&D Biosystems (Minneapolis, MN) following manufactures instructions.

2.2.6. Statistical Analysis: Values are expressed as the mean \pm SD. The difference between the means of the groups was tested for significance using Student's t-test. The level of statistical significance was set at $p < 0.05$. Based upon our sample size of twenty we had $>90\%$ power to detect a minimal difference 0.06 difference in XO activity, 87% power to detect a minimal difference of 0.72 for NAT2 activity and 47% power to detect a minimal difference of 1.35 for CYP 1A2 activity.

2.3. Results:

2.3.1. Participants' characteristics: Twenty-five pediatric volunteers between ages 6 - 10 yrs. were recruited for the study. Of these 25, 9 were classified as obese (% total body fat (TBF) > 38), while 16 were classified as lean (% TBF < 25 %). The median (range) age of volunteers in both groups was 9 yrs (6yrs – 10 yrs). The male: female ratio in the lean population was 7:9 while in the obese population it was 2:7 (Table 1). Laboratory tests confirmed that study subjects had no liver or kidney dysfunctions. Five out of 25 study subjects were excluded from the NAT2 analysis because they were classified as rapid acetylators (NAT2*4), and thus could not be compared with the individuals who were slow acetylators (NAT2*5, NAT2*6 and NAT2*14) based upon genotype. Additionally five study subjects were excluded from the CYP1A2 analysis due the presence CYP1A2 of polymorphisms that conferred a reduced activity genotype. Caffeine was well tolerated by all participants and no adverse events were observed. Biomarkers of obesity were measured to confirm subjects' metabolic obesity status (Table 1). As expected, the levels of leptin were 10 fold higher in the obese vs. lean population $p < 0.001$. Additionally IL- 6 and C-reactive protein were ~ 3 fold and 2 fold higher than in the lean population, respectively. However, adiponectin were highly variable in both groups and were unaltered ($p = 0.06$).

2.3.2. Assessment of CYP 1A2, XO, and NAT activity: The metabolic ratio (MR) for XO in the obese group (n=8) was 0.7 ± 0.06 whereas the MR for XO in the non-obese group (n=16) was 0.6 ± 0.047 , 95% CI (0.046,0.154) $p <0.001$. This suggests that obese subjects exhibit an increase in XO enzyme activity when compared to non-obese subjects

(Figure 1A). Similarly, the mean MR for NAT activity was observed to be 5 fold higher in the obese group (n=6) (1.01 ± 0.31 vs. 0.18 ± 0.13 , $p<0.05$) when compared to the non-obese group (n=14), thus indicating an increase in NAT2 activity in obese individuals, 95% CI (0.26,1.34) (Figure 1B.). In contrast, for CYP1A2, the mean MR in obese (n = 7) was found to be 5.4 ± 2.1 , whereas the MR in lean (n= 13) was 6.7 ± 1.7 , 95% CI (-2.72,0.12) ($p>0.05$, Figure 2).

2.4. Discussion:

Approximately 20-30% of children in the United States are classified as obese. Obesity is a major health concern, with various epidemiological studies linking obesity to coronary heart disease, stroke, type II diabetes and several forms of cancer. (22) Limited data are available regarding the impact of obesity on pharmacokinetics of drugs in children; however, obesity has been demonstrated to affect overall outcomes associated with the treatment of pediatric neurological disorders and cancer. (23)(24, 25) Because drugs can be metabolized by different drug metabolizing enzymes, extensive knowledge of how each enzyme is affected and the drugs metabolized by that enzyme is needed to fully understand the impact of obesity on drug metabolism of a particular agent. This is the first study to examine the effect of childhood obesity on CYP1A2, xanthine oxidase, and NAT2 activity as measured by administration of caffeine as a probe drug.

Xanthine oxidase catalyzes metabolism of endogenous substrates hypoxanthine and xanthine as well as the bioconversion of the exogenous anti-neoplastic agents, such as 6-mercaptopurine. We observed that obese children exhibited a 16% increase in xanthine oxidase activity compared to lean controls (Figure 1A.). This observed increase is relatively small compared to clinical data examining the pharmacokinetics of 6-mercaptopurine in lean and overweight pediatric cancer patients with acute lymphoblastic leukemia. Specifically, these studies have demonstrated that obese (as defined as BMI > 75%) pediatric cancer patients exhibited a twofold lower overall exposure to 6-mercaptopurine as compared to the lean children (as defined as BMI <75%).(26) Even as such the magnitude of difference in 6-mercaptopurine exposure as it pertains to obesity maybe in synergy with extrinsic factors (e.g. cancer) which also have the ability to alter

drug metabolism enzymes activity.(27) Albeit not conclusively, our finding still supports the hypothesis that increases in xanthine oxidase activity in obese subjects may play a role in the observed reduced exposure of 6-mercaptopurine in this population. A possible mechanism by which obesity can alter xanthine oxidase activity is through elevated cytokine concentrations. Cloning and characterization of the xanthine dehydrogenase gene revealed that there are cytokine specific regulatory response elements located in the promoter region of this gene. Specifically there were (3) IL-6, (5) INF- γ , (1) NF κ -B, (1) IL-1 and (1) TNF responsive elements identified.(28) In vitro studies in human mammary epithelial cells demonstrated that incubation with inflammatory cytokines, increases xanthine oxidase activity 2 to 8 fold. (29) Furthermore clinical studies quantifying the XO activity in the COPD airway epithelial lining fluid determined that XO activity was increased 4 fold in COPD subjects when compared to non-COPD subjects and this elevation in xanthine oxidase was highly correlated with increased concentrations of the proinflammatory cytokines TNF- α and IL- β .(30) We observed in our population that obese children had elevated IL-6, CRP, leptin levels, and decreased adiponectin levels when compared to the lean controls (Table 1). Therefore it is possible that these proinflammatory cytokines and adipokines which are excessively produced in the state of obesity upregulate XO gene expression and activity in these individuals. Another explanation for increase xanthine oxidase activity maybe due to an increase liver volume associated with obesity; thereby resulting in a higher metabolic activity in obese children when compared to lean children. Even though plausible, studies evaluating the pharmacokinetics and disposition of CYP3A4/5 substrates carbamazepine, methylprednisolone and triazalam reported that obese subjects exhibited a decrease in

clearance of these substrates by 35%, 38%, 36%, respectively, when compared to non-obese adults.(1, 3, 4)

N-Acetyltransferase 2 is responsible for the metabolism of some xenobiotics (e.g., metabolize isoniazid and procainamide) and the activation of some carcinogens (e.g. aromatic and heterocyclic amines). Additionally, this enzyme is responsible for the formation of the caffeine metabolite 5-acetylamino-6-formylamino-3-methyluracil (AFMU). Among the slow acetylators, we observed a 5-fold increase in NAT2 activity in the obese as compared to non-obese children. NAT2 activity has been reported to be associated with elevated risk of colon rectal cancer and breast cancer (31-33) in individuals who have slow acetylator phenotype. Additionally there is evidence that suggests that obese adults and children have increased cancer risks than non-obese counterparts.(34-36) This increase in cancer risk may be associated with alterations in oxidative and conjugative enzyme capacity for activating or inactivating carcinogenic agents. Thus the contribution of obesity to elevated enzyme activity may also be a predictor of increased risk for overall exposure to carcinogens activated through the NAT2 pathway.

CYP1A2 makes up approximately 10% of total liver CYP content (37) and is responsible for the metabolism of approximately ~4% of drugs used.(38) Common drugs that are substrates for the CYP1A2 isozyme include R-warfarin, theophylline, caffeine, and some benzodiazepines, antidepressants and antipsychotics. We observed that there was no difference in CYP1A2 enzyme activity between obese and non-obese children. Murine studies in the genetically obese mice (ob/ob) observed that obese mice exhibits reduced CYP1A activity when compared to lean controls.(39) However evidence of an effect of

obesity on CYP1A2 activity is inconclusive. (40) Alterations in both NAT and XO activity and not CYP1A2 activity demonstrates that the increase in metabolic ratio in the obese vs. non-obese is independent of the conversion of caffeine to paraxathine metabolite. Bracco et al. determined that parent caffeine urinary excretion was no different in lean vs. obese women; however urinary caffeine metabolites were more abundant in the obese women when compared to lean women. This suggests that dissimilarities in the production and excretion of these caffeine metabolites in obese vs. lean women may be a result of altered expression or activity of enzymes involved in caffeine metabolism similar to what was observed in our pediatric population. (41)

Despite our findings, our study does present some limitations. The first limitation being with respect to the metabolic ratios used to determine CYP1A2, NAT2, and XO activities. The metabolic ratios to determine CYP1A2, NAT2, and XO activities depend primarily on the urine collection time. Studies have shown that it is imperative to collect urine for long enough time periods so that caffeine metabolites can be formed. Rost et al. determined that the 5-8 hr (AMFU + 1U + 1X/ 17U) urinary ratio is a better correlative measure of CYP1A2 versus using urine collection times greater than 24 hours because it is independent of urine flow rate.(42) Furthermore Kennedy et al determined that 8 hour urine collection time is sufficient for pediatric phenotyping. (15, 42) Another limitation of the study is the inability to ascertain additional environmental factors (e.g. smoking, diet, exercise) that have been shown to be associated with altered enzyme activities.(43, 44)

In conclusion, xanthine oxidase and NAT2 enzyme activities are elevated in obese children. Future studies should be pursued to determine other drug metabolism enzymes

that may be altered by obesity in children. Additionally, studies should also be undertaken to determine the clinical implications of altered drug metabolism. These will enable us to develop dosing models that will help optimize dosing regimens in obese children with concomitant diseases.

Table 2.1: Demographic distribution of volunteer subjects.

Parameter	Lean	Obese	p-value
N	16	9	
Age median (range)	9 (6-10)	9 (8-10)	
Gender (m/f)	7/9	2/7	
Race/Ethnicity			
Caucasian	6	1	
African-American	9	6	
Hispanic	1	1	
Native American	0	1	
BMI percentile	<84%	>95%	<0.01
Baseline caffeine (nmoles)	0.37	0.72	0.829
Inter Leukin- 6 (pg/mL)	0.55 ± 0.26	1.56 ± 1.16	0.063
Adiponectin (ug/ml)	3.59 ± 0.65	3.14 ± 0.32	0.058
C-reactive protein (pg/mL)	179.02 ± 62.67	269.31 ± 34.07	0.001
Leptin (ug/ml)	6.56 ± 5.18	71.21 ± 15.28	<0.001

Scheme 1: Caffeine metabolism.

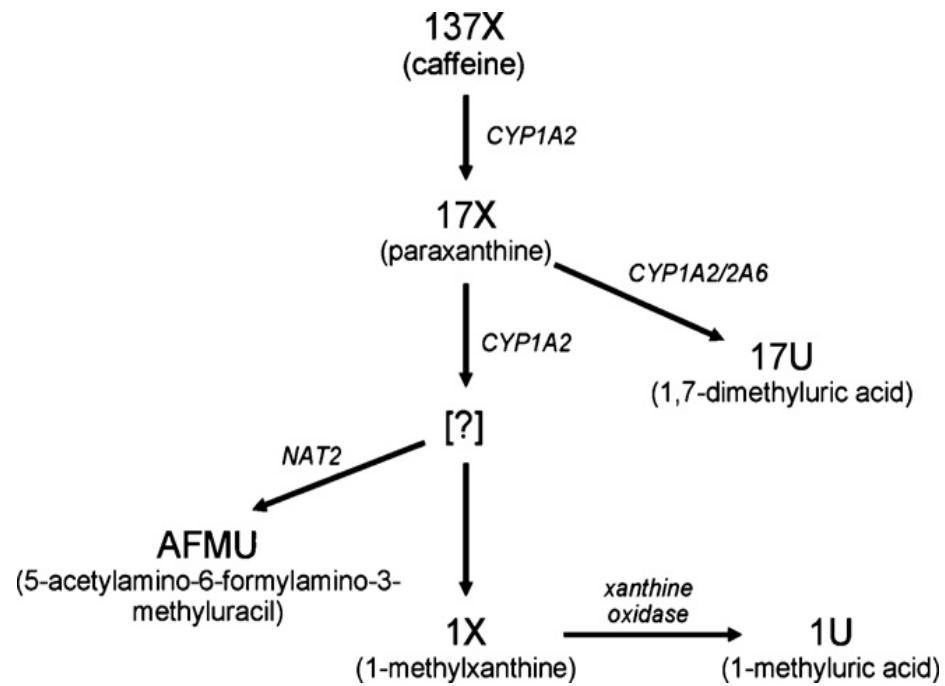
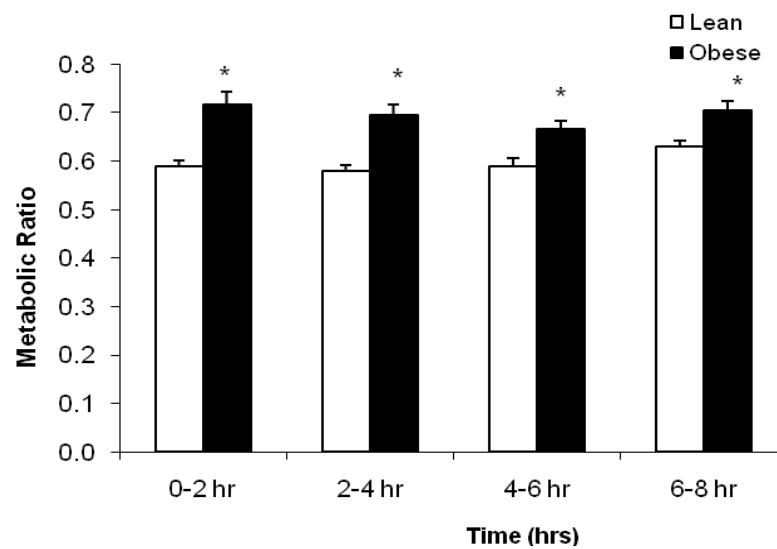


Fig 2.1.A-B. Metabolic Ratio of XO (A) and NAT2 (B) in obese and lean subjects.

A.



B.

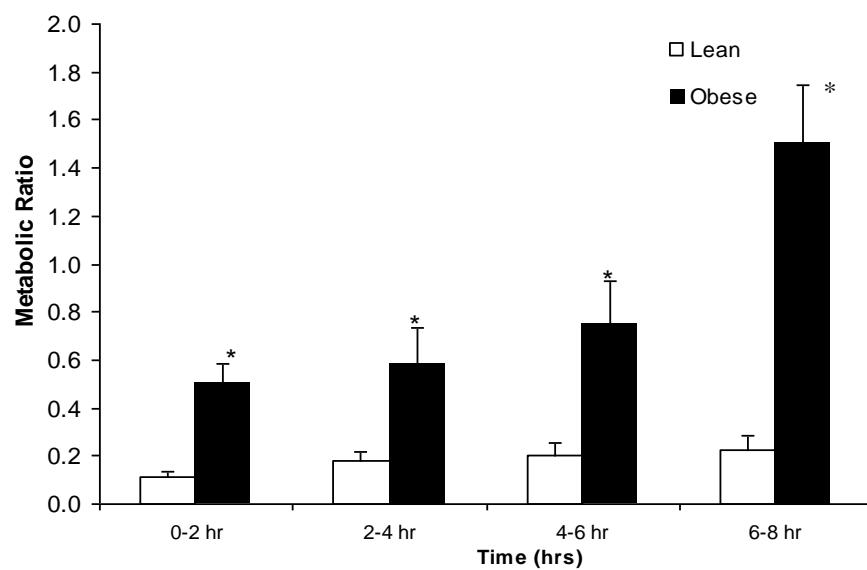
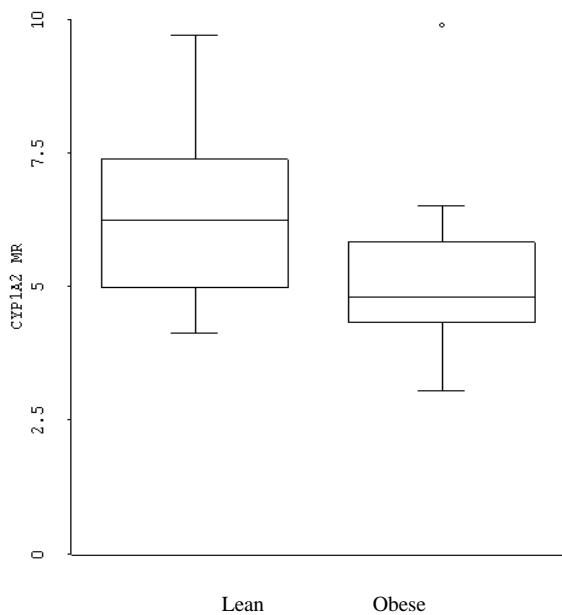


Fig 2.2. Metabolic ratio for CYP1A2 activity in obese and lean subjects.



Metabolic ratio for CYP1A2 activity in obese and lean subjects. CYP1A2 activity was determined by $(\text{AFMU} + 1\text{U} + 1\text{X}) / 17 \text{ U}$. Study subjects included in CYP1A2 analysis expressed normal activity genotype.

Addendum

Assessment of CYP2D6 and CYP3A activities using Dextromethorphan as the probe drug.

A.2.1.Introduction:

As described in section 2.1 obesity has been reported to alter the activity of various CYP P450s and UGTs. However, very few studies have been conducted in the obese pediatric population to determine the impact of obesity on CYP activity. The aim of the current study was to determine the activity of CYP2D6 and CYP3A in pediatric population using probe drug approach.

Dextromethorphan is an antitussive agent that is metabolized to dextrorphan and 3-methoxymorphinan by CYP2D6 and CYP3A respectively (Scheme A1). Urinary ratios of these metabolites have been validated as probes for both CYP2D6 and CYP3A activity. Apart from being a probe drug, dextromethorphan can be safely administered to pediatric population without any severe side effects. Hence, in our study we used dextromethorphan to assess changes in CY2D6 and CYP3A activity in obese children as compared to lean controls.

A.2.2 Methods:

The study was approved by the Institutional Review Board (IRB 0610M94328) and was conducted at the Clinical and Translational Sciences Institute of the University of Minnesota. All subjects 7 years of age and older signed an informed assent, where as all parents/ guardians signed an informed consent which was also approved by the IRB. The participants were included based on the following criteria: 1) Pre- pubertal children

between ages 6-10 yrs., 2) Children with BMI less than 85th percentile (control) or greater than 95th percentile (obese) for age and gender (<http://www.cdc.gov/obesity/childhood/defining.html>), 3) Children not allergic to dextromethorphan and 4) Children currently not on any medication that interferes with drug metabolism. Obese volunteers were recruited from the University of Minnesota Weight Management Clinic; whereas lean subjects were recruited randomly.

On the day of the study, urine and blood was collected from volunteers prior to oral administration of 0.5 mg/kg (30 mg max) of dextromethorphan (Robitussin® DM solution). During this study volunteers were also administered caffeine to assess CYP1A2, NAT2 and XO activity as described previously in chapter 2. Following administration of caffeine (11.5 mg as 4oz Coca Cola®) and dextromethorphan (0.5 mg/kg) urine samples were collected in two hour increments over 8 hours. Dextromethorphan and its metabolites were analysed using LC/MS based on previously described analytical methods. The patients were also genotyped for presence of low/high activity alleles in CYP2D6 (rs16947, rs3892097, rs1065852, rs28371706, rs59421388, and rs28371725)

The metabolic ratios in both the groups were estimated using equation 1 (for CYP3A activity) and equation 2 (for CYP2D6 activity).

Equation 1:

$$\text{CYP3A} = \frac{\text{Dextromethorphan}}{3 - \text{Methoxymorphinan}}$$

Equation 2:

$$\text{CYP2D6} = \frac{\text{Dextromethorphan}}{\text{Dextrorphan}}$$

A.2.3 Results:

The overall study demographics have been described in section 2.2.1. Of the total 25 volunteers, 16 were lean and 9 were obese. For CYP3A, there was no difference in the activity among the two groups, the mean MR in lean group was 2.9 ± 1.7 , while in the obese group it was 8.5 ± 8.3 ($p > 0.05$). Similarly for CYP2D6, the mean MR in the lean group was 0.09 ± 0.18 while in the obese group it was 0.2 ± 0.3 . Even after accounting for various genotypes in CYP2D6, the mean MR in lean group (0.06 ± 0.03) was similar to the obese group (0.29 ± 0.15).

A.2.4. Discussion:

In our current study the mean MR ratios for CYP2D6 and CYP3A were similar between the obese group as compared to the lean group. However, based on the sample size and the variability in activity associated with each group, the power analysis indicated that the study was not accurately powered to detect differences, if any, in the two groups. One of the major problems with the current clinical study was the difficulty in recruitment of pediatric volunteers. Based on this experience, further studies were planned to identify animal models or cell based techniques that could be used to study the impact of obesity on drug metabolism as well as to predict the changes in clearance in obese individuals.

Scheme A1: Metabolism of Dextromethorphan to its metabolites:

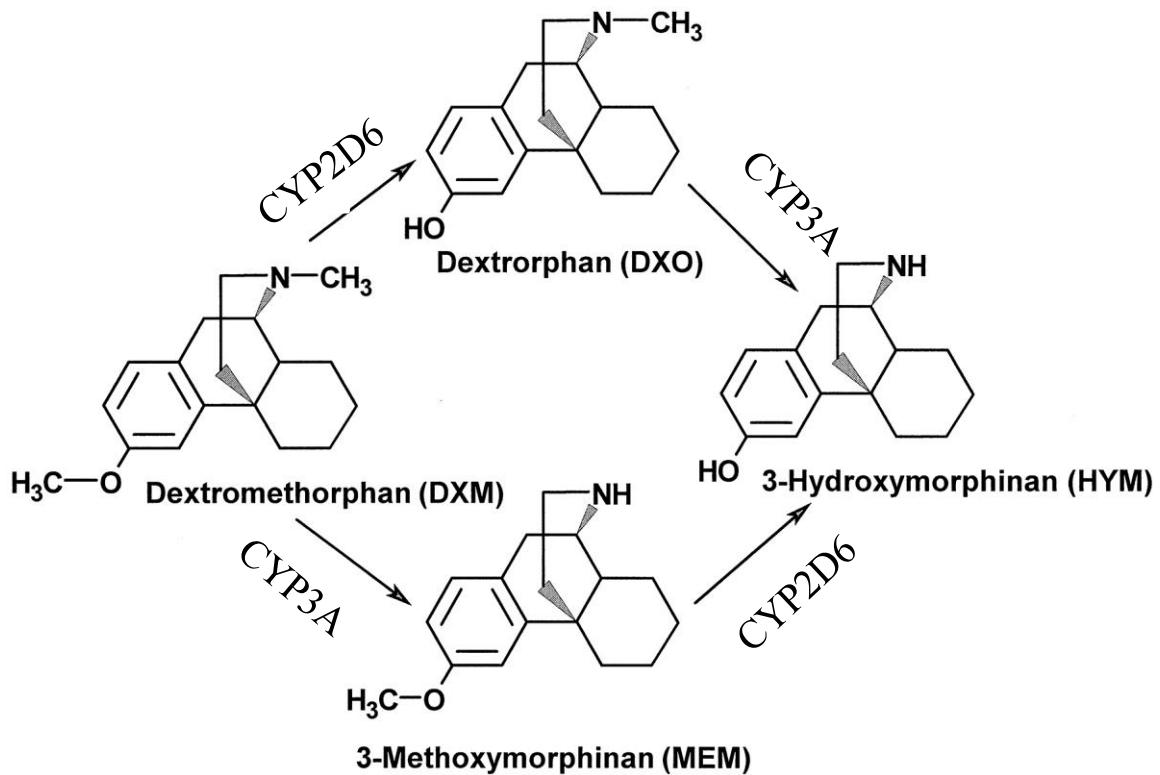


Fig A.1. Metabolic ratio for assessment of CYP3A activity in lean and obese group.

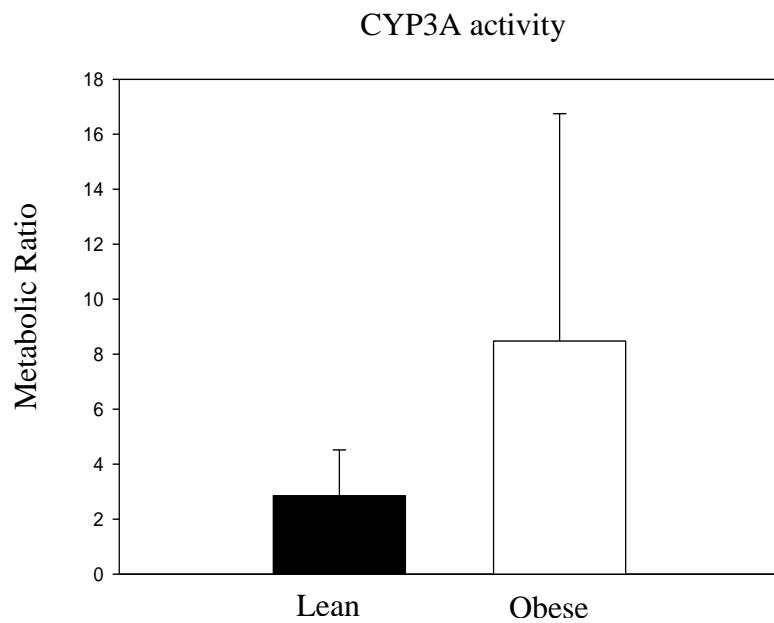
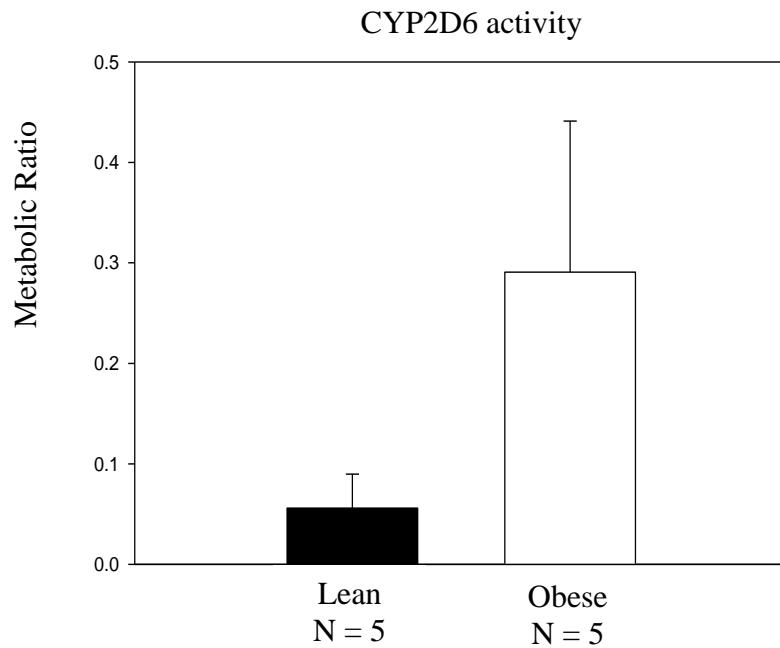


Fig A.2. Metabolic ratio for assessment of CYP2D6 activity in lean and obese group.



Chapter III

Comparison of Drug Metabolism Enzyme Activity in Mouse, Rat and Porcine

Animal Model of Obesity

3.1 Introduction:

Cytochrome P450 and UDP-glucuronosyl transferase family of enzymes are the major oxidative and conjugative hepatic enzymes that are responsible for the biotransformation of nearly 95 % currently marketed therapeutic agents.(1, 2) Expression and activity of these enzymes are important determinants of overall exposure of the body to these agents. Therefore, downregulation or upregulation in the expression and/or activity of these enzymes due to environmental and genetic factors can result in either suboptimal or toxic levels of therapeutic agents. Studies over the past two decades have shown that a host of environmental factors (i.e. disease states and genetic factors) regulate the expression and activity of these drug metabolizing enzymes. Specifically obesity, a growing epidemic in USA, has been shown to cause changes in expression and activity of these enzymes.(3) Recent clinical studies using probe drugs that are specific for particular P450 or UGT isoforms have shown that the obesity can cause clinically significant changes in the activity of major CYPs and UGTs. Studies by Abernethy et al have shown that the clearance of triazolam (CYP3A probe) was significantly lower in obese (340 ± 44 mL/min) as compared to lean control (531 ± 38 mL/min).(4) Additionally, studies have shown that there are significant changes in the CYP2E1, CYP2C9 and CYP2C19 activities in obese adults as compared to lean control. Also, studies have shown that the glucuronidation of oxazepam and lorazepam is higher in obese as compared to lean controls.(5) Thus, dosing therapeutic agents for the treatment of obesity associated diseases (hypertension, depression and gout) is clinically challenging and often results in failure of therapy leading to additional health care costs. (6)

Despite significant clinical evidence regarding obesity mediated changes in drug

metabolism, there is limited knowledge in regards to predicting these changes in humans. Recent studies have used various mouse and rat animal models to determine the effect of obesity on drug metabolism. These rodent models develop obesity due to, their genetic background, being fed high fat diet, or a combination of the two. There are two mouse models (ob/ob and db/db), and one rat model Zucker rats (fa/fa) that are commonly used genetic models of obesity. The fa/fa rats and db/db mouse lack the leptin receptor while the ob/ob mouse model lacks leptin.(7-9) Both of these deletions result in dysregulation of leptin signaling leading to hyperphagia induced obesity. Similar to humans, these models are characterized by perturbations in metabolic system leading to metabolic syndrome including insulin resistance. These models have therefore been used to determine the changes in drug metabolism and to establish a causal link to the observed changes. For e.g. studies in the rat Zucker model have shown that the mRNA levels of CYP1A are decreased in obese rats as compared to the lean rats. Also studies using the genetic (ob/ob) mouse model and the high fat diet model have shown that there is decreased protein and CYP1A activity in the obese group as compared to the lean group.(10) Using human liver samples, Donato et al. have shown decreased mRNA levels of CYP1A. Studies have also shown that CYP2A, CYP2B, CYP2C, CYP2E1 and CYP3A activities are altered in the rodent genetic and dietary model of obesity.(11) Though these changes in the mRNA, protein or activity are similar to the changes reported in humans, other studies have reported varying results in the same animal models of obesity.(12-14) One of the main reasons for these contradictory results is the differences in the genetic background of animals as well as use of variable high fat diet in these studies. Another important limitation is that the animal models do not share

homology with human drug metabolizing enzymes and also may have different mechanisms of enzyme regulation as compared to humans. For example in rats rifampicin is known to not induce rat CYP3A, while in humans it is a potent CYP3A inducer.(15) Thus, results from each of these individual rodent animal models may have limited utility in predicting changes in clearance in obese humans. However, the combined data obtained from these animal models may be used to predict clearance in humans using the allometric scaling approach.

Allometric scaling approach requires use of two rodents and one non-rodent species to estimate the clearance in humans.(16) Recently a novel porcine model using Ossabaw pigs has been developed by Sturek et al., and is reported to mimic human obesity. This porcine model apart from being a non-rodent model might be useful as predictor of changes in human drug metabolism since porcine CYPs share greater homology with human CYPs.(17, 18) However, apart from considering the homology with human CYPs, it is imperative to establish whether these models would mimic the changes that have been reported in obese humans, which would be based on the similarities of regulatory pathways among different species.(19) Thus, the aim of the current study was to evaluate the changes in activities in mouse, rat and porcine high fat diet model of obesity to determine if these models mimic the changes that have been reported in obese humans. The knowledge would help determine if conducting pharmacokinetic studies in these animals would provide an estimate of clearance in obese humans via allometric scaling.

3.2 Materials and Methods:

Animal Treatment: All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Missouri. Mice, rats and pigs were received at 6 weeks, 8 weeks and 5 weeks of age and maintained at a controlled temperature (22°C) and a 12 hour light: 12 hour dark cycle. Animals were either fed a low fat diet (LFD) containing 4 % fat or a high fat diet (HFD) containing 24 % fat. Food intake and body mass were measured weekly throughout the study.

Chemicals: NADPH was obtained from Calbiochem (La Jolla, CA). UDPGA, Acetyl CoA, PAPS, chlorzoxazone, dextromethorphan, dextrorphan, ethoxyresorufin, pentoxyresorufin, levallorphan, 7-hydroxycoumarin, resorufin, 6-hydroxychlorzoxazone, 4-methylumbellifrone (4-MU), 4-methylumbellifrone glucuronide, 6 β -hydroxytestosterone and 16 α -hydroxytestosterone were purchased from Sigma-Aldrich (St. Louis, MO). 6 β -hydroxytestosterone-d3 was obtained from Cerilliant (Round Rock, TX). Acetonitrile, ammonium acetate, phosphoric acid, and methanol for analytical assays were of high-performance liquid chromatography grade. All other chemicals were of ACS (American Chemical Society) grade and obtained from commercial sources.

Subcellular fraction isolation: Hepatic microsomes and cytosol from mouse, rat and porcine models of obesity and their lean controls was isolated by differential centrifugation method.(20) The protein content of the microsomal and cytosolic fractions was determined by Lowry method, using bovine serum albumin as the standard and the

Bio-Rad protein assay kit, as per the manufacturer's instructions. Microsomes and cytosol were stored at -80°C until incubation studies were performed.(21)

Enzyme Assays: The assay conditions for the enzymatic activity determinations were adapted from previously reported methods in the literature (22, 23) and are listed in Table 3.1. Briefly, incubations were performed in 50 mM potassium phosphate buffer in a total volume of 0.2 ml - 0.4 ml. Incubation mixtures were preincubated at 37°C for 3 min prior to the start of the reactions. Reactions were started by addition of cofactor and terminated using cold quenching agent after appropriate times (Table 3.1). Metabolite from these incubations was extracted using liquid-liquid extraction or by centrifugation at 13000 x g for 10 min. The extracted samples (reconstituted in mobile phase) or the supernatant were quantified by spectrofluorometric assay or HPLC based assays. The analytical assays described in Table 3.1 were conducted using LC/MS/MS equipped with an Agilent 1200 series autosampler/pump (Agilent Technologies, Santa Clara, CA) and Thermo Finnigan TSQ Quantum triple-quadrupole mass spectrometer (Thermo Fisher Scientific) (for 16 α , 6 β -hydroxytestosterone, 12-hydroxy lauric acid), a high-performance liquid chromatography system (Waters, Milford, MA) consisting of an Alliance 2695 autosampler/pump and 2487 dual-wavelength absorbance detector (for 6-hydroxychlorzoxazone, acetyl PABA and 7-hydroxycoumarin sulfate) or a 474 scanning fluorescence detector (for dextrorphan and 4- MU glucuronide) and Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VT) (for resorufin).

Data Analysis: Unless noted, all data consist of three independent experiments and are represented as the mean \pm standard error (S.E.). Kinetic parameters for the substrates were estimated by nonlinear regression analysis using Sigma Plot 10.0 (Systat Software, Inc., Chicago, IL). Data was fit to a typical Michaelis-Menten equation, and the intrinsic clearance was calculated from V_{max}/K_m ratio. The goodness of fit was determined by visual examination of the resulting curves, comparison of the coefficients of determination, and inspection of Eadie-Hofstee plots. Only for acetaminophen glucuronidation in pigs, CL_{int} was defined as the slope of the line when velocity was plotted against substrate concentration. Comparison between the groups was performed using the two-sided student's t-test (SigmaStat 3.1; Systat Software, Inc.). The level of significance for all the comparisons was set at $p < 0.05$.

3.3 Results:

Ethoxresorufin dealkylation activity was differentially altered in the three species, while the intrinsic clearance was lower in the obese mice and pigs as compared to their lean controls; it was higher in the obese rats as compared to lean controls (Fig. 3.1.a). For pentoxyresorufin dealkylation, mice and rats were found to have similar activity in lean phenotype as compared to obese phenotype. However, in porcine liver microsomes the CL_{int} for pentoxyresorufin dealkylation was lower in the obese group (0.1 ± 0.02 mL/min/mg protein) when compared to the lean control (0.15 ± 0.05 mL/min/mg protein). The testosterone 16α -hydroxylation was unchanged in the obese rats as compared to the lean control, however in the mouse model the CL_{int} was higher in the obese mice (0.68 ± 0.07 mL/min/mg protein) as compared to the lean control (0.25 ± 0.05 mL/min/mg protein). In the porcine model, there was decreased CL_{int} in the obese group (0.09 ± 0.01 mL/min/mg protein) as compared to lean group (0.28 ± 0.01 mL/min/mg protein). Also, testosterone 6β -hydroxylation was found to follow a similar pattern with higher intrinsic clearance in mouse and lower clearance in the porcine obese group, while there was no change in the rat obese group as compared to the lean controls (Fig. 3.1.b, Table 3.2). There was no difference in dextromethorphan O-demethylation (DMOD) activity between the lean and obese mouse and rat liver microsomes. However, the DMOD activity was nearly 3 – fold lower in obese porcine liver microsomes (0.23 ± 0.02 mL/min/mg protein) as compared to the lean control (0.67 ± 0.05 mL/min/mg protein) (Fig. 3.1.c). Xanthine oxidase activity was found to be higher in the obese mice and rats as compared to the lean controls, however interestingly the porcine xanthine oxidase activity was found to be lower in the obese group as compared to the lean control.

Apart from the oxidative enzymes, the conjugation of typical substrates was evaluated. 4-MU glucuronidation activity, which is considered to be a measure of general UGT activity, was same between the lean and obese phenotype in all the three models of obesity (Fig 3.2.a). The CL_{int} for estradiol was found to be lower in the obese mice and rats as compared to respective lean controls, while there were no changes in the porcine model of obesity (Table 3.3). The acetaminophen glucuronidation was found to be similar in obese mice and rats as compared to their respective control. However, in the obese pigs, the CL_{int} for acetaminophen glucuronidation (0.09 ± 0.04 mL/min/mg protein) was lower as compared to the lean pigs (2 ± 0.1 mL/min/mg protein). Testosterone glucuronidation which is mainly catalyzed by multiple UGT2Bs in humans and rats, was found to be higher in the obese rats as compared to the lean control while the CL_{int} was lower in obese mice (0.04 ± 0.01 mL/min/mg protein) as compared to the lean mice (0.1 ± 0.02 mL/min/mg protein). In porcine model, there was no difference in the CL_{int} in both the groups (Fig 3.2.b). The changes in morphine 3-O-glucuronidation activity were found to be species dependent, with lower glucuronidation in obese pigs ($CL_{int}: 11.5 \pm 2$ mL/min/mg protein v/s 33.1 ± 1.3 mL/min/mg protein) and higher activity in obese rats (20.3 ± 0.5 mL/min/mg protein v/s 11.08 ± 0.5 mL/min/mg protein) as compared to their respective control. There was no difference in morphine glucuronidation in obese mice as compared to lean mice (Fig. 3.2.c).

3.4. Discussion:

Various studies in animal models of obesity have reported changes in expression of drug metabolizing enzymes based on observed changes in mRNA levels or protein expression. However, it has been reported that the changes in mRNA and protein expression do not correlate with changes in the activity. The current study demonstrated using various substrates that obesity can alter the enzyme activity in a species and model dependent manner. However, in absence of literature regarding the specific enzymes responsible for the metabolism of these substrates in mice and pigs, the changes in intrinsic clearance of these substrates is not indicative of changes in activity of a particular enzyme in those species.

Ethoxresorufin O-dealkylation is known to be catalyzed mainly via CYP1A2 in humans. Using recombinant rat CYPs studies by Kobiyashi et al. have shown that apart from CYP1A2; CYP2C6 and CYP2C11 are also involved in the metabolism of ethoxresorufin, while there is no data regarding porcine and mice P450s.(24)(22) In our current study we observed lower intrinsic clearance of ethoxresorufin in obese mice and pigs, while the CL_{int} was higher in obese rats as compared to respective controls. The changes in intrinsic clearance in all the three species were due to changes in the V_{max} , which is indicative of expression of the enzymes responsible for dealkylation of ethoxresorufin.

Testosterone 16 α -hydroxylation and 6 β -hydroxylation activity was found to be altered in the mouse and porcine model of obesity; however we did not see any differences in the rat HFD model of obesity. Since, 16 α -hydroxylation and 6 β -hydroxylation of testosterone is known to be catalyzed by mainly CYP2C11 and CYP3A respectively in rats; our

studies indicate no differences in the activities of the enzymes in rat model of obesity. These results are in contrast to studies that have reported decreased mRNA expression of CYP2C11 and CYP3A in obese rats fed a high fat diet.(10) Also, the changes in intrinsic clearance of testosterone in mouse and porcine model of obesity were due to changes in the K_m of testosterone and not the V_{max} of the reaction, indicating minimal effect of obesity on expression of enzymes responsible for testosterone hydroxylation.

Dextromethorphan O-demethylation (DMOD) is a known marker substrate of human, rat and mouse CYP2D enzymes. In humans, there are no studies that have shown changes in CYP2D6 activity. In our current study we observed no changes in DMOD activity in the obese mice and rats; however the activity was significantly lower in the obese pigs as compared to lean pigs. Interestingly, in pigs this reaction is known to be catalyzed by the CYP2B enzymes and not by CYP2D enzyme.(25) Since, tolterodine has been validated as a marker for porcine CYP2D25 activity, additional studies need to be done in order to evaluate the effect of obesity on porcine CYP2D enzymes.(18)

Xanthine oxidase activity has been shown to be elevated in obese humans and various mouse and rat models of obesity.(26) In our studies we observed higher cytosolic xanthine oxidase activity in obese mice and rats while surprisingly the cytosolic XO activity was lower in obese pigs. These results indicate that despite greater homology between the porcine and human enzymes, the direction and magnitude of changes in obesity is dependent on the similarities of regulatory pathways influenced by obesity.

Apart from the changes in enzymes responsible for oxidation of various substrates, we also evaluated the changes in glucuronidation of variety of substrates in the three animal models of obesity. 4-methyumbelliferone is a known general UGT1A substrate. The

intrinsic clearance of 4-MU was similar in all the three models of obesity, indicating no changes in overall UGT1A activity. The results in the mouse model of obesity are consistent with a study by Watson et al. that reported no changes in the 4-MU glucuronidation in ob/ob mice as compared to the lean control.(27)

We observed that the estradiol 3 β -glucuronidation activity was lower in the obese mice and rats. While the change in CL_{int} in mice was due to lower V_{max} (indicating lower expression), the changes in obese rats were due to differences in K_m . In pigs, though the overall intrinsic clearance of estradiol was higher as compared to rats and mice, there were no phenotype based differences in the intrinsic clearance. Estradiol glucuronidation is mainly catalyzed by UGT1A1 and studies have shown that UGT1A1 expression is lower in obese mice and rats as well as in humans.(28)(29)

Acetaminophen is known to be glucuronidated by UGT1A6/7 and UGT2B in rats and humans.(30) However, the specific isoforms in mice and pigs, that glucuronidate acetaminophen are unknown. In this study there were no differences in the CL_{int} for acetaminophen glucuronidation in the obese mice and rats, indicating minimal changes in UGT1A6 and UGT2B activity, while in the obese pigs we observed a 50 % reduction in intrinsic clearance of acetaminophen. The results in mouse are consistent with reported lack of changes in mRNA levels of UGT1A6 and UGT2B in mouse HFD model of obesity. However the similar intrinsic clearance in rat HFD model of obesity is contradictory with the decreased mRNA levels of UGT1A6 and UGT2B reported by Osabe et al. in rats and increased glucuronidation of acetaminophen in obese humans.(31)(5)

The glucuronidation of testosterone (catalysed by multiple UGT2B enzymes) and morphine (UGT2B1 substrate in rats) was found to be higher in obese rats. In mice, there was decreased testosterone glucuronidation in the obese group while there were no changes in the extent of morphine glucuronidation. In the porcine model of obesity the morphine glucuronidation was lower in the obese group, while the testosterone glucuronidation was similar in both the groups. In humans studies have reported increased clearance of oxazepam which is a UGT2B substrate.(5) Thus based on the changes observed in the three animal models, the rat HFD model might provide information regarding clearance of therapeutic agents metabolized via UGT2Bs in obese individuals.

In conclusion, we observed species dependent differences in the three animal models of obesity, however not all the changes were due to the changes in the expression of enzyme (as assumed from changes in V_{max}), but some of the observed differences were due to changes in affinity of the enzyme for substrate (based on K_m estimates). Also, the changes in intrinsic clearance of various substrates were not in agreement with the changes in mRNA and protein expression reported by various authors. Considering the differences in the enzyme activities compared to obese humans in these animal models there appears to be a lack of utility in using this combination of animal models for predicting changes in clearance in obese individuals. However, based on the comparison of changes in the enzyme activities in humans and the animal models, we suggest using the rat model to further investigate the effect of obesity on bioactivation and DDI potential of therapeutic agents. Also, the rat model can be used to determine the effect of anti-obesity agent and age-obesity interaction on drug metabolism. However, additional

studies using specific probes and inhibitors of various DMEs would provide a better insight into the magnitude of differences in obese animals as compared to humans.

Fig 3.1.a: Comparison of CL_{int} for Ethoxresorufin metabolism in mouse, rat and porcine model of obesity. Values are mean \pm SE and ** indicates p-value < 0.05.

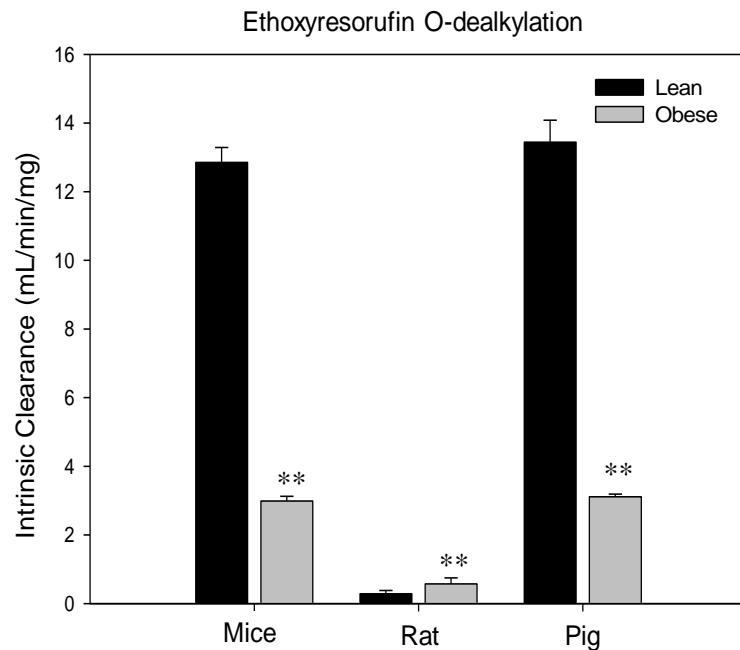


Fig 3.1.b: Comparison of CL_{int} for testosterone metabolism in mouse, rat and porcine model of obesity. Values are mean \pm SE and ** indicates p-value < 0.05.

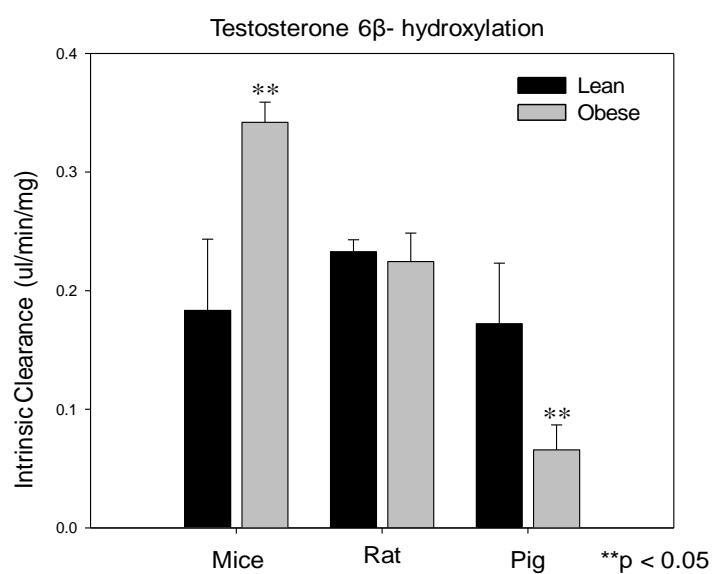


Fig 3.1.c: Comparison of CL_{int} for dextromethorphan metabolism in mouse, rat and porcine model of obesity. Values are mean \pm SE and ** indicates p-value < 0.05.

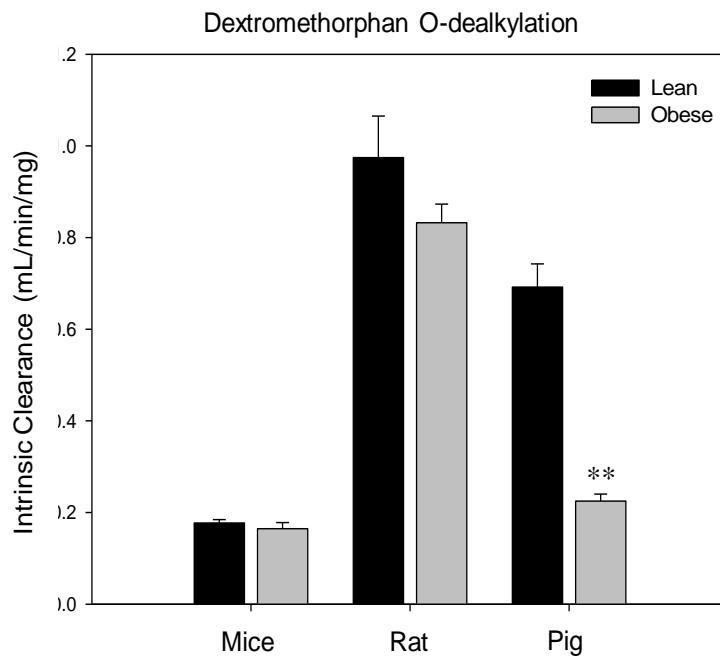


Fig 3.1.d: Comparison of xanthine oxidase activity in mouse, rat and porcine model of obesity. Values are mean \pm SE and ** indicates p-value < 0.05.

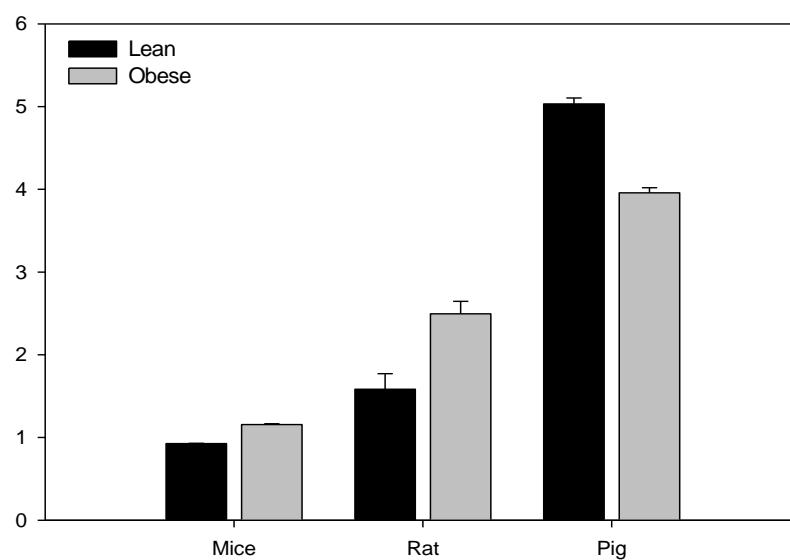


Fig 3.2.a.: Comparison of 4-MU glucuronidation in mouse, rat and porcine model of obesity. Values are mean \pm SE and ** indicates p-value < 0.05.

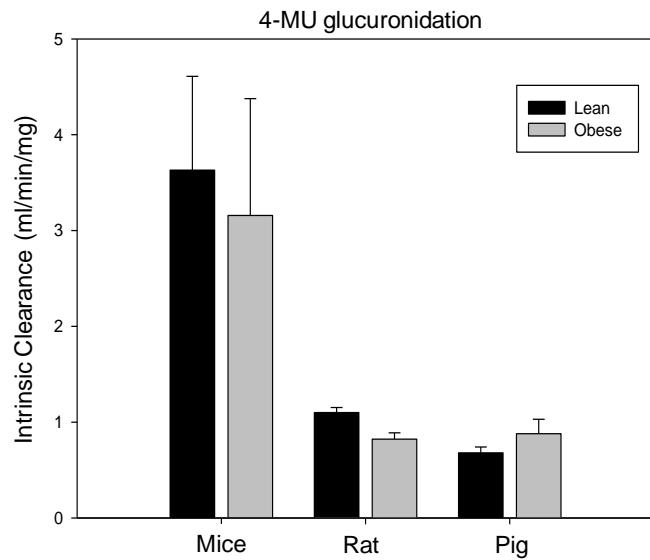


Fig 3.2.b. Comparison of Testosterone glucuronidation in mouse, rat and porcine model of obesity. Values are mean \pm SE and ** indicates p-value < 0.05.

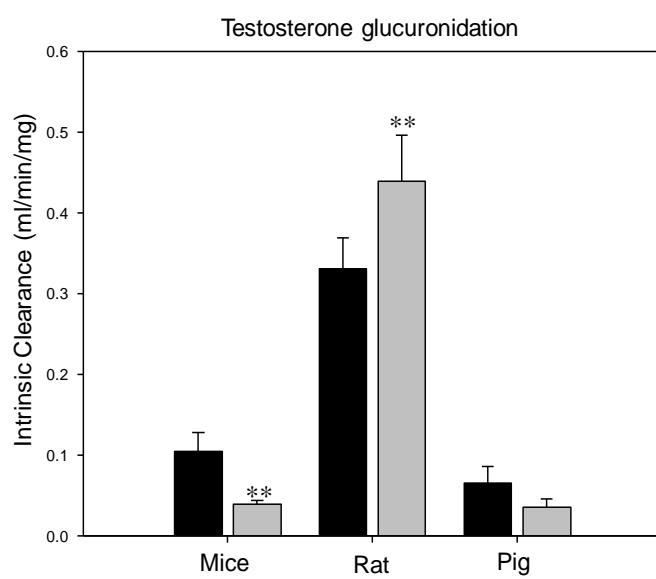


Fig 3.2.b. Comparison of Morphine glucuronidation in mouse, rat and porcine model of obesity. Values are mean \pm SE and ** indicates p-value < 0.05.

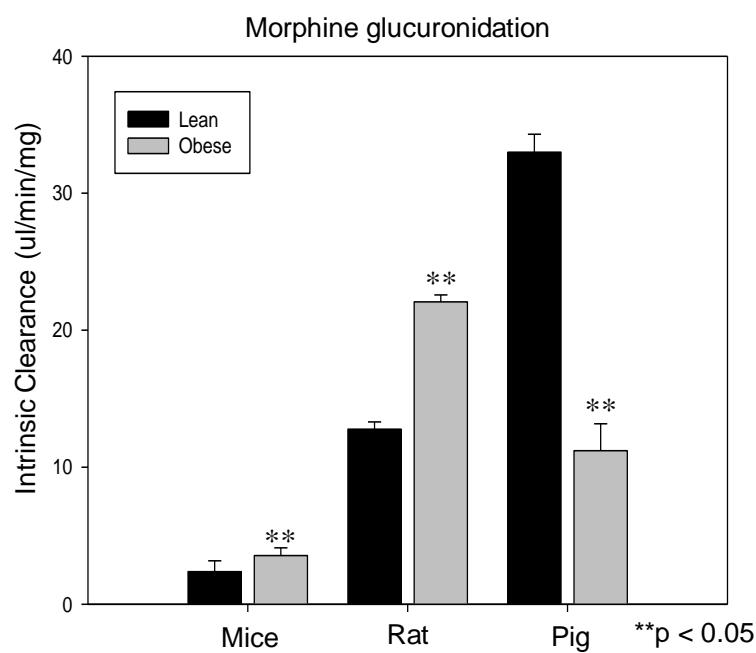


Table 3.1: Probe drugs and the incubation conditions for assessing various oxidative and conjugative enzyme activities.

MeCN: Acetonitrile; **PABA:** p-amino benzoic acid; **4-MU:** 4-methylumbelliferon.

Enzyme	Substrate	Metabolite	Incubation		Protein conc (mg/ml)	Co factor	Detection Method
			Vol (ul)	Time (min)			
CYP1A2	Ethoxresorufin	Resorufin	200	5	0.1	NADPH	Fluorescence
CYP2B	Pentoxyresorufin	Resorufin	200	5	0.1	NADPH	Fluorescence
CYP2C11	Testosterone	16 α -hydroxy testosterone	200	20	0.1	NADPH	LC/MS/MS
CYP2D2	Dextromethorphan	Dextrorphan	200	20	0.1	NADPH	HPLC-Fluorescence
CYP3A	Testosterone	6 β -OH testosterone	200	20	0.1	NADPH	LC/MS/MS
UGT	4-MU	4-MU glucuronide	200	20	0.1	UDPGA	HPLC-Fluorescence
	Estradiol	3 β -glucuronide	200	10	0.1	UDPGA	HPLC-Fluorescence
	Acetaminophen	Acetaminophen glucuronide	200	30	0.1	UDPGA	HPLC-UV
	Morphine	Morphine 3-O-glucuronide	200	30	0.1	UDPGA	HPLC-UV
	Testosterone	Testosterone β -D-glucuronide	200	15	0.1	UDPGA	LC/MS/MS
XO	Assay Kit					Fluorescence	

Table 3.2: Kinetic parameters of oxidative enzymes.

Substrate	Parameters	Mice		Rat		Pig	
		LFD	HFD	LFD	HFD	LFD	HFD
Ethoxyresorufin	V _{max}	5.6 ± 0.02	1.7 ± 0.02*	0.17 ± 0.05	0.33 ± 0.1*	2.1 ± 0.05	1.4 ± 0.1
	K _m	0.44 ± 0.01	0.59 ± 0.02	0.62 ± 0.03	0.57 ± 0.1	0.16 ± 0.1	0.45 ± 0.1
	CL _{int}	12.3 ± 0.43	2.9 ± 0.14*	0.27 ± 0.1	0.58 ± 0.2*	13.1 ± 0.6	3.1 ± 0.1*
Pentoxyresorufin	V _{max}	0.42 ± 0.04	ND	0.44 ± 0.03	0.42 ± 0.1	0.2 ± 0.02	0.15 ± 0.1
	K _m	3.3 ± 0.5	ND	1.2 ± 0.12	1.2 ± 0.3	1.3 ± 0.5	1.5 ± 0.3
	CL _{int}	0.13 ± 0.05	0.03 ± 0.01*	0.37 ± 0.04	0.35 ± 0.08	0.15 ± 0.05	0.1 ± 0.02
Testosterone 16α hydroxylation	V _{max}	2.9 ± 0.5	1.35 ± 0.2	12.7 ± 0.6	14.3 ± 0.6	7.4 ± 0.3	1.5 ± 0.2*
	K _m	11.5 ± 2.1	1.99 ± 1.3*	27.9 ± 4.9	27.1 ± 3.9	26.7 ± 6.9	16.6 ± 1.3
	CL _{int}	0.25 ± 0.05	0.68 ± 0.07*	0.46 ± 0.06	0.53 ± 0.06	0.28 ± 0.01	0.09 ± 0.01*
Dextromethorphan	V _{max}	0.66 ± 0.02	0.54 ± 0.03	1.8 ± 0.07	1.7 ± 0.04	4.9 ± 0.9	1.04 ± 0.4*
	K _m	3.7 ± 0.2	3.4 ± 0.4	1.8 ± 0.4	2.1 ± 0.1	7.3 ± 1	4.7 ± 0.6
	CL _{int}	0.18 ± 0.01	0.16 ± 0.01	1 ± 0.09	0.81 ± 0.1	0.67 ± 0.05	0.23 ± 0.02*
Testosterone 6β hydroxylation	V _{max}	11.3 ± 0.9	3.3 ± 0.3*	5.3 ± 1.8	7.6 ± 1.5	10.9 ± 1.8	2.4 ± 0.2*
	K _m	61.7 ± 17.7	9.7 ± 3.2	22.7 ± 5.5	33.8 ± 7.3	63.4 ± 15.6	36.5 ± 3.2
	CL _{int}	0.18 ± 0.06	0.34 ± 0.04*	0.23 ± 0.01	0.22 ± 0.02	0.17 ± 0.08	0.07 ± 0.03

V_{max} (nanomoles/min/mg); K_m (μM); * p value < 0.05

Table 3.3: Kinetic parameters of conjugative enzymes.

Substrate	Parameters	Mice		Rat		Pig	
		LFD	HFD	LFD	HFD	LFD	HFD
4-MU	V _{max}	166.1 ± 4.1	104.1 ± 1.62*	94.8 ± 5.5	50.1 ± 0.9*	74.8 ± 8.3	38.7 ± 3.23*
	K _m	45.7 ± 3.8	32.9 ± 1.45	86.9 ± 6.7	61.3 ± 3.7	113.8 ± 21.5	46.8 ± 10.2
	CLint	3.6 ± 0.9	3.2 ± 1.2	1.1 ± 0.1	0.82 ± 0.09	0.66 ± 0.06	0.83 ± 0.15
Estradiol	V _{max}	2.6 ± 0.4	1.36 ± 0.12*	1.8 ± 0.4	1.8 ± 0.3	24.7 ± 3.7	7 ± 1.6*
	K _m	27.4 ± 2.3	18.7 ± 2.85	31.7 ± 3.9	42.7 ± 6.2	0.6 ± 0.2	0.2 ± 0.09
	CLint	0.09 ± 0.06	0.07 ± 0.03	0.06 ± 0.03	0.04 ± 0.01	41.7 ± 1.1	35 ± 2.5
Acetaminophen	V _{max}	3.2 ± 0.8	2.6 ± 0.76	12.8 ± 0.5	10.3 ± 0.5	NE	NE
	K _m	844 ± 28	689 ± 37	9762 ± 1005	9689 ± 1367	NE	NE
	CLint (μL/min)	3.7 ± 0.2	3.7 ± 0.4	1.3 ± 0.3	1.1 ± 0.1	2 ± 0.1	0.09 ± 0.04*
Morphine	V _{max}	4.1 ± 1.1	1.32 ± 0.82	14.4 ± 0.2	24.3 ± 0.3	29.8 ± 3.6	13.8 ± 5.7
	K _m	1.7 ± 0.6	0.37 ± 0.1*	1.3 ± 0.7	1.2 ± 0.3	0.9 ± 0.3	1.2 ± 0.8
	CLint	2.4 ± 0.8	3.6 ± 0.6	11.1 ± 0.5	20.3 ± 0.5	33.1 ± 1.3	11.5 ± 2
Testosterone	V _{max}	4.1 ± 0.8	1.71 ± 0.4	8.8 ± 0.4	9.9 ± 0.6	1.2 ± 0.4	0.7 ± 0.2
	K _m	39.1 ± 6.8	43.42 ± 6.9	26.7 ± 4.8	22.5 ± 5.1	18.1 ± 2.5	19.2 ± 2.7
	CLint	0.1 ± 0.02	0.04 ± 0.01*	0.33 ± 0.04	0.44 ± 0.06	0.06 ± 0.002	0.04 ± 0.002

NE: Not Estimated, V_{max} (nanomoles/min/mg); K_m (μM)

Chapter IV.

**Sterculic Oil Prevents Obesity Mediated Alterations in Oxidative and Conjugative
Drug Metabolism Enzyme Activity.**

4.1. Introduction:

Obesity is a major health concern in the US with nearly 33.8 % adults and 17 % of children and adolescents being classified as obese (1). Obesity is characterized by abnormal fatty acid (FA) metabolism that leads to ectopic fat deposition. Increased accumulation of lipid in liver and muscle is associated with the development of insulin resistance, type 2 diabetes and other metabolic perturbations. These metabolic perturbations have been associated with changes in expression and activity of drug metabolizing enzymes (DMEs) and drug transporters in humans and in animal models of obesity (2). Clinical studies have shown changes in pharmacokinetics of various drugs like lorazepam, oxazepam etc. in obese adults due to changes in DME activity (3) . Recently, studies by our group have demonstrated that xanthine oxidase (XO) and N-acetyltransferase (NAT) enzyme activities were elevated in obese children compared to lean children (4).

Various animal models of obesity have also shown altered expression and activity of drug transporters, oxidative and conjugative enzymes. Specifically, studies in genetic and high fat diet (HFD) models of obesity have shown reduced expression of CYP1A, CYP2C11, and CYP3A and increased CYP2E1 enzyme activity in the obese group as compared to lean controls (5). Studies also have shown that conjugative enzymes like Ugt and Sults are altered in obese animals (6). However, these observed changes are variable and influenced by the model of obesity being studied. For example, hepatic Ugt1a1 expression was unchanged in Wistar rats fed a HFD (7), reduced in CD1 mice fed a HFD (6) and elevated in obese ob/ob mice (8). A limitation of these and other common

experimental models used to assess the effect of obesity on drug metabolism are the need to use diets with excessive fat content and/or the lack of an intact leptin signaling pathway. In addition to always being increased with obesity, leptin has been shown to regulate the expression of DMEs (9, 10), therefore studying changes in DME expression or activity in the presence of leptin is a more accurate reflection of the human obese hormonal environment. Hence, we sought to identify the effect of obesity on DME activity in the Otsuka Long Evans Tokushima Fatty (OLETF) rat model of obesity, which contains a functional leptin signaling pathway and exhibits hyperphagia and obesity due to a lack of the cholecystokinin-1 receptor. This model of obesity mimics human obesity in that these animals develop type II diabetes mellitus and hyperinsulemia (11, 12).

Using the OLEFT rat model, we recently demonstrated that supplementation of sterculic oil (SO), a natural inhibitor of stearoyl-CoA desaturase-1 (SCD1), resulted in improvements of the metabolic syndrome including reductions in hepatic lipid accumulation and increased glucose tolerance, without changes in the total body weight (13, 14). Stearoyl-CoA desaturase-1 is a major enzyme involved in the control of lipid metabolism (Fig. 1) and there is increasing interest in targeting this enzyme as a potential therapy for obesity and/or type 2 diabetes (15). Therefore, we were also interested in understanding the effect of long term treatment of SO on DME activity in OLETF rats.

Thus, the aim of the study was to characterize differences in drug metabolism between the hyperphagic obese OLETF rat and their nonhyperphagic lean Long-Evans Tokushima Otsuka (LETO) controls. Furthermore, we determined if SO-induced inhibition of SCD1 activity and improvements in metabolic state were associated with improvements in drug

metabolism in these animals.

4.2. Materials and Methods:

4.2.1. Animal Treatment: All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Missouri and are described previously (14). Briefly, animals were received at 4 weeks of age and maintained at a controlled temperature (22°C) and a 12 hour light/dark cycle. OLETF animals were housed individually while LETO animals were housed in pairs and all animals were fed for ad libitum food intake. Four-week old, male OLETF rats were assigned to one of two dietary treatment groups: 1) a standard AIN-93G rodent diet (OLETF) or 2) an AIN-93G diet supplemented with 0.5% sterculic oil (OLETF SO). Male LETO rats were used as a lean control and fed the AIN-93G diet (LETO). At 14 weeks of age, the animals were fasted for 6 h and sacrificed by CO₂ necrosis followed by exsanguination via cardiac puncture. Liver was dissected, weighed, and snap frozen in liquid nitrogen.

4.2.2. Chemicals: NADPH was obtained from Calbiochem (La Jolla, CA). UDPGA, Acetyl CoA, PAPS, chlorzoxazone, dextromethorphan, dextrorphan, ethoxyresorufin, pentoxyresorufin, levallorphan, 7-hydroxycoumarin, resorufin, 6-hydroxychlorzoxazone, para amino benzoic acid (PABA), Acetyl PABA, 4-methylumbelliferon (4-MU), 4-methyumbelliferon glucuronide, 7-hydroxycoumarin, 7-hydroxycoumarin sulfate, lauric acid and 12-hydroxylauric acid, 6 β -hydroxytestosterone and 16 α -hydroxytestosterone were purchased from Sigma-Aldrich (St. Louis, MO). 6 β -hydroxytestosterone-d3 was obtained from Cerilliant (Round Rock, TX). Acetonitrile, ammonium acetate, phosphoric

acid, and methanol for analytical assays were of high-performance liquid chromatography grade. All other chemicals were of ACS (American Chemical Society) grade and obtained from commercial sources.

4.2.3. Subcellular fraction isolation: Hepatic microsomes and cytosol from LETO, OLETF and OLETF (SO supplemented) rats ($n=3$) were isolated by differential centrifugation method (16). The protein content of the microsomal and cytosolic fractions was determined by Lowry method, using bovine serum albumin as the standard and the Bio-Rad protein assay kit, as per the manufacturer's instructions (17). Microsomes and cytosol were stored at -80°C until incubation studies were performed

4.2.4. Spectral Analysis of Total P450 content: The total CYP content in the microsomal fraction from 3 groups of rats was determined spectrophotometrically using the extinction coefficient of $91 \text{ mM}^{-1} \text{ cm}^{-1}$ (18).

4.2.5. Enzyme Assays: The assay conditions for the enzymatic activity determinations were adapted from previously reported methods in the literature (19, 20) and are listed in Table 1. Briefly, incubations were performed in 50 mM potassium phosphate buffer in a total volume of 0.2 ml - 0.4 ml. Incubation mixtures were preincubated at 37°C for 3 min prior to the start of the reactions. Reactions were started by addition of cofactor and terminated using cold quenching agent after appropriate times (Table 1). Metabolites from these incubations was extracted using liquid-liquid extraction or by centrifugation at

13 000 g for 10 min. The extracted samples (reconstituted in mobile phase) or the supernatant were quantified by spectrofluometric assay or HPLC based assays. The analytical assays described in Table 1 were conducted by LC/MS/MS analysis on a Thermo Finnigan TSQ Quantum triple-quadrupole mass spectrometer (Thermo Fisher Scientific) (for determination of 16 α , 6 β -hydroxytestosterone and 12-hydroxy lauric acid). A high-performance liquid chromatography system (Waters, Milford, MA) consisting of an Alliance 2695 autosampler/pump and 2487 dual-wavelength absorbance detector (for 6-hydroxychlorzoxazone, acetyl PABA and 7-hydroxycoumarin sulfate) or a 474 scanning fluorescence detector (for dextrorphan and 4- MU glucuronide) and Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VT) (for resorufin).

4.2.6. RT-PCR: Total RNA was isolated from whole liver homogenate with the RNeasy Miniprep Kit (QIAGEN, Valencia, CA). Total RNA was reverse-transcribed to cDNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Expression of rat *Abcb1*, *Abcc1*, *Abcc2*, *Slco1a4*, *Slc22a6* and *GAPDH* was quantified by qRT-PCR Assays-on-DemandTM Gene Expression (Applied Biosystems). Briefly, each reaction was performed in a 20 μ l volume with 50 ng of cDNA, 1 μ l of TaqMan[®] gene expression assay mix and 10 μ l of TaqMan Universal PCR Master Mix. All reactions were performed in triplicate on the ABI Prism 7300 Sequence Detection System (Applied Biosystems). The cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C followed by 60 s at 60°C. The target mRNA levels were normalized to *GAPDH* and expressed relative to the lean controls using the 2⁻

$\Delta\Delta C_t$ method (21).

4.2.7. Western Blotting: Liver lysate was resolved by SDS-PAGE (4-20% Tris-glycine) and blotted with 1:1000 dilution of anti-PPAR (α, β, γ), 1:200 dilution of anti-CAR and 1:500 dilution of anti-PXR antibody (Santa Cruz Biotechnology, Inc., CA). The positive control was rat liver extract (Santa Cruz Biotechnology, Inc., CA), and calnexin was used as an internal control. Bands were visualized with goat anti-rabbit horseradish peroxidase conjugated secondary antibody (1:8,000 dilution) for PPAR and PXR, and rabbit anti-goat horseradish peroxidase conjugated secondary antibody (1:8,000 dilution) for CAR with the ECL detection method. Quantification of the bands was performed by Adobe® Image software.

4.2.8. Data Analysis: Unless noted, all data consist of three independent experiments and are represented as the mean \pm standard error (S.E.). Kinetic parameters for the substrates were estimated by nonlinear regression analysis with SigmaPlot 10.0 (Systat Software, Inc., Chicago, IL). Data was fit to a typical Michaelis-Menten equation, and the intrinsic clearance was calculated from V_{max}/K_m ratio. Goodness of fit was determined by visual examination of the resulting curves, comparison of the coefficients of determination, and inspection of Eadie-Hofstee plots. Comparison between the groups was performed with the two-sided Student's t-test (SigmaStat 3.1; Systat Software, Inc.). The level of significance for all the comparisons was set at $p < 0.05$.

4.3 Results:

4.3.1. Effect of sterculic oil intervention on hepatic oxidative enzyme activities:

Total P450 content was found to be similar among the LETO, OLETF and OLETF SO groups (Table 3). CYP1A (Ethoxresorufin) and CYP2B (pentoxyresorufin) enzyme activity was unaltered in both the LETO and OLETF groups (Table 3). The addition of SO to diet of the OLETF group resulted in no changes in the dealkylation of CYP1A and CYP2B probes. CYP2C11 activity was found to be nearly 40 % lower in the OLETF group ($CL_{int} 0.48 \pm 0.06$ mL/min/mg) as compared to LETO group ($CL_{int} 0.64 \pm 0.02$ mL/min/mg). Interestingly, CYP2C11 activity in the OLETF SO group ($CL_{int} 0.59 \pm 0.07$ mL/min/mg) was found to be significantly higher than the OLETF group, and was similar to the activity in the LETO group ($CL_{int} 0.64 \pm 0.02$ mL/min/mg, $p > 0.05$), indicating prevention of obesity mediated changes in CYP2C11 activity following SO treatment.

CL_{int} for dextromethorphan was found to be lower in both the OLETF and OLETF-SO groups as compared to LETO group. This reduction in the CL_{int} resulted from a significantly lower V_{max} for CYP2D2-mediated dextrophan formation in the OLETF group (1.97 ± 0.03 nanomoles/min/mg protein) as compared to the LETO group (0.324 ± 0.04 nanomoles/min/mg protein, $p < 0.05$) , with no changes in the K_m (Fig 2). However, in the OLETF SO group, apart from the significant reduction in the V_{max} (0.682 ± 0.04 nanomoles/min/mg protein), the K_m was also found to be ~ 4-fold higher as compared to the LETO group (Fig 2). Contrary to the previous studies that have reported an elevated activity of CYP2E1 in obese rats, our study found that there was no difference in CYP2E1 activity among the LETO, OLETF and OLETF SO animals (Table 3). However,

CYP3A activity was approximately 30% lower in the OLETF group (CL_{int} 0.061 ± 0.02 mL/min/mg) as compared to the LETO group (CL_{int} 0.079 ± 0.01 mL/min/mg, $p < 0.05$). Similar to CYP2C11, obesity mediated reduction in Cyp3a activity was reversed upon addition of SO to the diet of OLETF group. The CL_{int} in the OLETF SO group (CL_{int} 0.092 ± 0.03 μ L/min/mg) was found to be similar to that of LETO group (0.08 ± 0.02 μ L/min/mg, respectively).

CYP4A activity (lauric acid 12-hydroxylation) in the OLETF rats (299.55 ± 6.825 nanomoles/min/mg protein) was similar to activity in LETO rats (310.55 ± 15.12 nanomoles/min/mg protein); however, SO treatment increased CYP4A activity (400.2 ± 7.82 nanomoles/min/mg protein, $p < 0.05$). Finally, the hepatic XO activity was 2.5 times higher in the OLETF obese rats when compared to the LETO lean rats (891.2 units/mg vs. 374.8 units/mg protein); SO treatment reversed the elevated XO activity (450.4 units/mg protein) to levels similar to those in LETO rats.

4.3.2. Minimal changes in UGT, SULT and NAT activity with Sterculic oil supplementation:

As described previously UGT, NAT, and SULT activity was measured with 4-MU, PABA, and 7-hydroxycoumarin respectively. The V_{max} of 4-MU glucuronidation in LETO rats (45.33 ± 2.24 nanomoles/min/mg protein) and OLETF rats (37.43 ± 2.32 nanomoles/min/mg protein) was similar. However, there was a significant reduction in V_{max} in the OLETF SO group (25.12 ± 1.26 nanomoles/min/mg protein) as compared to LETO and OLETF groups. There were no significant differences in K_m among any of the

three groups. Additionally, the NAT and SULT activities were not different among groups (Table 3).

4.3.3. Effect of Sterculic oil on mRNA expression of hepatic drug transporter:

Figure 3 shows the mRNA expression of various hepatic drug transporters in LETO, OLETF and OLETF SO groups. We observed no differences in mRNA levels of *ABCC1*, *ABCC2* and *SLC22A6* among the 3 groups. In OLETF rats, the mRNA levels of *OATPIA4* (uptake transporter) were found to be ~ 4-fold lower as compared to the LETO group. Treatment of OLETF rats with SO resulted in the prevention of the obesity mediated differences in mRNA levels of *OATPIA4* (Fig 3). For *ABCB1* (P-gP) the mRNA levels were similar between the lean and obese group, however exposure of the OLETF rats to SO resulted in elevated mRNA levels (~ 4-fold) of *abcb1*.

4.3.4. Expression of IL-6, CAR, PXR and PPAR in OLETF and SO treated OLETF rats:

Plasma levels of IL-6, TNF- α and leptin were determined as they have been shown to modulate the expression of various P450s and UGTs (22). As expected, IL-6 levels were found to be nearly 3-fold higher ($p < 0.05$) in the OLETF group (1014.7 ± 201.7 ng/mL) as compared to LETO group (363.9 ± 37.3 ng/mL). Treatment of OLETF groups with SO reduced the IL-6 concentration (402.76 ± 129.10 ng/mL) to levels that were comparable to those observed in the LETO group.

Transcription factors pregnane X receptor (PXR), constitutive androstane receptor

(CAR) and peroxisome proliferator-activated receptor (PPAR) s are known to regulate the expression of various CYPs and UGTs (23). In order to determine the probable mechanism of changes observed in various CYPs we determined the hepatic protein levels of these transcription factors. As shown in Figure 4, levels of PXR were 2-fold lower in the OLETF group as compared to LETO group; whereas, SO moderately reversed the reduced PXR levels seen in the OLETF group. However, CAR expression was similar between the LETO and OLETF groups, while SO treatment resulted in 1.8-fold increase in the protein expression (Fig. 4). PPAR α expression was ~ 2-fold higher in the OLETF group as compared to the LETO and OLETF SO groups. While, there was no significant difference in the expression of PPAR β , the expression of PPAR γ was found to follow a trend similar to that of PPAR α (Fig. 4).

4.4. Discussion:

Obesity has been shown to cause differences in the expression and activity of enzymes in various animal models of obesity (2). However, most of the reported studies have been done in the ob/ob mouse model of obesity or the Zucker rat model that lack the leptin receptor, which is not the predominant mechanism of obesity in humans (8, 24). This study demonstrated that the effects of obesity on DME activity profile were not similar in the OLETF rat model as compared to animal models with a dysfunctional leptin signaling pathway. Also, treatment of OLETF rats with SO was found to prevent the obesity mediated changes in activity of CYP2C11, CYP3A and XO.

CYP2C11 and CYP3A are the major isoforms in rat liver constituting nearly 50% of the total rat hepatic CYP P450s (19). We observed that CYP2C11-mediated testosterone 16 α -hydroxylation was nearly 1.5-fold lower in the OLETF group as compared to the LETO group. These phenotype-dependent changes in the CYP2C11 activity were consistent with the reported changes in hepatic Cyp2c mRNA levels in obese mice (25). Interestingly, the addition of SO to the diet of OLETF rats resulted in prevention of the obesity mediated changes in CYP2C11 activity (Fig 2). Similar to the changes observed in CYP2C11 activity, CYP3A-mediated testosterone 6 β -hydroxylation was lower in the OLETF group as compared to the LETO group, and was reversed in the SO treated OLETF group. These changes in CYP3A and CYP2C activity correlated with the changes in expression of PXR which is a known transcriptional regulator of these enzymes (Figure 4a) (23). Additionally the observed changes and prevention of DME activity may be influenced by the differences in the liver desaturation index in OLETF

and OLETF SO animals (14) and the metabolic differences between the groups (Table 2.) Our results also show reduced intrinsic clearance of dextromethorphan (CYP2D2 probe) in the OLETF and SO treated OLETF rats as compared to the lean controls. The decrease in CL_{int} in the OLETF group was due to a lower V_{max} ; whereas in the SO treated group CL_{int} was lower due to nearly 4-fold higher K_m . While cyclopropenoic fatty acids present in SO have been shown to inhibit SCD1 enzyme activity (a microsomal enzyme), fatty acid analysis of the microsomal fractions revealed that neither of these fatty acids were present in the microsomes from the OLETF-SO animals (data not shown) eliminating the possibility that the observed changes in K_m were due to inhibition of dextorphan formation by these fatty acids or changes in the enzyme orientation with chronic treatment of SO.

The activity of CYP4A (lauric acid hydroxylation) was found to be higher in the OLETF-SO group as compared to the LETO group (Table 3), while there was no difference in the activity between the LETO and OLETF groups. CYP4A has been known to metabolize arachidonic acid to epoxyeicosatrienoic acid (EETs) which may be anti-inflammatory in nature (26). Thus, higher activity of CYP4A in the SO treated OLETF group could explain the lowering of elevated IL-6 levels in this group as compared to the OLETF group. Previous studies have shown isoform specific changes in expression of CYP4A; with CYP4A2 in Zucker rats and Cyp4a10 in ob/ob mice being expressed at higher levels as compared to lean controls. However, these studies have also shown that CYP4A1 levels in the Zucker rats were unchanged while CYP4A12 activity was lower in the male ob/ob mice (27). These isoforms specific changes in CYP4A expression may be

attributed to differential activation of PPARs. In our study PPAR levels were not congruent with the changes in the CYP4A activity, which suggests that there may be other factors like protein degradation and mRNA instability that may play an important role in influencing regulation of CYP4A activity. Interestingly, the IL-6 levels correlated with the changes in the XO activity, with the XO activity being elevated in OLETF group as compared to the LETO group and normalization to the LETO group with SO treatment. IL-6 is known to alter the activity of XO by binding to the response element in the regulatory region of the XO gene. Additionally, clinical studies also have shown positive correlations between elevated IL-6 levels and xanthine oxidase activity (4).

Several studies have shown that the CYP2E1 activity is elevated in various animal models of obesity as well as in obese humans. We observed no change in CYP2E1 activity in either the OLETF rats or SO OLETF rats (Table 3). However, the lack of changes in the CYP2E1 activity was not completely unexpected since the OLETF and OLETF-SO group had nearly 2-fold higher circulating insulin levels (Ortinau et al. 2013). In a study by Woodcroft et al, higher insulin levels were found to reduce the activity of CYP2E1 in rat hepatoma cell lines by a post transcriptional mechanism (28). This also corroborates studies in obese diabetic animals by demonstrating no change or reduction in expression or activity of CYP2E1 (27).

Our results of unaltered Ethoxresorufin-dealkylation activity are consistent with previous studies that observed no changes in protein levels of CYP1A in male CD1 HFD mouse model of obesity (6). Also, the pentoxyresorufin-dealkylation activity was unaltered in OLETF and OLETF SO groups (Table 3). Some studies have shown either

decreased levels of CYP2B mRNA or increased activity based on the animal model being used (6, 9). However, despite the wide use of PROD activity as a measure of CYP2B1 activity it is also important to consider that pentoxyresorufin is not a very selective probe of rat CYP2B1, with rat CYP1A playing a significant role in its dealkylation (19). Hence, additional studies are needed to evaluate the differences in activity of CYP2B1 in OLETF rats.

In addition to the effect on oxidative drug metabolism in the OLETF and OLETF SO group, the activity of conjugative enzymes also was found to be altered in our study. The UGT mediated clearance of 4-MU (a general UGT substrate) was found to be lower in the SO treated group as compared to the LETO and OLETF group (Fig 2). However, there were no phenotype based differences in UGT activity. Previous studies have shown reduced UGT protein expression in animal models, while clinical studies in humans have shown higher UGT activity with obesity (3, 7). The species based differences in activity of UGTs could be attributed to differences in pathways involved in regulation of UGTs. Similar to the UGT activity, there was no phenotype based difference in the activity of NAT and no effect of SO treatment (Table 3). These results were surprising since we had previously observed significantly higher NAT activity in the obese children as compared to lean controls (4).

Finally, drug transporters have been found to influence the clearance of various therapeutic agents. For our study we compared the differences in expression of uptake transporters (*SLC22A6*, *OATPIA4*) and efflux transporters (*MDR1B*, *ABCC1* and *ABCC2*) in the 3 groups (Fig 4). We observed that the mRNA levels of uptake transporter

Oatp1a4 was nearly 4-fold lower in the OLETF group as compared to the lean group, consistent with previous reports (29). The promoter region of *OATPIA4* is known to contain binding sites for the transcription factor hepatocyte nuclear factor (HNF1) (30). Studies have shown that the levels of HNF1 are lower in obesity as well as in cholestasis and heptaosteatosis, which can explain the lower levels of *OATP* in obesity (29). Interestingly, treatment of obese animals with SO resulted in partial rescue of *OATPIA4* levels. Although we did not determine the HNF1 levels, the increased mRNA levels of *OATPIA4* could be due to increased HNF1 as a result of SO mediated improvement in metabolic status of these animals. The mRNA levels of *MDR1A* were found to be similar in the LETO and OLETF groups, which are similar to the previously published studies in mice (6). The addition of SO was found to increase the mRNA expression of *MDR1A* by nearly 5-fold in the OLETF group. Since we found that both the CAR and PXR levels were upregulated by SO, the additive effect of the increased transcription due to higher PXR and CAR could result in the increased levels of *MDR1* mRNA. However, additional studies need to be done in order to determine the changes in the transporter activity in both the OLETF and OLETF SO groups, since previous studies have shown a poor correlation between mRNA levels and activity of transporters (31). Based on the changes observed in DMEs, drug transporters and transcription factors, we propose a model that can partly explain the changes observed in OLETF rats that have been fed sterculic oil (Figure 4.5)

In conclusion our study indicates significant differences in the activity and expression levels of various DMEs and transporters in the hyperphagic obese OLETF rats

as compared to lean LETO rats. However, not all of the observed differences were congruent with those reported in literature indicating that the differences attributed to obesity in various animal models are dependent on the species being used. For the first time we demonstrated that treating obese rats with SO prevented the obesity mediated differences in activity of several drug metabolizing enzymes. Future studies are planned to identify the mechanism(s) of these changes and delineate the independent effects of SO in mediating changes in DME activity as against changes due to improved metabolic status in these animals. Such studies are important in light of the growing epidemic of obesity and given the increased interest in developing therapeutics for the treatment of obesity and its associated metabolic complications.

Fig 4.1. Mechanism of action of sterculic acid

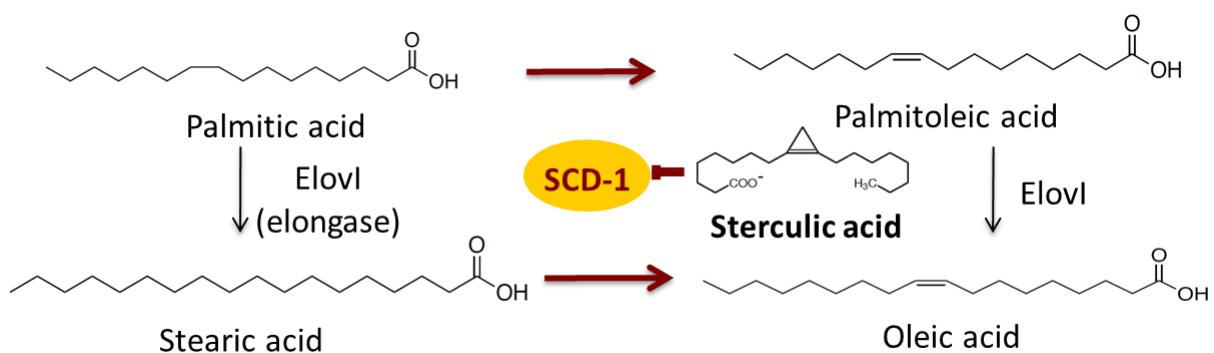


Fig 4.2. Enzyme kinetic parameter estimates for CYP2C11 (A), CYP2D (B), CYP3A (C) and UGT (D) in LETO (■) OLETF (□) and OLETF SO (■) groups. The data is represented as mean \pm SE. The alphabets represent statistical differences ($p < 0.05$) among groups. The V_{max} , K_m and CL_{int} were determined using the approach specified in the *Materials and Methods*.

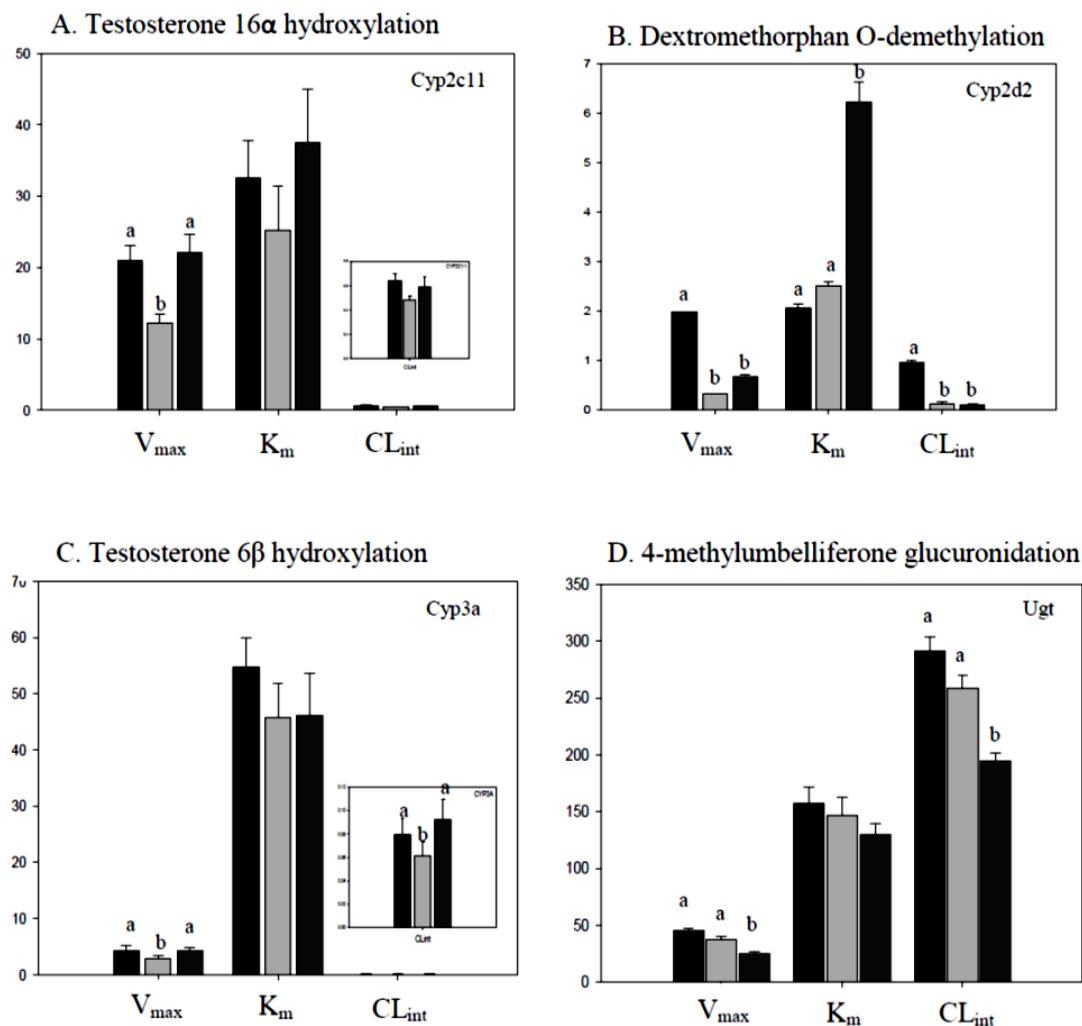


Fig 4.3. Relative mRNA levels of various influx and efflux transporters in LETO (■), OLETF (□) and OLETF SO (■) groups. The data is represented as Mean \pm SE. The alphabets represent statistical differences ($p < 0.05$) among groups.

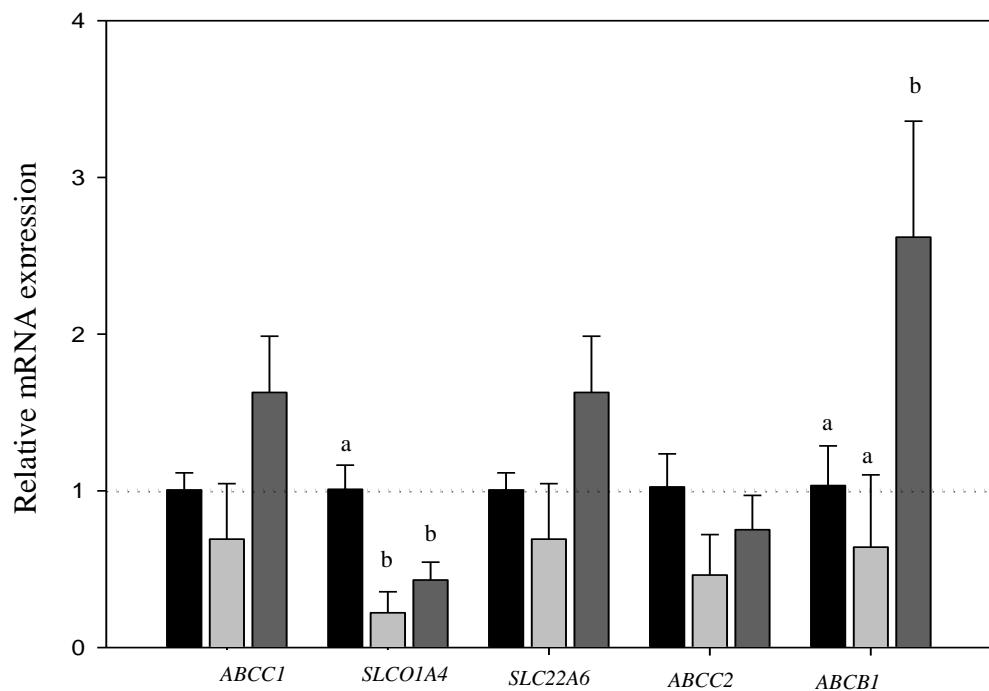


Fig 4.4. Relative protein content of hepatic transcription factors in LETO (■), OLETF (□) and OLETF SO (■) groups. The data is represented as mean \pm S.E. The alphabets represent statistical differences among groups ($p < 0.05$).

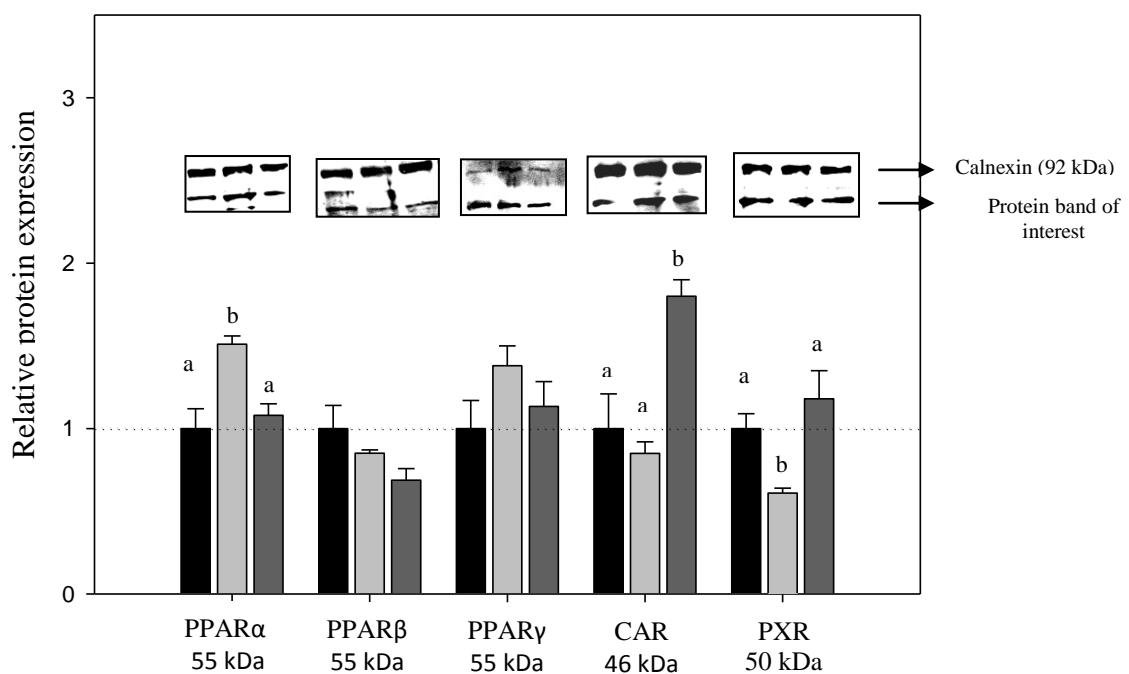


Fig 4.5. Proposed Model for the changes observed in OLETF rats treated with sterculic oil.

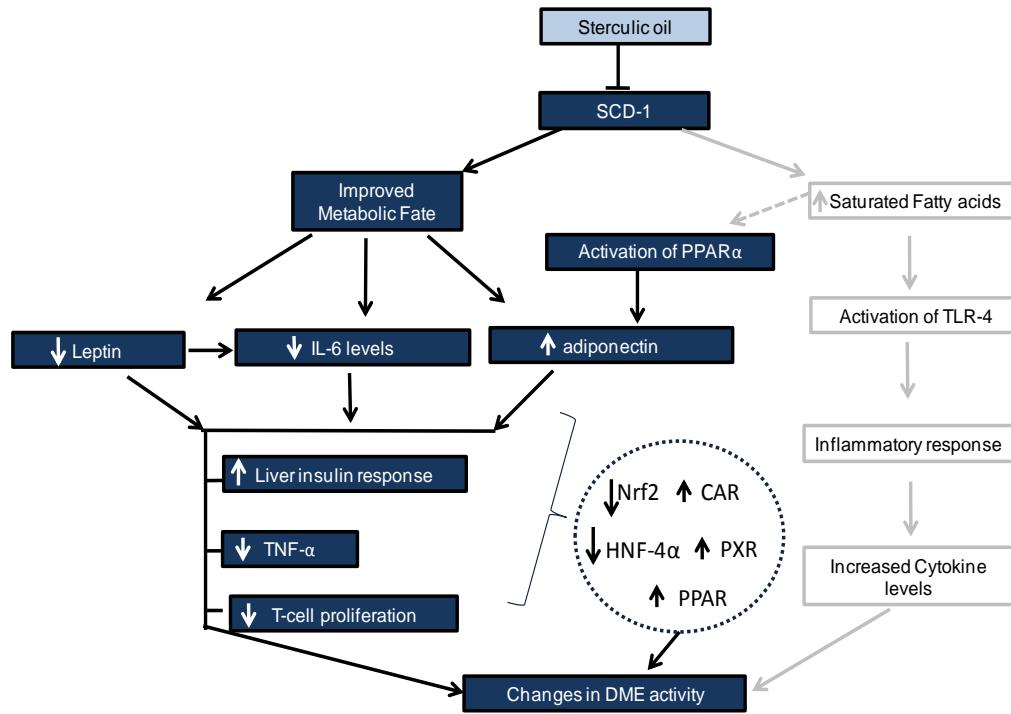


Table 4.1: Probe drugs and the incubation conditions for assessing various oxidative and conjugative enzyme activities. **MeCN:** Acetonitrile; **PABA:** p-amino benzoic acid; **4-MU:** 4-methylumbelliflferone.

Enzyme	Substrate	Metabolite	Incubation		Protein conc (mg/ml)	Co factor	Quenching agent	Detection Method
			Vol (ul)	Time (min)				
CYP1A2	Ethoxresorufin	Resorufin	200	5	0.1	NADPH	Methanol	Fluorescence
CYP2B	Pentoxyresorufin	Resorufin	200	5	0.1	NADPH	Methanol	Fluorescence
CYP2C11	Testosterone	16 α -hydroxy testosterone	200	20	0.1	NADPH	MeCN	LC/MS/MS
CYP2D2	Dextromethorphan	Dextrorphan	200	20	0.1	NADPH	Phosphoric acid	HPLC-Fluorescence
CYP2E1	Chlorzoxazone	6-OHChlorzoxazone	400	20	0.4	NADPH	MeCN	HPLC - UV
CYP3A	Testosterone	6 β -OH testosterone	200	20	0.1	NADPH	MeCN	LC/MS/MS
CYP4A	Lauric acid	12-hydroxylauric acid	200	20	0.1	NADPH		LC/MS/MS
NAT	PABA	AcetylPABA	90	20	1	Acetyl CoA	Methanol	HPLC-UV
UGT	4-MU	4-MU glucuronide	200	20	0.1	UDPGA	Methanol	HPLC-Fluorescence
SULT	7-HC	7-HC sulfate				PAPS		HPLC-UV
XO		Assay Kit						Fluorescence

Table 4.2: Animal characteristics.

Variable	LETO	OLETF	OLETF SO	P value
Body weight (g)	391.5 ± 7.7^b	515.9 ± 11.7^a	501.2 ± 9.3^a	< 0.01
Food intake (g/day)	17.2 ± 1.3^b	25.0 ± 1.7^a	25.0 ± 1.6^a	< 0.01
Liver tissue weight (g)	12.9 ± 0.4^c	18.8 ± 0.7^a	16.4 ± 0.5^b	< 0.05
Insulin (ng/mL)	2.0 ± 0.1^b	3.8 ± 0.8^a	3.9 ± 0.3^a	< 0.01
IL-6 (pg/mL)	363.9 ± 37.3^a	1014.71 ± 201.7^b	402.8 ± 129.1^a	< 0.05
Leptin (ng/mL)	5.57 ± 0.83^a	18.6 ± 2.7^b	16.6 ± 3.48^b	< 0.01
TNF- α (pg/mL)	14.95 ± 1.92^a	9.25 ± 1.69^a	4.69 ± 1.43^a	0.08

Data are presented as Mean \pm SE. Statistical difference ($p < 0.05$) between groups indicated by different alphabets.

Table 4. 3: Kinetic parameters for various oxidative and conjugative enzyme activities.

Enzyme	Parameters	Units	LETO	OLETF	OLETF_SO
Total CYP content	Amount	picmoles/mg	95 ± 13	134 ± 25	145 ± 28
CYP1A	V _{max}	nanomoles/min/mg	0.46 ± 0.3	0.44 ± 0.09	0.68 ± 0.33
	K _m	μM	0.69 ± 0.02	0.68 ± 0.03	0.92 ± 0.02
	CLint	mL/min/mg	0.69 ± 0.05	0.65 ± 0.03	0.74 ± 0.04
CYP2B	V _{max}	nanomoles/min/mg	0.39 ± 0.03	0.36 ± 0.01	0.41 ± 0.03
	K _m	μM	0.61 ± 0.04	0.68 ± 0.09	0.63 ± 0.06
	CLint	mL/min/mg	0.64 ± 0.01	0.53 ± 0.06	0.65 ± 0.03
CYP2E1	V _{max}	nanomoles/min/mg	0.7 ± 0.18	0.75 ± 0.28	0.66 ± 0.11
	K _m	μM	158.2 ± 16.3	148.2 ± 12.7	166.3 ± 17.3
	CLint	μL/min/mg	4.4 ± 1.1	5.1 ± 2.1	4 ± 1.01
CYP4A	Activity	nanomoles/min/mg	299.5 ± 6.8	310.6 ± 15.1	400.2 ± 7.8*
NAT	Activity	picmoles/min/mg	12.4 ± 2.1	12.1 ± 5	11.8 ± 2
XO	Activity	units/mg	374.8 ± 25.3	891.2 ± 78.4*	450.4 ± 24.9
SULT	Activity	nanomoles/min/mg	4.46 ± 0.8	4 ± 0.4	4.34 ± 0.8

* p value < 0.05. For CYP4A, N-acetyltransferase, xanthine oxidase and sulfonyltransferases the activities were determined at the reported K_m

CHAPTER V

Effect of Biochemicals on Drug Metabolizing Enzyme Expression and Development of Biochemically Obese Hepatocytes

5.1 Introduction:

The global epidemic of obesity "globesity" – is one of the major health problems in many parts of the world. Efficaciously dosing obese individuals is a challenge since clinical data shows obesity to be associated with altered clearance of drugs.(1) Studies have shown that factors elevated in obesity, specifically interlukin-6 (IL-6) and fatty acids, can modulate the expression and activity of drug metabolizing enzymes (DMEs) like xanthine oxidase (XO) and uridine glucuronosyltransferases (UGTs) thereby leading to altered clearance of therapeutic agents.(2, 3) Specifically, studies have shown that, when compared to lean individuals, obese adults have reduced midazolam (CYP3A substrate), and cyclophosphamide (CYP2B6 substrate) clearance, while the clearance of glipizide (CYP2C9 substrate), chlorzoxazone (CYP2E1 substrate) is elevated.(4) Also, clinical studies have shown that glucuronidation of drugs like acetaminophen, oxazepam and lorazepam is elevated in obese individuals as compared to lean individual. However, most of these enzymes are also known to be regulated by co-morbidities like Type 2 diabetes, associated with obesity as well as genetic polymorphisms.(1) Hence, it is challenging to conduct well controlled, adequately powered clinical studies to determine the effect of obesity on drug clearance.

Recently, animal models of obesity have been used to fill the knowledge gaps associated with clinical studies in obese individuals.(5-7) Though, results from these studies have been used to understand the mechanism of changes in some DMEs, they do not provide conclusive results regarding effect of obesity on drug disposition. This may, in part, be attributed to the fact that most of these studies use models that are

characterized by non-functional leptin receptors or are polygenetic (unknown mechanism of obesity). Also, due to species differences, some of the changes in DMEs observed in animal models are not consistent with the changes seen in human clinical studies.(8)

Considering the limitations associated with the use of animal models, human hepatocytes over the last two decades have been extensively developed as an *in vitro* tool to study bioactivation, metabolite formation and potential of drug-drug interactions. Hepatocytes are also used to predict the clearance of various therapeutic agents.(9) Studies have shown that the predicted hepatic clearance of drugs is within two-fold of the reported *in vivo* hepatic clearance. With advent of newer technologies like co-culture systems, microfluidic systems, the accuracy of prediction has increased considerably. Thus hepatocytes can be used as a tool to predict the clearance in obese individuals. Hepatocytes have also been used to determine the effect of various cytokines as well as hormones on expression of DMEs. Dickmann et al. have shown that incubating human hepatocytes with IL-6 results in downregulation of CYP3A and UGT activity,(10) while Su-Young Choi et al. have shown isoform specific regulation by estradiol and progesterone.(11) However, there are no studies that have sought to identify the effect of adipokines on drug metabolism. Leptin and Resistin are two important adipokines that are elevated in obesity. Also, leptin and resistin are known to influence of the levels of IL-6 and TNF- α (Scheme 5.1), and since both IL-6 and TNF- α are potent regulators of DME expression, the current study sought to identify the effect of elevated leptin and resistin concentrations on DME expression.

Because obesity is known to modulate the clearance of therapeutic agents, it is imperative

to obtain hepatocytes from obese individuals in order to accurately predict clearance in this cohort. However, such hepatocytes are not always available and if available are not of optimum quality. Hence we propose to biochemically modulate “quality” hepatocytes in order to obtain obese hepatocytes by incubating them in presence of biochemical combination that mimics the plasma profile of obese individuals. To our knowledge this is the first attempt to biochemically modulate hepatocytes to represent an obese phenotype. Considering the cost involved in working with human hepatocytes, the proof-of-concept would be established in rat hepatocytes. We hypothesize that incubating hepatocytes from lean animals in presence of biochemicals would cause modulation of enzyme expression to levels similar to obese hepatocytes. However validating the biochemically obese hepatocytes is beyond the scope of this thesis and Scheme 5.2 provides the steps that would help generate and validate such biochemically obese hepatocytes.

5.2 Materials and Methods:

5.2.1. Animal treatment and hepatocyte isolation: All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Briefly, Obesity prone and Obesity resistant animals were received at 5 weeks of age from Charles River and maintained at a controlled temperature (22°C) and a 12 hour light/dark cycle. Obesity prone animals were given a commercial high fat diet (HFD) containing 60 % fat, while obesity resistant animals were fed a low fat diet (LFD) containing 4 % fat. At 19-20 weeks of age hepatocytes were isolated using collagenase perfusion method. Animals were anesthetized using sodium pentobarbital (50 mg/kg). Portal vein was cannulated and the liver was initially perfused with Ca^{2+} free HBSS buffer containing 0.25 mM EDTA for 15 mins followed by HBSS buffer containing collagenase and CaCl_2 . Following liver perfusion, the liver cells were suspended in Williams E Medium supplemented with 5 % fetal bovine serum (FBS) and Penicillin/Streptomycin. The hepatocytes were isolated and separated from the suspension via series of centrifugation steps at $50 \times g$ and by using Percoll®. The viability of the isolated hepatocytes was assessed using Tryphan blue solution and was always greater than 85 %. The cells were plated on a 24-well plate at a density of 0.8×10^6 cells/mL and were treated with leptin, resistin, IL-6 and TNF- α at varying concentrations. The RNA was isolated at 72 hrs after plating.

5.2.2. Treatment of hepatocytes with biochemicals: Hepatocytes were treated with either leptin, resistin, IL-6 or TNF- α at 4 different concentrations as detailed in Table 5.1.

Additionally, hepatocytes were also treated with a biochemical combination of these four biochemicals at 4 different concentrations. The four biochemicals were mixed such that the final concentration of individual biochemicals was similar to the concentrations when each was used individually. The media was replaced with fresh media containing biochemicals every 12 hr. in order to maintain the levels of these biochemicals.

Table 5.1: Concentrations of biochemicals used to treat hepatocytes.

Biochemical	Concentration			
	1	2	3	4
Leptin (ng/mL)	5	10	15	20
Resistin (ng/mL)	20	50	75	100
IL-6 (pg/mL)	30	50	100	150
TNF-α (pg/mL)	5	10	15	20
	Combination 1	Combination 2	Combination 3	Combination 4

5.2.3. Determination of acetaminophen metabolism: Following incubation with various biochemicals for 0, 24, 48 and 72 hr. the media was replaced with fresh media containing Acetaminophen (final conc. 600 μ M) and the cells were incubated at 37°C for a period of 30 mins. Following 30 mins incubation, the media was collected and placed in tubes containing MgCl₂ in order to stabilize the acetaminophen glucuronide. The media

was stored at -80°C until further HPLC analysis. Acetaminophen glucuronide was quantified using previously described HPLC method.(12) Briefly, 200 µL of sample media was thawed and 20 µL of 20 % TCA was added along with 30 µL of 1 mM caffeine (internal standard). The samples were centrifuged at 8000 rpm for 10 mins at 4°C. The supernatant was analyzed using HPLC (Waters, Milford, MA) consisting of an Alliance 2695 autosampler/pump and 2487 dual-wavelength absorbance detector. The injection volume was 100 µL and separation of acetaminophen glucuronide, IS and acetaminophen was achieved using a mobile phase consisting of acetonitrile and 1% glacial acetic acid. The flow rate was 0.8 mL/min and the detection wavelength was set at 250 nm.

5.2.4. Isolation of RNA and qRT-PCR: Total RNA was isolated from cells using the Qiagen RNeasy Mini Kit® according to manufacturer's protocol. The quality of the isolated RNA was evaluated using NanoDrop (Agilent Technologies, Santa Clara, CA). All RNA samples were then normalized to 5 ng/µl with nuclease-free water. cDNA was synthesized using Qiagen RT² First Strand cDNA Kit® according to manufacturer's protocol. The cDNA was added to RT² Profiler™ PCR Array plated containing primers for 12 drug metabolizing enzyme genes and four controls. The controls were used to check for genomic DNA contamination, quality of cDNA and reverse transcription, while GAPDH was used as an internal control. All reactions were performed on the ABI Prism 7300 Sequence Detection System (Applied Biosystems). The cycling conditions were as follows: 1 min at 95°C, and 40 cycles of 15 s at 95°C followed by 60 s at 60°C. The target

mRNA levels were normalized to *GAPDH* and expressed relative to the controls using the $2^{-\Delta\Delta C_t}$ method.(13)

5.3. Results:

5.3.1. Time dependent effect of leptin, resistin, IL-6 and TNF- α on acetaminophen metabolism:

In order to identify optimum duration of treatment of hepatocytes with biochemicals, the effect of biochemicals on acetaminophen metabolism (acetaminophen glucuronide formation) over time was determined. Treatment of hepatocytes with leptin resulted in 17.7 % loss inactivity during the first 24 hr as compared to untreated controls. The activity remaining was 62.3 ± 6.4 % and 60.3 ± 5.7 % following 48 hr and 72 hr treatment respectively (Fig 5.1). Treatment of hepatocytes with resistin and IL-6 resulted in increased acetaminophen glucuronide formation, and this increased activity was similar at 48 hr and 72 hr time points as compared to the respective untreated controls. Similarly for TNF- α loss in activity over time was similar at 48 hr and 72 hr and was 29.6 % and 31.3 % respectively as compared to the untreated controls. All additional experiments were conducted using 48 hr as standard treatment time.

5.3.2. Effect of obesity on mRNA expression of major DMEs in rat hepatocytes:

The mRNA expression of major DMEs was determined in hepatocytes isolated from obese rats and compared to lean controls (Fig 5.2). The expression of CYP1A, CYP2E1 and CYP3A in the obese rats was nearly 9-fold, 10-fold and 13-fold lower respectively, while mRNA expression of CYP2B1 was only 2-fold lower. Interestingly, only mRNA levels of CYP2D2 were found to be elevated in the obese group as compared to the lean controls. There was nearly 1.5-fold decrease in mRNA expression of UGT1A1 and

UGT1A6, while there was a 5-fold decrease in mRNA expression. No phenotype dependent changes were observed in SULT1A1 mRNA expression (Fig 5.2.).

5.3.3 Effect of biochemicals on mRNA expression of oxidative and conjugative genes:

The expression of DMEs in hepatocytes isolated from lean animals was determined following treatment with biochemicals at various concentrations (as described in Table 5.1). Leptin was found to decrease the mRNA expression of all the major DMEs at concentration of 20 ng/mL (Fig 5.3.A). The mRNA expression of CYP2E1, UGT1A6 and UGT2B1 was nearly 1.8-fold lower at higher concentrations of leptin. Also, leptin treatment was associated with a dose dependent linear decrease in mRNA expression of CYP2B1, CYP2C11, CYP2D2 and UGT1A1 (Fig 5.3.B.). Incubation of hepatocytes in presence of resistin resulted in isoforms specific concentration dependent changes. At lower concentrations of resistin, there was nearly 2-fold decrease in mRNA expression of CYP1A2, while mRNA expression of CYP2D2 was found to be higher by 60 %. (Fig 5.4.A). Higher concentrations of resistin were associated with nearly 2-fold increased expression of UGT1A6, UGT2B1 and SULT1A1. Interestingly, correlation of resistin concentration with mRNA expression of these genes indicated a non-linear relationship, with only higher concentrations being associated with increased mRNA levels. TNF- α was found to cause modest alterations in the mRNA levels of DMEs. At highest concentration of 20 pg/mL, mRNA expression of CYP1A2 and CYP2C11 was found to be 1.8 fold lower. For CYP2C11, the decrease in mRNA expression was linear over

increasing the concentration of TNF- α treatment. Similarly, for UGT1A1, the mRNA levels decreased in presence of increasing TNF- α concentrations (Fig 5.5.B). However, changes in UGT1A1 mRNA expression were modest with only 60 % decrease in mRNA expression at highest TNF- α concentration (Fig 5.5.A). IL-6 was found to upregulate the mRNA expression of CYP2D2, UGT1A6 and SULT1A1 by nearly 2-fold at highest concentration, with modest changes in mRNA expression of these enzymes at lower concentrations. However, interestingly, at higher concentrations, there was a 2-fold decrease in mRNA expression of CYP2E1, with no changes at lower concentrations (Fig 5.6.A). Unlike other biochemicals, there was no linear relationship between IL-6 levels and mRNA expression of DMEs. (Fig 5.6.B)

Since these biochemicals are present in combination in plasma; we evaluated the effect of combination on DME expression in hepatocytes. Hepatocytes were incubated in presence of different combinations (Table 5.1) and relative expression of DMEs compared to untreated lean hepatocytes was determined as described previously. The biochemical combination 1 did not cause significant changes in mRNA expression of any of the genes. However, combination 2 resulted in 40 % increase in CYP2D2 mRNA expression, and approximately 30 % increase in UGT1A1, UGT1A6 mRNA expression. Interestingly, biochemical combination 3 resulted in modest decrease in mRNA expression. However, biochemical combination 4 resulted in decreased mRNA expression of CYP1A2 (34 %), CYP2B1 (43 %), UGT1A1 (35 %) and UGT1A6 (36%) (Fig 5.7).

5.3.4 Development of biochemically obese hepatocytes:

Hepatocytes isolated from lean animals were incubated in presence of biochemical combinations as described in Table 5.1. The mRNA expression of various DMEs was determined and compared with mRNA expression of hepatocytes isolated from obese animals. Genes which had no change in expression in lean hepatocytes when treated with biochemicals compared to untreated obese hepatocytes were considered to have been biochemically modified to represent the obese phenotype. Based on our results, we observed lower than 2-fold difference in mRNA expression between the biochemically obese and obese for CYP2B1, CYP2C11, CYP2D2, UGT1A1, UGT1A6, UGT2B1 and SULT1A1. (Fig 5.8) However, for other genes there was a significant difference in the mRNA expression of treated hepatocytes from lean animals and that of untreated hepatocytes from obese animals (Fig 5.8).

5.4 Discussion:

Various studies have shown the impact of cytokines on expression and activity of drug metabolizing enzymes and drug transporters. Cytokines are a common feature of inflammation and are either pro-inflammatory or anti-inflammatory in nature.(14) Obesity a state of chronic inflammation has been shown to be associated with increased levels of pro-inflammatory cytokines. Obesity is also known to alter the expression and activity of various DMEs.(15, 16) However, apart from cytokines various adipokines (secreted by adipose tissue) are known to be elevated in obese individuals. In our current study we demonstrated that the adipokines leptin and resistin, which are elevated in obese individuals, modulate the mRNA levels of DMEs in hepatocytes and these changes are concentration dependent and are isoform specific.

The effect of obesity on various DMEs was determined by comparing the mRNA expression of DMEs in hepatocytes isolated from lean rats and obese rats. Our overall results indicated that there was greater than 2-fold decrease in mRNA levels of major DMEs in hepatocytes isolated from obese as compared to the lean. The observed differences in mRNA levels were in agreement with those reported in literature.(17, 18) For example, we observed that obese rats had reduced CYP1A2 mRNA levels when compared to lean rats. These studies were in agreement with Suh et al who also reported decreased mRNA levels in obese rats.(19) Studies using human livers report decreased mRNA levels and activity of CYP1A in obese humans as compared to lean.(20) These changes in CYP1A2 have been attributed to changes in binding of aryl hydrocarbon receptor to promoter region of CYP1A1 by cytokine activated transcription factor NF-

κ B.(21)(22) Another possible mechanism by which CYP1A1 expression can be downregulated is through the nuclear factor-1 via oxidative stress pathways. In our study we observed that CYP2C11, CYP2B1, and CYP3A1 expression was downregulated in obese rats when compared to lean rats. These changes in mRNA expression were consistent with the literature which reports downregulation of these genes in either the HFD animal model of obesity, the Zucker rat model of obesity, or livers obtained from obese humans.(6, 20, 23) A possible mechanism for these changes has been reported to be via decreased expression of PXR and CAR. (20) Interestingly, in our studies we observed a 2-fold increase in mRNA expression of CYP2D2 in obese rats which is in contrast with studies that have reported decreased CYP2D expression in human hepatocytes treated with free fatty acids.(24) However, in absence of any literature regarding specific mechanism of regulation, additional studies would provide an insight into the mechanism of observed upregulation of CYP2D2 expression.

In regards to conjugative enzymes we observed that mRNA expression of UGT2B1 was downregulated 4.5-fold in obese hepatocytes as compared to lean hepatocytes. While, this observation is in agreement with reported downregulation of mRNA expression of UGT isoforms in obese rats,(17, 25) our studies (section 3.2) using morphine as probe drug indicate increased UGT2B activity in the obese rats. These results clearly indicate the possibility of posttranscriptional or posttranslational events as additional factors influencing the changes in phenotype. Additionally, we also observed a 70% and 50% downregulation of UGT1A1 and UGT1A6 respectively. It has been hypothesized that downregulation of UGT expression is due to alterations in the transcriptional factor

expression of CAR and PXR.(26) In obese insulin resistant patients studies have demonstrated that the Sterol-regulatory element binding protein (SREBP) is upregulated and inhibits the activity of both PXR and CAR resulting in downregulation of target genes.(27) Also, changes in FOXO1 and AMPK have been reported to cause changes in CAR levels leading to downregulation of UGTs and also other CYPs. (28)

Despite the current knowledge regarding how obesity mediates changes in DME expression, few studies have sought to predict the changes which could help determine changes in clearance of therapeutic agents in obese individuals. As stated previously, adipokines and cytokines represent a family of biochemicals that are altered during obesity. Studies have demonstrated that cytokines (specifically IL-6, interferon and TNF- α) induce changes in hepatic DME expression during acute and chronic inflammatory states.(29) Studies involving treatment of hepatocytes with IL-6 and TNF- α have reported changes in mRNA expression of all the major P450s. Morgan et al have reported 2 to 3-fold downregulation of CYP1A mRNA expression.(29) In our study, treatment of hepatocytes with IL-6 and TNF- α resulted in no changes in CYP1A mRNA expression and 2-fold decreased mRNA expression of CYP1A2, respectively. Also, CYP2C11 and UGT1A1 mRNA expression was reduced in hepatocytes treated with high concentrations of TNF- α . These results are consistent with Monshouwer et al who have reported 50 – 60% decreased glucuronidation in porcine hepatocytes following 48 hr treatment with TNF- α . Also, studies by Ziad Abdel-Razzak et al have reported lower mRNA expression of CYP2C in human hepatocytes treated with TNF- α .(30) Similarly for IL-6, studies have reported downregulation of major P450s in acute models of inflammation. For example

studies by Aitken et al. in human hepatocytes have shown 20 - 65% reduction in mRNA expression of major human P450s, with only 5% reduction in CYP3A4 mRNA expression.(31) In contrast, Schmitt et al. reported a marked reduction in the mRNA expression of CYP3A4 following treatment of human hepatocytes with IL-6.(32) In our study we observed a 50% increase in the mRNA expression of CYP3A and increasing concentrations of IL-6 were found to reduce the extent of mRNA upregulation. IL-6 was also found to upregulate the mRNA expression of UGT1A6 and SULT1A1 at higher concentrations in a dose dependent manner. Studies reported 40% decrease in UGT1A9-dependent propofol glucuronidation at higher concentrations of IL-6, with no changes in any other UGT isoforms activities. Also, most of the studies involving animal models of obesity have reported decreased mRNA levels of various UGT isoforms.(17, 25) While changes in UGT and SULT mRNA expression in our study were unexpected, it is important to note that treatment of hepatocytes in our study was performed at IL-6 concentrations that were most physiologically relevant to obesity rather than acute inflammation. Also, previous studies by Xu et al. in mouse model of steatosis (low grade inflammation) have reported increased mRNA levels of these enzymes due to oxidative stress. The authors of this study attribute these changes to the fasting induced upregulation of CAR, PPARs and Nrf2.(33) However, additional studies would be needed in order to determine the exact mechanism of these changes.

Novel to our current study we also determined the effect of leptin and resistin of mRNA expression of various CYPs (Fig 5.3 and 5.4). Both these adipokines are upregulated in obese individuals and are known to be proinflammatory in nature. Apart from activating

inflammatory pathways, these adipokines are also known to increase the secretion of IL-6 and TNF- α . However, despite reported 4- 5 fold increase in plasma levels of leptin in obese individuals, only one study has been reported regarding changes in DMEs following leptin administration. In our study we observed 1.6 – 2 fold decrease in most major drug metabolizing enzymes (Fig 5.3.A), with minimal changes in CYP3A mRNA expression. Also, leptin was found to cause a dose dependent decrease in mRNA expression of CYP2D2, CYP2B1 and UGT1A1. Watson et al. reported decreased CYP1A1 activity (EROD), CYP2B1 (PROD activity) and UGT activity (4-MU glucuronidation), while testosterone 6 β -hydroxylation was higher in ob/ob mice treated with leptin.(34) While, the authors in this study have not reported potential mechanism of these changes, we hypothesize that in our study the observed decrease in mRNA expression could be due to activation of inflammatory pathway by leptin (Scheme 5.1). These activated inflammatory pathways lead to the activation of transcriptional pathways that are known to suppress the expression of major DMEs (detailed in Section 1.2.4.). Resistin has been shown to be elevated in obese individuals and despite being an activator of proinflammatory pathways, no studies have been reported that explore the role of resistin in mediating changes in drug metabolism. In our current study we observed modest (20 % - 60 %) increase in mRNA levels of most of CYPs at the highest concentrations. However, for the conjugative enzymes we observed nearly 2 fold increase in mRNA expression. Interestingly for both the CYPs and UGTs there was a nonlinear relationship between resistin concentration and mRNA expression. For CYPs, there was no change in mRNA expression at lower concentration; however increased concentration

resulted in decreased mRNA expression that was similar across all high concentrations of resistin used, implying that mRNA levels in overweight individuals might not be different than in obese individuals. Similarly for UGTs, there was no change in mRNA expression at lower concentrations, however at highest concentration there was nearly a 80 % increase in mRNA expression of UGT1A6, UGT2B1 and SULT1A1 (Fig 5.4.B).

The effect of resistin on DME expression was surprisingly similar to the effect of IL-6 (Fig 5.4.A), and was completely opposite to that of leptin (Fig 5.3.A). Thus, all biochemicals, at physiologically relevant concentrations, despite being proinflammatory resulted in changes in DME expression that were peculiar to each. These results clearly indicate that there is a crosstalk between different pathways activated by individual biochemicals that results in changes in gene expression that are not specific and not predictive by a single biochemical.

Since these biochemicals are all present in the plasma, we also determined the expression of various DMEs following incubation of hepatocytes in the presence of various combinations of these biochemicals (Table 5.1 and Fig. 5.7). In our study we observed differences in mRNA expression of CYP2D2, CYP1A2, CYP2B1, UGT1A1, and UGT1A6. However, the magnitude of these differences was not greater than that seen with individual biochemicals. Considering the opposite effects of IL-6 and resistin as compared to TNF- α and leptin, the decreased effect of biochemical signature was not completely unexpected. However, differences in magnitude are not uncommon; as several studies have reported marked differences in effect of single biochemical as compared to a cocktail approach. For eg., in a study by Monshouwer et al. in porcine

hepatocytes, the authors reported 48% reduction in 6 β -hydroxy testosterone formation following treatment with IL-6. However, treatment of hepatocytes with cocktail containing IL-6, TNF- α and IFN- γ resulted in only 31% reduction in formation.(35)

Apart from determining the effect of biochemicals on hepatic DMEs, another important aim of the current study was to evaluate the possibility of developing biochemically obese hepatocytes. In absence of quality hepatocytes from obese humans, the development of biochemically obese hepatocytes would provide an attractive *in vitro* tool to study the bioactivation potential and DDI potential of therapeutic agents in obese individuals. Currently, in absence of such a tool, animal models are being used to predict changes in DDI and bioactivation of drugs, however, results using animal models are confounded by species differences and the type of obesity model. For example, in a study by Aubert J et al., the authors reported differences in hepatotoxicity associated with acetaminophen bioactivation between db/db and ob/ob mice, with higher liver injury in db/db mice as assessed by hepatic glutathione levels, plasma transaminases and liver histology.(36) Thus, determining the best animal models for predicting changes in humans is challenging. We believe development of bioengineered obese hepatocytes would help overcome some of the challenges associated with the use of animal models. In our current study we observed that treating lean hepatocytes with biochemicals resulted in changes in mRNA expression of CYP2B1, CYP2C11, CYP2D2, UGT1A1, UGT1A6 and SULT1A1 to levels similar to those in hepatocytes isolated from obese rats. However, mRNA levels of CYP1A2, CYP3A, CYP2E1 and UGT2B1 were not similar. Based on our results we propose incubating hepatocytes with biochemical combination 3,

consisting of Leptin (15 ng/mL), Resistin (75 ng/mL), IL-6 (100 pg/mL) and TNF- α (15 pg/mL), so as to best mimic the changes seen in obesity. However, for other enzymes, the use of additional/other biochemicals might provide a better alternative to replicate the obese phenotype. One of the major hurdles in development of biochemically obese hepatocytes is that most of these biochemicals also act on other cell types normally found in the liver, such as Kupffer cells. Also, cultured hepatocytes are viable for a relatively short duration of time. Thus, future studies should aim at developing biochemically obese hepatocytes in co-culture systems involving Kupffer cells that are engineered to last for longer duration. Such a system would help better mimic the changes in obesity, since obesity is a chronic low grade inflammation.

In conclusion, our studies indicate that resistin and leptin play an important role in mediating changes in DME expression. Also, based on our preliminary results, we believe that it is possible to bioengineer lean hepatocytes to mimic the obese phenotype.

Fig. 5.1. Effect of biochemicals on acetaminophen glucuronidation in hepatocytes isolated from lean animals.

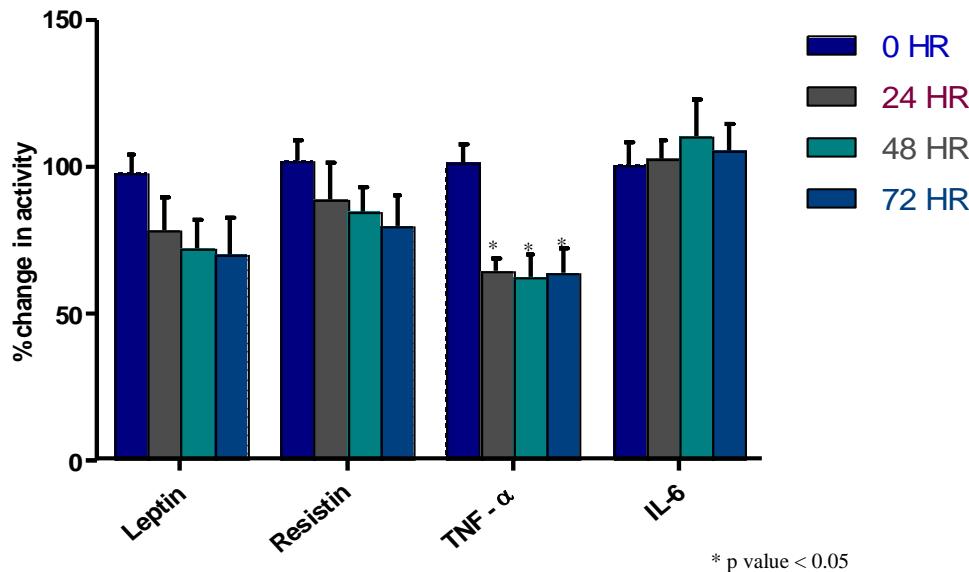


Fig 5.1. Hepatocytes were incubated in presence of Leptin (20 ng/mL), Resistin (100 ng/mL), IL-6 (150 pg/mL) and TNF- α (20 pg/mL) for 0 hr, 24 hr, 48 hr and 72 hr. Acetaminophen glucuronidation was measured and % change in activity as compared with the non-treated hepatocytes was determined over 72 hrs. Each value represents the mean \pm SE of three independent incubations.

Fig 5.2. Effect of obesity on mRNA expression of major DMEs.

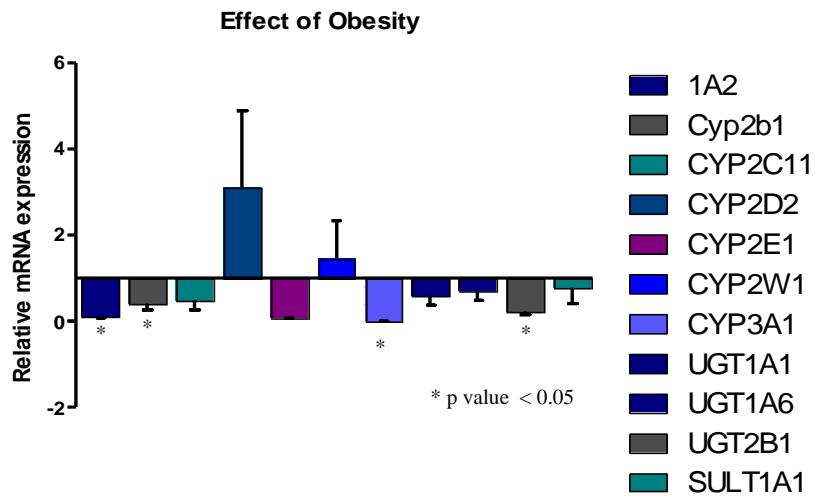


Fig 5.2. Relative mRNA expression of various DMEs in hepatocytes isolated from obese rats as compared to lean controls, following 72 hr. of plating. Relative mRNA expression of 1 represents no difference in mRNA expression in obese when compared to lean controls. Each value represents the mean \pm SE of three different rats.

Fig 5.3.A. Dose–response effects of Leptin on mRNA expression of DMEs in lean rat hepatocytes.

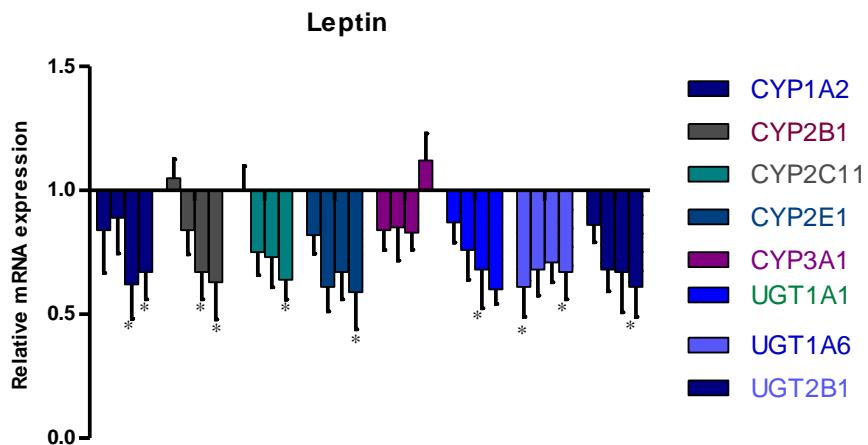


Fig 5.3.A. Hepatocytes were treated with increasing concentrations of leptin (Table 5.1) for a period of 48 hr. For each gene data are expressed as relative expression to untreated lean hepatocytes (control) and each column represents different concentrations of leptin (increasing from left to right). Each value represents the mean \pm SE of three different rats and the values which are significantly different from controls are shown by * ($p < 0.05$).

Fig 5.3.B. Correlation of Leptin with mRNA expression of DMEs.

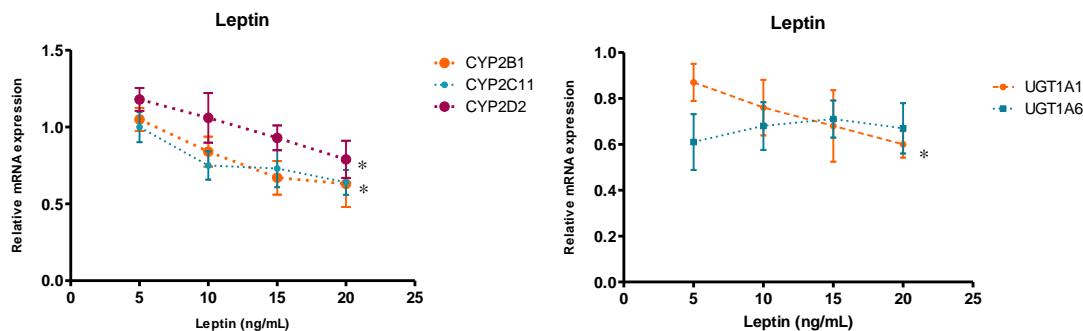


Fig 5.3.B. The mRNA expression of various genes was correlated with the different leptin concentrations. Correlations with significant slope (*) are shown above.

Fig 5.4.A. Dose-response effects of Resistin on mRNA expression of DMEs in lean rat hepatocytes.:

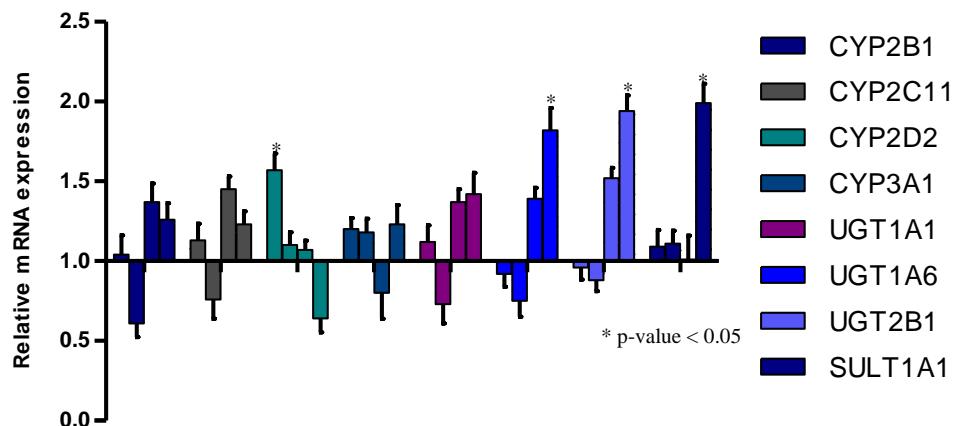


Fig 5.4.A. Dose-response effects of Resistin on mRNA expression of DMEs in lean rat hepatocytes. Hepatocytes were treated with increasing concentrations of resistin (Table 5.1) for a period of 48 hr. For each gene data are expressed as relative expression to untreated lean hepatocytes (control) and each column represents different concentrations

of resistin (increasing from left to right). Each value represents the mean \pm SE of three different rats and the values which are significantly different from controls are shown by * ($p < 0.05$).

Fig 5.4.B. Correlation of Resistin with mRNA expression of DMEs.

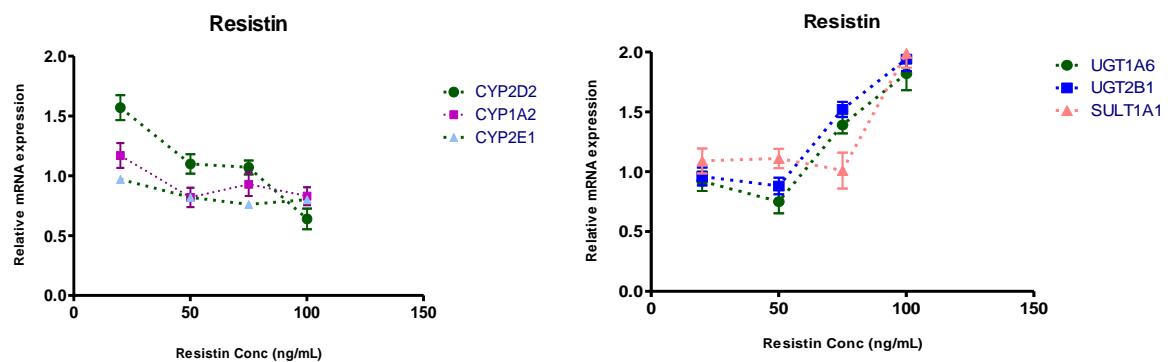


Fig 5.4.B. The mRNA expression of various genes was correlated with the different resistin concentrations.

Fig 5.5.A. Dose–response effects of TNF- α on mRNA expression of DMEs in lean rat hepatocytes.

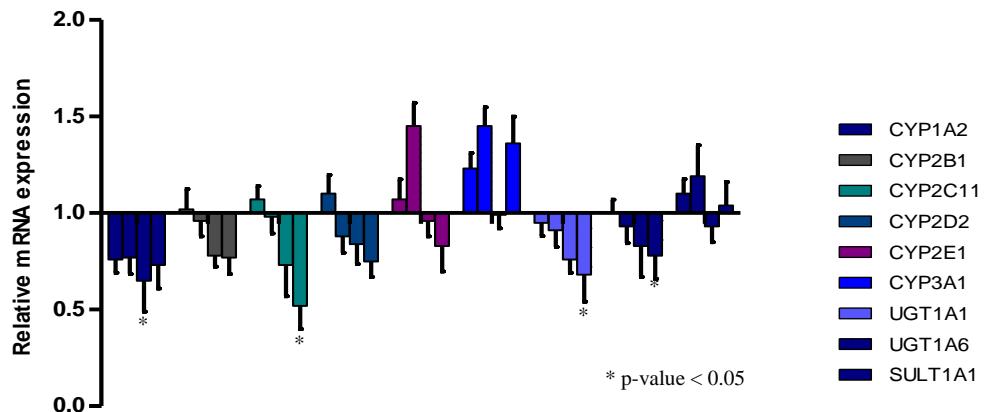


Fig 5.5.A. Dose–response effects of TNF- α on mRNA expression of DMEs in lean rat hepatocytes. Hepatocytes were treated with increasing concentrations of TNF- α (Table 5.1) for a period of 48 hr. For each gene data are expressed as relative expression to untreated lean hepatocytes (control) and each column represents different concentrations of TNF- α (increasing from left to right). Each value represents the mean \pm SE of three different rats and the values which are significantly different from controls are shown by * ($p < 0.05$).

Fig 5.5.B. Correlation of TNF- α with mRNA expression of DMEs.

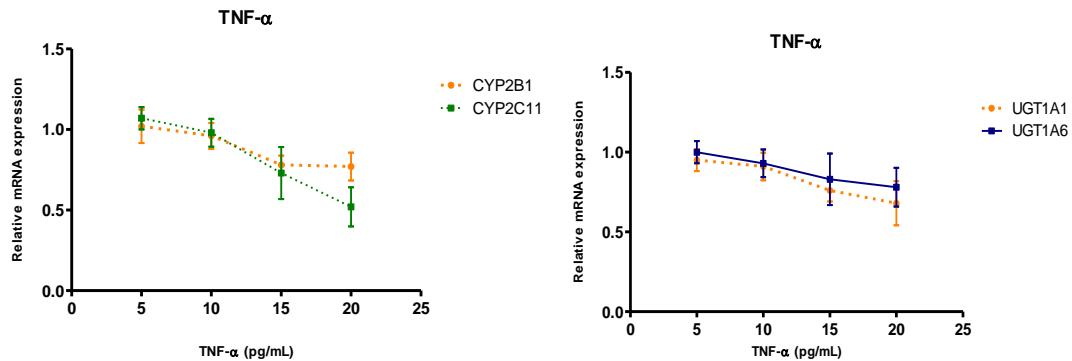


Fig 5.5.B. The mRNA expression of various genes was correlated with the different TNF- α concentrations. Correlations with significant slope are shown above.

Fig 5.6.A. Dose-response effects of IL-6 on mRNA expression of DMEs in lean rat hepatocytes.

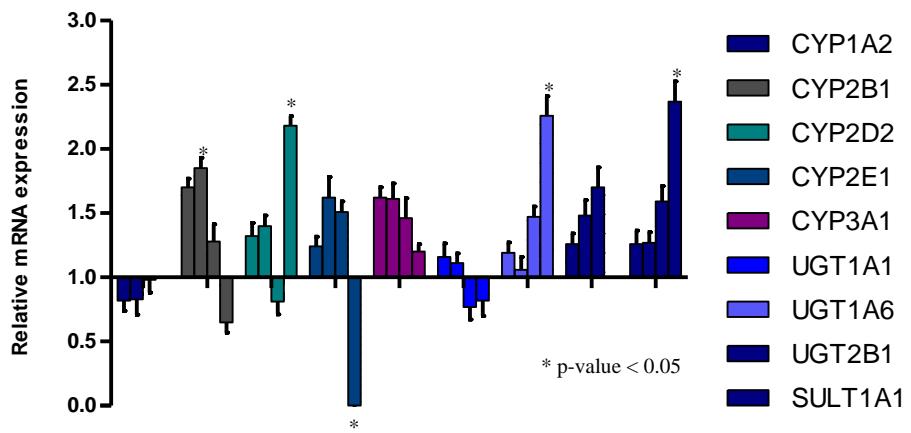


Fig 5.6.A. Dose-response effects of IL-6 on mRNA expression of DMEs in lean rat hepatocytes. Hepatocytes were treated with increasing concentrations of IL-6 (Table 5.1) for a period of 48 hr. For each gene data are expressed as relative expression to untreated

lean hepatocytes (control) and each column represents different concentrations of IL-6 (increasing from left to right). Each value represents the mean \pm SE of three different rats and the values which are significantly different from controls are shown by * ($p < 0.05$).

Fig 5.6.B. Correlation of IL-6 with mRNA expression of DMEs.

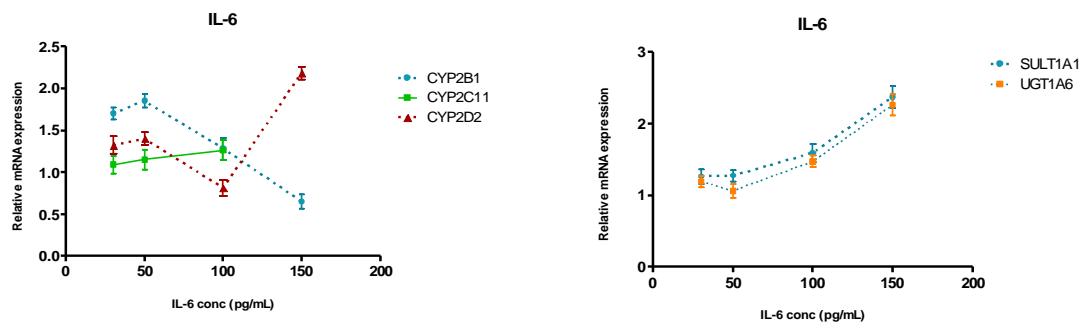


Fig 5.6.B. The mRNA expression of various genes was correlated with the different IL-6 concentrations.

Fig: 5.7. Effect of Biochemical combination on DMEs.

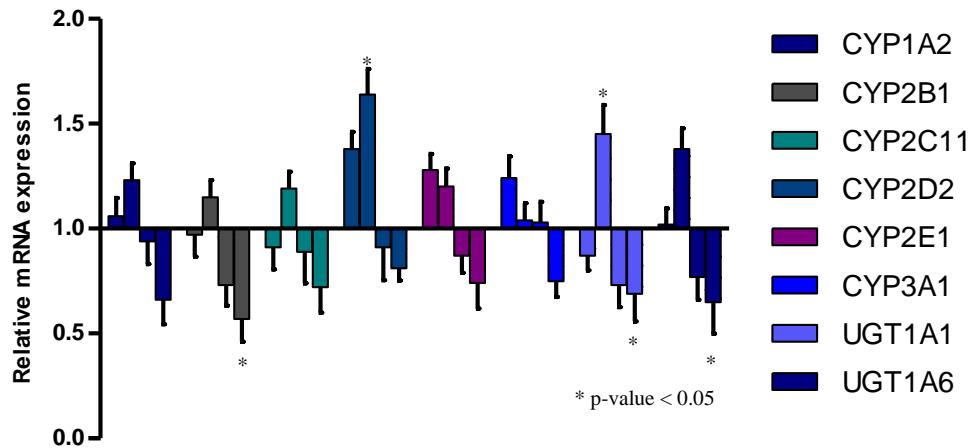


Fig 5.7: Effects of biochemical combinations on mRNA expression of DMEs in lean rat hepatocytes. Hepatocytes were treated with 4 different combinations of biochemicals BC1-BC4 (Table 5.1) for a period of 48 hr. Data are expressed as relative expression to untreated lean hepatocytes (control). Each value represents the mean \pm SE of three different rats and the values which are significantly different from controls are shown by * ($p < 0.05$).

Fig 5.8. Development of biochemically obese hepatocytes.

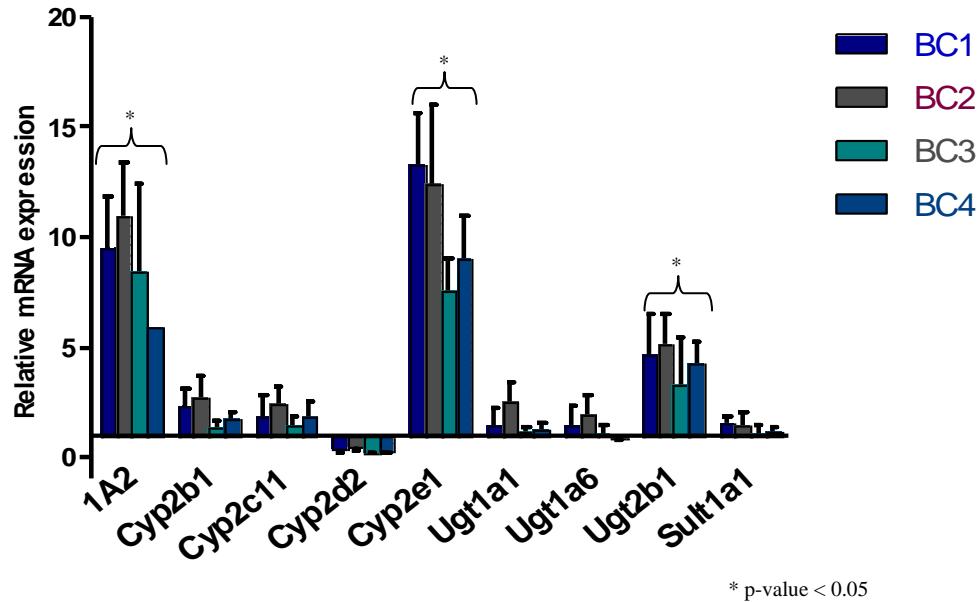
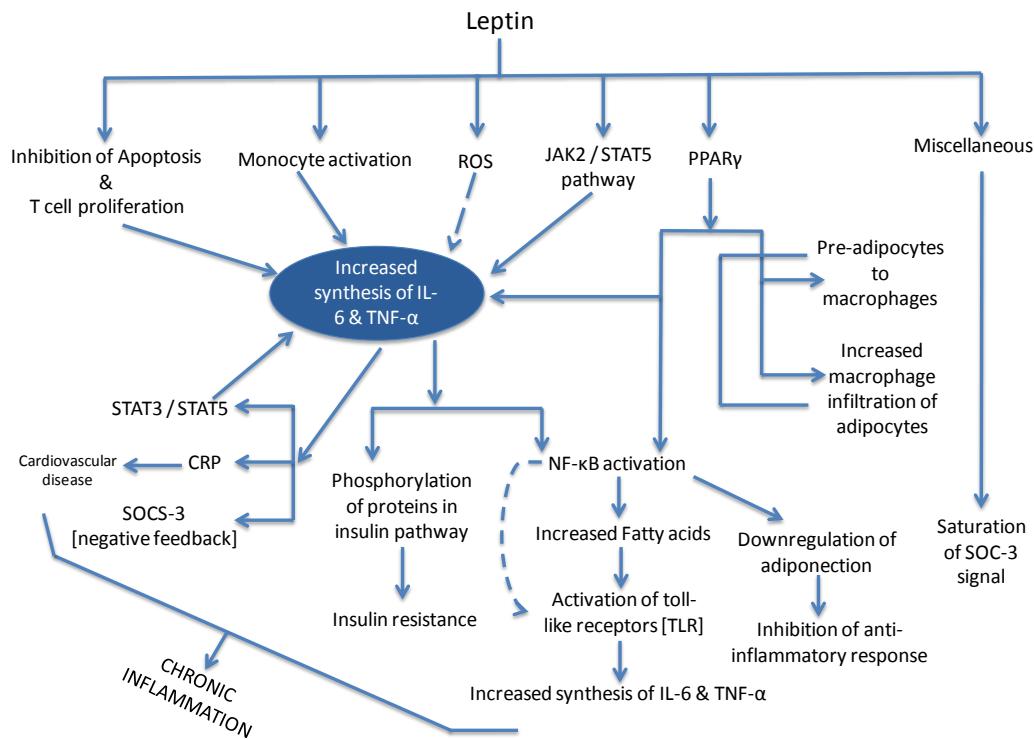
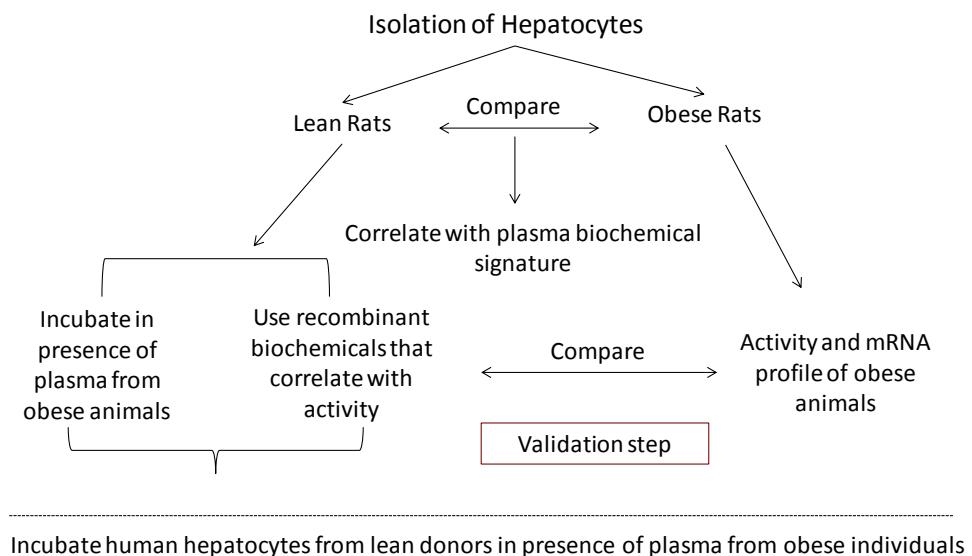


Fig 5.8. The mRNA expression of DMEs in lean rat hepatocytes, following 48 hr treatment with biochemicals (at 4 different combinations) (Table 5.1) was compared with mRNA expression of hepatocytes obtained from obese rats. Each value represents the mean \pm SE of three different rats. Hepatocytes would be considered to mimic obese phenotype for a gene if the relative mRNA expression for that gene is not significantly different than 1.

Scheme 5.1: Role of leptin in mediating changes in TNF- α and IL-6



Scheme 5.2: Steps in development of biochemically obese hepatocytes



Chapter VI

Summary and Conclusions

Obesity is a growing epidemic in the US with nearly 35 % adults and 20 % children being classified as obese. It is associated with various co-morbidities and alterations in the pharmacokinetics of therapeutic agents used to treat these diseases. Despite the extensive knowledge regarding obesity mediated changes in pharmacokinetics in adults, dosing obese adults is challenging and is often associated with either lack of efficacy or toxicity.(1) Considering the increase in prevalence of pediatric obesity, the problem is further compounded since only few studies have evaluated the impact of obesity on clearance in this population and the data from these studies indicates that similar to obese adults, there are differences in pharmacokinetics of drugs in obese versus healthy weight children. For example, Zuccaro demonstrated that of children being treated for acute lymphocytic leukemia (ALL) with 6-mercaptopurine, subjects who had a BMI \geq 75th percentile exhibited a 59% and 57% reduction in 6-mercaptopurine AUC_{0- ∞} and C_{max}, respectively compared to subjects with a BMI < 75th percentile; whereas, Cl and V_d were increased by 121% and 66%, respectively.(2) Thompson et al., demonstrated that when dosing pediatric cancer patients with doxorubicin without taking into account ideal body weight, doxorubicinol metabolic clearance was decreased by 42% in patients with >30% body fat whereas doxorubicin clearances were not affected.(3) Notably, this is in direct contrast to results observed in obese adult cancer patients treated with doxorubicin wherein doxorubicin clearance was reduced by 43%, however there was no difference in CL or AUC the doxorubicinol metabolite.(4) It has also been reported that obese children are more likely to experience adverse outcomes (5). Specifically, two studies described a correlation of obesity with

therapeutic outcome, wherein overweight children with ALL or acute myeloid leukemia (AML) exhibited reduced survival and increased incidence of regimen-related toxicities. The field of pediatric oncology is just one of many instances where there is a need to determine the effect of obesity on drug metabolism in order to develop and evaluate tools that would predict changes in clearance in all obese individuals.

Therefore this dissertation aimed to 1) understand what DME enzymes are altered in pediatric obesity, 2) identify appropriate animal models that would be able to predict obesity mediated alterations in human adults and children, 3) evaluate alternative strategies that would reverse the obesity mediated changes in drug metabolism, and 4) develop model and validate a model system that would be able to predict the obesity mediated changes in drug disposition for various classes of pharmacotherapies.

In chapter 2 we determined the impact of obesity on CYP1A2, CYP2D6, CYP3A, NAT and XO activity using probe drug approach in the pediatric population. CYP1A2, 2D6, 3A are responsible for the metabolism of 55% of the currently market drugs and thus understanding the changes in activity of these enzymes would provide the foundational information necessary to elicit change in dosing guidelines of therapeutic agents which go through these metabolism pathways. Our study reported increased xanthine oxidase and N-acetyl transferase activity in obese children as compared to lean control while taking into account the genotype. The elevation in xanthine oxidase activity was the first evidence based finding that explains differences in 6-mercaptopurine disposition between obese and lean pediatric ALL patients. Therefore, identifying changes in xanthine oxidase activity, apart from TMPT genotype, maybe an additional

strategy to ensure 6-mercaptopurine efficacy in children. In our study we did not observe any significant differences in the CYP1A2 activity. Since CYP1A2 metabolizes < 5% of the current drugs on the market further exploration of the effect of obesity on modulation of this enzyme is not warranted. Due to the effect of genotype and difficulty in recruiting volunteers in each genotype, our study was not adequately powered to detect changes in CYP3A and CYP2D6 activity in this population. The choice of in vivo probes to assess CYP3A and CYP2D6, even though not ideal was most suitable choice considering the population. However, based on our results we intend to conduct well powered clinical studies in lean, overweight and obese individuals to determine the impact of obesity on drug metabolism and correlate the changes in DME activity with plasma biochemicals using multiple regression analysis. In light of difficulty associated with conducting clinical studies in this specialty population, we decided to evaluate animal models of obesity as tools to predict clearance in obese individuals.

Previous studies using rodent animal models have reported differences in mRNA expression of DMEs. However, few studies have determined the changes in activity of various DMEs towards substrates. Evaluation of activities would help identify animal models that can be used to predict clearance in obese humans using allometric scaling approach. In Chapter 3, we compared the activities of various DMEs in mouse, rat and porcine model of obesity. While the rodent models of obesity are commonly used, we were the first group to evaluate differences in DME activity in pediatric porcine model of obesity. The choice of porcine model was based on the fact that there is a great homology between human and porcine DMEs. However, despite the homology between human and

porcine enzymes, we found significant differences in DME activity pattern in obese pigs as compared to humans. One of the major differences involved decreased xanthine oxidase activity in obese pigs vs. the increase in xanthine oxidase (XO) activity which was observed in our clinical study (Chapter 2). Since XO is known to be induced under inflammatory conditions; the lack of upregulation in porcine model indicates differences in regulatory pathways between humans and porcine model. Apart from porcine model, the high fat diet mouse model was also found to show DME activity pattern which was not similar to humans (Table 3.1 and Table 3.2). Interestingly, in the rat model of obesity we observed changes in DME activities which were similar to those seen in humans. Thus, our study indicates that the rat model of obesity would be the best choice to pursue additional studies in obesity model development (Chapter 5) and determine strategies that would normalize the obesity mediated changes in drug metabolism (Chapter 4).

One way to efficaciously dose obese patients with disease is to mitigate the obesity mediated alterations in drug disposition. In chapter 4 , we evaluated the changes in DME activity in rats fed with sterculic oil, a novel nutraceutical that has been shown to improve metabolic syndrome associated with obesity. In our study, we observed reversal of CYP2C, CYP3A and xanthine oxidase activity treatment with anti-obesity agents. Being able to normalize CYP2C and CYP3A is critical for drugs whose clearance depends on metabolism by these enzymes in order to appropriately dose them. For example, in the cancer population, paclitaxel and doxorubicin are associated with wider variability and part of the variability could be explained by obesity mediated changes in CYP3A metabolism.(6) Thus, normalization of CYP3A activity in obese population

using anti-obesity agent might help reduce the variability associated with the use of these agents. However, sterculic oil itself was found to influence the activities of CYP2D, CYP4A and mRNA expression of efflux transporter Pgp. Thus, long term dosing of this agent in humans would be beneficial, but might lead to nutraceutical-drug interactions.

Though we believe that the rat model of obesity can be used to address certain research questions, by itself it would be inadequate to predict the clearance of therapeutic agents in humans. Hepatocytes are an attractive tool to predict clearance of drugs, and are commonly used at the drug discovery and drug development stage. However, the use of hepatocytes is limited due to supply and quality of hepatocytes. Also, in order to predict clearance in obese patients it necessary to obtain hepatocytes from obese individuals. The availability of such hepatocytes is limited and not certain; hence we decided to bioengineer obese hepatocytes by treating the hepatocytes from lean individuals with biochemicals so as to replicate the obese phenotype. Considering the expense associated with working with human hepatocytes, we decided to test this proof of concept in rat hepatocytes. Based on the preliminary studies, we propose using a biochemical combination containing Leptin (15 ng/mL), Resistin (75 ng/mL), IL-6 (100 pg/mL) and TNF- α (15 pg/mL), to best mimic the changes seen in obesity. However, future studies using multiple biochemicals, are needed to establish an obese phenotype for the enzymes. Also, it is important to validate these hepatocytes by comparing predicted clearance using these hepatocytes with the in vivo clearance. Development of such a system would not only help in predicting the clearance in obese individuals, but also help predict bioactivation potential and drug-drug interaction potential in this population.

In conclusion, over the last three decades various *in vitro*, animal and clinical studies have reported the impact of obesity on drug metabolism and its role in influencing the pharmacokinetics of therapeutic agents used in the obese population. However, there is a clear need to establish a system that can predict changes in clearance in this growing population. Based on the different studies reported in this thesis, we believe that development of biochemically obese hepatocytes is an innovative and feasible way forward to predict changes in clearance in the obese population.

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