COORDINATED REGULATION OF HEPATIC AND RENAL MEMBRANE TRANSPORTERS IN EXPERIMENTAL NONALCOHOLIC STEATOHEPATITIS

by

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DEDICATION

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## TABLE OF CONTENTS

| LIST OF FIGURES | ................................................................................................................ | 11 |
| LIST OF TABLES | ............................................................................................................... | 14 |
| ABSTRACT | ............................................................................................................... | 15 |

### CHAPTER 1: XENOBIOTIC PHARMACOKINETICS AND FACTORS INFLUENCING INTER-INDIVIDUAL VARIABILITY IN DRUG RESPONSE .................................. 17

#### Pharmacokinetics- Background and Principles ............................................................................................................... 17
#### Xenobiotic Metabolism ...................................................................................................................................................... 19
    - Phase I Metabolism ......................................................................................................................................................... 20
    - Phase II Metabolism ......................................................................................................................................................... 21
#### Membrane Transporters .................................................................................................................................................... 24
    - The ATP-binding Cassette (ABC) Superfamily .................................................................................................................. 25
    - The Solute Carrier (SLC) Superfamily of Transporters .................................................................................................. 28
#### Membrane Transporter Regulation .................................................................................................................................... 30
    - Transcriptional Regulation .................................................................................................................................................. 31
    - Post-translational Regulation ............................................................................................................................................ 34
#### The Liver- Physiology and Role in Xenobiotic Metabolism and Disposition ................................................................. 36
#### Liver Anatomy ................................................................................................................................................................. 36
    - Architecture and Blood Supply ....................................................................................................................................... 36
    - Cellular Components of the Liver .................................................................................................................................... 37
    - The Hepatic Lobule and Hepatic Acinus .......................................................................................................................... 38
    - The Role of the Liver in Xenobiotic Metabolism and Disposition .................................................................................. 42
#### Inter-Individual Variability in Xenobiotic Response ........................................................................................................ 45
    - Adverse Drug Reactions: Prevalence and Sources ............................................................................................................ 45
    - Genetic Polymorphisms in XMEs and Membrane Transporters ....................................................................................... 46
    - Role of Liver Disease in the Inter-Individual Variability of Xenobiotic ADME ................................................................. 49
    - Nonalcoholic Fatty Liver Disease: Prevalence and Causes ............................................................................................... 52
    - Effects of NASH on Xenobiotic Metabolism and Disposition .......................................................................................... 54
    - Extra-Hepatic Regulation of Xenobiotic Disposition: Role of the Kidneys ........................................................................ 56
#### Present Study ........................................................................................................................................................................ 60
TABLE OF CONTENTS - Continued

CHAPTER 2: MODELING HUMAN NONALCOHOLIC STEATOHEPATITIS-ASSOCIATED CHANGES IN DRUG TRANSPORTER EXPRESSION USING EXPERIMENTAL RODENT MODELS ................................................. 64
   Introduction ........................................................................................................................................ 64
   Materials and Methods ...................................................................................................................... 67
      Materials ......................................................................................................................................... 67
      Animals .......................................................................................................................................... 67
      Tissue Harvesting ............................................................................................................................. 68
      Plasma Chemistries .......................................................................................................................... 68
      Tissue Staining and Evaluations ....................................................................................................... 69
      RNA Purification .............................................................................................................................. 69
      Branched Chain DNA (bDNA) Analysis ............................................................................................ 69
      Protein Preparations ......................................................................................................................... 70
      Immunoblot Protein Analysis ......................................................................................................... 71
      mRNA and Protein Concordance Analysis Across Human and Rodent Models ............................ 72
      Statistical Analysis .......................................................................................................................... 73
   Results .............................................................................................................................................. 73
      Rodent Body Weights, Tissue Weights and Clinical Chemistries .................................................... 73
      NASH Histology and Pathological Assessment in Rodent Models ................................................ 77
      Hepatic mRNA Expression of Drug Transporters in Experimental NASH Models ...................... 80
      Hepatic Protein Expression of Drug Transporters in Experimental NASH Models ..................... 82
      Concordance Analysis Across Human and Rodent mRNA and Protein Expression in NASH ......... 84
   Discussion .......................................................................................................................................... 90

CHAPTER 3: IDENTIFICATION OF A FUNCTIONAL ANTIOXIDANT RESPONSE ELEMENT WITHIN THE EIGHTH INTRON OF THE HUMAN ABCC3 GENE-MECHANISTIC INSIGHT INTO HEPATIC ABCC3 INDUCTION IN NONALCOHOLIC STEATOHEPATITIS ........................................ 96
   Introduction ...................................................................................................................................... 96
   Materials and Methods ..................................................................................................................... 98
      Materials ....................................................................................................................................... 98
**TABLE OF CONTENTS - Continued**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Culture</td>
<td>99</td>
</tr>
<tr>
<td>Chromatin Immunoprecipitation (sequencing)</td>
<td>99</td>
</tr>
<tr>
<td>In Silico Analysis</td>
<td>100</td>
</tr>
<tr>
<td>Molecular Cloning of ABCC3 Intron</td>
<td>100</td>
</tr>
<tr>
<td>Sub-cloning of ABCC3 Intron in Reporter Vectors</td>
<td>101</td>
</tr>
<tr>
<td>PCR Mutagenesis of ARE2 and ARE5 within Intron</td>
<td>101</td>
</tr>
<tr>
<td>Transfection and Luciferase Reporter Gene Assay</td>
<td>102</td>
</tr>
<tr>
<td>Nrf2 DNA Pull-down Assay</td>
<td>103</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>104</td>
</tr>
<tr>
<td>Results</td>
<td>104</td>
</tr>
<tr>
<td>ChIP-sequencing Analysis</td>
<td>104</td>
</tr>
<tr>
<td>ABCC3 Gene Sequence Alignment across Mouse and Human</td>
<td>108</td>
</tr>
<tr>
<td>In Silico Identification of Putative ARE Elements within Eighth Intron of ABCC3 Gene</td>
<td>111</td>
</tr>
<tr>
<td>Luciferase Reporter and Nrf2 Pulldown Assays</td>
<td>113</td>
</tr>
<tr>
<td>Discussion</td>
<td>116</td>
</tr>
<tr>
<td>CHAPTER 4: RENAL XENOBIOTIC TRANSPORTER EXPRESSION IS ALTERED IN MULTIPLE EXPERIMENTAL RODENT MODELS OF NONALCOHOLIC STEATOHEPATITIS</td>
<td>122</td>
</tr>
<tr>
<td>Introduction</td>
<td>122</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>124</td>
</tr>
<tr>
<td>Materials</td>
<td>124</td>
</tr>
<tr>
<td>Animals</td>
<td>125</td>
</tr>
<tr>
<td>Tissue Harvesting</td>
<td>125</td>
</tr>
<tr>
<td>Tissue Staining and Evaluations</td>
<td>126</td>
</tr>
<tr>
<td>RNA Purification</td>
<td>126</td>
</tr>
<tr>
<td>Branched DNA (bDNA) Analysis</td>
<td>126</td>
</tr>
<tr>
<td>Protein Preparations</td>
<td>127</td>
</tr>
<tr>
<td>Immunoblot Protein Analysis</td>
<td>128</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>129</td>
</tr>
<tr>
<td>Chapter</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>ALTERED ARSENIC DISPOSITION IN A MOUSE MODEL OF NONALCOHOLIC FATTY LIVER DISEASE: THE ROLE OF LIVER AND KIDNEY TRANSPORTERS</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
</tr>
<tr>
<td></td>
<td>Materials</td>
</tr>
<tr>
<td></td>
<td>Animals</td>
</tr>
<tr>
<td></td>
<td>Arsenic Disposition Study</td>
</tr>
<tr>
<td></td>
<td>Sample Preparation</td>
</tr>
<tr>
<td></td>
<td>Determination of Arsenic Species in Urine and Tissue</td>
</tr>
<tr>
<td></td>
<td>Protein Preparations</td>
</tr>
<tr>
<td></td>
<td>Immunoblot Protein Analysis</td>
</tr>
<tr>
<td></td>
<td>Statistical Analysis</td>
</tr>
<tr>
<td></td>
<td>Results</td>
</tr>
<tr>
<td></td>
<td>Mouse Liver NAFLD Histology</td>
</tr>
<tr>
<td></td>
<td>Urinary Excretion of Arsenic in Experimental NAFLD</td>
</tr>
<tr>
<td></td>
<td>Renal and Hepatic Accumulation of Arsenic in Experimental NAFLD</td>
</tr>
<tr>
<td></td>
<td>As3mt and Mrp1 Expression in Mouse Liver</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
</tr>
<tr>
<td>Chapter 6</td>
<td>SUMMARY OF CURRENT STUDY AND FUTURE DIRECTIONS</td>
</tr>
<tr>
<td></td>
<td>Summary and Current Perspective</td>
</tr>
<tr>
<td></td>
<td>Future Studies</td>
</tr>
<tr>
<td></td>
<td>Study 1: Investigate the Role of the Pro-Inflammatory Cytokine, TNF-α, in Mediating Liver and Kidney Transporter Regulation in NASH</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS - Continued

Study 2: Investigate the effect of NASH on Intestinal Membrane Transporter Expression and Function .......................................................................................................................... 191

APPENDIX A: ALTERED REGULATION OF HEPATIC EFFLUX TRANSPORTERS DISRUPTS ACETAMINOPHEN DISPOSITION IN PEDIATRIC NONALCOHOLIC STEATOHEPATITIS ................................................................. 195

Introduction .......................................................................................................................... 195

Materials and Methods .......................................................................................................... 197
  Materials .............................................................................................................................. 198
  Clinical subjects ................................................................................................................ 198
  Visit procedures ................................................................................................................ 199
  Human Liver Samples ........................................................................................................ 200
  Tissue Preparations .......................................................................................................... 201
  MRP3 Immunoblot Analysis .............................................................................................. 201
  MRP2 Liver Immunohistochemistry .................................................................................. 202
  APAP and APAP Metabolite Quantification ...................................................................... 203
  Microarray Expression Analysis of Sulfur Activation Genes ............................................. 203
  Statistical Analysis ........................................................................................................... 203

Results ................................................................................................................................ 203
  Patient Demographics and Serum Chemistry ................................................................. 204
  Serum and Urine APAP and APAP Metabolites in Pediatric NAFLD Patients ............... 206
  Hepatic MRP3 and MRP2 Regulation in Human NASH Patients .................................... 208
  Sulfur Activation and Utilization Gene Expression .......................................................... 211

Discussion ............................................................................................................................. 213

APPENDIX B: SUPPLEMENTAL DATA FOR CHAPTER 2 ......................................................... 219

APPENDIX C: SUPPLEMENTAL DATA FOR CHAPTER 5 ....................................................... 223

APPENDIX D: FUNDING ACKNOWLEDGEMENTS ............................................................... 225

REFERENCES ....................................................................................................................... 226
LIST OF FIGURES

Figure 1.1: Hepatic Lobule and Acinus.................................................................41
Figure 1.2: Mechanisms Mediating Liver Disposition........................................44
Figure 1.3: Renal Membrane Transporter Orientation.....................................58
Figure 2.1: Liver Histopathology of Rodent NASH Models...............................78
Figure 2.2: Liver mRNA Expression of Drug Transporters in Rodent NASH........81
Figure 2.3: Liver Protein Expression of Drug Transporters in Rodent NASH........83
Figure 2.4: Effect Size Analysis of Human and Rodent NASH mRNA Expression of Drug Transporters..............................................................86
Figure 2.5: Effect Size Analysis of Human and Rodent NASH Protein Expression of Drug Transporters..............................................................89
Figure 3.1: Chromatin Immunoprecipitation (sequencing)..................................107
Figure 3.2: ABCC3 Gene Comparison Across Human and Mouse......................110
Figure 3.3: Comparison of Putative ABCC3 Intronic AREs with Consensus ARE .................................................................................................................112
Figure 3.4: Luciferase Reporter Activity of ABCC3 Intron in HEK293 Cells........114
Figure 3.5: DNA Pull-down of Nrf2..................................................................115
Figure 4.1: Liver Histology in Rodent NASH......................................................131
LIST OF FIGURES - Continued

Figure 4.2: Kidney Histology in Rodent NASH………………………………………………..134
Figure 4.3: Kidney Uptake Transporter mRNA Expression in Rodent NASH………..138
Figure 4.4: Kidney Efflux Transporter mRNA Expression in Rodent NASH……….140
Figure 4.5: Kidney Protein Expression of Membrane Transporters in Rodent NASH………………………………………………………………………………143
Figure 5.1: Liver Histopathology of Mice fed a Control, High Fat and MCD Diet………………………………………………………………………………..158
Figure 5.2: Total Arsenic Elimination in Urine………………………………………………….161
Figure 5.3: Urine Profile of Arsenic Metabolites……………………………………………….162
Figure 5.4: Differential Elimination of Trivalent and Pentavalent iAs in Urine………………………………………………………………………………………163
Figure 5.5: Liver Accumulation of Arsenic Metabolites……………………………………166
Figure 5.6: Kidney Accumulation of Arsenic Metabolites…………………………………..167
Figure 5.7: Differential Accumulation of Arsenic Metabolites in Liver……………...168
Figure 5.8: Hepatic Protein Expression of As3mt and Mrp1…………………………..170
Figure 6.1: Working Model Summarizing Dissertation Studies………………………187
Figure A.1: Plasma and Urine APAP, APAP-gluc, and APAP-sulf in Pediatric NAFLD …………………………………………………………………………………….207
Figure A.2: Hepatic MRP3 Protein Induction in NASH…………………………………..209
Figure A.3: Hepatic MRP2 Localization in Patients with NASH……………………….210
Figure A.4: Normalized Gene Expression of Sulfur Activation and Utilization Pathways
Figure C.1: Chemical Structure of Inorganic and Methylated Arsenic Species……224
LIST OF TABLES

Table 2.1: Rat Body Weight, Liver Weight and Plasma Chemistries..................75
Table 2.2: Mouse Body Weight, Liver Weight and Plasma Chemistries...............76
Table 2.3: Liver Pathology Scoring of NASH Rodent Models.........................79
Table 4.1: Kidney Pathology Scoring in NASH Models...............................135
Table A.1: Study Participant Demographics and Blood Chemistry..............205
ABSTRACT

Inter-individual variability in drug response is a significant clinical concern and may lead to the development of adverse drug reactions, which are currently a top-ten cause of death in the United States. Recently, the manifestation of disease, which may alter normal physiological function, has gained increased attention for its role as a contributing factor in the development of inter-individual responses to drugs. One such disease, known as nonalcoholic fatty liver disease (NAFLD), is the most common chronic liver disease in Western society and represents a spectrum of clinical morbidities that range from the usually benign simple fatty liver to the more advanced nonalcoholic steatohepatitis (NASH). Prior investigations have identified liver-specific alterations in xenobiotic transporter and metabolizing enzymes in NASH, which lead to the functional disruption of drug disposition. To identify a useful model(s) that is representative of hepatic transporter expression profiles in humans with NASH, gene and protein expression profiles of liver membrane transporters were assayed across several commonly used experimental rodent models of the disease. NASH models that were representative of the human condition developed global, adaptive changes in transporter regulation in the liver, which was not present in models that failed to recapitulate human profiles. Specifically, decreased expression of hepatic uptake transporters was coupled with an induction of efflux transporters, which may serve as a hepatoprotective response by limiting hepatic exposure to potentially harmful substances during times of tissue stress. To link a possible molecular mechanism for these hepatic adaptations in NASH, the role of the oxidative stress-activated transcription factor, Nrf2, was investigated. A functional Nrf2 regulatory element was
identified within the eighth intron of the human \textit{ABCC3} transporter gene, implicating Nrf2 activation in NASH as a contributor to the coordinated induction of hepatic efflux transporters in the disease. To further clarify the effects of NASH on renal membrane transporter regulation, a thorough analysis of gene and protein expression was conducted with the validated rodent models used previously. Following the manifestation of disease, a global induction of renal efflux was observed, suggesting a compensatory, coordinated response of membrane transporters in the kidney upon disease induction. The functional consequences of liver and kidney xenobiotic transporter dysregulation was shown to disrupt the disposition of the environmental toxicant, arsenic. Specifically, NASH results in increased excretion of arsenic into urine as well as altered hepatic and renal exposure. These findings are associated with hepatic and renal transporter dysregulation and demonstrate for the first time that NASH alters the disposition of environmental toxicants. In summary, these studies contribute novel findings that identify liver and kidney-specific adaptations in disease that may contribute to global alterations in xenobiotic disposition thereby increasing the likelihood of developing adverse drug reactions in patients with NASH.
CHAPTER 1: XENOBIOTIC PHARMACOKINETICS AND FACTORS INFLUENCING INTER-INDIVIDUAL VARIABILITY IN DRUG RESPONSE

Biological organisms are exposed to a continuous onslaught of exogenous substances, known as xenobiotics, which may include environmental contaminants, pesticides, food additives, and/or pharmaceutical drugs. Many of these substances can cause harm if allowed to accumulate and as such, most organisms, including prokaryotes, have evolved specialized cellular mechanisms that mediate the detoxification and excretion of xenobiotics. However, these molecular and cellular mechanisms may be perturbed, leading to inter-individual variability in biological responses following exposure. These variations may result in toxicity and the studies conducted in this dissertation aim to increase the current understanding of how disease alters normal liver and kidney function in respect to handling xenobiotics.

Pharmacokinetics - Background and Principles

The study of pharmacokinetics (PK) describes the movement of xenobiotics (drugs, toxicants, compounds, etc.) throughout the body (Fan & de, I, 2014). More specifically, pharmacokinetics refers to the collective biological processes that act on a xenobiotic that mediate its absorption, distribution, metabolism, and excretion (ADME) from the body. These four fundamental principles influence the overall exposure level of xenobiotics to bodily tissues, which ultimately dictate the compound’s pharmacological and/or toxicological effect. As Hodgson once said - “A chemical cannot be a drug, no matter how active nor how specific its action, unless it is also taken appropriately into the body.
(absorption), distributed to the right parts of the body, metabolized in a way that does not instantly remove its activity, and eliminated in a suitable manner- a compound must get in, move about, hang around, and then get out” (Hodgson, 2001).

The importance of ADME in clinical drug development can be clearly highlighted by examining the causes of clinical drug attrition over a ten year period, from 1990-2000. In the mid-1990’s, nearly 40% of all drug attrition from clinical phases was attributable to PK and bioavailability concerns (Segall & Barber, 2014; Kola & Landis, 2004). This, in turn, resulted in the development of high-throughput ADME screening assays as well as the introduction of advanced computational modeling algorithms that could better predict ADME properties of novel chemical compounds early in pre-clinical phases (Tarbit & Berman, 1998; Ekins et al., 2002). These advances in the field helped reduce the overall cause of clinical drug failures due to PK from 40% to 10% in the year 2000 as well as saving millions in lost revenue due to early drug withdrawals (Kola & Landis, 2004). However, overcoming the challenges associated with factors that govern ADME still remains a major hurdle in drug development today.

Several factors, both intrinsic and extrinsic, may govern the absorption, distribution, metabolism and excretion of xenobiotics. Intrinsic factors include the physiochemical properties of a compound (liphophilicity, size, ionization, etc.), which influence its ability to passively move across non-aqueous biological membranes into cells to elicit biological effects (Fan & de, I, 2014); however, the vast majority of xenobiotic disposition is mediated by extrinsic factors, which use a functionally diverse set of cellular mechanisms that actively transport and metabolize xenobiotics to facilitate their
disposition. Specifically, these specialized mechanisms include the biochemical modification of xenobiotics by enzymatic systems as well as membrane transporters, which promote the movement of substrates across biological membranes for absorption and ultimate excretion from the body.

**Xenobiotic Metabolism**

Xenobiotic metabolism is mediated by a diverse set xenobiotic metabolizing enzymes (XME) that facilitate the elimination and/or detoxification of both endogenous and exogenous compounds in the body (Meyer, 1996). Because the chemical nature of compounds largely dictate its ability to freely diffuse across biological membranes, the overall goal xenobiotic metabolism is to convert hydrophobic chemical molecules into more hydrophilic compounds, which are more readily excreted from the body. XMEs are typically described as being either Phase I or Phase II systems, depending on the type of enzymatic reactions they catalyze. Phase I metabolism involves the biochemical modification of substrates, typically via oxidation, reduction, and hydrolysis, which functionally adds and/or exposes reactive polar groups on substrates. In contrast, Phase II metabolic reactions are mediated by multiple superfamilies of proteins that catalyze the enzymatic conjugation of substrates with bulky and typically polar side groups such as sulfate, glucuronic acid and glutathione (Xu et al., 2005). In general, the enzymatic conjugation mediated by Phase II reactions results in significant structural changes to the substrate as well as increasing its hydrophilicity, which leads to less pharmacologically/toxicologically active metabolites and enhanced excretion from the body, respectively (Xu et al., 2005).
Phase 1 Metabolism

Several Phase I superfamilies of enzymes exist; however, by far the most intensely studied and unarguably most clinically important system are the cytochrome P-450 (CYP) superfamily of enzymes. The CYP enzymes dominate nearly all Phase I metabolic reactions and are present in all eukaryotic organisms as well as some bacteria, which clearly exemplifies their importance in maintaining cellular and whole-body homeostasis (Meunier B, 2004). Also known as monooxygenases, CYPs are microsomal enzymes that catalyze the oxidative biotransformation of many xenobiotics and endogenous substrates (Zanger & Schwab, 2013; Meunier et al., 2004). The catalytic capacity of CYPs is largely dictated by the incorporation of a prosthetic heme structure, which coordinates the transfer of one atom of molecular oxygen to its substrate whereas the other is reduced to water by a 2-electron transfer by NAD(P)H (Meunier et al., 2004). As such, CYPs are able to catalyze a variety of different reactions, including dehydrogenation, dealkylation, epoxidation, and hydroxylation (Zanger & Schwab, 2013).

CYP enzymes are intracellularly localized to the inner membrane of the endoplasmic reticulum and their substrates vary considerably, which include endogenous compounds such as sterols, fatty acids, vitamins, and xenobiotics, such as pharmaceuticals and environmental toxicants (Guengerich, 2008) Currently, 18 families and 44 sub-families of CYP enzymes have been described, with a total of 57 genes identified across the human genome (Zanger & Schwab, 2013). The CYP enzymes are predominantly expressed in the liver, although extra-hepatic sites such as the kidney, small intestine and lung also have some metabolic capacity (Guengerich, 2008). Moreover, the majority of CYP families are
involved in the metabolism of endobiotics, whereas families 1-3 account for nearly all xenobiotic biotransformation and metabolize 75-90% of pharmaceutical compounds currently in clinical use (Zanger & Schwab, 2013). Furthermore, a total of only 5 CYP genes (CYP3A4/5, CYP2D6, CYP2C9, CYP2C19, and CYP1A2) contribute to 95% of all CYP-mediated metabolism of pharmaceuticals and other exogenous compounds (Guengerich, 2008). Although CYP-mediated metabolism is generally considered a detoxification mechanism, resulting in biologically inactive metabolites, the formation of highly reactive metabolites is possible, which may have increased pharmacological and/or toxicological activity by a process known as activation (Rowland et al., 2013).

**Phase II Metabolism**

In contrast to Phase I metabolism, Phase II metabolic enzymes are more functionally diverse and catalyze the covalent linkage of xenobiotic substrates with endogenous molecules that generally yield more polar, hydrophilic metabolites that are readily excreted (Jancova et al., 2010). Chemical sites on substrates that are targets for conjugation include electrophilic and/or nucleophilic functional groups such as carboxyls (-COOH), hydroxyls (-OH), aminos (-NH₂), and sulfhydryls (-SH) (Jancova et al., 2010). As such, the products of Phase I reactions commonly serve as further substrates for Phase II enzymes; however, conjugation reactions can occur on any hydrophobic compound harboring these functional groups. Typical Phase II conjugation reactions include sulfonation, glucuronidation, glutathione conjugation, methylation, acetylation, and amino acid conjugation; however, the former three are the most clinically significant enzymatic
pathways and together, represent more than 50% of all Phase II reactions of xenobiotics (Jancova et al., 2010).

Glucuronidation represents an important detoxification and elimination pathway for many xenobiotics. It is the most clinically significant of all Phase II reactions and approximately 40-70% of all drugs currently used in the clinic are subjected to glucuronide conjugation (Jancova et al., 2010). These reactions are catalyzed by members of a superfamily of enzymes known as UDP-glucuronosyltransferases (UGT), which facilitate the covalent linkage of the glucuronic acid to reactive nucleophiles (-OH, -COOH, -NH₂, -SH) located on substrates using the cofactor, UDP-glucuronic acid (Rowland et al., 2013). The resulting chemical conjugate is considerably more hydrophilic than its parent compound and therefore more readily excreted.

On the basis of gene similarity, four UGT enzyme families have been identified (UGT1, UGT2, UGT3, and UGT8) and comprise a total of 22 individual genes in humans (Mackenzie et al., 2005). The UGT1 and UGT2 families are the most significant in regard to xenobiotic metabolism and mediate the metabolism of pharmaceuticals across several drug classes including anticonvulsants, antivirals, and antipsychotics (Rowland et al., 2013). Additionally, glucuronidation serves as a significant route of metabolism for many endobiotics such as bilirubin, bile acids, fatty acids, and steroid hormones (Rowland et al., 2013). The liver contains the greatest diversity and absolute levels of UGT expression and therefore represents an important site for UGT-mediated xenobiotic metabolism (Ohno & Nakajin, 2009; Rowland et al., 2013). More importantly, the intracellular localization of UGTs on the inner membrane of the endoplasmic reticulum ideally positions them for
subsequent metabolism of CYP-catalyzed metabolites, facilitating the orchestrated elimination of exogenous and endogenous molecules. Together, these two xenobiotic metabolizing (CYPs and UGTs) pathways account for 90% of the clearance of all pharmaceuticals that are dependent on hepatic elimination (Rowland et al., 2013).

Sulfonation serves as another important Phase II xenobiotic metabolizing pathway for several small endogenous compounds and exogenous molecules such as drugs and environmental contaminants. Sulfonation reactions are mediated by a superfamily of enzymes known as the sulfotransferases (SULTs), which are ubiquitously expressed in tissues throughout the body, including the liver, kidney, lung, brain, and GI tract (Nowell & Falany, 2006). SULTs catalyze the conjugation of sulfonate (SO$\text{_{2}O^-}$), derived from the cofactor 3'-phosphoadenosine 5'-phosphosulfate (PAPS), to either hydroxyl (-OH) or amino (-NH$_2$) functional groups on substrates (Gamage et al., 2006). A total of 4 SULT families have been identified thus far (SULT1, SULT2, SULT4, SULT6), consisting of 13 individual gene members (Jancova et al., 2010). Moreover, SULTs are categorized into two broad classes based on their subcellular localization, which can either be cytosolic or membrane-bound in the Golgi apparatus of the cell (Gamage et al., 2006). The cytosolic SULTs represent a class with broad substrate specificity and are responsible for the metabolism of several endogenous molecules such as steroids, bile acids, neurotransmitters as well as pharmaceuticals and environmental contaminants (Jancova et al., 2010).

Glutathione (L-$\gamma$-glutamyl-L-cysteinylglycine; GSH) is the most abundant cellular tripeptide and is an important cellular antioxidant (Pastore et al., 2003). Intracellular GSH concentrations typically range in millimolar levels and is synthesized via the covalent
linkage of glutamate-cysteine-glycine. The sulfhydryl group within the cysteine residue serves as a potent nucleophile that is capable of binding to and neutralizing electrophiles, therefore functioning as an important first-line defense mechanism against cellular oxidative stressors (Pastore et al., 2003). Although GSH can react with electrophilic substrates non-enzymatically, GSH conjugation is commonly mediated by the glutathione $\delta$-transferases (GST); a large superfamily of enzymes present throughout the animal kingdom, including all mammals, insects, plants and certain microbes (Board & Menon, 2013).

A total of three families of human GSTs have been described: cytosolic, mitochondrial, and membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) (Wu & Dong, 2012). The cytosolic GSTs are further divided into seven classes (alpha, mu, pi, theta, omega, sigma, and zeta) and represent the largest and most diverse family. The GST enzymes are important in detoxifying electrophilic chemicals (drugs, endogenous oxidants, and environmental carcinogens) via the covalent linkage of these compounds with the sulfhydryl moiety of GSH (Wu & Dong, 2012). The liver retains the highest concentrations of the tripeptide cofactor, GSH, and therefore represents an important site for GST-mediated detoxification (Pastore et al., 2003).

**Membrane Transporters**

The biotransformation of endogenous and exogenous substrates by Phase I/II metabolizing enzymes represents an important elimination and detoxification mechanism. However, the entry and further excretion of these substrates into/out of cells depends on their passage across the hydrophobic microenvironment of the cell’s plasma membrane.
Mechanisms such as passive diffusion across these biological barriers may be sufficient; however, factors such as molecular size and hydrophilicity may limit the ability of compounds to successfully diffuse across these biological barriers. Moreover, the inherent nature of Phase I/II metabolic pathways to biotransform substrates into more hydrophilic, excretable products necessitates their assistance to move across lipophilic membrane barriers. In these circumstances, the cell uses specialized membrane-spanning proteins known as membrane transporters that facilitate the entry and exit of substrates in and out of cells. Transporters therefore represent an important class of proteins that are essential in mediating the absorption and excretion of endogenous and exogenous substrates. Depending on the mechanism by which they function, membrane transporters are divided into two primary superfamilies, the solute carrier (SLC) superfamily and the ATP binding cassette (ABC) superfamily (Klaassen CD, 2010).

The ATP-binding Cassette (ABC) Superfamily

The ABC superfamily of transporters represents one of the largest and oldest families of proteins with individual gene members found throughout all kingdoms of life (Rees et al., 2009). ABC gene members function as primary active transporters, which use the power generated by the hydrolysis of ATP to actively transport substrates, including sterols, nutrients, and xenobiotics across biological membranes, typically against steep concentration gradients (Rees et al., 2009). Eukaryotic ABC transporters primarily function as efflux pumps, and are important players in the cellular extrusion and ultimate excretion of xenobiotics and their metabolic products from the body (Klaassen & Aleksunes, 2010).
Structurally, all ABC transporter members contain, at a minimum, four protein domains that are characteristic of the superfamily: two transmembrane domains (TMD), which are localized within the membrane bilayer as well as two nucleotide binding domains (NBD) located within the cytosol (Rees et al., 2009; Jones & George, 2004). The NBD domains act as molecular motors, facilitating protein conformation changes via the binding and subsequent hydrolysis of ATP within highly conserved Walker A and B consensus motifs (Rees et al., 2009). In contrast, TMDs typically contain 6 membrane-spanning helices (12 total), forming a channel (TM channel) through the cell’s membrane, and are structurally and functionally diverse, which reflect the broad-substrate specificity that is typically seen across ABC family members (Jones & George, 2004).

Based on sequence homology, a total of 7 subfamilies of ABC transporters, arranged from ABCA to ABCG, have been characterized (Jones & George, 2004; Szakacs et al., 2008). The majority of members within these subfamilies function in maintaining normal physiological homeostasis, such as lipid trafficking (Albrecht & Viturro, 2007); however, several members, including the ABCC family, ABCB1, and ABCG2 are clinically important xenobiotic transporters that participate in the cellular and whole-body excretion of numerous, structurally unrelated pharmaceutical agents. (Klaassen & Aleksunes, 2010).

The ABCC subfamily, also known as the multidrug resistance-associated proteins (MRP), is arguably the largest and most clinically significant subfamily of ABC transporters (Klaassen & Aleksunes, 2010). Their name is derived from the original observation that overexpression of these transporters, particularly MRP1, is associated with
increased resistance to chemotherapeutic agents in several cancer cell lines (Hipfner et al., 1999). Nine ABCC gene members are identified thus far and four of them, ABCC1-4 (MRP1-4), have been extensively investigated in relation to their capacity to transport pharmaceutical agents and other xenobiotics. MRP1 transports a broad range of substrates including vinca alkaloids, glutathione and glutathione conjugates, and other chemotherapeutics (Klaassen & Aleksunes, 2010; DeGorter et al., 2012). In contrast, MRP2 and MRP3 primarily transport sulfonate and glucuronide conjugates, chemotherapeutics, and conjugated bile acids and share significant substrate overlap (DeGorter et al., 2012). MRP4 substrates include a number of endogenous signaling molecules such as cyclic nucleotides, bile acids, and eicosanoids, as well as antivirals and conjugated xenobiotics (DeGorter et al., 2012). Tissue expression of these transporter proteins is quite ubiquitous, but highly expressed in sites such as the liver, kidney, lung and GI tract (Klaassen & Aleksunes, 2010).

ABCB1, or P-glycoprotein (P-gp), was the first xenobiotic transporter identified by discovering that a population of Chinese hamster ovary cells was resistant to the effects of a wide-range of amphiphilic compounds (Juliano & Ling, 1976). Since the identification that these earlier observations were mediated by a membrane transporter, extensive investigations into its role in normal physiology and drug resistance have been undertaken. In general, P-gp functions in limiting cellular accumulation of xenobiotics and therefore plays a profound role in modulating toxicological and pharmacological responses (DeGorter et al., 2012). P-gp has broad substrate-specificity, which includes several hydrophobic and cationic drugs such as protease inhibitors, chemotherapeutics, calcium
channel blockers, and vinca alkyloids (Klaassen & Aleksunes, 2010). Expression of P-gp is high in the liver and kidneys as well as in the brain, where it functions in limiting exposure of potentially harmful compound to the sensitive brain tissue (Klaassen & Aleksunes, 2010).

Like P-gp, ABCG2, also known as breast cancer resistance protein (BCRP), is functionally important for transporting several therapeutic agents and toxicant including tyrosine kinase inhibitors, statins, irinotecan, etoposide, environmental contaminants and endogenous hormone molecules (DeGorter et al., 2012). In particular, BCRP and P-gp have important roles maintaining physiological tissue barrier function, such as the blood brain barrier, by facilitating the efflux of potentially harmful substances to protect tissues from xenobiotic and toxicant exposure (DeGorter et al., 2012).

**The Solute Carrier (SLC) Superfamily of Transporters**

The SLC superfamily of transporters comprises the largest family of transporters in the human genome, with at least 384 unique protein members identified (Rask-Andersen et al., 2013). Unlike the ABC family, which function as primary active transporters, members of the SLC superfamily are categorized as either facilitative transporters (transport substrates down an electrochemical or concentration gradient) or secondary active transporters, which allow the transport of substrates up their concentration and/or electrochemical gradient by coupling it with the transport of a secondary solute, usually an inorganic ion, down its gradient (electrochemical and/or concentration). SLC transporters are arranged into 52 distinct families and primarily function as uptake transporters, facilitating the entry of substrates and ions into cells (Rask-Andersen et al., 2013).
Moreover, the majority of SLC families harbor members that function in maintaining cellular and physiological homeostasis by transporting various endogenous macromolecules, inorganic ions, and nutrients, which include nucleotides, amino acids, fatty acids, neurotransmitters, and sugars (Hediger et al., 2004; Rask-Andersen et al., 2013). As a result of their importance in various physiological functions, several SLC members are molecular targets for pharmacological therapy by drugs classes such as antidepressants, diuretics, and antipsychotics (Rask-Andersen et al., 2013). In addition, members of two SLC families, the SLC22 and SLCO, serve clinically important roles in the disposition of several xenobiotics, namely pharmaceuticals (Koepsell, 2013).

The SLC22 family is a group of poly-specific transporters that share a predicted membrane topology consisting of 12 α-helical transmembrane domains. Members include the organic cation transporters (OCT) 1-3 (SLC22A1-3) and the organic anion transporters (OAT) 1-3 (SLC22A6-8) (Koepsell & Endou, 2004; Koepsell, 2013). The OCTs are considered uniporters and function by facilitative diffusion, transporting substrates into cells via the inward-facing electrochemical gradient (Koepsell & Endou, 2004). OCT1 and OCT2 mediate the first step in the hepatic and renal uptake of various cationic drugs, respectively, which include agents such as metformin and cimetidine (Koepsell, 2013; Klaassen & Aleksunes, 2010). In contrast, members of the OAT subgroup mediate the exchange of anionic compounds bidirectionally by coupling the transported substrates with the transport of small organic anions. OATs mediate the renal and hepatic uptake of several small anions such as antivirals, chemotherapeutic agents, and nonsteroidal anti-inflammatory drugs for ultimate excretion form the body (Koepsell, 2013).
In addition to the SLC22 family, the SLCO family, also known as the organic anion transporting polypeptide (OATP) family of transporters, have been extensively investigated for their role in the disposition of drugs as well as several endogenous compounds. Originally named as SLC21A family, the OATPs represent a large, multispecific, family of uptake transporters that are phylogenetically distinct from other members of the SLC superfamily. The exact mechanism driving OATP-mediated transport remains elusive, but it appears to be sodium and ATP-independent as well as involve the exchange of small intracellular molecules such as bicarbonate and glutathione (Roth et al., 2011). A total of 11 human OATP proteins are identified, categorized into 6 families; however, the OATP1 family, consisting of OATP1A2, OATP1B1, OATP1B2, and OATP1C1, represents the best characterized and studied family (Roth et al., 2011). Tissue-specific expression of individual OATP genes is ubiquitous; however, the most abundant is found in the liver (Klaassen & Aleksunes, 2010). OATPs are capable of transporting a variety of structurally unrelated compounds such as hormones, bile acids, and xenobiotics (Shitara et al., 2013). Specifically, the disposition and hepatic elimination of several members belonging to a class of drugs known as statins are dependent on OATP function and is important for statin-mediated pharmacological responses (Klaassen & Aleksunes, 2010).

Membrane Transporter Regulation

Membrane transporters are regulated by several mechanisms, which include transcriptional, post-transcriptional, chromosomal (epigenetic), translational (mRNA stability), and post-translational methods. In general, the focus has significantly leaned
toward transcriptional regulators such as nuclear receptors and transcription factors; however, post-translational modifications, such as phosphorylation and glycosylation, have become increasingly recognized as important functional determinants of membrane transporters.

**Transcriptional Regulation**

Transcriptional regulation of membrane transporters has been extensively investigated and currently remains an active area of research. Nuclear receptors, such as the constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are well established as major players in the transcriptional activation of membrane transporter and XME genes. Specifically, the activation of these nuclear receptors causes a coordinated response in the induction of XMEs and transporters that promote the metabolism and subsequent excretion of xenobiotics and endobiotics, respectively (Nakata et al., 2006). Moreover, emerging roles of transcription factors such as the nuclear factor erythroid 2-related factor 2 (Nrf2) in genomic regulation of transporter genes are developing, particularly in pathological conditions.

Both PXR and CAR belong to the nuclear receptor superfamily: a large family of proteins that are important in orchestrating intracellular signaling events that ultimately trigger the activation of gene transcription. Like other nuclear receptors, PXR and CAR function as ligand-activated transcription factors and belong to a subclass known as orphan receptors, which lack an identified endogenous ligand(s) (Tolson & Wang, 2010). Upon activation, PXR heterodimerizes with the retinoid X receptor (RXR) and translocates to the nucleus where it associates within genomic regulatory regions known as xenobiotic
response elements (XRE) located within the DNA promoter regions of target genes, activating their transcriptional machinery (Tolson & Wang, 2010). Due to PXR’s versatile and flexible ligand-binding domain, it can accommodate a variety of structurally diverse ligands, which include food additives, environmental toxicants, and xenobiotics such as drugs (Tolson & Wang, 2010; Chen et al., 2012). PXR-target genes include Phase I XMEs including CYPs, Phase II XMEs such as UGTs, and efflux membrane transporters such as MRP2 and P-gp (Nakata et al., 2006).

In contrast to PXR, CAR activation is more complex. As its name suggests, CAR is constitutively active and does not require ligand binding for full activation; however, several known xenobiotic ligands can bind to and activate CAR. Similar to PXR, CAR heterodimerizes with RXR and then enters the nucleus where it binds and transactivates CAR-inducible response elements (Tolson & Wang, 2010). CAR-target genes share considerable overlap with PXR genes and include Phase I/II XMEs, such as CYP isoforms, as well as membrane transporters, including MRP2, MRP3, MRP4, and P-gp (Assem et al., 2004; Kast et al., 2002; Tolson & Wang, 2010).

Together, CAR and PXR act as “xenosensors” that function in cytoprotective mechanisms by regulating the detoxification and excretion of compounds from the body. These receptors share considerable cross-talk and initiate a coordinated response by up-regulating Phase I/II XMEs and efflux transporters to facilitate the metabolism and subsequent excretion of compounds, respectively. Moreover, the structural diversity in ligand-recognition, which include anti-cancer compounds, herbal extracts, pesticides, environmental pollutants and several other classes of xenobiotics, allows for flexible, yet
global, responses in these cellular defense mechanisms. However, due to the global adaptations in XME and membrane transporter expression in response to CAR and PXR activation, these nuclear receptors are implicated in the development adverse drug reactions as a result of aberrant drug metabolism upon concomitant exposure to chemical activators (Tolson & Wang, 2010). Moreover, activation of these xenosensors, such as in the case of acetaminophen exposure, may result in heightened toxicity by creating a more reactive metabolite. CAR−/− mice are resistant to acetaminophen-induced hepatotoxicity, suggesting that, in special circumstances, CAR activation may potentiate toxicity (Zhang et al., 2002).

In addition to nuclear receptors, the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), has gained increased attention for its role in membrane transporter regulation. Nrf2 is the master regulator of the antioxidant response pathway and orchestrates the transcription of a battery of genes involved in the detoxification of oxidative insults such reactive oxygen species and electrophiles (Zhang, 2006). Nrf2-target genes include Phase I detoxification enzymes such as heme oxygenase 1 (HO-1) as well as Phase II enzymes, including NAD(P)H: quinone oxidoreductase (NQO1), GSTs, and the enzyme that catalyzes the rate-limiting step in glutathione synthesis, glutamate-cysteine ligase (GCL) (Zhang, 2006; Klaassen & Aleksunes, 2010). Therefore, the activation of Nrf2 is considered a cellular defense mechanism by coordinately activating the induction of cytoprotective genes.

Nrf2 is tightly regulated by modulating its cellular accumulation. Under basal conditions, Nrf2 is associated with the actin-binding protein, kelch-like ECL associating
protein 1 (Keap1), which acts as an adaptor protein for an E3 ubiquitin ligase complex, targeting Nrf2 for ubiquitin-mediated proteasomal degradation (Kobayashi et al., 2004). However, upon the induction of oxidative stress the interaction between Keap1 and Nrf2 is disrupted, leading to the cytosolic accumulation of Nrf2 and its subsequent translocation into the nucleus. In the nucleus, Nrf2 heterodimerizes with small musculo-aponeurotic fibrosarcoma (Maf) proteins and binds to regulatory regions of target genes known as antioxidant response elements (ARE) (Klaassen & Aleksunes, 2010).

In addition to Phase I/II metabolizing enzymes, several lines of evidence suggest that xenobiotic membrane transporter genes are also molecular targets of Nrf2-dependent transcriptional activation. By using genetically altered experimental animals and Nrf2 chemical activators, it was demonstrated that Nrf2 activation induces the expression of efflux transporters, most notably Mrp2, 3 and 4 (Maher et al., 2005). In line with previous observations of CAR and PXR-dependent regulation of drug metabolism and disposition, these findings suggest that Nfr2 also contributes to the coordinated regulation of both Phase I/II and membrane transporters. However, current knowledge on the precise molecular mechanisms mediating the Nrf2-dependent induction of membrane transporters is lacking. Maher et al. has previously identified a functional ARE within the mouse promoter region that may be responsible for the Nrf2-dependent induction of Mrp3 in rodents (Maher et al., 2007); however, the identification of a functionally active ARE within the human MRP3 (ABCC3) gene is still ongoing, despite clear evidence of Nrf2-dependent regulation (Adachi et al., 2007; Sasaki et al., 2012).

Post-translational Regulation
Post-translational modifications of transporters is an area of active investigation. In contrast to transcriptional regulation, post-translational regulation involves the modulation of transporter function without altering its rate of transcription. This is accomplished primarily by phosphorylating and/or glycosylating the transporter proteins directly, which may affect their function and/or subcellular trafficking.

Transporter phosphorylation has been shown to alter the function of several proteins, particularly members of the OAT and OCT families; however, efflux transporters belonging to the ABC superfamily are also subject to phosphorylation events. Specifically, the activation of protein kinase C (PKC) was shown to decrease OAT3-mediated transport via protein kinase C-dependent internalization of OAT isoforms into the cytosol (Soodvilai et al., 2004; Wolff et al., 2003). In contrast, PKC and protein kinase A (PKA) activation stimulate OCT2 and OCT1 transport function, suggesting that PKA and PKC-dependent phosphorylation may have differential effects on transport function (Mehrens et al., 2000; Wilde et al., 2009). More recently, it was shown that PKC activity alters MRP2 function by regulating its internalization from the plasma membrane in an oxidative stress-dependent manner (Ito et al., 2005; Sekine et al., 2011).

Like phosphorylation, glycosylation is also important in regulating the activity of membrane transporters. OCT and OAT isoforms are extensively glycosylated and their function in response to altered glycosylation status has been previously reviewed. OCT2 function decreases in response to mutating critical asparagine residues that serve as glycosylation sites, resulting in both reduced sub-cellular targeting of the transporter to the plasma membrane and increased transporter turnover (Pelis et al., 2006). Moreover,
mutating a critical glycosylation site within the OAT1 reduces functionality without altering membrane localization (Tanaka et al., 2004). Interestingly, the same amino acid residue is not glycosylated in mouse Oat1; however, mutating it causes a similar decrease in Oat1 function, implicating that glycosylation may be important in substrate recognition as well as membrane trafficking.

**The Liver- Physiology and Role in Xenobiotic Metabolism and Disposition**

Only second to the skin, the liver is the largest organ in the human body, representing up to 5% of the total body weight (McCuskey, 2008). The liver is critical in a diverse array of functions that range from maintaining whole-body energy homeostasis to participating in the synthesis and excretion of bile acids into the gut for facilitating dietary fat absorption. In addition, the liver’s strategic positioning in the body allows it to act as a first-line of defense by receiving all of the portal blood supply, making it crucially important in protecting the body from exposure to gut-derived endotoxins, microbes, and other particulate matter that include environmental contaminants, food stuffs, and xenobiotics.

**Liver Anatomy**

*Architecture and Blood Supply*

The liver’s unique architecture, anatomical position, and microvasculature allows it to efficiently carry out its roles as a biological filter. Receiving 25% of the total cardiac output, the liver collects blood from two sources; 80% of which is derived from low oxygenated, nutrient rich blood from the portal tract (portal vein) and the remaining 20% from well oxygenated arterial blood (hepatic artery) (McCuskey, 2008; Eipel et al., 2010).
Branches of the hepatic artery and portal vein coarse through the tissue in parallel and continue branching to form terminal microvascular vessels known as sinusoids, where both venous and arterial blood mix. Sinusoids represent the principal site in which the exchange of solutes and other compounds occur with the tissue’s parenchymal cells (McCuskey, 2008). Portal and arterial blood within the sinusoids flow to central venules, which in turn branch into central veins that ultimately collect into the vena cava for systemic circulation.

**Cellular Components of the Liver**

Sinusoids contain three principal cell populations: fenestrated endothelial cells, Kupffer cells and stellate cells. The endothelial cells form the sinusoidal lining and have natural fenestrations to allow for the passive exchange of solutes between the blood and parenchymal cells (McCuskey, 2008). Similarly, Kupffer cells also take up residence on the sinusoidal wall; however, function as resident phagocytic macrophages that are important in clearing foreign matter, particularly endotoxins and other microbe-derived compounds. Hepatic stellate cells reside in the space of Disse, a region defined between endothelial cells and parenchymal cells, and are important in storing lipids as well as initiating tissue repair mechanisms that include fibrogenic responses (McCuskey, 2008).

The liver parenchymal tissue represents approximately 80% of the total volume and consists of cells known as hepatocytes (McCuskey, 2008). Hepatocytes form an epithelium that is one cell thick and is the principal site in which the vast majority of liver-dependent processes occur. Moreover, to facilitate many of its functions, hepatocytes form a functional barrier, separating two fluid filled compartments, which are the sinusoids and the bile canaliculi (McCuskey, 2008). These small tubular-like structures form between
two adjacent hepatocytes and collect secreted bile acids and bile constituents synthesized and/or secreted within hepatocytes. The direction of bile flow in bile canaliculi is anti-parallel to sinusoidal blood flow, flowing toward the branching venules and arterioles, collecting into a branch of the hepatic bile duct, which joins the common bile duct that is then merged into the duodenum. Because of their unique position between two compartments, hepatocytes are polarized, with the apical face oriented towards the bile canaliculus and the basolateral membrane facing the sinusoid (McCuskey, 2008). Like any polarized epithelia, the apical and basolateral domains of hepatocytes are physiologically and biochemically distinct from one another by harboring specific membrane transporters that facilitate the vectorial movement of endogenous and exogenous compounds from the blood (sinusoid) to the bile (canaliculus).

The Hepatic Lobule and Hepatic Acinus

The unique structural architecture of the liver is traditionally described by two organizational units known as the hepatic lobule and acinus (Figure 1.1). Also regarded as the functional unit of the liver, the lobule is organized as a hexagonal structure with its central axis consisting of the hepatic venule and its periphery containing branches of the hepatic artery, portal vein and hepatic bile duct, collectively known as the portal triad (McCuskey, 2008). Therefore, the hepatic lobule represents all hepatocytes, drained by a single central venule.

In contrast to the lobule, the hepatic acinus does not contain distinct morphological boundaries. It’s an irregularly shaped structure formed with its central axis lining two portal tracts and its secondary axis joining two central venules of adjacent lobules. The acinus
can be further divided into three zones, which is based on the level of oxygenation of the
blood being filtered through that region (Figure 1.1). Zone 1 consists of hepatocytes near
the periportal region and encounter the highest oxygen supply whereas hepatocytes in the
intermediate (zone 2) and distal (zone 3) regions receive the lowest. As a consequence of
these oxygen gradients within the lobule, hepatocytes across all three zones differ quite
dramatically in their metabolic function and sensitivity to chemical stressors (McCuskey,
2008).
Figure 1.1: Hepatic Lobule and Acinus. The hepatic lobule is a polygonal structure that represents the functional unit of the liver. The lobule is bordered by portal tracts (PT) around its periphery, which contain a branch of the hepatic artery, portal vein and bile duct. Blood (red arrow) flows from the portal tract, through sinusoids (S) and collects in a central venule (CV). Bile (green arrow) is produced in hepatocytes and is secreted into bile canaliculi, formed between two adjacent hepatocytes and flows to the branch of the bile duct in a portal tract. The acinus contains its axis around portal tracts of two adjacent lobules. This creates zonal distribution of hepatocytes within the lobule of oxygen level they are exposed to. Zone 1 is closest to the portal tract and receives the most oxygen; zone III is furthest from the portal tract and receives the least amount of oxygen.
The Role of the Liver in Xenobiotic Metabolism and Disposition

The liver is the principal site of metabolism within the body and represents an important excretory route, via the bile, for many endogenous and exogenous compounds. Both Phase I and Phase II metabolic enzymes that are important for xenobiotic metabolism are most abundant in the liver. Moreover, the liver’s anatomical position between the portal and systemic blood supply, coupled to its high perfusion rate, creates a defensive barrier by detoxifying and eliminating potentially harmful compounds that would otherwise be exposed systemically. This, of course, functions as a protective barrier, but can also be a clinical burden with pharmaceuticals that undergo extensive first pass metabolism. This is a result of substrates that are absorbed from the GI tract and are extensively metabolized and subsequently eliminated prior to reaching systemic circulation (Zanger & Schwab, 2013).

The management of xenobiotics by the liver can be generalized into 4 functional mechanisms (Figure 1.2). The first stage is uptake of the compound into hepatocytes. This is primarily mediated by uptake transporters belonging to the OATP family, but OCT1 and OAT2 are also clinically important (Klaassen & Aleksunes, 2010). The second step is metabolism by Phase I/II XMEs, resulting in the formation of usually inactive, polar metabolites that have two potential routes of excretion: biliary efflux (step 3) into the bile and/or sinusoidal efflux (step 4) into the blood. Efflux transporters localized to the apical membrane (MRP2, P-gp, and BCRP) and basolateral membrane (MRP3, MRP4) facilitate the biliary and sinusoidal efflux, respectively. Importantly, basal MRP3 and MRP4 expression is lower in the liver and therefore biliary efflux represents the primary route of
excretion; however, during times of hepatic stress, excretion into sinusoidal blood provides an alternate route of efflux via the induction of MRP3 and MRP4 (Mennone et al., 2006; Keppler, 2014). It is important to note that these four mechanisms are mutually exclusive and are not dependent on each other for proper function (i.e. metabolism doesn’t have to occur prior to transporter-mediated efflux).
Figure 1.2: Mechanisms Mediating Liver Disposition. Four mechanisms that mediate the disposition of xenobiotics in the liver. Uptake occurs from the blood within the sinusoid (1). Metabolism within the hepatocyte (2) followed by biliary efflux (MRP2, BCRP, P-gp) into the bile canaliculus (3) or sinusoidal efflux (MRP1, MRP3, MRP4) into the blood (4)
Inter-Individual Variability in Xenobiotic Response

The cellular and molecular mechanisms that mediate the ADME of xenobiotics govern the ultimate fate of a compound’s disposition within the body. Their proper function is critical for effectively distributing a pharmaceutical or toxicant to its molecular site of action, therefore ultimately facilitating its pharmacological and/or toxicological effects. Humans display a large degree of variability in their biological responses following exposure to toxicants, pharmaceuticals, pollutants, and other xenobiotics. Importantly, these inter-individual differences in drug responses may lead to unwanted biological side effects, also known as adverse drug reactions (ADR), which have become a significant clinical burden over the recent years. Several factors contribute to inter-individual variability in drug responses, the most notable of which include genetic polymorphisms within genes that regulate ADME processes as well as the manifestation of inherent disease states that may ultimately alter normal physiological function.

Adverse Drug Reactions: Prevalence and Sources

An adverse drug reaction (ADR), defined by the World Health Organization, is “any response to a drug which is noxious and unintended, and which occurs at doses normally used in humans for prophylaxis, diagnosis, or therapy of disease, or for the modification of physiological function.” (Nebeker et al., 2004). Over the years, our society has had an increase in the development and dependence on pharmacological therapy, which has led to a rise in the incidence of ADRs. These events have become an increasing cause of morbidity and mortality across the world and in particular, within the United States alone, ADRs are one of the top ten causes of death, accounting for more than 700,000
hospitalizations and 100,000 deaths annually (Bond & Raehl, 2006; Howard et al., 2007; Wooten, 2010b). Moreover, in addition to their detrimental effects on public health, ADRs significantly burden the economy by being responsible for up to $4 billion in annual healthcare costs (Howard et al., 2007).

Causes contributing to the development of ADRs vary and can be attributed to factors such as patient compliance, medication errors, and/or idiosyncratic reactions to the medication itself. Importantly, it is estimated that up to 70% of ADR cases can be preventable with the utilization of improved medication administration and diligent pharmacovigilant practices within the healthcare industry (Bond & Raehl, 2006; Wooten, 2010b; Sultana et al., 2013). However, given the enumerable variables and mechanisms that mediate ADME, inherent factors in an individual’s ability to properly metabolize and/or excrete pharmaceuticals represent a greater challenge to predict. Specifically, genetic variability, in the form of gene polymorphisms, within XME and membrane transporter genes as well as the manifestation of disease are recognized as significant contributors to the development of variable pharmacokinetic and pharmacodynamics responses. Together, these genetic and environmental/physiological factors cause extensive functional variation in XMEs and membrane transporters, which affect the ADME of xenobiotics.

*Genetic Polymorphisms in XMEs and Membrane Transporters*

By recognizing the significance of pharmacogenetics in clinical practice, the FDA has incorporated warnings on over 100 drug labels that inform health care practitioners of specific genotype-dependent interactions in drug response (Wei et al., 2012). Genetic
variation in gene expression and ultimately protein function can result from single-nucleotide polymorphisms (SNPs), insertions, deletions, and duplications (Ma & Lu, 2011). SNPs tend to be the most common polymorphisms and to date, nearly 400 unique alleles are characterized across members of the CYP1-3 families (Sim et al., 2013). Moreover, the individual genes encoding for CYP3A4, CYP2D6, CYP2C9, CYP2C19, and CYP1A2, which account for 95% of the CYP-dependent metabolism of xenobiotics, are polymorphic (Zanger & Schwab, 2013). More importantly, the frequency by which these alleles are present across distinct racial populations can vary extensively. CYP2D6, for example, is highly polymorphic and the frequency of reduced and non-functional alleles within Black and Asian populations can be as high as 35% (Bradford, 2002). Additionally, the CYP2D6*10 allele has a frequency of 51% in Chinese populations and homozygous carriers of this allele had measurable reductions in the clearance of tramadol (Xu et al., 2014). In contrast to loss of function alleles, some allelic variants lead to increased CYP enzymatic activity, resulting in a spectrum of metabolic phenotypes that range from poor to ultra-metabolizers (Bradford, 2002; Neafsey et al., 2009). Like CYP2D6, CYP2C19 is also highly polymorphic and individuals harboring the CYP2C19*2 allele, which creates a splicing defect resulting in the null-expression of the CYP2C19 gene, have decreased anti-coagulation responses to the drug, clopidogrel, and are associated with an increased risk in developing serious adverse cardiovascular events (Sofi et al., 2011; Jang et al., 2012; Bauer et al., 2011; Holmes et al., 2011). These findings have led to the inclusion of a Black Box warning for clopidogrel, informing health care professionals of the decreased

In contrast to CYPs, genetic variability within Phase II xenobiotic conjugation enzymes is not as prevalent and their clinical significance is not as well established. Genetic polymorphisms have been identified in several UGT isoforms, including UGT1A1, 1A3, 1A4, 2B7, and 2B17; however, with the exception of UGT1A1, unequivocal evidence that demonstrates the clinical relevance of these polymorphisms in xenobiotic metabolism is lacking (Rowland et al., 2013). UGT1A1 is important for the conjugation of bilirubin and polymorphisms within this gene are associated with decreased UGT1 function that leads to conditions known as Gilbert’s and Crigler-Najjar syndromes (Rowland et al., 2013). Genetic variability in the SULTs and GST superfamilies are rare and although some genes are polymorphic (SULT1A2, GST-M1), interest has been primarily focused on their roles in the pathogenesis of several cancers rather than xenobiotic metabolism (Jancova et al., 2010; Nowell & Falany, 2006).

Much of the attention in characterizing inter-individual variability in xenobiotic disposition has been within genes encoding XMEs; however, genetic variability within membrane transporter genes has become increasingly appreciated and is quickly being recognized as a factor contributing to the development of ADRs. Several genetic polymorphisms have been identified in both the ABC and SLC superfamilies. Recently, it was shown that an ABCC2 polymorphism (-24C>T) was highly associated with an increased risk of developing methotrexate toxicity in pediatric patients receiving high-dose treatment for acute lymphoblastic leukemia (Liu et al., 2014). Moreover, these observations
correlate with higher plasma retention of the drug. Decreased ABCC2 function as a result of certain genetic polymorphisms is also associated with the development of Dubin-Johnson syndrome, a form of conjugated hyperbilirubinemia resulting from decreased bilirubin excretion into the bile (Devgun et al., 2012). In addition to ABCC2, a recent study has shown a 3 fold increase in plasma diflomotecan levels as a result of a loss of function polymorphism within the ABCG2 gene (Sparreboom et al., 2004). P-gp is also found to be polymorphic, but its clinical significance is still under investigation (Dessilly et al., 2014).

Genetic polymorphisms in SLC transporters, most notably the OATP family, are also a significant cause for inter-patient variability in drug responses with emerging evidence linking OATP polymorphisms with drug efficacy and toxicity. In particular, OATP1B1 is expressed exclusively in the liver and contributes to the hepatic uptake of drugs such as antibiotics, anti-cancer agents, and statins (Gong & Kim, 2013). A total of 14 non-synonymous SNPs are identified within the coding regions of OATP1B1, many of which have been shown to alter protein function (Gong & Kim, 2013). By far the most intensely studied class of drugs in respect to OATP1B1 genetic variability are the statins. Several functional OATP1B1 allelic variants are associated with altered statin disposition. For example, loss of function OATP1B1 polymorphisms are associated with decreased hepatic clearance of pravastatin leading to nearly a 2.3-fold increase in its plasma area under the curve (AUC) (Nishizato et al., 2003; Niemi et al., 2004). Moreover, genome-wide association studies have also found a correlative link between OATP1B1 polymorphisms and the risk for developing stain-induced myopathy (Gong & Kim, 2013).
Human disease vary significantly in their complexity as well as severity and elicit a large spectrum of effects on the basal expression and regulation of genetic information. This, in turn, may have detrimental consequences to routine physiological functions, which can ultimately lead to increased morbidity and/or mortality. In particular, the manifestation of diseases in tissue sites that are important in mediating ADME processes may increase the likelihood of developing ADRs and contribute to inter-individual variability in xenobiotic responses. Given the common practice of treating disease with pharmacological intervention, a more thorough understanding of disease-mediated contributions to drug response and/or toxicity is necessary.

Altered liver function, as a result of underlying disease, is of particular concern to the health care community given the important role of this site in mediating xenobiotic metabolism and excretion. Several cases from the literature clearly highlight the impact of hepatic impairment on the pharmacokinetics of drugs, which ultimately lead to variable systemic exposure, affecting drug efficacy and/or toxicity (Eley et al., 2014; Weil et al., 2010; Tortorici et al., 2011). Until recently, there is increased awareness and appreciation of the link between liver-related disorders and dysregulation of XMEs and membrane transporters.

Cholestasis is a condition characterized by decreased or impaired bile flow, which results in accumulation of bile acids (BA) and other biliary constituents within the liver and causing toxicity. The etiology can be due to two sources: physical obstruction of the common bile duct (gallstones), termed extra-hepatic cholestasis, or the development of congenital and/or autoimmune disorders such as primary biliary cirrhosis (intra-hepatic
cholestasis). In response to increased hepatic BA levels, several molecular and physiological adaptations occur to further limit intra-hepatic exposure to toxic BA levels. Such molecular adaptations include the repression of hepatic BA synthesis via down-regulating CYP7A1 expression, decreasing the expression of BA acid uptake transporters and the induction of hepatic efflux transporters such as MRP3, MRP4, and MRP2, which help facilitate the removal of intracellular BAs (Chai et al., 2012; Gartung et al., 1996; Zollner et al., 2001; Slitt et al., 2007; Dumont et al., 1997). Although these adaptive changes in membrane transporter and biotransformation enzyme expression are protective by limiting BA-induced liver injury, they also cause disturbances in the pharmacokinetic fate of drugs and other xenobiotics.

Clinical evidence of cholestasis affecting the ADME and/or the pharmacological effectiveness of drugs is lacking; however, several investigations using rodent models have shown a link between cholestatic disease and altered drug PK. Specifically, intra and extra-haptic cholestasis in rodents cause decreased biliary efflux of methotrexate coupled with a concomitant increase in its plasma levels, which was attributed to altered function of hepatic uptake and efflux transporter expression (Bračkova et al., 2009). In addition, Hasegawa et al. demonstrated that morphine administration to bile duct ligated rats, a common extra-haptic cholestasis model, causes a significant increase in systemic levels of the glucuronidated morphine metabolite, morphine-3-glucuronide (M3G). These findings are presumably due to an induction of hepatic Mrp3 and Ugt2b1 protein expression, resulting in increased efflux of M3G into systemic blood and increased morphine metabolism, respectively (Hasegawa et al., 2009). More recently, Jin et al.
demonstrated reduced hepatic uptake of the oral anti-diabetic, metformin, in an ethynlyestradiol (EE)-induced rat model of cholestasis (Jin et al., 2009). This observed decrease in metformin uptake in the liver resulted in diminished metformin-stimulated glucose utilization, demonstrating altered therapeutic responsiveness to metformin as a result of altered disposition. In addition to membrane transporter dysregulation, altered expression and function of CYPs in cholestatic rats results in a 22% decrease in the metabolism of doxorubicin (Fukushima et al., 2008; Hasegawa et al., 2009; Choi et al., 2013). Together, these findings suggest that liver dysfunction may result in altered xenobiotic PK through the differential regulation of hepatic membrane transporters and XMEs. These findings have since fueled novel investigations into the effects of other disease conditions, such as nonalcoholic fatty liver disease, on xenobiotic PK.

Nonalcoholic Fatty Liver Disease: Prevalence and Causes

The metabolic syndrome encompasses a gamut of clinical morbidities that include obesity, hypercholesterolemia, hypertension, and type II diabetes. The prevalence of metabolic disorders is growing, and in the United States alone, an estimated 25% of adults suffer symptoms associated with the metabolic syndrome (Falkner & Cossrow, 2014). Moreover, symptoms of the metabolic syndrome predispose individuals to more chronic illnesses such as nonalcoholic liver disease (NAFLD). Often regarded as the hepatic manifestation of the metabolic syndrome, NAFLD is the most common form of chronic liver disease in Western society and is highly associated with conditions such as obesity and type II diabetes (Lomonaco et al., 2013). Current prevalence rates for NAFLD are estimated to be within 6-33% worldwide and can be as high as 50% in certain regions and
NAFLD pathogenesis is multi-faceted and originates as simple fatty liver, termed steatosis, which can serve as the primer in the development of the more severe form known as non-alcoholic steatohepatitis (NASH) (Marra et al., 2008). NASH is characterized by an increase in hepatic inflammation, oxidative stress, and varying degrees of fibrosis (Marra et al., 2008; Kleiner et al., 2005). Limitations in accurate, non-invasive diagnostic measures, makes it difficult to accurately determine the prevalence of NASH; however, it is predicted to be as high as 12.2% in the general population, and as high as 37% in morbidly obese individuals (Rahimi & Landaverde, 2013). More concerning, NASH may progress to end-stage liver disease such as cirrhosis and was found to be the most common etiologic risk factor in developing hepatocellular carcinoma (Rahimi & Landaverde, 2013).

Approximately 23%-40% of patients with NAFLD progress to developing NASH; however, the mechanisms mediating the transition from simple fatty liver to NASH is not entirely clear (Rahimi & Landaverde, 2013). It is generally well recognized that following the development of hepatic steatosis NASH pathogenesis involves several parallel “hits” that may act independently or in concert to drive disease progression (Lall et al., 2008; Rahimi & Landaverde, 2013). Specifically, the manifestation of insulin resistance within adipose tissue appears to be a central feature that is believed to be the primary mechanism in developing simple fatty liver via increased free-fatty acid flux to hepatocytes from fat-laden adipose tissue (Rahimi & Landaverde, 2013). This “lipotoxic” environment in the liver results in the induction of oxidative stress pathways, pro-inflammatory cytokines and mitochondrial dysfunction, which are thought to be key mediators in NASH pathogenesis.
(Rahimi & Landaverde, 2013; Ali & Cusi, 2009). These physiological changes that occur in NASH lead to extensive dysregulation of hepatic gene expression profiles, which may have significant implications on the normal liver function, including xenobiotic metabolism and disposition (Moylan et al., 2014).

**Effects of NASH on Xenobiotic Metabolism and Disposition**

Over the past several years, research into the identification and understanding of how NASH affects xenobiotic disposition has grown quite considerably. The Cherrington lab, in particular, has been at the forefront of these investigations and is the pioneer in identifying clinically relevant adaptations in membrane transporter and XME regulation throughout the progressive stages of the disease. Early findings demonstrate that the development of NASH causes a coordinated up-regulation of hepatic efflux transporters and a global down-regulation of uptake transporters (Lake et al., 2011; Hardwick et al., 2011; Fisher et al., 2009a; Clarke et al., 2014a). It is interesting to note that these initial observations are similar to what occur in cholestatic liver diseases and may represent a protective mechanism in the liver by limiting its exposure to toxicants and endogenous waste products when in a stressed state.

These studies have since initiated investigations into the functional consequences of NASH on xenobiotic disposition. In particular, acetaminophen disposition and its metabolite, acetaminophen-glucuronide, are altered in an experimental model of NASH. Specifically, a shift from biliary excretion to plasma retention of acetaminophen-glucuronide was documented, which was shown to be due to the up-regulation of the basolateral efflux transporters, Mrp3 and Mrp4, in the liver (Lickteig et al., 2007a). These
same findings were then confirmed in humans by using a cohort of pediatric patients diagnosed with NAFLD, who were found to have a higher systemic burden of acetaminophen-glucuronide following a single dose of acetaminophen (Barshop et al., 2011). Similar observations were also noted with the cholesterol-uptake inhibitor, ezetimibe, which serves as a substrate for MRP4. Specifically, plasma levels of ezetimibe-glucuronide were increased whereas biliary efflux decreased (Hardwick et al., 2012). This functional shift from biliary to plasma excretion was found to be due to 1) Induction of Mrp4 at the basolateral membrane of hepatocytes and 2) Decreased trafficking of Mrp2 to the canalicular membrane, which appeared to be selectively disrupted in NASH (Hardwick et al., 2012). Although the glucuronidated metabolites of both acetaminophen and ezetimibe lack toxicological activity, the increase in plasma levels of potentially toxic metabolites is particularly concerning.

NASH also leads to decreased expression of the hepatic uptake transporters, Oatps, leading to increased simvastatin exposure (Clarke et al., 2014a). Moreover, the combination of NASH and genetic loss of OATP function has a synergistic effect on pravastatin excretion, suggesting the potential for environmental and genetic interactions (Clarke et al., 2014b). In addition to transporters, the function and activity of hepatic Phase I enzymes, such as CYPs, and the Phase II UGT and SULT conjugation enzymes is altered in human and animal NASH models (Hardwick et al., 2013; Patoine et al., 2013; Fisher et al., 2008). Taken together, these findings implicate a clear role in NASH disrupting the functional regulation of hepatic Phase I/II XMEs and in particular, membrane transporters; however, knowledge regarding the effects of NASH on extra-hepatic sites such as the
kidsneys is lacki

Like the liver, the kidneys serve as an important site of excretion for endogenous and exogenous compounds.

*Extra-Hepatic Regulation of Xenobiotic Disposition: Role of the Kidneys*

The kidneys play an essential role in maintaining whole-body homeostasis by regulating fluid status, electrolyte balance, and acid-base balance in addition to producing or activating several hormones that regulate blood pressure and blood flow. The kidneys also serve as specialized filters by removing exogenous and endogenous waste products from the blood that are to be excreted into the urine (Morrissey et al., 2013). To accomplish this task, the kidneys are composed of approximately 1 million individual functional units known as nephrons (Morrissey et al., 2013). A nephron is divided into two functional units: the glomerulus and the tubule, which is further segmented into three distinct regions known as the proximal tubule, the loop of Henle, and the distal tubule.

The glomerulus acts as a filtration mechanism and consists of a specialized cluster of blood vessels harboring a fenestrated epithelium, which allow for the passive filtration of solutes and other small molecules into the lumen of tubule, forming a filtrate. The filtrate passes along each functional segment of the tubule and is acted upon by the epithelial cells that line the lumen, prior to collection into the collecting ducts for excretion as urine. One of the primary functions of this epithelium is to recover vital nutrients and ions that were freely filtered, such as glucose, amino acids, and small inorganic ions, in a process known as re-absorption (Morrissey et al., 2013). Likewise, these cells also mediate the active secretion of compounds, including xenobiotics, via the vectoral transport of substrates from the peritubular capillaries into the tubule lumen for urinary excretion. Both reabsorption
and secretion mechanisms are mediated by membrane transporters, whereas filtration within the glomerulus is a passive process largely dictated by the molecular size of the solute. Together, the sum of the effects of glomerular filtration, secretion, and reabsorption dictate the renal clearance of a particular compound (Morrissey et al., 2013).

The kidneys serve as an important excretory route for exogenous compounds, such as drugs. In 2010, 200 of the top-prescribed drugs were excreted, unchanged, by renal mechanisms (Morrissey et al., 2013). Moreover, the epithelial cells lining the tubules express an abundance of membrane transporters that include members from both the ABC and SLC superfamilies. These transporters, like the liver, are uniquely expressed on both the basolateral (capillary side) and apical (tubule lumen side) membranes of tubular epithelial cells to facilitate the active secretion of xenobiotics into the tubule lumen (Morrissey et al., 2013) (Figure 1.3). In general, efflux transporters belonging to the ABC superfamily are primarily expressed on the apical membrane of tubular cells and function in extruding compounds into the filtrate whereas SLC transporters are primarily expressed on the basolateral membrane, with the exception of OATP1A1, which is localized to the apical membrane (Figure 1.3).
**Figure 1.3: Renal Membrane Transporter Orientation.** Basolateral uptake transporters (OCT1, OCT2, OAT1, and OAT2) and apical efflux transporters (P-gp, BCRP, MRP4, and MRP2) facilitate the active secretion of substrates from the blood into the filtrate formed in the nephron. OATP1A1, in contrast, is an uptake transporter localized to the apical membrane and promotes reabsorption.
Although direct kidney injury and/or insufficiency may result in deficiencies in the renal excretion of xenobiotics, it was demonstrated that hepatic impairment may lead to adaptive responses in renal membrane transporter expression and function. In cholestasis, for example, extra-hepatic regulation of renal membrane transporters is well documented and is hypothesized to function as a mechanism that helps reduce the overall BA load by promoting the active secretion of BAs into urine in response to hepatic dysfunction. In general, the efflux transporters, Mrp1-5, are induced in the kidney along with the up-regulation of the basolateral uptake transporter, Oat3 (Slitt et al., 2007; Chen et al., 2008). Moreover, bile-duct ligation in rats causes an induction of the renal-specific organic cation transporter, Oct2, resulting in a functional increase in the renal secretion of cimetidine (Kurata et al., 2010). Disturbances in the renal secretion of p-aminohippurate and bromosulphophthalein have also been reported, which were linked to disrupted Oat membrane transporter expression and function in the kidney (Brandoni et al., 2006a; Brandoni & Torres, 2009).

The mechanisms that govern these adaptive changes in renal drug transport expression in cholestasis are currently under investigation and more information regarding compensatory alterations in renal transporter expression in humans with cholestatic liver disease is still needed. Taken together, however, these observations suggest that during times of hepatic stress, an overall increase in the renal secretion of xenobiotics occurs, which may act as an adaptive response to facilitate the secretion of xenobiotics and endogenous waste products when the liver injury occurs. The effects of hepatic dysfunction
on kidney transporter regulation in NASH are currently unknown and form the basis for the investigations proposed in the present study.

**Present Study**

Inter-individual variability in responses to xenobiotics, including drugs, is a major cause for morbidity and mortality across the world (Bond & Raehl, 2006). The research conducted in the Cherrington Laboratory has established that a disease known as nonalcoholic fatty liver disease (NAFLD) is a significant source in contributing to inter-individual responses in xenobiotic exposure, increasing the likelihood in developing adverse reactions. NAFLD is the most common form of liver disease in Western society and represents a spectrum of liver conditions that range from the benign simple fatty liver, to the more advanced inflammatory condition known as nonalcoholic steatohepatitis (NASH). Moreover, NASH is highly associated with symptoms of the metabolic syndrome and as such, its prevalence is only expected to increase in the near future given the increasing trend in obesity and type 2 diabetes (Ali & Cusi, 2009). Novel investigations into human and rodent models of NASH have revealed liver-specific alterations in the regulation of xenobiotic membrane transporters, leading to functional disturbances in pharmaceuticals such as acetaminophen, ezetimibe, and simvastatin (Lickteig et al., 2007a; Hardwick et al., 2012; Clarke et al., 2014a). However, further understanding of whether NASH disrupts the regulation of xenobiotic transporters in extra-hepatic sites, such as the kidney, is lacking. Previous investigations using rodent models of cholestasis reveal a specific, coordinated response in kidney transporter regulation, leading to a disruption of xenobiotic secretion in the kidney (Ikemura et al., 2013; Brandoni et al., 2006a).
Specifically, these adaptations in kidney transporter function may facilitate bile acid excretion in the kidneys during times of hepatic stress. In addition to cholestasis, a nearly 6-fold and 3-fold mRNA induction of Mrp4 and Mrp2, respectively, was observed in obese, leptin-deficient mice (Cheng et al., 2008). These findings suggest that metabolic disease may also have the capacity to alter renal transporter expression and potentially function. Given the close association of NAFLD with the metabolic syndrome, *I hypothesize that the manifestation of NASH leads to coordinated, adaptive alterations in hepatic and renal xenobiotic membrane transporter regulation that ultimately affect the disposition of xenobiotics.*

The following aims were designed to test this hypothesis:

*Aim 1 (Chapter 1) - Experimentally Profile Hepatic Membrane Transporter Expression Across Several Experimental Rodent Model(s) of NASH.*

Using several, commonly used rodent models of NASH, hepatic gene and protein expression of membrane transporters are analyzed and characterized to determine the coordinated regulation of transporters in the disease. Moreover, these results are statistically compared and cross-referenced to data derived from human NASH, which provides experimentally relevant, working models that are consistent with known hepatic expression profiles in the human disease. Given the complete lack of data describing the effects of the disease on renal transporter regulation in the human, a reliance on rodent models is necessary for these studies to occur. These validated models can be used with greater confidence when investigating kidney and liver membrane transporter expression profiles in NASH.
Aim 2 (Chapter 2) - Determine the Molecular Mechanism of Hepatic MRP3 Up-Regulation in Human NASH.

The transcription factor, Nrf2, is emerging as a key regulator of transporter gene expression in NAFLD. The development of oxidative stress is a critical component of NASH pathogenesis and several reports have identified Nrf2 and Nrf2 target gene activation during the progressive stages of the disease. However, the molecular mechanisms of Nrf2-dependent gene induction of the transporter, MRP3, are still largely unknown. MRP3 is induced in NASH and its up-regulation is shown to alter the disposition of xenobiotics. By utilizing ChIP-sequencing technology, the studies outlined in this aim provide the identification and characterization of novel Nrf2 genomic regulatory elements within the human MRP3 gene. Importantly, these results aim to demonstrate a mechanistic link between Nrf2 activation and MRP3 gene induction in NASH.

Aim 3 (Chapter 3) - Determine the Effects of NASH on Renal Membrane Transporter Regulation in Multiple Experimental NASH Models.

Utilizing the rodent models outlined and characterized in the studies within Aim1, membrane transporter expression profiles are determined in the kidneys, which yield insight into the global adaptations of membrane transporter regulation in NASH.

Aim 4 (Chapter 4) - Determine the Effects of NASH on the Disposition of the Environmental Toxicant Arsenic in an Experimental Model of the Disease.

Previous work has consistently demonstrated that NASH alters the disposition of pharmaceutical drugs; however, evidence contributing to the notion that the disease can also impact the disposition of environmental toxicants, such as arsenic, is absent. Taking
advantage of a previously characterized rodent model, arsenic excretion into the urine as well as liver and kidney retention of its methylated metabolites is thoroughly investigated and linked with renal and hepatic transporter deregulation in the disease. Given the multi-organ toxicity profile of arsenic, NASH-mediated disruption of its disposition can lead to aberrant toxicity and is therefore a significant public health concern.
CHAPTER 2: MODELING HUMAN NONALCOHOLIC
STEATOHEPATITIS-ASSOCIATED CHANGES IN DRUG
TRANSPORTER EXPRESSION USING EXPERIMENTAL RODENT
MODELS

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a complex, multi-faceted disease that encompasses a spectrum of liver pathologies including simple fatty liver (hepatic steatosis) and nonalcoholic steatohepatitis (NASH). NASH is the more pathologically advanced stage of the disease and is characterized by increased hepatocellular damage, chronic liver inflammation, and fibrosis (Feldstein, 2010; Masuoka & Chalasani, 2013; Ali & Cusi, 2009). Recently, NAFLD has quickly increased in prevalence and is now considered the most common form of chronic liver disease in Western Society, with up to 50% of certain populations being afflicted with some form of the disease (McCullough, 2006; Lomonaco et al., 2013).

As a result of the significant hepatocellular damage caused by oxidative stress and chronic inflammation in NASH, hepatic biotransformation and transport mechanisms are
dysregulated, which can alter the absorption, distribution, metabolism and excretion. (ADME) of xenobiotics and lead to altered drug exposure. It was previously established that xenobiotic transporter mRNA and protein expression in the liver is altered in human NASH. Moreover, these findings were confirmed in rodents by using a methionine and choline deficient (MCD) diet model of NASH, leading to perturbations in the disposition of several pharmaceutical agents (Hardwick et al., 2010; Fisher et al., 2009b; Lickteig et al., 2007a). These NASH-associated changes in pharmacokinetics may impact drug efficacy and/or toxicity potentially requiring greater pharmacovigilant practices in the clinic. Therefore, identifying experimental models that more accurately reflect the pharmacokinetic parameters of the human disease, such as transporter expression, is critical in predicting drug disposition in human NASH.

Due to ethical and practical limitations, rodent models are used to further understand and characterize the functional aberrations of xenobiotic disposition in NASH. Dietary models, where rodents are fed specialized diets, are the most common NASH models because they may accurately reproduce the clinical and/or histopathological features of the disease (Schattenberg & Galle, 2010; Hebbard & George, 2011). These models include the methionine and choline deficient (MCD) diet as well as a modified high fat diet with supplemented cholate and cholesterol (atherogenic diet). Both of these diets are capable of recapitulating the histopathological features of NASH; however the MCD diet, in contrast to the atherogenic diet, fails to fully capture the metabolic disorders that frequently accompany NASH such as insulin resistance, dyslipidemia, and type II diabetes; however, the quick disease onset (8 weeks) as well as the severity of NASH that occurs
with the MCD diet makes it a practical and popular model in NASH research (Larter & Yeh, 2008; Matsuzawa et al., 2007).

In addition to dietary models, genetically obese rodents that carry deficiencies in leptin signaling, such as ob/ob and db/db mice and fa/fa rats, are also used as NASH models. Due to their inherent leptin dysregulation, these animals are hyperphagic, obese, and develop insulin resistance and therefore are often considered more advantageous models that provide a full spectrum of the clinical morbidities that frequent NAFLD (Larter & Yeh, 2008; Takahashi et al., 2012). However, the ob/ob and db/db mice do not develop NASH spontaneously and require an additional hit, usually in the form of a four week course of MCD feeding, to develop severe disease pathology (Larter & Yeh, 2008).

The purpose of this current study is to determine which experimental NASH models best recapitulate the mRNA and protein expression profile of clinically relevant drug transporters altered in human disease. The MCD and atherogenic diet were used as dietary NASH models and the ob/ob and db/db mice and fa/fa rat were used as genetic NASH models. Clinical biomarkers of metabolic syndrome were measured as well as histological analyses were conducted to confirm NASH. Additionally, rodent mRNA and protein expression of hepatic drug transporters were measured and compared to previously published human NASH expression profiles using concordance and effect size statistical analyses. This study will aid in determining more appropriate NASH models for use in future pharmacokinetic studies that can be translated to the clinical setting. Moreover, given that human data concerning extra-hepatic effects on membrane transporters is
lacking in NASH, these findings will provide experimental platforms to investigate the
effects of NASH on renal membrane xenobiotic transporter regulation.

**Materials and Methods**

**Materials**
Tris-HCl, Ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), glycerol,
potassium phosphate (KPO₄), potassium chloride (KCl), sodium pyrophosphate
(decahydrate), and Nonidet P-40 were obtained from Sigma-Aldrich (St. Louis, MO).
Neutral buffered formalin (10%) was obtained from Fisher Scientific (Pittsburgh, PA).

**Animals**
Eight to ten week old, male, C57BL/6J, B6.Cg-Lep<ob>/J (ob/ob), and B6.BKS(D)-Lepr<db>/J (db/db) mice were obtained from Jackson Laboratories (Bar Harbor, ME).
Eight to ten week old, male, Sprague Dawley and Crl:ZUC-Lepr<fa> fatty (fa/fa) rats were obtained from Charles River Laboratories (Wilmington, MA). All animals were acclimated in 12 hour light and dark cycles in a University of Arizona AAALAC-certified animal facility for at least one week prior to initiation of experiments and were given access to standard chow and water *ad libitum*. Housing and experimental procedures were in accordance with NIH guidelines for the care and use of experimental animals and were approved by the University of Arizona Institutional Animal Care and Use Committee. To model NASH, C57BL/6J mice and Sprague Dawley rats (N=4-7) were fed either a methionine and choline deficient (MCD) diet (#518810) (Dyets, Inc., Bethlehem, PA), or
an atherogenic diet (#D06061401) (Research Diets Inc., New Brunswick, NJ) for 8 weeks. As a control, C57BL/6J mice (N=4-7) and Sprague Dawley rats (N=4-7) were fed a methionine and choline re-supplemented diet (#518754) (Dyets, Inc., Bethlehem, PA). The ob/ob (N=4) and db/db (N=4-7) mice were fed a MCD diet for 4 weeks to induce NASH. The fa/fa rats were provided a modified high fat diet (#101447) for eight weeks (Dyets, Inc., Bethlehem, PA). Animals were weighed prior to diet start to record a baseline body weight.

_Tissue Harvesting_

At the conclusion of dietary feeding, the animals were weighed to record a final body weight then euthanized using CO₂ asphyxiation. Terminal blood was collected via cardiac puncture and plasma was extracted by centrifugation at 9,500 x g for 5 minutes using a tabletop centrifuge (4°C). The resulting plasma was stored at -20°C until analysis. The liver was immediately harvested, weighed, and a small portion was fixed for two days in 10 % neutral buffered formalin (4°C), followed by tissue processing and paraffin-embedding at the University of Arizona Histology Core Facility. The remaining tissue was snap frozen in liquid nitrogen and stored at -80°C for future analyses.

_Plasma Chemistries_

Rodent plasma samples were submitted to the pathology lab at University Animal Care facility, University of Arizona Health Science Center for determination of plasma ALT and
glucose levels. Plasma insulin was determined using a rodent enzyme-linked immunosorbent assay (Millipore, St. Charles, MO) per the manufacturer’s protocol.

**Tissue Staining and Evaluations**

Hematoxylin and eosin (H & E) stains were performed on formalin fixed, paraffin-embedded liver sections at the University of Arizona Histology Core according to the facility’s common practice. Masson’s trichrome staining was performed using the Masson Trichrome Stain Kit (Sigma Aldrich, St. Louis, MO) according to the manufacture’s protocol. H & E stained liver sections were submitted to the Arizona Health Sciences Center Animal Facility for pathological scoring evaluations according to a previously validated NASH scoring system (Kleiner et al., 2005). All samples were evaluated blindly and excluded disease and animal information.

**RNA Purification**

Total RNA was extracted and isolated from rat and mouse liver using RNAzol B reagent (Tel-Test Inc., Friendswood, TX) per the manufacturer's protocol. RNA concentrations were determined using UV spectrophotometry, and the integrity of the RNA was confirmed by ethidium bromide staining after agarose gel electrophoresis.

**Branched Chain DNA (bDNA) Analysis**

bDNA analysis was used to determine mRNA transcript levels of transporter genes and was previously shown to be highly specific method for mRNA quantification with a high
degree of accuracy and reproducibility (Hardwick et al., 2010; Hardwick et al., 2011; Lu et al., 2009; Lee et al., 2008). Specific oligonucleotide probes for Mrp1–4, Mdr1a, Mdr1b, Bcrp, Oatp1a1, 1a4, 1b2, and 2b1 were diluted in lysis buffer supplied by the Quantigene HV Signal Amplification Kit (Genospectra, Fremont, CA). Substrate solution, lysis buffer, capture hybridization buffer, amplifier, and label probe buffer used in the analysis were all obtained from the Quantigene Discovery Kit (Genospectra). The assay was performed in 96-well format with 10 µg of total RNA added to the capture hybridization buffer and 50 µl of the diluted probe set. The total RNA was then allowed to hybridize to the probe set overnight at 53°C. Hybridization steps were performed per the manufacturer's protocol the following day. Luminescence of the samples was measured with a Quantiplex 320 bDNA luminometer interfaced with Quantiplex Data Management Software, version 5.02 (Bayer, Walpole, MA).

Protein Preparations

Whole cell lysate preparations of mouse and rat liver were prepared from ~200 mg of tissue homogenized in NP-40 buffer (20 mM Tris HCl, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, and 2 mM EDTA) with 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) per 25ml at 4°C. Homogenized tissue was then agitated at 4°C for 2 hours, centrifuged at 10,000 x g for 30 minutes, and the supernatant transferred to a clean collection tube. Liver microsomal fractions were prepared from ~200 mg of frozen tissue. Briefly, tissue was homogenized in buffer A (50 mM Tris HCl pH 7.4, 1 mM EDTA, and 154 mM KCl) with added Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) per 25 ml at 4°C.
The resulting homogenate was centrifuged at 10,000 x g for 30 minutes at 4°C and the supernatant was collected into ultra-centrifuge tubes and centrifuged at 100,000 x g for 70 minutes at 4°C. The resulting pellet was resuspended in 600 µl of buffer B (100 mM sodium pyrophosphate pH 7.4 and 0.1 mM EDTA) and subjected to a second 100,000 x g centrifugation for 70 minutes at 4°C. The resulting pellet was resuspended in 100 µl of buffer C (10 mM KPO₄ pH 7.4, 1 mM EDTA, and 20% glycerol). Protein concentrations for both whole cell and microsomal fractions were determined using the Pierce BCA Protein Quantitation Assay (Thermo Scientific, Rockford, IL) per the manufacturer’s protocol and stored at -80°C until further analysis.

**Immunoblot Protein Analysis**

Whole cell lysate or microsomal proteins (50 µg/well) were prepared in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) with or without β-mercaptoethanol and heated at 37°C for 30 minutes prior to separation by SDS-PAGE on 7.5% gels. Resolved protein was transferred to polyvinylidene fluoride (PVDF) membranes for 70 min at 350 mAmperes at 4°C. Following transfer, the membranes were blocked in 5 % nonfat dry milk diluted in phosphate-buffered saline-tween 20 for 1 hr at room temperature. To determine relative protein levels the following primary antibodies were used: Mrp2, sc-5770; Pgp, sc-8313; Mrp3, sc-5775; Oatp1b2, sc-376904 (rat) and sc-47270 (mouse) (Santa Cruz Biotechnology, Santa Cruz, CA); Mrp4, ab15602 (Abcam, Cambridge, MA); Oatp1a4, OATP21-A (Alpha Diagnostics Intl., Inc., San Antonio, TX); Bcrp, MC-981 (Kamiya Biomedical Co., Seattle, WA). The blots were incubated with primary antibody overnight
at 4°C with constant rocking. The following HRP-conjugated secondary antibodies were used: anti-rat (sc-2065), anti-rabbit (sc-2004), anti-goat (sc-2350), and anti-mouse (sc-2005) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Quantification of relative protein expression was determined using image processing and analysis with Image J software (NIH, Bethesda, MD) and normalized to β-actin protein (whole cell lysate) (sc-47778, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or pan-cadherin (microsomal fraction) (Ab16505, Abcam, Cambridge, MA).

**mRNA and Protein Concordance Analysis Across Human and Rodent Models**

Concordance analyses to compare human and rodent mRNA and protein expression in NASH were performed by measuring the effect size of NASH vs. control for each gene. The effect size (estimated by Glass’s Δ) is the standardized mean difference between two populations and can be calculated using the following equation:

$$\Delta = \frac{\bar{X}_1 - \bar{X}_2}{S_p}$$

Where $\bar{X}_1$ and $\bar{X}_2$ are the sample means for two groups, and $S_p$ is the pooled standard deviation for both. Data from bDNA analysis were used for mRNA comparisons whereas normalized densitometry data were used for protein comparisons. All raw human data (mRNA and protein) used in this analysis was previously published (Hardwick et al., 2011; Clarke et al., 2014b). The analyses were performed using R version 3.0.2 (http://www.r-project.org/).
Statistical Analysis

Data were analyzed using one-way ANOVA to determine significant differences between model groups with a Bonferroni post-hoc analysis. A significance level of \( p \leq 0.05 \) was used for all analyses. All analyses were carried out using GraphPad Prism software Version 5 (GraphPad Software, Inc., La Jolla, CA).

Results

Rodent Body Weights, Tissue Weights and Clinical Chemistries

To determine and confirm the clinical features that are normally associated with NASH, body and tissue weight as well as plasma chemistry profiles were measured. Body weight, tissue weight and liver to body weight ratios for rats and mice are shown in Tables 2.1 and 2.2, respectively. No significant change in body weight compared to control was observed among the rat models, although the MCD rats trended towards a decrease whereas the \( fa/fa \) and atherogenic models trended towards an increase in body weight (Table 2.1). In contrast, the MCD and atherogenic mice had a significant reduction in body weight while the \( ob/ob \) mice had increased in body weight compared to control mice (Table 2.2).

To assess the magnitude of either weight gain or loss, change in body weight from start to finish of the study was measured. The magnitude of weight change between MCD and control rats was significantly different due to the loss in body weight in the MCD rats. The atherogenic and \( fa/fa \) rats tended to increase in weight more than controls but the magnitude of change was not statistically significant (Table 2.1). In contrast, the
magnitude of weight change that occurred in the MCD, atherogenic, ob/ob, and db/db mice was significantly different from control mice (Table 2.2). Liver weight was measured and the atherogenic and fa/fa rats, as well as the db/db mice had increased liver mass compared to controls (Tables 2.1 and 2.2). Liver to body weight ratios indicate that the atherogenic rat model and db/db mice as having increased liver mass in relation to body mass.

NASH is clinically associated with a variety of metabolic disorders including hyperglycemia and diabetes. Therefore, blood glucose and insulin were measured to determine if these experimental NASH models parallel the conditions typically present in the human NASH condition. Of the rat models, only the fa/fa rats developed hyperglycemia and hyperinsulinemia compared to controls (Table 2.1). In contrast, the rat MCD model demonstrated significantly reduced plasma glucose levels compared to control. No significant changes were identified in plasma glucose and insulin across the mouse models, although glucose levels tended to increase in the ob/ob and db/db mice (Table 2.2). Alanine aminotransferase (ALT) plasma levels were significantly increased in the rat MCD, atherogenic, fa/fa, as well as the mouse MCD, ob/ob, and db/db models.
Table 2.1: Rat Body Weight, Liver Weight and Plasma Chemistries. Body weight, weight gain, liver weight, liver to body weight ratios and plasma chemistries of rat NASH models. ALT; alanine aminotransferase. Data represent the mean ± S.E.M. from 3-7 rats.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>CONTROL</th>
<th>MCD</th>
<th>A Th ero</th>
<th>fa/fa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g, N=3)</td>
<td>522.67 ± 9.01</td>
<td>308 ± 2.65</td>
<td>594 ± 24.17</td>
<td>733.33 ± 10.18</td>
</tr>
<tr>
<td>Weight Gain (g, N=3)</td>
<td>+169.33 ± 7.46</td>
<td>-47.00 ± 6.28 *</td>
<td>+270.67 ± 17.98</td>
<td>+205 ± 6.61</td>
</tr>
<tr>
<td>Liver Weight (g, N=3)</td>
<td>21.24 ± 0.30</td>
<td>16.09 ± 0.33</td>
<td>43.62 ± 1.90 *</td>
<td>38.32 ± 0.44 *</td>
</tr>
<tr>
<td>Liver to Body Weight Ratio (%) , N=3</td>
<td>4.09 ± 0.08</td>
<td>5.24 ± 0.12</td>
<td>7.32 ± 0.03 *</td>
<td>5.24 ± 0.07</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ANALYTE</th>
<th>CONTROL</th>
<th>MCD</th>
<th>A Th ero</th>
<th>fa/fa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl, N=4)</td>
<td>157.5 ± 4.7</td>
<td>112.1 ± 7.3 *</td>
<td>159.3 ± 10.5</td>
<td>199.8 ± 11.1 *</td>
</tr>
<tr>
<td>Insulin (ng/ml, N=3)</td>
<td>3.7 ± 0.9</td>
<td>1.3 ± 0.6</td>
<td>9.0 ± 2.5</td>
<td>28.6 ± 1.2 *</td>
</tr>
<tr>
<td>ALT (U/L, N=7)</td>
<td>19.1 ± 1.1</td>
<td>136.1 ± 15.0 *</td>
<td>51.9 ± 5.5 *</td>
<td>53.5 ± 4.9 *</td>
</tr>
</tbody>
</table>

* p ≤ 0.05 to control; + weight gain; - weight loss
Table 2.2: Mouse Body Weight, Liver Weight and Plasma Chemistries. Body weight, liver weight, liver to body weight ratios and plasma chemistries of mouse NASH models. ALT; alanine aminotransferase Data represent the mean ± S.E.M. from 3-7 mice.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>CONTROL</th>
<th>MCD</th>
<th>AHERO</th>
<th>ob/ob</th>
<th>db/db</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal Body Weight (g, N=3)</td>
<td>35.17 ± 0.83</td>
<td>17.53 ± 0.64*</td>
<td>26.8 ± 1.22*</td>
<td>45.82 ± 1.17</td>
<td>38.97 ± 1.14</td>
</tr>
<tr>
<td>Weight Gain (g, N=3)</td>
<td>+9.5 ± 1.04</td>
<td>-10.83 ± 1.70*</td>
<td>+1.8 ± 0.12*</td>
<td>-5.82 ± 1.42*</td>
<td>-6.67 ± 0.60*</td>
</tr>
<tr>
<td>Liver Weight (g, N=3)</td>
<td>2.04 ± 0.09</td>
<td>0.80 ± 0.03*</td>
<td>1.74 ± 0.01</td>
<td>2.80 ±0.39</td>
<td>3.09 ± 0.08*</td>
</tr>
<tr>
<td>Liver to Body Weight Ratio (%)</td>
<td>5.79 ± 0.19</td>
<td>4.59 ± 0.11</td>
<td>6.52 ± 0.34</td>
<td>6.12 ± 0.77</td>
<td>7.96 ± 0.44*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ANALYTE</th>
<th>CONTROL</th>
<th>MCD</th>
<th>AHERO</th>
<th>ob/ob</th>
<th>db/db</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl, N=3)</td>
<td>121.1 ± 56.5</td>
<td>53.1 ± 27.7</td>
<td>111.8 ± 59.3</td>
<td>182.9 ± 6.4</td>
<td>137.4 ± 19.5</td>
</tr>
<tr>
<td>Insulin (ng/ml, N=3)</td>
<td>3.2 ± 0.3</td>
<td>0.5 ± 0</td>
<td>2.5 ± 1.4</td>
<td>3.8 ± 0.9</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>ALT (U/L, N=7)</td>
<td>46.02 ± 7.7</td>
<td>211.9 ± 15.2*</td>
<td>90.14 ± 22.4</td>
<td>274.99 ± 48.4*</td>
<td>343.457 ± 33.0*</td>
</tr>
</tbody>
</table>

*p ≤ 0.05 to control; + weight gain; - weight loss
**NASH Histology and Pathological Assessment in Rodent Models**

H & E stained liver sections as well as Masson’s trichrome staining from mouse and rat NASH animals are shown in Figure 2.1. Macrovesicular steatosis, a common pathological lesion that accompanies NASH, is clearly present in the livers of rat MCD as well as mouse MCD, *ob/ob*, and *db/db* models (Figures 2.1A and 2.1B, black arrowhead). To determine the extent of liver fibrosis, Masson’s trichrome staining was used on formalin fixed, paraffin embedded tissue samples. The results clearly show significant branching fibrosis (blue staining, arrow) in rat MCD liver (Figure 2.1C). Masson’s trichrome stain in mouse livers did not reveal any fibrotic tissue (Figure 2.1D).

In order to quantify the severity of NASH within each model, H & E stained samples were evaluated according to a previously validated NASH pathology scoring rubric. The total sum of the scores measured for characteristic NASH lesions yields a total NASH activity score (NAS), with scores at or above 4 being defined as NASH (Table 2.3). This assessment shows that rat MCD and atherogenic as well as mouse *db/db* models have NASH (Table 2.3). Interestingly, although the *ob/ob* model has severe pathology (macrovesicular lipid deposits and inflammation) this model fails to fully develop advanced NASH due to lower levels of inflammation and the absence of fibrosis.
Figure 2.1: Liver Histopathology of Rodent NASH Models. Representative hematoxylin and eosin stained liver sections from rat (A) and mouse (B) NASH models. Macrovesicular steatotic deposits, a distinguishable lesion seen in NASH, are shown by the black arrowhead. Masson’s trichrome staining in rat (C) and mouse (D) NASH models. Branching fibrosis is indicated by the black arrow. Images were taken at 20x magnification.
Table 2.3: Liver Pathology Scoring of NASH Rodent Models. NASH Activity Scores (NAS) were tabulated by summing the numerical grades of steatosis (0-3), inflammation (0-2), hepatocyte ballooning (0-2), and fibrosis (0-4) present within the liver. A total NAS score above four is a positive NASH diagnosis.

<table>
<thead>
<tr>
<th></th>
<th>Steatosis</th>
<th>Inflammation</th>
<th>Fibrosis</th>
<th>Ballooning</th>
<th>Total NAS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0.25 ± 0.06</td>
<td>0</td>
<td>0</td>
<td>0.25 ± 0.25</td>
</tr>
<tr>
<td>MCD</td>
<td>3</td>
<td>1</td>
<td>0.75 ± 0.06</td>
<td>0</td>
<td>4.75 ± 0.25</td>
</tr>
<tr>
<td>Athero</td>
<td>1.25 ± 0.06</td>
<td>1.25 ± 0.06</td>
<td>0.5 ± 0.07</td>
<td>0</td>
<td>4 ± 0.58</td>
</tr>
<tr>
<td>fa/fa</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Mice</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1 ± 0.14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 ± 0.58</td>
</tr>
<tr>
<td>MCD</td>
<td>2</td>
<td>1.25 ± 0.06</td>
<td>0</td>
<td>0</td>
<td>3.25 ± 0.25</td>
</tr>
<tr>
<td>Athero</td>
<td>2</td>
<td>0.75 ± 0.06</td>
<td>0.25</td>
<td>0</td>
<td>3 ± 0.41</td>
</tr>
<tr>
<td>ob/ob</td>
<td>3</td>
<td>0.5 ± 0.07</td>
<td>0</td>
<td>0</td>
<td>3.5 ± 0.29</td>
</tr>
<tr>
<td>db/db</td>
<td>3</td>
<td>1</td>
<td>0.5 ± 0.07</td>
<td>0</td>
<td>4.5 ± 0.29</td>
</tr>
</tbody>
</table>
**Hepatic mRNA Expression of Drug Transporters in Experimental NASH Models**

Previous studies have shown that mRNA expression of drug transporters is altered in the liver of both human and MCD diet-induced rodent NASH (Hardwick et al., 2010; Hardwick et al., 2011; Fisher et al., 2009b). To determine if gene expression is altered in other experimental NASH models, the mRNA of clinically relevant drug transporters was measured via bDNA analysis (Figure 2.2). mRNA expression of the efflux transporters Mrp1, Mrp2, Mrp3, Mrp4, Bcrp, Mdr1a and Mdr1b were all significantly induced in the rat MCD model whereas only Mrp2 and Mdr1b were induced in the atherogenic rat model (Figure 2.2A). Of the mouse NASH models, the efflux transporters Mrp1 (db/db), Mrp2 (MCD, athero, ob/ob, and db/db), Mrp3 (ob/ob and db/db), Mrp4 (MCD, ob/ob and db/db) and Mdr1a (MCD, ob/ob, db/db) were induced at a significant level (Figure 2.2B).

Hepatic Oatp transporter mRNA expression was measured and shown in Figure 2.2C and 2.2D. Oatp1a1 was significantly down-regulated in the fa/ta rat model as well as the mouse MCD, ob/ob, and db/db models, whereas the MCD rat and db/db mouse models had a significant down-regulation of the Oatp1b2 isoform (Figure 2.2C and 2.2D). Conversely, hepatic Oatp1a4 displayed an opposite effect and was significantly induced in the MCD and atherogenic rat models as well as the MCD and db/db mice.
Figure 2.2: Liver mRNA Expression of Drug Transporters in Rodent NASH. mRNA expression of rat efflux (A) and uptake (C) transporters as well as mouse efflux (B) and uptake (D) transporters in rodent NASH models via branched DNA gene analysis. Data represent the mean ± S.E.M from 4 animals. * p ≤ 0.05 versus control within each group.
**Hepatic Protein Expression of Drug Transporters in Experimental NASH Models.**

To verify whether the observed alterations to transporter mRNA expression translates to altered protein expression, Western Blot analyses were performed to determine relative protein expression levels of select hepatic transporters across the NASH models (Figure 2.3). Protein expression of Mrp2, Mrp3, Mrp4, and Pgp were all up-regulated in the MCD rat model, (Figure 2.3A and 2.3B). In contrast, hepatic Oatp1a4 protein expression was down-regulated in all models whereas Oatp1b2 protein expression was down-regulated in the MCD and atherogenic models but induced in fa/fa rats.

Among the mouse models, Mrp3 and P-gp were significantly induced in the ob/ob mice whereas Mrp4 was significantly induced in the MCD mouse model (Figure 2.3C and 2.3D). The ob/ob and db/db mice trended towards an induction of Mrp4 protein, but this did not reach statistical significance. Alternatively, Oatp1b2 was down-regulated in the MCD, ob/ob and db/db models. No change in Bcrp protein expression was observed across all rodent models.
Figure 2.3: Liver Protein Expression of Drug Transporters in Rodent NASH.

Representative Western blot of rat (A) and mouse (C) transporters in rodent NASH models. Densitometry analysis of Western blot data of rat (B) and mouse (D) blots is shown. β-actin was used a loading control for whole cell lysates, whereas pan-cadherin was used for microsomal preparations. Data represent the mean ± S.E.M from 4 animals. * p ≤ 0.05 versus control within each group.
Concordance Analysis Across Human and Rodent mRNA and Protein Expression in NASH.

To determine how well the rodent NASH models recapitulate human NASH mRNA and protein expression changes, a concordance analysis was performed using the data derived from this study with previously published data from human NASH mRNA and protein expression data (Hardwick et al., 2011; Clarke et al., 2014b). Due to the lack of orthology in human OATP1B1 and OATP1B3 to rodents, rodent Oatp1b2 was compared to both human OATP1B1 and OATP1B3 separately. Additionally, human MDR1 (P-gp) mRNA expression was compared to rodent Mdr1a and Mdr1b orthologs separately. Figures 4 and 5 show human mRNA and protein data as a function of rodent mRNA and protein effect sizes, respectively. Effect sizes in the same direction (positive or negative) represent similar trends in the direction of gene expression whereas the magnitude of change corresponds to the statistical power in detecting a difference in expression in NASH versus control. The rat and mouse MCD model, as well as mouse ob/ob and db/db have the most abundant transporter genes that share a positive effect size across both human and rodent (top right quadrant of graphs, Figure 2.4A and 2.4B). Human OATP1B3 shares a negative effect size (down-regulation), which is also present in rat and mouse MCD, along with mouse ob/ob and db/db models (lower left quadrant of graphs). In contrast, the mouse and rat atherogenic models along with the rat fa/fa model show opposite effect sizes compared to human mRNA expression for several transporter genes, including Mdr1a (rat atherogenic), Mrp2 (fa/fa), Mrp4 (fa/fa), and Mrp1 (mouse atherogenic). (Figure 2.4A and 2.4B).
Figure 2.4: Effect Size Analysis of Human and Rodent NASH mRNA Expression of Drug Transporters. Effect size of human transporter mRNA expression as a function of rat (A) and mouse (B) mRNA expression in NASH. Positive effect changes reflect induction of gene expression whereas negative effect changes reflect down-regulation of gene expression. The magnitude of the effect change (positive or negative) reflects the power of the disease (human or rodent model) to detect a change in gene expression over control. Values were calculated by the method described in the materials and methods section.
Figure 5 shows the effect sizes of human protein as a function of rat (Figure 2.5A) and mouse (Figure 2.5B) transporter protein expression. The rat MCD model shows a similar effect size distribution compared to human for all transporters except OATP1B1, which is up-regulated in human but Oatp1b2 is down-regulated in rat MCD (Figure 2.5A). Similarly, the mouse ob/ob model shares positive effect size changes compared to human for all transporters except for OATP1B1 which is induced in human NASH and down-regulated in the ob/ob model (Figure 2.5B). The mouse db/db and MCD models do share a similar effect change to human protein expression for all transporters except for Bcrp, which shows a negative effect size in these rodent models whereas a positive effect size is observed in human NASH (Figure 2.5B). Similar to the mRNA effect size comparison, rodent atherogenic, as well as fa/fa rats do not share similar protein effect size changes to human NASH across all transporters investigated. For a list of raw effect size data see supplemental Tables 1-4 (Appendix B).
Figure 2.5: Effect Size Analysis of Human and Rodent NASH Protein Expression of Drug Transporters. Effect size of human transporter protein expression as a function of rat (A) and mouse (B) protein expression in NASH is shown. Positive effect changes reflect induction of protein expression whereas negative effect changes reflect repression in protein expression. The magnitude of the effect change (positive or negative) reflects the power of the disease (human or rodent model) to detect a change in protein expression over control. Values were calculated by the method described in the materials and methods section.
Discussion

With the increasing dependency on pharmacotherapy to manage symptoms associated with disease, adverse drug reactions (ADRs) have become a significant cause for morbidity and mortality worldwide. In the United States alone, ADRs are one of the top ten causes of death, accounting for ~100,000 deaths annually and over 700,000 hospitalizations per year (Wooten, 2010a; Lazarou et al., 1998; Valente & Murray, 2011). The causes for ADRs are multi-faceted and include idiosyncratic drug reactions as well as inter-individual variations in the metabolism and elimination of drugs (Shepherd et al., 2012; Valente & Murray, 2011). It is well established that genetic polymorphisms that exist within drug transporters and drug metabolizing enzymes have a role in determining the pharmacokinetics of drugs, thereby impacting the development of clinical ADRs (Daly, 2012; Yiannakopoulou, 2013; Clarke & Cherrington, 2012). However, genetic polymorphisms within genes that mediate ADME processes are estimated to account for less than 20% of ADRs suggesting that other host factors, such as diseases, may be significant in the development of ADRs (Ingelman-Sundberg & Rodriguez-Antona, 2005). Therefore, it is important to investigate patients with diseases such as NASH as being an at-risk population for developing drug-induced ADRs.

The purpose of this study was to examine experimental models of NASH and determine which of these models accurately represents the expression patterns of hepatic drug transporters in human NASH. This information will allow for meaningful predictions of drug disposition in NASH that could identify potential ADRs in preclinical studies. However, rodent NAFLD models vary dramatically in their ability to reproduce both the
clinical and histopathological features of the disease, making selection of the appropriate model difficult. For example, the MCD diet model is criticized for failing to recapitulate the natural progression of the disease along with lacking common aberrant clinical features such as obesity and hyperglycemia (Tahan et al., 2004; Rinella & Green, 2004). Our data confirm that mice and rats fed a MCD diet fail to develop metabolic aberrations such as obesity and hyperglycemia suggesting that metabolic alterations are not a consequence of MCD feeding. However, both rats and mice fed a MCD diet develop histopathological features associated with NASH such as lobular inflammation, macrovesicular steatosis and varying degrees of fibrosis, which is consistent with previous findings (Leclercq et al., 2000; Fisher et al., 2009b). Interestingly, however, our results suggest that rats are more sensitive to the effects of MCD feeding than mice. MCD rats scored higher in steatosis and fibrosis grades compared to mice in addition to histological evaluations confirming these findings by the appearance of a greater number of macrovesicular lipid and collagen deposits. A previous study showed that Wistar rats develop more pronounced steatotic deposits in the liver compared to the C57BL/6 mouse strain (Kirsch et al., 2003). Our results are in agreement with these findings, but in contrast to our observation that rats developed more severe NASH, Kirsch et al. reported that mice were more sensitive to the MCD diet. A possible explanation for this discrepancy may lie in the differences in pathological markers measured (NAS scoring versus lipid peroxidation bi-products and mitochondrial injury) or the duration of MCD feeding (4 weeks versus 8 weeks). Certainly, the longer diet regimen used in our study may drive NASH to a more advanced pathology that resembles human disease.
In response to the criticism that the MCD diet model fails to represent the spectrum of clinical features of NASH, several other rodent NASH models were developed and investigated. Genetically obese rodents having dysregulated leptin signaling, such as the fa/fa rats as well as the ob/ob and db/db mice have increased in popularity due to their inherent nature in developing clinical features associated with the metabolic syndrome including obesity, dyslipidemia, and hyperglycemia (Bray & York, 1979; Schattenberg & Galle, 2010; Carmiel-Haggai et al., 2005). However, ob/ob and db/db mice do not develop NASH spontaneously and must be exposed to a ‘second hit’ such as short term MCD feeding to propagate the manifestation of NASH (Takahashi et al., 2012). In our study, ob/ob and db/db mice fed a MCD diet for four weeks develop histopathological features consistent with NASH such as macrovesicular steatosis and inflammation. In addition, the db/db mice had enlarged livers, increased liver to body weight ratios and were obese despite a reduction in body weight. As expected, MCD feeding failed to maintain the metabolic disturbances seen in these strains such as hyperglycemia and hyperinsulinemia. These observations are consistent with previous findings and are a negative consequence of the MCD diet (Sahai et al., 2004; Yamaguchi et al., 2007). Interestingly, despite a previous report of fa/fa rats developing NASH upon high fat diet feeding (Carmiel-Haggai et al., 2005), our study did not find the full development of NASH in these animals. While these rats are significantly obese and clinical markers are suggestive of the presence of the metabolic syndrome, the histopathological analysis reveals a lack of NASH diagnostic markers present despite having hepatic steatosis.
Despite the differences in NASH manifestations, the ability to recapitulate human gene expression in the liver is most valuable in translational research in the ADME of pharmaceuticals and toxicants. Membrane drug transporters are important mediators of xenobiotic disposition (Klaassen & Aleksunes, 2010) and therefore alterations in the expression and/or function of transporters can impact the pharmacokinetics of drugs, potentially increasing the likelihood of developing ADRs. In the present investigation, we report both mRNA and protein expression profiles of clinically important hepatic drug transporters across several NASH rodent models. Our results suggest that the rat and mouse MCD, along with the db/db and ob/ob mouse models significantly alter the expression profiles of both uptake and efflux transporters in the liver that is consistent with human NASH. Specifically, efflux transporters belonging to the ATP-binding cassette family of transporters are generally induced whereas uptake transporters belonging to the solute carrier family of transporters are repressed, which is consistent with previous analyses conducted in MCD-fed rodents (Fisher et al., 2009b; Lickteig et al., 2007a; Hardwick et al., 2012). It is interesting to note that Oatp1a4 mRNA expression is induced in NASH, whereas protein expression is down-regulated in MCD rats but for the purpose of this investigation, protein expression will be taken into greater consideration since protein levels will have a functional impact on ADME processes. Taken together, these uniform responses to hepatic injury by NASH suggest a coordinated response in the regulation of hepatic drug transporters similar to what is observed in human NASH (Hardwick et al., 2011; Lake et al., 2011). These results are consistent with previous findings suggesting that this may be a coordinated response that acts as a protective mechanism, limiting further
xenobiotic exposure to the liver by decreasing uptake and facilitating efflux (Lake et al., 2011). Interestingly, the MCD, ob/ob, and db/db models share histopathological features that are consistent with NASH but lack clinical aberrations associated with the metabolic syndrome. This suggests that the pathological lesions sustained by the liver in NASH, rather than the metabolic aberrations, may be the major driving force in regulating the transporter gene expression changes observed in the disease, although this needs to be examined further.

Using our previously published mRNA and protein expression data from human livers diagnosed as healthy or NASH, we performed a statistical analysis comparing the mean effect size of gene expression changes between each of the rodent models investigated and our published human data. The results from this analysis suggest that the rat and mouse MCD models as well as the mouse ob/ob and db/db models had the highest power in detecting gene expression changes that reflect the alterations in human transporters. The atherogenic models, as well as the rat fa/fa model share similar changes in transporter expression with human NASH in both direction and magnitude, but other transporters are inconsistent and fail to parallel human NASH transporter expression. Interestingly, the models that were fed an MCD diet share the most similarity to drug transporter expression in human NASH. It is well known that MCD feeding causes significant induction of oxidative stress as well as the release of pro-inflammatory cytokines, both of which are mediators of drug transporter gene regulation (Leclercq et al., 2000; Chowdhry et al., 2010; Cherrington et al., 2013; Ikemura et al., 2013). Additionally, hepatic oxidative stress is increased in humans with NASH leading to aberrations in
oxidative stress-mediated gene regulation (Hardwick et al., 2010). These observations further suggest that the pathological consequences sustained throughout the progressive stages of NASH likely play a major role in the dysregulation of ADME in NASH, whereas metabolic perturbations are less influential.

In conclusion, the rat and mouse MCD as well as the mouse \textit{ob/ob} and \textit{db/db} NASH models best represent the drug transporter expression changes seen in the livers of humans with NASH. We observe a similar coordinated response in hepatic xenobiotic transporter expression in these NASH models, suggesting a similar conserved mechanism of regulation in the disease. Future investigations into the effects of NASH on the disposition of xenobiotics that share similar transporter kinetic profiles across humans and rodents are encouraged to use these models. Lastly, given the close association of these models to the human condition, translative investigations into the regulation of extra-hepatic alterations in xenobiotic transporter expression may be made possible.
CHAPTER 3: IDENTIFICATION OF A FUNCTIONAL ANTIOXIDANT RESPONSE ELEMENT WITHIN THE EIGHTH INTRON OF THE HUMAN ABCC3 GENE - MECHANISTIC INSIGHT INTO HEPATIC ABCC3 INDUCTION IN NONALCOHOLIC STEATOHEPATITIS

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Introduction

Membrane transporters, such as ABCC3 (MRP3), serve important roles in mediating the absorption, distribution and excretion of endo and xenobiotic substrates. ABCC3 encodes for an organic anion transporter belonging to the ATP-binding cassette (ABC) superfamily of transporters (MRP3) and transports a variety of endogenous and exogenous compounds such as bile acids, bilirubin, and chemotherapeutic agents (methotrexate, etoposide), as well as glutathione, sulfate, and glucuronide conjugates (Borst et al., 2007). ABCC3 is almost exclusively expressed on the basolateral membrane of polarized epithelial cells and tissue distribution in humans is primarily confined to the adrenal glands, kidney, liver, small intestine, colon, pancreas and the bladder (Borst et al., 2007; Klaassen & Aleksunes, 2010). ABCC3 is also highly expressed in several forms of
cancer including non-small-cell lung carcinoma, gallbladder cancer, and hepatocellular carcinoma (Young et al., 2001; Nies et al., 2001; Wang et al., 2010).

In addition to being over-expressed in several malignant tumors, expression of ABCC3, particularly in the liver, is highly inducible in other non-malignant conditions (Borst et al., 2007). Hepatic ABCC3 was shown to be up-regulated in primary biliary cirrhosis, hepatitis C viral infections, nonalcoholic steatohepatitis (NASH), and rodent models of cholestasis (Zollner et al., 2001; Ros et al., 2003; Hardwick et al., 2011). The mechanisms that mediate ABCC3 induction are hypothesized to be hepatoprotective in nature and primarily involve the activation of transcriptional regulators such as nuclear receptors and transcription factors. It was demonstrated that nuclear receptors such as the constitutive androstane receptor (CAR) and pregnane X receptor (PXR) as well as the transcription factor nuclear factor (erythroid 2)-like 2 (Nrf2) are important mediators of the xenobiotic-dependent induction of the Abcc3 gene (Klaassen & Aleksunes, 2010).

Nrf2 is a master regulator of the antioxidant response pathway and orchestrates the transcriptional activation of cellular defense mechanisms in combating oxidative stress. Nrf2 activation is highly regulated and is controlled by the Kelch-like ECH-associated protein (Keap1), an adapter protein that belongs to an E3 ubiquitin ligase complex (Zhang, 2006). Under basal conditions, Nrf2 associates as an inactive complex with Keap1 and is targeted for ubiquitin-mediated proteasomal degradation. Under periods of oxidative/electrophilic stress, however, the Keap1/Nrf2 interaction is disrupted, leading to cytosolic accumulation and subsequent nuclear translocation of Nrf2 where it can drive the transcription of target genes (Zhang, 2006; Jaiswal, 2004).
Activation of Nrf2 is largely regarded as a cytoprotective mechanism and target genes consist of a battery of phase I/II detoxifying enzymes and xenobiotic transporters that ultimately aid in defending the cell in response to oxidative stress (Shen & Kong, 2009). Multiple investigations using Nrf2 chemical activators have shown a clear role in the Nrf2-dependent induction of xenobiotic transporters (Maher et al., 2005; Maher et al., 2008; Aleksunes et al., 2008). Importantly, this has resulted in the identification of an ARE upstream of the promoter of the mouse Abcc3 gene (Maher et al., 2007); however, identification of a functional ARE in the human gene is lacking despite clear evidence of Nrf2-dependent regulation of human ABCC3 (Wang et al., 2010; Sasaki et al., 2012; Mahaffey et al., 2012; Mahaffey et al., 2009).

The purpose of the current study was to identify and characterize the functional ARE(s) responsible for the Nrf2-dependent induction of the human ABCC3 gene. Chromatin immunoprecipitation (ChIP) assays followed by sequencing analysis in Nrf2 overexpressing cell lines will be used to identify Nrf2-specific interactions within the human ABCC3 gene. The results obtained from this study will contribute novel knowledge by providing mechanistic insight into membrane transporter regulation in conditions associated with Nrf2 activation, such as NASH.

**Materials and Methods**

**Materials**

Unless otherwise specified, all other materials were obtained from Sigma Aldrich (St. Louis, MO).
Cell Culture

HEK293 cells used were a gift from the Zhang laboratory at the University of Arizona, Tucson AZ. Cells were grown in Dulbecco’s Modified Eagles Media supplemented with 10% fetal bovine serum, glutamine, and penicillin-streptomycin. Cultures were grown at 37°C in 5% CO₂ atmosphere.

Chromatin Immunoprecipitation (sequencing)

Cell Culture. A549 cells (ATCC CCL-185) were passaged in DMEM 5% FBS with 1% penicillin/streptomycin. A 10 cm plate was grown to approximately 90% confluence, translating to roughly 1.5x10⁶ cells. Cross-linking and Sonication. Formaldehyde (~270 μL) was added to cell media to reach a final concentration of 1%. Cells were incubated at room temperature while shaking. Cross-linking was stopped by adding 1 ml of 1.25 M glycine, and cells were scraped and centrifuged at 800 RPM. Media was aspirated completely, rinsed with phosphate-buffered saline, then re-suspended in 1 mL Cell Lysis Buffer (5 mM 1,4-piperazinediethanesulfonic acid, pH 8.0; 85 mM KCl; 0.5% NP40) for 10 min on ice. Samples were centrifuged at 2000 RPM, the supernatant was removed and re-suspended in 1 L Nuclei Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0) for 10 min on ice. Sonication of samples was conducted according to the following parameters: Output 4, Duty Cycle 50%, 20 sec., 12 repetitions. Samples were centrifuged at full speed for 30 min at 4°C, then diluted with Dilution Buffer (1.1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0) Immunoprecipitation. Pre-clearing was conducted for 12 h at 4°C using 5 µg rabbit IgG and 300 µl of pre-blocked protein A/G fast-flow sepharose
beads. Samples were centrifuged at 3000 RPM for 5 min, and then transferred to new tube. This process was repeated for a total of 4 times. Subsequently, into 1 mL of sample, the antibody of interest was added at the following amounts: IgG (2 µg), Nrf2 (2 µg), and dH3K4dimethyl (3 µg). Samples were rotated for 24 h at 4°C, and 60 µl of pre-cleared beads were added. Samples were centrifuged at 3000 RPM, and the supernatant was removed. Beads were washed with a series of low salt, high salt, and LiCl and TE buffers. To elute, 250 µl of Elution Buffer was added (1% SDS, 0.1 M NaHCO3, 0.25 µM DTT) at 65°C overnight. Samples were treated with proteinase K (10 mg/ml), then a phenol-chloroform extraction for final re-suspension into 30 µl of PCR-grade water. Library Construction and Sequencing. Library creation was accomplished using adapter ligation methods. Each unique library was sequenced along with an input control library using the Solexa Genome Analyzer (Illumina, San Diego, CA), and sequences were aligned to the human genome (www.genome.ucsc.edu) using ELAND software. Peaks were visualized using the Integrated Genome Browser (BioViz, UNC, Charlotte, North Carolina).

In Silico Analysis

Identification of putative AREs within the eighth intron was performed using CLC Sequence Viewer v6.4 (CLC bio, Denmark). Species alignments were performed using comparative genomics tools available at ensemble.org.

Molecular Cloning of ABCC3 Intron
A DNA construct containing the eighth intron (1.2 kb) of the ABCC3 gene was produced by PCR amplification of the RP11 BAC clone, 121F10. Primers used included exogenous restriction sites (underlined) and are as follows: forward primer 5’-CGCCTCGAGTGGCTGGCTAGCCCAGAGGA-3’ (XhoI site), and reverse primer 5’-CGCAAGCTTGCACCCTGGCCCCACATGA-3’ (HindIII site). The resulting PCR product was cloned into TOPO vector using the TOPO TA Cloning Kit (Invitrogen) per the manufacture’s recommendations.

Sub-cloning of ABCC3 Intron in Reporter Vectors

The cloned ABCC3 intron (~1.2 kb) was sub-cloned from TOPO vector into the pGL3 Basic Reporter Vector (Promega Corp, Madison WI) using the XhoI/HindIII restriction sites within the multiple cloning region upstream of the luciferase gene (1.2 kb). The intronic insert cloned into pGL3 Basic was subsequently cloned into the pT81luc reporter vector (containing a HSV thymidine kinase promoter) upstream of the promoter by PCR amplification of the insert using the following primers: forward primer 5’-TCGCGGATCCGAGGAAGGAACAACGTACAG-3’ and reverse primer 5’-ATAGCAAGCTTAGATCTTTGAAGCCAGATG-3’. The underlined sequences represent exogenous restriction sites that were inserted for ligation of insert into pT81luc vector (BamHI for forward and HindIII for reverse). The resulting PCR amplicon is ~900 bp and was inserted into pT81luc reporter vector using BamHI/HindIII restriction sites.

PCR Mutagenesis of ARE2 and ARE5 within Intron
Mutagenesis of ARE2 and ARE5 was carried out by PCR using a proof-reading polymerase to reduce sequencing errors. Briefly, reverse-complementary primers (primer pairs) containing the mutated sequence of interest were used and were as follows: ARE2 mutant, 5’-

CAGGAACTCAGAGGAGTGAGAGCGGTGTTCAAGGGCACACAGCAAAGAAGC

-3’ (along with reverse complement) and ARE5 mutant; 5’-

AACAGGGAAAGTTGCAGCCTCAAAATTAGCAGCAAAAGCCCTGAAACAGCATG

AC-3’ (as well as its reverse complement). Underlined sequences represent the ARE sequence whereas the bolded sequences are mutated (TGACTTGTC is wt sequence for ARE2 and AGCTTAGTCA is wt sequence for ARE5). The mutagenesis reaction was carried out using the cloned intron in pT81luc as a template (50 ng/reaction) and the PCR cycle was as follows: 95°C for 1 min, 95°C for 30 sec, 55°C for 1 min, 68°C for 10 min, steps 2-4 repeated 24 more times. The reactions were cooled to room temperature followed by the addition of 1 µl DpnI restriction enzyme and incubated at 37°C for 1 h to cleave parental plasmid DNA (and leave newly, synthesized nascent strand intact). Following incubation, 5 µl was transformed into competent bacteria cells and subsequently screened and sequenced for mutant sequence.

Transfection and Luciferase Reporter Gene Assay

HEK293 cells were transfected with pT81 constructs using Fugene 6 (Roche, Germany) transfection reagent per the manufacturer’s protocol, including the use of pRL-tk transfection control (Promega Corp, Madison WI). Twenty-four hours post-transfection,
the cells were treated with either 50 µM tert-butyl-hydroquinone (tBHQ) (in DMSO), or DMSO control for 17 h. Following treatment, luciferase activity was determined using the Dual-Glo Luciferase Assay (Promega Corp, Madison WI) per manufacturer’s instructions and normalized to transfection control. Each reporter construct was assayed in triplicate wells, and luciferase activity was measured using a GloMax® 20/20 Luminometer (Promega Corp, Madison WI).

*Nrf2 DNA Pull-down Assay*

The pull-down assay was performed *in vitro* using recombinant GST-tagged Nrf2 and Histagged MafG protein, which were a kind gift from the laboratory of Dr. Donna Zhang at the University of Arizona, Department of Pharmacology and Toxicology. Briefly, GST-Nrf2 and His-MafG (5:1 ratio) were incubated in microcentrifuge tubes with RIPA buffer (10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1% sodium deoxycholate, 2 mM EDTA, 0.1% SDS, 1% NP-40) and double-stranded (hybridized), 5’ biotin-labelled oligonucleotide probes containing ARE2, ARE5, or NQO1 promoter as a positive control as well as their mutated counterparts. The sequences of the probes used were as follows (lower case are nucleotides that were mutated):

ARE2wt: 5’-CTCAGAGAGGTGAAGTGACTTGGCCAAGGGCACCACAGCAGAAA-3’
AREmut: 5’-CTCAGAGAGGTGAAGcGgCTTGttCAAGGGGCACACAGCAGAAA-3’
AREwt: 5’-GGAAAGTTCAGCCTCAGCTTAGTCACAAAGCCTGAAACAGC-3’
AREmut: 5’-GGAAAGTTCAGCCTCaaTTAGcCgCAAAGCCCTGAAACAGC-3’
NQO1wt: 5’-AAATCGCAGTCACAGTGACTCAGCAGAATCCTGAGCCTAGGG-3’
NQO1mut: 5’-AAATCGCAGTCACAGactCTCAcgAGAATCTGAGCCTAGGG-3’

Tubes were incubated at 4°C under constant rotation for 2 h to allow for complexes to form. Following incubation, 30 µl of streptavidin agarose resin (pre-washed with RIPA buffer to remove alcohol) was added to each reaction vial and incubated overnight at 4°C under constant rotation to precipitate the bound complexes. After incubation, the beads were centrifuged, supernatant removed, and beads washed with RIPA buffer to remove any unbound protein. This previous step was performed three times to ensure removal of all unbound protein. After the final centrifugation, all the remaining supernatant was removed and the complexes were eluted by the addition of Laemmli sample buffer (20 µl) to each reaction tube and heated at 95°C for 5 min. The beads were then centrifuged and the supernatant was subjected to SDS-PAGE and Western blot analysis using a primary antibody directed against GST (Nrf2) (sc138) and an anti-mouse secondary (sc2005) (Santa Cruz Biotechnology, Santa Cruz, CA). Densitometry analyses were performed using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical Analysis

Data was analyzed using one-way ANOVA to determine significant findings in luciferase assay across reporter vectors with a Bonferroni post-hoc analysis. A significance level of $p \leq 0.05$ was used for all analyses. All analyses were carried out using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

Results

**ChIP-sequencing Analysis**
To determine the relative location of Nrf2 association within the ABCC3 gene, a ChIP assay followed by sequencing analysis was performed using A549 cells, a human lung carcinoma cell line that has constitutively active Nrf2. The results reveal a specific, robust interaction of Nrf2 within a genomic region located -26258 to -28823 upstream from the transcription start site, which corresponds to the eighth intronic region of the ABCC3 gene (green arrow), whereas no Nrf2 binding was detected within the promoter region (red arrow) (Figure 3.1). Furthermore, histone 3, lysine 4 (H3K4) methylation status was assessed and was found to be hypermethylated within the intronic region corresponding to Nrf2-association as well as the promoter region of the ABCC3 gene.
Figure 3.1: Chromatin Immunoprecipitation (sequencing). Results obtained from a chromatin immunoprecipitation assay in A549 cells (Nrf2 constitutively active) followed by sequencing analysis. The DNA sequence of the *ABCC3* gene is depicted as the green line, with exons shown as green bars. Positive results from the ChIP sequencing analysis are shown as binding intensity from two separate ChIP assays (teal, row 3; purple, row 4) and represents a strong Nrf2 interaction within the eighth intron of the *ABCC3* gene (green arrow) (intron located -26258 to -28823 from TSS. Histone 3, lysine 4 (H3K4) methylation status is highlighted in teal on the top row of the figure (color represents positive methylation status) (Row 1). Input control is also shown as teal in row 2. No Nrf2 interaction was observed within the promoter region of the *ABCC3* gene (red arrow).
*ABCC3 Gene Sequence Alignment across Mouse and Human*

Gene comparison analyses between human and mouse ABCC3 show that the previously characterized ARE within the mouse promoter region (Maher et al., 2007) (green arrow) is located within a non-conserved genomic region suggesting that this ARE is possibly unique to the mouse species (*Figure 3.2*). In contrast, the genomic region corresponding to Nrf2 association within the eighth intron of ABCC3 appears to be in a region that is conserved across human and mouse (*Figure 3.2, red arrow*).
Figure 3.2: ABCC3 Gene Comparison Across Human and Mouse. Gene comparison of human (top) and mouse (bottom) ABCC3 is shown. Brown squares represent protein coding regions and pink represents conserved regions across both species. The previously identified mouse ARE is in a region shown by the green arrowhead, which is located in a non-conserved genomic region. Contrastingly, the intronic region corresponding with the Nrf2 association in human ABCC3 is located in a genetically conserved locus (red arrowhead).
In Silico Identification of Putative ARE Elements within Eighth Intron of ABCC3 Gene

To identify potential ARE(s) within the eighth intronic region of ABCC3 gene, *in silico* and sequencing analyses were performed using CLC sequence viewer software. A total of six putative ARE and ARE-like elements were identified within the eighth intronic region; however, only two (ARE2 and ARE5) matched the core ARE consensus sequence and are separated by ~400 bp (Figure 3.3). Furthermore, comparative analysis of the ARE2 and ARE5 across several eutherian mammal species showed that ARE5, and not ARE2, is evolutionarily conserved (data not shown).
Figure 3.3: Comparison of Putative ABCC3 Intronic AREs with Consensus ARE.

Comparison of the six ARE and ARE-like elements located within the eighth intron of the ABCC3 with the consensus ARE sequence is shown. For additional comparison purposes, the ARE of known Nrf2-target genes, NQO1, GLC, GCLM are also shown. The underlined, bold sequences of the consensus sequence are required for activity. A total of two (ARE2 and ARE5) AREs from the six contain the required consensus sequence.
Luciferase Reporter and Nrf2 Pulldown Assays

To confirm ARE functionality, luciferase reporter constructs containing a ~900 bp region of the ABCC3 eighth intron were transfected into HEK293 cells and treated with 50 µM of the Nrf2 activator, tert-butyl-hydroquinone (tBHQ) (Figures 3.4A and 3.4B). tBHQ treatment of pT81-Intron (ABCC3 intron 8) transfectants resulted in a near four-fold induction of luciferase activity compared to the vehicle treated transfectants (Figure 3.4A). This result suggested the presence of at least one functional ARE within this region. To further characterize the ARE(s) mediating the Nrf2-dependent induction of luciferase activity, mutant ARE2 and ARE5 constructs were generated (Figure 3.4). Mutating ARE2 had no effect on luciferase activity, whereas mutation of ARE5 caused a three-fold decrease and a near complete ablation of the Nrf2-dependent induction of luciferase activity (Figure 3.4B).

To further elucidate interaction of Nrf2 with the intronic DNA region, DNA-pulldown assays were conducted using biotin-labelled DNA probes containing the ARE2 and ARE5 genomic sequences (Figures 3.5A and 3.5B). A strong, positive interaction of Nrf2 was observed with the ARE5 probe that was ~75% of the positive control NQO1wt (Figure 3.5B). Interestingly, Nrf2 interaction with the ARE2 probe was ~50% of ARE5 whereas mutating both the ARE5 and ARE2 sequences significantly reduced the enrichment of Nrf2 (Figure 3.5B).
Figure 3.4: Luciferase Reporter Activity of ABCC3 Intron in HEK293 Cells. Luciferase reporter activity of the eighth ABCC3 intron cloned into pT81 reporter vector and transfected into HEK293 cells. Nrf2 was subsequently activated by treatment of cells with 50 µM tBHQ. Constructs containing mutations of the two identified intronic AREs (ARE2 and ARE5) were also used to independently determine functional activity of each (A). Data represents the results of three independent experiments done in triplicate for each group. Fold-change from treated pT81-intron is depicted in panel B. * p ≤ 0.05 versus DMSO treatment, † p ≤ 0.05 versus tBHQ treated intron.
**Figure 3.5: DNA Pull-down of Nrf2.** Recombinant Nrf2 (GST-tagged) and its heterodimeric partner, MafG were incubated with 5’ biotin-labelled oligonucleotide probes containing ARE2 or ARE5 along with their native flanking sequences. The complex was “pulled-down” using streptavidin beads and the precipitated complex was subjected to Western blot analysis using a commercially available antibody against GST (Nrf2). Probes containing the NQO1 wild-type and NQO1 mutant ARE were used as positive and negative controls, respectively. Input represents the amount of Nrf2-GST used in each incubation and used directly in SDS-PAGE and Western blot. Results are shown in panel A with respective densitometry analysis in panel B.
Discussion

The purpose of the current study was to identify the ARE(s) responsible for the Nrf2-dependent induction of the human ABCC3 gene and our investigation herein describes a novel mechanism responsible for this induction. Using high-throughput ChIP-sequencing followed by in silico analyses we have successfully identified two putative AREs located within the eighth intron of the ABCC3 gene. Functional characterization using gene reporter assays confirms a functional ARE response element which is unresponsive to Nrf2 activity and binding upon mutation. Furthermore, comparative sequencing analyses identify one of these AREs, ARE5, as being evolutionarily conserved across several species, suggesting a potentially important role in mediating the Nrf2-dependent regulation of human ABCC3.

Xenobiotic membrane transporters are pivotal players in mediating the absorption, distribution and excretion of both endo- and xenobiotics. The function of these transporters may directly impact the biological effectiveness and toxicity of their chemical substrates, and understanding the multiple levels of membrane transporter regulation is crucial in determining functionality. There are multiple lines of evidence to support Nrf2-mediated transcriptional regulation of several ABCC gene family members, including Abcc3, in mice. Studies conducted in mice and rats treated with known Nrf2 activators such as oltipraz, butylated hydroxyanisole and ethoxyquin have shown significant gene induction of Abcc3 along with other Abcc family members such as Abcc2 and Abcc4 (Maher et al., 2005; Merrell et al., 2008; Slitt et al., 2003). Additionally, several investigators have reported a loss of Abcc3 induction in Nrf2-null mice following treatment with a variety of
Nrf2 chemical activators (Okada et al., 2008; Tanaka et al., 2009; Aleksunes et al., 2008; Maher et al., 2008; Maher et al., 2007). Together, these findings suggest a clear role for the Nrf2-dependent regulation of Abcc3 in rodents; however, the mechanistic understanding of this regulation of human ABCC3 still remains unclear.

Investigation into the mechanism that drives the regulation of human ABCC3 by Nrf2 is limited. However, several correlative reports strongly demonstrate the role of Nrf2-dependent induction of the ABCC3 gene. Previous observations in primary human hepatocytes show ABCC3 up-regulation following treatment with the Nrf2 activator oltipraz (Jigorel et al., 2006). Similarly, human derived HepG2 cells treated with tBHQ increased levels of ABCC3 mRNA (Adachi et al., 2007). Lastly, several malignancies that have Nrf2 mutations are associated with high levels of ABCC3 expression (Wang et al., 2010; Sasaki et al., 2012). The first mechanistic investigation into Nrf2-dependent induction of ABCC3 was performed in human lung cancer cell lines in which the investigators describe the identification of four putative AREs upstream of the TSS, but did not attempt to functionally assess the contributions of these AREs (Mahaffey et al., 2009). Using ChIP PCR analyses, Mahaffey et al later demonstrated that upon treatment of a lung carcinoma cell line with the Nrf2 activator, 4-hydroxynonenol, a specific interaction occurred between Nrf2 and a genomic region -805 bases from the transcription start site of the ABCC3 gene (Mahaffey et al., 2012). However, our results in A549 cells reveal no interaction between Nrf2 and the promoter region of the ABCC3 gene. This discrepancy may be due to the different approach used to investigate the interaction between Nrf2 and the ABCC3 gene. The investigators use a ChIP-PCR method and limited
their genomic region of interest to just the promoter region. In contrast, using ChIP, followed by sequencing analyses, allows for a higher throughput method that does not limit the investigation to a particular chromosomal region.

In addition to Nrf2 association within the eighth intron, our analysis yields novel information regarding H3K4 methylation status of the \textit{ABCC3} gene. Our data suggests that the \textit{ABCC3} gene is hypermethylated within several regions including the promoter and the eighth intron where we have identified Nrf2 association. Interestingly, unlike other methylation sites, methylation of H3K4 is almost exclusively found within regions of transcriptionally active genes (Shilatifard, 2008). The identification of H3K4 hypermethylation within the eighth intron further supports our findings that a functional ARE is present within this region. Recent studies have implicated an important role of the H3K4 methyltransferase, mixed lineage leukemia 3, in the transcriptional regulation of several clinically important membrane transporters including MDR1 and the Farsenoid X receptor-dependent transcriptional regulation of hepatic \textit{ABCC2}, \textit{ABCB11}, and \textit{SLC10A1} (Ananthanarayanan et al., 2011; Huo et al., 2010). However, the role of H3K4 methylation and the Nrf2-dependent induction of \textit{ABCC3} has not been investigated and further analyses are necessary to elucidate the importance of histone 3 methylation within the eighth intron of this gene.

A recent investigation by Malhotra et al. utilized a ChIP-seq analysis approach in Keap1-null mice (constitutive Nrf2 activation). Although the regulation of mouse \textit{Abcc3} gene was not specifically examined, these studies did reveal interesting information concerning the ARE consensus sequence (Malhotra et al., 2010). Historically, the
definition of the ARE is a core sequence of “TGACnnnGC” (see Figure 3). Using a larger data set of 410 DNA sequences, these new studies reveal an expanded role of the intervening three base pairs (between TGAC and GC), with TCA being the most common sequence observed. Interestingly, the intervening bases (nnn) of this consensus sequence (TCA) are more homologous to the ARE5 core sequence (TAA) than the ARE2 (TTG). It is possible that this difference seen in the intervening bases between ARE2 and ARE5 may partially explain why Nrf2 binds more strongly to ARE5 as well as it being the functionally dominant ARE. It is interesting to note, however, that mutating ARE5 did not completely abolish reporter activity upon Nrf2 activation. This observation may be partially explained by the fact that mutant ARE5 still confers slight affinity for Nrf2 as seen by the DNA-binding assay.

The identification of putative AREs within the eighth intron of the ABCC3 gene occurred within a region that is conserved across mouse and human suggesting that this region within the mouse genome may have ARE(s) as well. A previous study conducted in a mouse liver cell line has identified an ARE upstream of the promoter region using ChIP-PCR analysis (Maher et al., 2007). Interestingly, this region is not conserved in the human (see Figure 2) despite a 3-4 fold induction in Abcc3 following oltipraz treatment in two, independent studies in mice (Aleksunes & Klaassen, 2012; Maher et al., 2007). Using primary human hepatocytes, Jigorel et al showed a 1.5 fold induction of ABCC3 upon oltipraz treatment suggesting that mice may be more sensitive to chemical induction of ABCC3 by Nrf2 (Jigorel et al., 2006). Together, these findings support the need for further
investigation into whether additional functional ARE(s) are present within the eighth intron of the mouse *Abcc3* gene.

The identification of gene regulatory elements within regions downstream of the TSS is a rare occurrence, but has been reported in various genes by various transcription factors. Many of these downstream elements are located in the first intron, however, there are several examples revealing distal elements that can regulate the transcription of genes. ChIP-seq experiments have reported that 38% of ERα binding elements were found within introns along with an additional 38% being further than 10kb from the TSS (Levy et al., 2008). Other examples of nuclear receptors with identified response elements within an intronic region include the androgen receptor (Zheng et al., 2006), the vitamin D receptor (Zella et al., 2006), and PPARα (Sun et al., 2008). In addition, androgen regulation of Nrf2-target gene GSTP1 appears to be dependent upon a 500bp region of the fifth intron of the gene (Ikeda et al., 2002); however, to our knowledge, this is the first example of Nrf2 itself potentially regulating a target gene through an intronic response element.

In addition to the role of Nrf2 in the induction of *ABCC3* in cancerous tumors, Nrf2 activation and subsequent gene expression alterations is thought to be a cornerstone in the pathogenesis of NASH. Nrf2 activation was previously reported in mouse models of nonalcoholic steatohepatitis (NASH) with a concomitant increase in expression of *Abcc3* (Lickteig et al., 2007b; Lickteig et al., 2007a). Similarly, humans diagnosed with NASH have increased hepatic Nrf2 activation along with increased *ABCC3* expression in the liver (Hardwick et al., 2010; Hardwick et al., 2011). Identifying specific AREs responsible for the Nrf2-dependent induction of *ABCC3* and other transporters may provide important
information that may provide potential avenues for therapeutic intervention to modulate
ABCC3 expression in diseases such as NASH.

In conclusion, we have identified two putative AREs in the eighth intron of the human ABCC3 gene. In silico analyses have shown that this region is highly conserved across several mammalian species suggesting an important role for this genomic region in the Nrf2-dependent induction ABCC3. Furthermore, functional characterization of these novel elements revealed that one of these AREs is functionally important for the Nrf2-dependent induction of the ABCC3 gene. These findings are the first to our knowledge to identify an Nrf2 regulatory element within an intronic region of a gene. This novel finding may promote the future identification of AREs within non-flanking regions of genes and further elucidate non-traditional mechanisms of gene regulation. Moreover, the results obtained in this study functionally link Nrf2 activation and ABCC3 induction, suggesting a potential mechanism for ABCC3 gene induction observed in NASH.
CHAPTER 4: RENAL XENOBIOTIC TRANSPORTER

EXPRESSION IS ALTERED IN MULTIPLE EXPERIMENTAL RODENT MODELS OF NONALCOHOLIC STEATOHEPATITIS

Text and Figures in this section are derived from: Canet MJ, Hardwick RN, Lake AD, Dzierlenga AL, Clarke JD, Goedken MJ, and Cherrington NJ. 2014. Submitted to Drug Metabolism and Disposition for publication.

Introduction

Nonalcoholic fatty liver disease (NAFLD) represents a range of distinct liver histopathologies, ranging from simple steatosis to the more advanced nonalcoholic steatohepatitis (NASH) (Lomonaco et al., 2013). Although the mechanisms are not entirely understood, it is generally well accepted that NASH progression involves several “hits,” such as inflammation and oxidative stress, that may act independently or in parallel to drive disease progression (Rahimi & Landaverde, 2013; Lomonaco et al., 2013). The role of adipocytes has recently emerged as being instrumental in the development and propagation of inflammation in NASH. In obese states, fat-laden adipocytes are a primary source for the secretion of pro-inflammatory cytokines, including tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), and interleukin-1β (IL-1β), which contribute to the increased systemic levels readily observed in NASH (Hotamisligil et al., 1993; Tilg, 2010; Kochi et al., 2014). The induction of these pro-inflammatory cytokines within adipose tissue may act in a paracrine fashion by targeting the liver and propagating the development of NASH
pathogenesis (Tilg, 2010). Moreover, inflammatory mediators, particularly TNF-α, activate various intracellular signaling cascades and may contribute to the extensive dysregulation of hepatic gene expression profiles observed in the disease (Moylan et al., 2014).

NASH causes extensive alterations in the regulation of hepatic xenobiotic transporters of both human and rodent models, leading to the functional disruption of acetaminophen, ezetimibe, and simvastatin (Hardwick et al., 2012; Clarke et al., 2014a; Lickteig et al., 2007a). Moreover, the results highlighted in Chapter 2 clearly demonstrate a coordinated response in membrane transporter regulation across several rodent models. However, little information is currently known regarding the regulation of xenobiotic transporters in distal sites during times of hepatic stress, which may further contribute to altered xenobiotic disposition in hepatic disease states.

The kidneys serve as important sites for xenobiotic excretion, and an estimated 30% of the top 200 prescribed drugs in 2010 are eliminated by renal clearance (Morrissey et al., 2013). Studies using experimental models of cholestasis have linked liver dysfunction with compensatory responses in the regulation of xenobiotic transporters in the kidneys. Particularly, liver-specific adaptations in xenobiotic transporters occur to restrict further hepatic exposure to toxic levels of bile acids, resulting in higher systemic levels of potentially toxic bile acids (Keppler, 2011). However, cholestatic stress also results in compensatory adaptations in the expression of renal efflux and uptake transporters that may function to facilitate the secretion of bile acids into the urine (Slitt et al., 2007; Brandoni et al., 2006b). A recent study confirmed the functional outcome of these
adaptations by demonstrating increased cimetidine clearance as a result of kidney Oct2 induction in a rat model of cholestasis (Kurata et al., 2010). Together, these findings demonstrate the influence of liver dysfunction on kidney-specific handling of xenobiotics, which may impact their renal secretion.

The purpose of the current study was to investigate the effects of NASH on xenobiotic transporter expression in the kidneys. Several rodent models used in the studies outlined in Chapter 1 were subsequently used and a comprehensive analysis of kidney mRNA and protein expression profiles across these models was performed. These studies contribute to the hypothesis that during times of hepatic stress, adaptations in kidney function also occur to limit the accumulation of endogenous and exogenous compounds in systemic circulation. Moreover, these findings will change the manner in which we investigate disease-mediated effects on xenobiotic disposition by confirming that physiological adaptations occur in distal tissue sites, which can ultimately contribute to inter-individual variability in response to xenobiotics.

**Materials and Methods**

**Materials**

Tris-HCl, ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), glycerol, potassium phosphate (KPO₄), potassium chloride (KCl), sodium pyrophosphate (decahydrate), and Nonidet P-40 were obtained from Sigma-Aldrich (St. Louis, MO). Neutral buffered formalin (10%) was obtained from Fisher Scientific (Pittsburgh, PA).
**Animals**

Eight to ten week old, male, C57BL/6J, B6.Cg-Lep<ob>/J (ob/ob), and B6.BKS(D)-Lepr<db>/J (db/db) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Eight to ten week old, male, Sprague Dawley and Crl:ZUC-Lepr<fa> fatty (fa/fa) rats were obtained from Charles River Laboratories (Wilmington, MA). All animals were acclimated in 12 hour light and dark cycles in a University of Arizona AAALAC-certified animal facility for at least one week prior to initiation of experiments and were given access to standard chow and water *ad libitum*. Housing and experimental procedures were in accordance with NIH guidelines for the care and use of experimental animals. To model NASH, C57BL/6J mice and Sprague Dawley rats (N=4) were fed either a methionine and choline deficient (MCD) diet (#518810) (Dyets, Inc., Bethlehem, PA), or an atherogenic diet (#D06061401) (Research Diets Inc., New Brunswick, NJ) for 8 weeks. As a control, C57BL/6J mice (N=4) and Sprague Dawley rats (N=4) were fed a methionine and choline re-supplemented diet (#518754) (Dyets, Inc., Bethlehem, PA). The ob/ob (N=4) and db/db (N=4) mice were fed a MCD diet for 4 weeks to induce NASH. The fa/fa rats were provided a modified high fat diet (#101447) for eight weeks (Dyets, Inc., Bethlehem, PA).

**Tissue Harvesting**

At the conclusion of dietary feeding, the animals were euthanized via CO₂ asphyxiation. The kidneys were immediately harvested and a small portion of each was fixed for two days in 10 % neutral buffered formalin (4°C), followed by tissue processing and paraffin-
embedding at the University of Arizona Histology Core Facility. The remaining tissue was
snap frozen in liquid nitrogen and stored at -80°C for future analyses.

*Tissue Staining and Evaluations*

Paraffin-embedded kidney and liver sections were stained with hematoxylin and eosin
(H&E) at the University of Arizona Histology Core. Kidney sections were evaluated and
scored for renal injury. Liver sections were injury scored according to a previously
validated NASH scoring method (Kleiner et al., 2005) and the results have been shown
previously (Chapter 2).

*RNA Purification*

Total RNA was extracted and isolated from rat and mouse kidney using RNAzol B reagent
(Tel-Test Inc., Friendswood, TX) per the manufacturer's protocol. RNA concentrations
were determined using UV spectrophotometry, and the integrity of the RNA was confirmed
by ethidium bromide staining after agarose gel electrophoresis.

*Branched DNA (bDNA) Analysis*

bDNA analysis was used to determine mRNA transcript levels of transporter genes.
Specific oligonucleotide probes for Mrp2, Mrp4, Mdr1a, Bcrp, Oatp1a1, Oat1, Oat3, Oct1,
and Oct2 were diluted in lysis buffer supplied by the Quantigene HV Signal Amplification
Kit (Genospectra, Fremont, CA). Substrate solution, lysis buffer, capture hybridization
buffer, amplifier, and label probe buffer used in the analysis were all obtained from the
Quantigene Discovery Kit (Genospectra). The assay was performed in 96-well format with 10 µg of total RNA added to the capture hybridization buffer and 50 µl of the diluted probe set. The total RNA was then allowed to hybridize to the probe set overnight at 53°C. Hybridization steps were performed per the manufacturer's protocol the following day. Luminescence of the samples was measured with a Quantiplex 320 bDNA luminometer interfaced with Quantiplex Data Management Software, version 5.02 (Bayer, Walpole, MA).

Protein Preparations

Whole cell lysate preparations of mouse and rat kidney were prepared from ~200 mg of tissue homogenized in NP-40 buffer (20 mM Tris HCl, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, and 2 mM EDTA) with 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) per 25 mL at 4°C. Homogenized tissue was then agitated at 4°C for 2 hours, centrifuged at 10,000 x g for 30 minutes, and the supernatant transferred to a clean collection tube. Rat kidney crude membrane fractions were prepared from ~100 mg of frozen tissue. Briefly, tissue was homogenized in homogenization buffer (100 mM Tris HCl pH 7.4) with added Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) per 25 ml at 4°C. The resulting homogenate was centrifuged at 1500 x g for 10 minutes at 4°C and the supernatant was collected into ultra-centrifuge tubes and centrifuged at 100,000 x g for 70 minutes at 4°C. The resulting pellet was resuspended in 500 µl of homogenization buffer from above. Protein concentrations for both whole cell and microsomal fractions were determined using the Pierce BCA Protein Quantitation Assay (Thermo Scientific,
Rockford, IL) per the manufacturer’s protocol, and all samples were stored at -80°C until further analysis.

**Immunoblot Protein Analysis**

Whole cell lysate or membrane fraction proteins (50 μg/well, 20 μg/well, respectively) were prepared in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) with or without β-mercaptoethanol and heated at 37°C for 30 minutes prior to separation by SDS-PAGE on 7.5 % gels. Resolved protein was transferred to polyvinylidene fluoride (PVDF) or nitrocellulose membranes for 70 min at 350 mAmps at 4°C. Following transfer, the membranes were blocked in 5 % nonfat dry milk diluted in phosphate-buffered saline-tween 20 for 1 hr at room temperature. To determine relative protein levels the following primary antibodies were used: P-gp, sc-8313; Oct1, sc-133866; Oatp1a1, sc-47265, (Santa Cruz Biotechnology, Santa Cruz, CA); Mrp2, ab15603; Mrp4, ab15602; Oat1, ab183086, (Abcam, Cambridge, MA); Bcrp, MC-981 (Kamiya Biomedical Co., Seattle, WA). The blots were incubated with primary antibody overnight at 4°C with constant rocking. The following HRP-conjugated secondary antibodies were used: anti-rat (sc-2065), anti-rabbit (sc-2004), anti-mouse (sc-2005), and anti-goat (sc-2350 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Quantification of relative protein expression was determined using image processing and analysis with Image J software (NIH, Bethesda, MD) and normalized to β-actin protein (whole cell lysate) (sc-47778, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or pan-cadherin (microsomal fraction) (ab16505, Abcam, Cambridge, MA).
Statistical Analysis

Data were analyzed using one-way ANOVA to determine significant differences between model groups with a Bonferroni post-hoc analysis. Histological scores were rank-ordered prior to analysis via ANOVA. A significance level of \( p \leq 0.05 \) was used to determine experimental significance. All analyses were carried out using GraphPad Prism software Version 5 (GraphPad Software, Inc., La Jolla, CA).

Results

Rodent NASH Liver Pathology

To determine NASH severity, pathological evaluations were conducted according to a validated scoring rubric (Kleiner et al., 2005), and are described in Chapter 2 (Table 2.3). Figure 4.1 shows representative H & E stained images of liver sections across all rodent models investigated. Diffusely, hepatocytes of MCD rats and mice, along with the \( ob/ob \) and \( db/db \) mice (Figure 4.1A and 4.1B) contained clear, well-defined macrovesicular vacuoles (i.e. hepatic steatosis). Prior pathological assessments had increased inflammation and mild perisinusoidal fibrosis in the rat MCD, rat and mouse atherogenic, and mouse \( db/db \) models (Table 2.3). In contrast, the rat and mouse atherogenic as well as \( fa/fa \) rat models had diffuse microvesicular clear vacuoles (i.e. lipid deposits) within the liver (Figure 4.1A and 4.1B). Incidence and severity scores confirmed that the rat MCD and atherogenic model as well as the \( db/db \) mice developed definitive NASH whereas the mouse MCD, atherogenic, and \( ob/ob \) models developed minor grades of the disease. Interestingly, the \( fa/fa \) rats failed to develop NASH.
**Figure 4.1: Liver Histology in Rodent NASH.** Representative hematoxylin and eosin stained liver sections from rat (A) and mouse (B) NASH models. Diffuse macrovesicular vacuoles are shown by the black arrowhead and represents a common pathological lesion in NASH. Images were taken at 20X magnification.
Rodent Kidney Pathology in NASH

Histological assessments were conducted to investigate the effect of NASH on renal morphology. The incidence and severity of kidney pathology (including tubular degeneration, necrosis and regeneration, parenchymal inflammation and glomerular mesangial expansion and/or hypertrophy) are summarized in Table 4.1. Across the rat models, a significant increase in overall severity scores was observed in the MCD, atherogenic, and fa/fa animals (Figure 4.2C). Glomerular changes (mesangial expansion and/or hypertrophy) were the most common observation(s) in MCD and atherogenic rats, whereas the fa/fa rats additionally developed parenchymal tubular changes (degeneration/necrosis/regeneration, protein casts) and parenchymal inflammation (Figure 4.2A, arrow, inserts; Table 1). Similarly, glomerular finding were more common than tubular injury in the mouse models (Figure 4.2, inserts). All mouse groups had increased overall severity scores with the exception of the db/db mice (Figure 4.2C and Table 4.1).
Figure 4.2: Kidney Histology in Rodent NASH. Representative hematoxylin and eosin stained kidney sections from rat (A) and mouse (B) NASH models. A significant number of protein casts was present in the kidneys of fa/fa rats (4.2A; arrow). Images were taken at 20X magnification. Higher magnification (100X) images of glomerular changes were captured and shown as an insert to each figure. Pathological scoring evaluations describing total kidney injury scores (C) are shown. Horizontal bar represents the median of data (N=4 animals) and * p ≤ 0.05 versus control within each group. Data was ranked-ordered prior to statistical analysis. Scoring key: 0- none; 1- minimal (<10% affected); 2- mild (10-25% affected); 3- moderate (25-40% affected); 4- marked (40-50% affected); 5- severe (>50% affected).
Table 4.1: Kidney Pathology Scoring in NASH Models. Kidney pathology was assessed via the lesions described below. Values represent the median (range) of N=4 animals and were rounded to next whole number (median of 1.5 is reported as 2). Scoring key: 0- none; 1- minimal (<10% affected); 2- mild (10-25% affected); 3- moderate (25-40% affected); 4- marked (40-50% affected); 5- severe (>50% affected).

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Control</th>
<th>MCD</th>
<th>Athero</th>
<th>fa/fa</th>
<th>Mice</th>
<th>Athero</th>
<th>ob/ob</th>
<th>db/db</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubular Degeneration</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (1-2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Regeneration</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (0-1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Inflammation</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (2-4)</td>
<td>1 (0-1)</td>
<td>0 (0-1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Mesangial Expansion</td>
<td>0 (0)</td>
<td>1 (1-2)</td>
<td>1 (1-2)</td>
<td>1 (0-1)</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
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<tr>
<td>Glomerular Hypertrophy</td>
<td>0 (0)</td>
<td>0 (0)</td>
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Kidney Transporter mRNA Expression in Rodent NASH

mRNA quantification analyses were conducted on renal transporter genes to determine the effect of NASH on their regulation (Figure 4.3 and Figure 4.4). Among the uptake transporters investigated in rats, no significant changes in gene expression were noted with the exception of Oat3, which was induced in rat MCD whereas Oatp1 was repressed in the MCD, athero, and fa/fa rat models (Figure 4.3A). In contrast, Oct2 and Oat1 were down-regulated in the MCD, ob/ob, and db/db mice along with an induction seen in the atherogenic model (Figure 4.3B). Like the rats, Oat 3 was significantly induced in the ob/ob and db/db mouse models whereas Oatp1 expression was repressed in the MCD, ob/ob and db/db models and up-regulated in the atherogenic mice (Figure 4.3B).

In contrast to uptake transporters, efflux transporter mRNA was generally up-regulated in the rodent models with confirmed-NASH (Figure 4.4). Mrp2, Mrp4, Bcrp, and Mdr1a (P-gp) genes were all induced in the rat MCD model whereas no changes were observed in the atherogenic and fa/fa rats (Figure 4.4A). Similarly, Mrp2 was induced in the ob/ob mice whereas Mdr1a expression was up-regulated in the MCD, atherogenic, ob/ob and db/db mouse models (Figure 4.4B). No changes were observed in Bcrp expression; however, Mrp4 was induced in the kidney of MCD, ob/ob, and db/db mice (Figure 4.4B).
Figure 4.3: Kidney Uptake Transporter mRNA Expression in Rodent NASH. mRNA expression of rat uptake (A) and mouse uptake (B) transporters in the kidneys of rodent NASH models via bDNA gene analysis. Data represent the mean ± S.E.M from 4 animals. * p ≤ 0.05 versus control within each group.
4.4: Kidney Efflux Transporter mRNA Expression in Rodent NASH. mRNA expression of rat efflux (A) and mouse efflux (B) transporters in the kidneys of rodent NASH models via bDNA gene analysis. Data represent the mean ± S.E.M from 4 animals. * p ≤ 0.05 versus control within each group.
Kidney Transporter Protein Expression in Rodent NASH

Protein expression of membrane transporters across rodent models was also investigated and shown in Figure 4.5. Consistent with the mRNA analyses, protein expression of the renal efflux transporters Mrp2 and P-gp was induced in the MCD rats (Figure 4.5A). Mrp4 expression was also up-regulated, but these results did not reach statistical significance (Figure 4.5A). In addition, P-gp expression was induced in the atherogenic rat model. Interestingly, protein expression of Bcrp was down-regulated in the kidney of fa/fa rats (Figure 4.5A). Oatp1a1, Oat1, and Oct1 renal uptake transporter expression was not significantly changed at the protein level in the rat models. Oatp1 expression tended to decrease in the MCD model, but these results did not reach statistical significance (Figure 4.5A).

Similar to the rats, efflux transporter expression in the kidney was generally induced at the protein level in mice with NASH (Figure 4.5B). Specifically, Mrp4 and P-gp protein expression was induced in the mouse MCD, ob/ob, and db/db models. Bcrp expression did not change across models except for atherogenic mice, which had a significant decrease in Bcrp protein expression in the kidney (Figure 4.5B). In contrast, Oatp1 protein expression was significantly reduced in the mouse ob/ob and db/db models. No change in Oat1 expression was observed in the mice; however, it tended to decrease in the db/db model.
**Figure 4.5: Kidney Protein Expression of Membrane Transporters in Rodent NASH.**

Western blot analyses depicting rat (A) and mouse (B) transporter expression in the kidneys of rodent NASH models. Densitometry analysis and representative Western blots are shown. β-actin was used as a loading control for whole cell lysates, whereas pan-cadherin was used for crude membrane preparations. Rat Mrp2 and Oct1 protein were analyzed using crude membrane preparations whereas the remainder (mouse and rat) were analyzed in whole cell fractions. Data represent the mean ± S.E.M from 4 animals. * p ≤ 0.05 versus control within each group.
Discussion

The increase in adverse drug reaction incidents has become a significant health concern worldwide. In the United States, ADRs have become one of the top-10 causes of death, clearly highlighting the need for more effective pharmacovigilant practices within the healthcare industry (Wooten, 2010b; Valente & Murray, 2011). Many ADRs can be attributed to inter-individual variations in xenobiotic disposition, which may be preventable by identifying factors that contribute to these variations. In addition, recent advances in our understanding of the impact of disease states on xenobiotic pharmacokinetics have helped identify populations that may be at risk for developing ADRs. In particular, diseases that manifest in the liver have gained increased attention given the importance of the liver in mediating xenobiotic metabolism and disposition. Despite previous evidence demonstrating adaptive responses in the kidney in cholestatic disease, our understanding of how NASH affects renal clearance is lacking and necessitates further investigation.

The purpose of this study was to determine the effects of NASH on the regulation of renal membrane transporters. The use of several models in these investigations allows for a more comprehensive profile that strengthens our findings and allows translating these findings to the human condition more feasible, considering it is currently unknown how the regulation of renal xenobiotic transporters is altered in human NASH. Our results demonstrate that the development of NASH causes significant alterations in the expression of several membrane transporters in the kidneys of various rodent models. In particular, we observe a coordinated up-regulation of Mrp2, Mrp4, and Pgp, suggesting that during
times of hepatic stress, the kidneys may compensate by facilitating the renal secretion of xenobiotics and endogenous substrates, such as bile acids, into the urine. Similar compensatory changes are observed in other models of liver injury (Slitt et al., 2007). Using bile duct-ligated mice, Slitt et al. demonstrated an induction of renal Mrp1-5 mRNA and a reduction in Oatp1a1 mRNA expression. Interestingly, our data also show a down-regulation of renal uptake transporter Oatp1a1 expression in NASH, which facilitates the reabsorption of compounds from the renal tubule filtrate, suggesting an overall shift from renal reabsorption to renal secretion of organic anions in NASH. Furthermore, Mrp2 induction in the kidney was observed in various rat models of cholestasis (Lee et al., 2001) as well as liver ischemia-reperfusion injury (Tanaka et al., 2008), which is consistent with our findings in the pathologically-confirmed NASH models (MCD, ob/ob, and db/db rodents).

The induction of renal Mrp2 is a common observation across several cholestatic injury models. It is proposed that Mrp2 induction in the kidneys may serve as an alternative route of elimination of bile acids in situations in which liver function is compromised (Klaassen & Aleksunes, 2010). Similar to cholestasis, hepatic Mrp2 function is significantly reduced in NASH whereas Mrp4 is induced leading to a functional shift in the disposition of xenobiotics from bile to plasma (Hardwick et al., 2011; Hardwick et al., 2012; Lickteig et al., 2007a). The similarity in the changes to hepatic and renal transporter gene expression between cholestasis and NASH is suggestive of a common mechanism mediating these effects. Recent findings show that mice fed an MCD diet develop intrahepatic cholestasis, leading to increased plasma bile acid levels (Wu et al., 2014).
Furthermore, Tanaka et al. has demonstrated that Mrp2 expression is elevated following treatment of renal proximal tubule cells with conjugated bilirubin or human bile suggesting that bile acids and/or bile constituents may directly regulate Mrp2 gene transcription, possibly through the nuclear receptors farnesoid X receptor and/or pregnane X receptor (Tanaka et al., 2002; Kast et al., 2002). Together, these results demonstrate that the up-regulation of renal Mrp2 may partially be explained by direct exposure of bile acids to the kidneys due to the development of cholestasis secondary to NASH.

Similar to renal efflux transporters, the expression of Oat3 in the kidney is significantly elevated in rodents with NASH. In particular, we observe an induction of renal Oat3 in the rat MCD as well as the mouse ob/ob and db/db models. In contrast, our data suggests that Oat1 protein expression is not changed, although levels tend to decrease in the ob/ob and db/db models. Similar findings were reported previously in experimental models of cholestasis. Chen et al. demonstrated an induction of Oat3 protein in the kidney whereas Oat1 levels did not change in Eisai hyperbilirubinemic (EHBR) rats, which lack functional Mrp2 and serve as a cholestatic model (Chen et al., 2008). However, Oat3 mRNA in EHBR did not change whereas we observed an induction, suggesting an alternative mechanism of regulation in NASH. Several investigations have shown that members of the Oat transporter family, in particular Oat1 and Oat3, are post-transcriptionally regulated by intracellular phosphorylation events mediated by PKC and PKA. PKC activation causes a down-regulation of Oat3-mediated uptake of esterone sulfate, whereas PKA activation leads to increased transport activity, suggesting that differential activation of protein kinase cascades may cause tissue and disease-specific
transporter regulation (Soodvilai et al., 2004). The effect of NASH on renal PKC and PKA regulation is not completely understood and further investigations are needed to clarify the mechanistic role of renal Oat3 induction in NASH.

Liver damage sustained in NASH results in inflammation, and oxidative stress, leading to hepatocellular damage, and the subsequent activation of cellular defense mechanisms such as the nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor (Hardwick et al., 2010). Nrf2 is known to regulate several membrane transporter genes and its activation in NASH may partially explain the induction of hepatic efflux transporters, particularly Mrp3 and Mrp4 (Klaassen & Aleksunes, 2010). However, our results suggest that renal injury in the rodent models with NASH is minimal and lack significant evidence of inflammation and oxidative kidney injury. In contrast, the fa/fa, rat model, which failed to develop pathological NASH, had significant renal injury, and yet renal transporter expression in this rodent model did not vary significantly from control rats. Together, these findings suggest that direct renal injury is not a primary factor in membrane transporter regulation in NASH and that the minimal renal injury sustained in rodents with NASH may be due to liver-derived inflammation.

Alternatively, the pathological disturbances that occur in the liver may secondarily affect distal tissue function through the release of cellular mediators that may act in a paracrine signaling fashion. Chronic liver inflammation observed in NASH is associated with increased systemic levels of pro-inflammatory cytokines such as TNF-α and IL-6 (Alaaeddine et al., 2012; Kochi et al., 2014; Carter-Kent et al., 2008). Moreover, several independent studies have shown a direct role of pro-inflammatory cytokines in mediating
the regulation of membrane transporters. Treatment of primary hepatocytes with TNF-α and IL-6 results in marked alterations in membrane transporter expression (Le et al., 2009). Additionally, IL-1β exposure results in the down-regulation of membrane transporters in human hepatocytes (Le et al., 2008). Together, these findings suggest pro-inflammatory cytokines as a potential mediator in the regulation of hepatic and renal membrane transporters in NASH. Furthermore, TNF-α and IL-6 induction in NASH may regulate renal transporter expression and function in a paracrine fashion. It has recently been shown that both TNF-α and IL-6 have differential effects on transporter expression across cell lines differing in tissue origin as well as different cell lines derived from the same tissue (Le et al., 2009; Mosaffa et al., 2009; Malekshah et al., 2012). Further investigation is needed to characterize the differential effects of pro-inflammatory cytokines on hepatic and renal xenobiotic transporter expression in NASH.

The altered regulation of renal transporters in disease states such as cholestasis has resulted in functional disturbances in xenobiotic disposition. Induction of Oct2 in the kidney following bile duct-ligation increases cimetidine clearance in rats (Kurata et al., 2010). Additionally, disturbances in the renal secretion of bromosulphophthalein and p-aminohippurate in rodent cholestatic models were linked to altered transporter function in the kidney (Brandoni & Torres, 2009; Brandoni et al., 2006a). However, information is lacking regarding the function of membrane transporters in the kidney during diseases such as NASH.

In conclusion, we have demonstrated that rodent models with NASH pathology cause significant alterations to membrane transporter expression in the kidney. In
particular, we observe a general induction of renal apical efflux transporters as well as the basolateral uptake transporter, Oat3, whereas Oatp1 expression is significantly down-regulated. Together, these data suggest a coordinated regulation of renal membrane transporters in NASH that favors renal secretion. This may serve as an adaptive response mechanism that facilitates the elimination of xenobiotics and bile acids during times of hepatic stress. Furthermore, our data demonstrate that the manifestation of NASH fails to cause significant pathology in the kidney, suggesting that direct tissue injury is not responsible for the changes observed in transporter regulation. These findings highlight the importance of investigating the contribution of renal elimination mechanisms during hepatic disease states, which may globally affect xenobiotic disposition and contribute to the development of adverse drug reactions.
CHAPTER 5 - ALTERED ARSENIC DISPOSITION IN A MOUSE MODEL OF NONALCOHOLIC FATTY LIVER DISEASE: THE ROLE OF LIVER AND KIDNEY TRANSPORTERS

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is now recognized as the most common cause of liver disease (Marra et al., 2008). It is comprised of a spectrum of liver pathologies that vary in severity from simple fatty liver (hepatic steatosis), to the more advanced form of nonalcoholic steatohepatitis (NASH), which is characterized by an increase in hepatocellular damage, inflammation, and varying degrees of fibrosis (Feldstein, 2010; Ali & Cusi, 2009).

The liver plays a crucial role in the metabolism and elimination of many environmental toxicants and clinically relevant drugs from the body. Hepatic metabolizing enzymes as well as membrane transporters are pivotal in governing the pharmacokinetic fate of many xenobiotics. The liver damage manifested throughout the progressive stages of NAFLD was shown to disrupt the expression of clinically important membrane transporters and phase I/II drug metabolizing enzymes in the liver of both rodent models
and human NASH, leading to the disruption in the pharmacokinetics of drugs (Hardwick et al., 2011; Fisher et al., 2009a; Lickteig et al., 2007a). Although knowledge is emerging on the functional capacity of the liver to properly eliminate pharmaceuticals in the context of NASH, little information is currently available regarding the effect of NASH on the metabolism and excretion of environmental toxicants, such as arsenic.

Arsenic is a toxic metalloid found naturally at various levels in water, soil, and food. Chronic exposure to arsenic, namely via contaminated drinking water, is associated with a host of health-related effects including peripheral vascular diseases and various forms of cancer (Yoshida et al., 2004). Paradoxically, arsenic trioxide was used for centuries in the management of various diseases including psoriasis and syphilis and is currently approved by the US Food and Drug Administration for the treatment of relapsing acute promyelocytic leukemia (Dilda & Hogg, 2007).

In mammals, inorganic arsenic is primarily metabolized in the liver via an oxidative biomethylation scheme sequentially yielding both monomethyl-As (MMA) and dimethyl-As (DMA) metabolites (Drobna et al., 2010) (see Appendix C for chemical structure of species). The methylation of arsenic is catalyzed by a single enzyme, arsenic (3+ oxidation state) methyltransferase (As3MT) (Lin et al., 2002a), leading to the formation of both trivalent and pentavalent MMA and DMA metabolites, which are predominantly excreted in the urine (Thomas, 2007a). Although biomethylation of arsenic was once viewed as a detoxication process, the formation of the intermediate trivalent species is now considered to be an activation mechanism due to the more potent toxicity of these species compared to the pentavalent form (Wang et al., 2007a).
There is currently a limited amount of data concerning how pathophysiological factors such as disease may influence the metabolism and/or excretion of environmental toxicants. The purpose of the current study was to determine whether experimentally-induced NASH in mice alters the disposition of arsenic. Sodium arsenate was used to model environmental exposure, whereas arsenic trioxide was used to replicate clinical exposure in this study. Understanding the effects of liver disease on arsenic disposition may have important implications in predicting toxicity following exposure to environmental toxicants in individuals with NASH.

**Materials and Methods**

*Materials*

Sodium arsenate (Acid Heptahydrate Sodium Salt) and HPLC-grade H2O was obtained from Fisher Scientific (Pittsburgh, PA). Arsenic trioxide was a kind gift from Michael J. Kopplin at the University of Arizona. Both arsenical compounds used in dosing were determined to be >99.8% pure. HClO4, HgCl2, KOH, Tris-HCl, EDTA, NaCl, glycerol, and Nonidet P-40 were obtained from Sigma-Aldrich (St. Louis, MO).

*Animals*

Male C57BL/6 mice weighing 20-25 grams were obtained from Harlan Laboratories (Indianapolis, IN). All animals were acclimated in 12 hour light and dark cycles in a University of Arizona AAALAC-certified animal facility for one week prior to initiation of experiments and were allowed standard chow and water *ad libitum*. Housing and
experimental procedures were in accordance with NIH guidelines for the care and use of experimental animals. Mice were fed an isolcaloric diet (N=3) (#180820) as a control or a high fat diet (N=5) (high cholesterol, 18% butter fat) diet (#112280) for five weeks to induce simple steatosis (Dyets, Inc., Bethlehem, PA). Mice were placed on a methionine and choline deficient (MCD) diet (N=5) (#518810) for eight weeks to induce NASH or a methionine and choline re-supplemented diet (N=3) (#518754) as a control (Dyets, Inc., Bethlehem, PA). Due to no histopathological changes observed between both control diets (isocaloric and methionine choline re-supplemented diets) they were both combined as a single control group in statistical analyses for all experiments conducted in this study.

*Arsenic Disposition Study*

Following the time allotted on the respective diets, the mice were given a single, oral dose of either 0.75 mg/kg sodium arsenate or 0.2 mg/kg arsenic trioxide dissolved in HPLC-grade H₂O. Dosing was performed using 20 gauge feeding needles (Fisher Scientific, Pittsburgh, PA). Following dosing, the mice were placed in Nalgene metabolic cages, where total urine was collected for 24h. At the conclusion of the 24h period, the mice were euthanized by CO₂ asphyxiation followed by collection of livers and kidneys. A section of each liver was cut and fixed with 10% neutral-buffered formalin overnight followed by paraffin embedding for hematoxylin and eosin staining. The remaining liver, along with kidneys were snap-frozen in liquid nitrogen, and stored at -80º C until analysis. Total urine was transferred to a sterile collection tube and stored at -80º C prior to analysis.
Sample Preparation

The extraction of arsenical species from liver and kidney was carried out using previously described methods (Csanaky & Gregus, 2003). All reagents were prepared in HPLC-grade H₂O. Briefly, ~200 mg of tissue was homogenized in 1 ml of cold 0.4 M HClO₄. Five hundred micro liters of homogenate was transferred to a sterile collection tube containing 50 µl of aqueous 150 mM HgCl₂ then gently mixed and kept on ice for one minute. The samples were centrifuged at 15,850 x g for two minutes at 4°C. The supernatant was removed and transferred to a sterile collection tube and the pH was adjusted to ~2 using 1 M KOH. Samples were centrifuged at 15,850 x g for two minutes at 4°C and the supernatant was removed and stored at -80°C prior to analysis for arsenic speciation. It must be noted that although these samples were treated with HClO₄ and HgCl₂ per the extraction method, there is a lack of evidence regarding the effect of these compounds on the arsenic oxidation state present in the tissue homogenates. Therefore, there may be an effect of these treatments on the valence state of arsenic and it may not accurately reflect the levels of trivalent and pentavalent arsenic present in the unperturbed tissue. However since all the samples were treated and processed in the same manner, it is conceivable that any difference in the pattern of arsenic oxidation state across the samples tested cannot be explained by HgCl₂ and HClO₄ treatment but rather the inherent differences in the tissue samples themselves.

Determination of Arsenic Species in Urine and Tissue
The arsenic speciation method is adapted from Gong, Z., *et al.*, 2001. Urine and previously extracted tissue homogenates were filtered through 0.45 µm nylon centrifuge filters and diluted prior to injection into the HPLC system. The HPLC system consisted of an Agilent 1100 HPLC (Agilent Technologies Inc., Santa Clara, CA) with a reverse-phase C18 column (Gemini 5µm C18 110Å, 150x4.60mm, Phenomenex, Torrance, CA) and guard cartridge. The mobile phase (pH 5.85) contained 4.7mM tetrabutylammonium hydroxide, 2mM malonic acid and 4% (v/v) methanol at a flow rate of 1.2 ml/min. The column temperature was maintained at 50ºC and samples were kept at 4ºC in a thermally controlled autosampler. An Agilent 7500ce inductively coupled plasma mass spectrometer (ICP-MS) with a Conikal nebulizer (Glass Expansion) was used as the detector. The operating parameters were as follows: Rf power 1500 watts, plasma gas flow 15 L/min, carrier flow ~0.9 L/min, 0.15 L/min makeup, and arsenic was measured at 75 m/z.

**Protein Preparations**

Whole cell lysate preparations of mouse liver were prepared from ~250 mg of tissue homogenized in NP-40 buffer (20 mM Tris HCl, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, and 2 mM EDTA with 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) per 50mL) at 4ºC. Homogenized tissue was then agitated at 4ºC for 2 hours, centrifuged at 10,000 x g for 30 minutes, and the supernatant transferred to a clean collection tube. Protein concentrations were determined using the Pierce BCA Protein Quantitation Assay (Thermo Scientific, Rockford, IL) per the manufacturer’s protocol.
**Immunoblot Protein Analysis**

Whole cell lysate proteins (75 μg/well) were prepared in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) with β-mercaptoethanol and heated at 90°C for ~7 minutes prior to separation by SDS-PAGE on 10% gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes overnight at 4°C. Following transfer, the membranes were blocked in 5% nonfat dry milk diluted in either phosphate-buffered saline-tween 20 (Mrp1) or tris-buffered saline-tween 20 (As3mt) for 45 minutes at room temperature. To determine relative protein levels of As3mt, a rabbit polyclonal antibody developed with full-length human As3MT and containing cross-reactivity towards both rat and mouse As3mt was used (kindly donated by Walter Klimecki at the University of Arizona, Tucson). Relative protein levels of Mrp1 was performed using a rat monoclonal antibody against Mrp1 (clone MRPr1; generated by George L. Scheffer, Amsterdam, The Netherlands) with known cross-reactivity against both human and mouse Mrp1 (Aleksunes et al., 2008). The blots were incubated with primary antibody overnight at 4°C with constant rocking. The following HRP-conjugated secondary antibodies were used: anti-rat (sc-2065) and anti-rabbit (sc-2004) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Quantification of relative protein expression was determined using image processing and analysis with Image J software (NIH, Bethesda, MD) and normalized to ERK 1 protein (C-16, Santa Cruz, CA). Erk protein was previously validated and used to normalize protein expression data from NASH samples (Hardwick et al., 2010). The use of more customary proteins such as GAPDH is inconsistent, perhaps due to the association of NASH with fibrosis.
**Statistical Analysis**

Data was analyzed using one-way ANOVA to determine significant findings among diet groups with a Bonferroni post-hoc analysis. A two-way ANOVA was used to determine significance between arsenic metabolites found in urine and tissue amongst the diet groups followed by a Bonferroni post-hoc analysis. A significance level of \( p \leq 0.05 \) was used for all analyses. All analyses were carried out using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

**Results**

*Mouse Liver NAFLD Histology*

Representative images of hematoxylin and eosin stained liver sections from mice fed a control diet, high fat diet, and MCD diet are shown in Figure 5.1. Microvesicular lipid deposits are clearly visible in the liver of mice fed a high fat diet for five weeks (white arrowhead), whereas macrovesicular steatosis was primarily observed within the livers of MCD-fed mice (black arrowheads). Inflammation, a key distinguishing feature of NASH, is present in the livers of MCD-fed mice (circles). These lesions are consistent with the hepatic histopathological progression of human NAFLD from simple fatty liver (steatosis) to the more advanced form of NASH and the histopathological assessment of these two dietary models in rodents was previously characterized by a veterinary pathologist at the University of Arizona using a validated NAFLD scoring system (Kleiner et al., 2005).
Figure 5.1: Liver Histopathology of Mice fed a Control, High Fat and MCD Diet. Representative hematoxylin and eosin stained liver sections from control (A), high fat (B), and MCD (C) fed mice. Microvesicular steatosis identified in the high fat fed mice is indicated by the white arrow, whereas macrovesicular steatosis after a MCD diet is shown by the black arrow. Inflammation is clearly visible in MCD-fed mice evident by the presence of inflammatory cells (circle). Images were taken at 20x magnification.
Cumulative 24h arsenic excretion in the urine of control, steatosis, and NASH mice is shown in Figure 5.2. Mice with NASH excreted a significantly higher proportion (p < 0.05) of total arsenic in 24h compared to control following a single, oral dose of 0.2 mg/kg arsenic trioxide (Figure 5.2A). No statistical difference in the excretion of total arsenic was observed between steatosis mice and controls. Mice with NASH given a single oral dose of 0.75 mg/kg sodium arsenate did not excrete significantly higher levels of arsenic in their urine compared to control (Figure 5.2B).

A breakdown of the relative amount of arsenic metabolites excreted in 24h urine of control, steatosis, and NASH mice dosed with arsenic trioxide and sodium arsenate is shown in Figure 5.3. The dimethylated arsenic metabolite (DMA) was the predominant metabolite recovered in the urine of both treatment groups, and its excretion was not affected by disease state. However, mice with NASH excreted significantly less (p < 0.05) inorganic arsenic (iAs) compared to controls following arsenic trioxide treatment (Figure 5.3A). No difference in the relative amounts of metabolites excreted in 24h urine was detected amongst disease groups in animals treated with sodium arsenate (Figure 5.3B).

To determine whether the valence state had an effect on the disposition of arsenic metabolites in NASH, the amount of trivalent and pentavalent iAs was quantified. Mice with NASH were found to differentially excrete trivalent and pentavalent iAs in their urine (Figure 5.4). Specifically, mice with NASH excreted a statistically higher (p < 0.05) percentage of the dose as trivalent iAs compared to control following a single, oral dose of arsenic trioxide (Figure 5.4B). However, urinary excretion of trivalent iAs in NASH versus
control mice given sodium arsenate did not reach statistical significance. (Figure 5.4D).

No change in the excretion of pentavalent iAs in urine was observed among disease states (Figure 5.4A and Figure 5.4C).
Figure 5.2: Total Arsenic Elimination in Urine. Percent of total arsenic dose excreted in 24h urine from control, steatosis, and NASH mice given a single oral dose of arsenic trioxide (A), or sodium arsenate (B). Data represent the mean ± S.E.M from 4-6 mice. * p < 0.05 versus control mice.
Figure 5.3: Urine Profile of Arsenic Metabolites. Relative amount of arsenic metabolites recovered in 24h urine from control, steatosis, and NASH mice given a single, oral dose of arsenic trioxide (A), or sodium arsenate (B). Data represent the mean ± S.E.M. from 4-6 mice. * p < 0.05 versus control mice.
Figure 5.4: Differential Elimination of Trivalent and Pentavalent iAs in Urine. Percent of the arsenic dose excreted as pentavalent (A, C) and trivalent (B, D) iAs in 24h urine from control, steatosis, and NASH mice. Mice were given either a single, oral dose of arsenic trioxide (A, B), or sodium arsenate (C, D). Data represent the mean ± S.E.M. from 4-6 mice. * p < 0.05 versus control mice.
Renal and Hepatic Accumulation of Arsenic in Experimental NAFLD

Arsenic accumulation in the liver and kidneys of mice was evaluated 24h after dosing with either arsenic trioxide or sodium arsenate to determine disease-dependent retention of arsenical species in these tissues. No difference in total arsenic accumulation in the liver was identified amongst disease groups (Figure 5.5A and Figure 5.5C). In contrast, the accumulation of specific arsenic metabolites was altered in a disease-dependent fashion (Figure 5.5B and Figure 5.5D). Mice with NASH that were dosed with arsenic trioxide and sodium arsenate accumulated significantly higher (p < 0.05) MMA levels in liver compared to controls as well as a significant reduction in the level of DMA.

Accumulation of total arsenic and arsenic metabolites in the kidneys of control, steatosis and NASH mice is shown in Figure 5.6. No significant difference in the renal levels of total arsenic was identified in the kidneys 24h post-dose for either arsenic trioxide or sodium arsenate between disease groups (Figure 5.6A and Figure 5.6C). However, similar to what was observed in the liver, the retention of arsenic metabolites in kidney was altered in a disease-dependent fashion (Figure 5.6B and Figure 5.6D). Specifically, retention of iAs was significantly increased (p<0.05) with a concomitant decrease (p<0.05) in the renal levels of DMA in mice with NASH treated with arsenic trioxide and sodium arsenate.

To further investigate the effects of NAFLD on the disposition of arsenic, the retention of both trivalent and pentavalent iAs was measured in livers of control, steatosis and NASH mice 24h post dose with either arsenic trioxide or sodium arsenate (Figure 5.7).
NASH livers had a significantly higher proportion (p<0.05) of the iAs content as pentavalent iAs compared to control (Figure 5.7A and Figure 5.7C). Conversely, no statistically significant difference in the levels of trivalent iAs was observed amongst disease groups in both arsenic trioxide and sodium arsenate treated mice (Figure 5.7B and Figure 5.7D).
Figure 5.5: Liver Accumulation of Arsenic Metabolites. Total arsenic retained in the livers of control, steatosis, and NASH mice 24h post-dose given a single, oral dose of arsenic trioxide (A, B), or sodium arsenate (C, D). A and C depict total arsenic retained in liver, while B and D depict the percent of arsenic species present in the liver 24h post-dose. Data represent the mean ± S.E.M. from 4-6 mice. * p < 0.05 versus control mice.
Figure 5.6: Kidney Accumulation of Arsenic Metabolites. Total arsenic retained in the kidneys of control, steatosis, and NASH mice 24h post-dose given a single, oral dose of arsenic trioxide (A, B), or sodium arsenate (C, D). A and C depict total arsenic retained in kidneys. B and D depict percent of arsenic species present in the kidneys 24h post-dose. Data represent the mean ± S.E.M. from 4-6 mice. * p < 0.05 versus control mice.
Figure 5.7: Differential Accumulation of Arsenic Metabolites in Liver. Percent of the arsenic dose retained in the liver as pentavalent (A, C) and trivalent (B, D) iAs 24h post-arsenic dose from control, steatosis, and NASH mice. Mice were given a single, oral dose of arsenic trioxide (A, B) or sodium arsenate (C, D). Data represent the mean ± S.E.M from 4-6 mice. * p < 0.05 versus control mice.
As3mt and Mrp1 Expression in Mouse Liver

To explore a possible mechanism responsible for the altered disposition of arsenic metabolites, metabolizing enzyme and transporter protein levels were measured in the livers of control, steatosis, and NASH mice (Figure 5.8A). No significant change in relative protein expression of As3mt was found between control and NASH mice, however As3mt protein was significantly (p<0.05) downregulated in the livers of mice with steatosis compared to control. Furthermore, the protein expression of Mrp1, a membrane transporter known to transport arsenical species, was significantly (p<0.05) induced in the livers of mice with steatosis and NASH, compared to control.
Figure 5.8: Hepatic Protein Expression of As3mt and Mrp1. Relative protein expression of As3mt (A) and Mrp1 (B) in the liver of control, steatosis, and NASH mice. Data is normalized to Erk protein expression. Data represent the mean ± S.E.M. from 4-6 mice. * p < 0.05 versus control mice
Discussion

Millions of people worldwide are exposed to high levels of arsenic, placing them at risk for developing various forms of cancer as well as non-malignant diseases (Thomas et al., 2001; Rosen & Liu, 2009). With the extensive worldwide prevalence of NAFLD, it is reasonable to suspect that individuals exposed to high levels of arsenic may also have NAFLD. However, little information is currently known regarding the effect of NAFLD on the fate of arsenic within the body.

The purpose of the current study was to determine whether experimentally-induced NAFLD alters the normal metabolism, disposition and/or excretion of the environmental toxicant arsenic. Our results clearly demonstrate that mice with the most progressive form of NAFLD, NASH, were found to excrete significantly more total arsenic in their urine after a single, oral dose of arsenic trioxide. A similar profile was seen in mice administered sodium arsenate; however the results did not reach statistical significance. Previous studies investigating the effects of hepatitis, steatosis, and alcoholic cirrhosis in humans have also reported an increase in the urinary elimination of arsenic compared to healthy individuals (Buchet et al., 1984). This suggests that the manifestation of disease in the liver may have a considerable influence on the elimination of this toxic metalloid.

In addition to the intrinsic liver damage sustained throughout the progressive stages of NAFLD, associated metabolic disorders such as obesity may further impact the elimination of arsenic. A recent study conducted in a female population in southwest Arizona and northern Mexico determined a positive correlation between arsenic methylation efficiency and body mass index (Gomez-Rubio et al., 2011). Specifically, a
lower MMA coupled to a higher DMA/MMA ratio was measured in the urine of these women. In contrast, our results suggest that although total arsenic elimination is increased in mice with NASH, no significant aberration in methylation efficiency was observed. These findings may be partly explained by the observation that the majority of metabolites recovered in the urine of mice were dimethylated, suggesting higher methylation efficiencies in these animals.

Arsenic is primarily metabolized in the liver by As3mt and uses the methyl donor s-adenosyl methionine (SAM) to produce both mono and dimethyl arsenic metabolites (Lin et al., 2002b). To investigate a potential mechanism for the observed increase in arsenic found in the urine of mice with NASH, we measured relative protein expression of As3mt in the liver. No significant change in protein expression of hepatic As3mt was detected in NASH animals, suggesting that the increase in total urinary excretion is not due to alterations in metabolism. These findings coincide with the observation that the relative amounts of both mono and dimethylated arsenic metabolites recovered in the urine were not altered by disease state. Interestingly, a significant downregulation of As3mt protein expression was seen in mice with steatosis without any concomitant aberration in total arsenic, MMA, or DMA recovery in the urine. However, a decrease in DMA was present in livers of mice with steatosis. A potential explanation for the observation that no differential effects on arsenic elimination were seen in steatosis mice, despite the down regulation in As3mt expression, may lie in alternative methods for arsenic methylation in vivo. A recent study using As3mt-knockout mice given an oral dose of arsenate revealed the presence of fully dimethylated metabolites in urine (Drobna et al., 2009). These results
suggest that alternative mechanisms for arsenic methylation may exist, which may partially compensate for the decrease in As3mt expression seen in mice with steatosis. Additionally, pre-systemic metabolism by gut microflora may further contribute to the appearance of methylated urinary metabolites in the current study. Various microorganisms normally found in the gastrointestinal tract have the capacity to methylate arsenic (Bentley & Chasteen, 2002). Nonetheless, these data suggest for the first time that As3mt protein expression may be altered in different pathophysiological states. Further investigation is needed to identify whether dysregulation of As3mt protein expression also occurs in humans with NASH.

In addition to the direct effects of protein expression on As3mt function, aberrations in the levels of the necessary methyl donor SAM may indirectly alter the function of this enzyme. The amino acid methionine is a necessary cofactor in the biosynthesis of SAM (Loenen, 2006) and dietary intake of methionine was shown to impact the methylation efficiency of arsenic in humans as well as animals (Heck et al., 2009; Jin et al., 2010). In addition, the effects of the MCD diet on hepatic SAM levels in mice remains controversial as some groups have reported depletion in hepatic SAM whereas others report no change in hepatic SAM after 6 weeks of MCD diet (Caballero et al., 2010; Gyamfi et al., 2008). However, our results show that despite the depletion in dietary methionine, the majority of excreted arsenic recovered in the urine from mice fed the MCD diet was dimethylated. This observation suggests that methylation efficiency was not impaired by the MCD diet in light of the potential confounding influences of SAM depletion. Prior evidence from rabbits fed diets deficient in methionine or choline showed a decrease in the urinary excretion of
methylated arsenic metabolites coupled with higher tissue retention of arsenic in a 72h period (Vahter and Marafante, 1987). In contrast, the results presented in this study do not suggest that dietary methionine and choline depletion in mice alters the methylation capacity of arsenic as no change in total hepatic arsenic retention or urinary excretion of arsenic metabolites was observed. Interestingly, lower rates of methionine transmethylation were shown to occur in humans with NASH implicating a lower rate of SAM biosynthesis in this population of individuals (Kalhan et al., 2011). Whether this alters the metabolism of arsenic in humans with NASH is unknown.

A key factor in the overall toxicity of arsenic is the formation of the trivalent MMA (MMA\textsuperscript{III}) and DMA (DMA\textsuperscript{III}) metabolites, which were shown to be significantly more toxic than their pentavalent counterparts (Kligerman et al., 2003; Wang et al., 2007b). The identification of these toxic metabolites in human urine suggests that they are sufficiently stable to distribute into target tissues and elicit a toxicologic response (Mandal et al., 2001). Due to limitations and restrictions of the current analysis, we could not determine the valence state of the methylated species in the urine or tissues sampled. However, this analysis was able to differentiate and quantify pentavalent and trivalent inorganic arsenic (iAs). Interestingly, we identified a significant shift in the renal elimination and liver accumulation of these arsenical species between different disease groups. Mice with NASH had significantly higher levels of the more toxic trivalent iAs in urine, whereas livers accumulated significantly higher levels of the pentavalent form.

The role of membrane transporters in regulating the disposition of arsenic is slowly emerging as an important mechanism in governing its disposition. In particular, one
member of the ABCC subfamily of efflux transporters, the multidrug-resistance associated protein 1 (MRP1), is implicated in the cellular transport of arsenic (Lorico et al., 2002; Thomas, 2007b). Interestingly, MRP1 was shown to transport arsenite\textsuperscript{III} but not arsenate\textsuperscript{V} in the presence of glutathione (Leslie \textit{et al.}, 2004), suggesting that transport of arsenic by MRP1 is dependent on the valence state, with MRP1 preferentially transporting trivalent inorganic arsenic over its pentavalent counterpart. Additionally, an MRP1 over-expressing confers resistance to MMA\textsuperscript{III} but not MMA\textsuperscript{V} in a glutathione-dependent fashion suggesting that MMA\textsuperscript{III} is also a MRP1 substrate and further lending support to the valence-specificity of arsenic transporters (Carew \textit{et al.}, 2011). To investigate whether the increase in elimination of trivalent inorganic arsenic in urine of mice with NASH could be partially explained by dysregulation of Mrp1, we measured its relative protein expression in the liver. Our results indicate that Mrp1 is significantly induced in the liver of mice with NASH and steatosis suggesting a possible mechanism for the increase in trivalent iAs urinary excretion observed in NASH mice. Furthermore, arsenic is a known substrate for the hepatobiliary transporter MRP2 and its function is required for the biliary elimination of arsenic-glutathione conjugates (Kala \textit{et al.}, 2000; Carew & Leslie, 2010; Kala \textit{et al.}, 2004). Previous reports have demonstrated that MRP2 localization on the bile canalicular membrane is altered in both human and rodent models of NASH thus inhibiting its ability to properly excrete compounds into the bile (Hardwick \textit{et al.}, 2012; Hardwick \textit{et al.}, 2011). However, further investigation is needed to confirm whether biliary excretion of arsenical metabolites is altered in NASH.
The membrane transporter, MRP4, has recently emerged as an important player in mediating arsenic transport. In particular, it was demonstrated that MRP4 has a high affinity for transporting dimethylated arsenical species (Banerjee et al., 2014). It is interesting to note that decreased levels of DMA were reported in both the liver and kidney of mice with NASH. The studies outlined in Chapters 2 and 4 demonstrated that NASH results in the induction of Mrp4 in both the liver and kidney, respectively. Together, these results suggest that the induction of Mrp4 in both of these tissues leads to decreased tissue exposure of DMA species and potentially lessening the possibility of hepatic and renal, DMA-induced toxicity.

Future investigations need to be conducted to determine the functional and toxicological effects of altered arsenic disposition in NAFLD. In this study, we have demonstrated that NASH increases total arsenic elimination in the urine, along with specific alterations identified in the elimination and tissue accumulation of arsenic metabolites. However, whether these aberrations manifest in an altered toxicodynamic and/or pharmacodynamic effect is yet to be determined. We have identified specific molecular alterations that may provide novel insights into the mechanisms by which NASH alters the disposition of arsenic while emphasizing the importance of hepatic and renal membrane transporters in influencing the pharmacokinetics and tissue accumulation of this toxic metalloid.
CHAPTER 6: SUMMARY OF CURRENT STUDY AND FUTURE DIRECTIONS

Summary and Current Perspective

The primary objective of the current study was to determine the effects of NASH on liver and kidney membrane transporter regulation. Specifically, the working hypothesis proposed is that the manifestation of NASH leads to coordinated, compensatory alterations in hepatic and renal xenobiotic membrane transporter regulation that ultimately affect the disposition of xenobiotics. With the use of several experimental animal models of NASH, these studies not only expand our knowledge of disease-mediated effects on hepatic gene and protein regulation, but also contribute novel insight into the idea of extra-hepatic tissue compensation during times of hepatic stress. Together, these adaptations during disease states lead to global, tissue-specific alterations in the disposition of xenobiotics, which include pharmaceuticals and environmental toxicants, such as arsenic.

Reliable in vitro models used to study the complex biology of disease are lacking and therefore research investigating the physiological effects and pathogenesis of disease are almost exclusively performed using rodent models. This is especially true for NASH, and over the years, several models have been developed and characterized, making the selection of choosing one that is physiologically and experimentally relevant an arduous task. The use of multiple experimental models in both Chapters 1 and 3 is a distinguishing feature of these studies that strengthens the hypothesis presented. The use of these models offers two significant advantages. First, the manifestation and progression of disease varies
significantly across models and by including several in this study, a more accurate determination of disease-specific effects versus model-specific effects can be determined. Second, a thorough comparison of transporter deregulation can be made with changes in human gene expression, which can help determine the most representative model for future ADME studies in NASH.

Characterization of membrane transporter expression in human NASH liver was previously reported and can therefore be used as a reference when choosing a rodent model that reflects similar membrane transporter expression profiles. However, due to the difficulty in acquiring human kidney tissue from patients with NASH, directly cross-referencing results in renal transporter regulation to humans with the disease is not feasible. Therefore, relying on an experimental model that recapitulates known human membrane transporter expression profiles is important when considering the effects of the disease on ADME mechanisms in extra-hepatic tissues. This forms the basis and rationale for the studies performed in Chapter 1; to characterize the effects of the disease on hepatic membrane transporter regulation across rodent models, which will serve as a platform for comparison to the human condition. This, in turn, provides more relevant and useful information when conducting ADME studies in NASH followed by attempting to translate extra-hepatic pharmacokinetic disturbances to humans.

Several commonly used rodent NASH models were used in the studies highlighted in Chapter 1. Dietary models, such as the familiar MCD diet as well as the more recently developed atherogenic diet, were included along with genetically obese rodents such as the ob/ob, db/db mice and fa/fa (zucker) rat. A comprehensive analysis on hepatic mRNA and
protein expression profiles of xenobiotic membrane transporters was performed, followed by a statistical comparison of the findings to human data. An interesting observation that initially resulted from these experiments was that the degree and magnitude of membrane transporter dysregulation appeared to correlate with disease severity. For example, MCD diet rats, as well as ob/ob and db/db mice all developed significant liver pathology as a result of disease manifestation and these models had the most numerous and robust changes in membrane transporter expression. In contrast, the fa/fa rats failed to develop NASH as well as lacked statistically significant changes in gene and protein expression. These observations suggest that the severity of disease pathology may be the primary driver in hepatic dysfunction. This is consistent with previous findings reporting gene expression profiles are more significantly disrupted in the progressive stages of NAFLD (Lake et al., 2011; Moylan et al., 2014).

A major contribution resulting from the experiments highlighted in Chapter 1 is the finding that the MCD diet as well as ob/ob and db/db models recapitulate human gene and protein expression profiles of hepatic xenobiotic transporters in NASH. Importantly, these models also develop significant disease pathology and therefore, future studies investigating the effects of NASH on ADME are encouraged to use these experimental platforms. Moreover, the consistent pattern of membrane transporter expression across these models is particularly peculiar and deserves more thorough discussion. The overall induction of hepatic efflux transporters (Mrp2, Mrp3, Mrp4, P-gp, Bcrp) and repression of uptake transporters (Oatps) is a common theme present across the above mentioned models. This is in contrast to the atherogenic and fa/fa rodents, which do not develop these
patterns of regulation. In conclusion, this suggests that the manifestation of NASH initiates a coordinated response in membrane transporter regulation that functions in facilitating the excretion of substrates from the liver while concomitantly inhibiting their entry. This was reported previously in humans with NASH as well as models of cholestatic disease and is hypothesized to be a protective mechanism limiting hepatic exposure to potentially harmful substances in times of tissue stress (Hardwick et al., 2011; Keppler, 2014). However, it is important to emphasize that although these mechanisms are intended to be hepatoprotective in nature, the decrease in hepatic exposure increases the systemic burden of endo and xenobiotics, placing individuals at risk for extra-hepatic and systemic toxicity.

Genomic regulation of membrane transporter genes is extensive and can be influenced by multiple cellular signal transduction. In particular, transcription factors, such as Nrf2, are reported to play a major role in the induction of transporter genes. Nrf2 functions as the master regulator of the anti-oxidant response pathway and its activation leads to the induction of a battery of cytoprotective genes that include Phase I/II enzymes and membrane transporters (Jaiswal, 2004; Zhang, 2006). The experiments outlined in Chapter 2 aimed to contribute novel information regarding Nrf2 regulation by investigating the link between its activation and the induction of the membrane transporter, MRP3, in humans. Under basal conditions, MRP3 expression in hepatocytes is low, but can be significantly up-regulated by chemical and physiological stressors (Cui et al., 2009; Klaassen & Aleksunes, 2010). As evidenced by the results herein, Mrp3 is significantly induced in rodent NASH as well as humans (Lickteig et al., 2007a) (Appendix A). Previous studies in our laboratory have also identified Nrf2 activation in the liver of rodents
and humans with NASH, implicating a potential role of the anti-oxidant response pathway in the regulation of ADME genes, particularly membrane transporters in the disease (Lickteig et al., 2007b). Nrf2 genomic regulatory elements, known as antioxidant response elements (ARE), within the promoter region of the rodent Mrp3 gene have been previously characterized; however, the mode of regulation in humans has remained elusive.

Using high-throughput ChIP sequencing analyses, several putative ARE and ARE-like elements were identified within the eighth intron of the human MRP3 (ABCC3) gene whereas the promoter region lacked genomic enrichment of Nrf2 association. This was also supported by the finding of histone 3, lysine 4 (H3K4) methylation enrichment within this intronic region, which is suggestive of an active chromatin state. Further functional characterization of these elements using reporter gene assay and DNA pull-down experiments confirm the identification of a functional ARE within this region. This is the first report of Nrf2 regulating a target gene through an intronic response element. Importantly, these findings link MRP3 induction to Nrf2 activation in humans, which may have functional implications in disease states that are associated with oxidative stress, such as NASH. The significance of MRP3 induction in NASH is highlighted by the studies performed in Appendix A, which serves to connect the functional consequences of MRP3 induction to xenobiotic disposition by investigating the effects of NASH on acetaminophen disposition in the clinical setting.

Using a small pilot study design, Appendix A serves to expand the findings obtained in rodents on xenobiotic disposition in NASH to the clinical setting. Pediatric patients, diagnosed with having simple fatty liver or NASH, were recruited and challenged
with a dose of acetaminophen (APAP). APAP and its two primary metabolites, APAP-glucuronide (APAP-gluc) and APAP-sulfate (APAP-sulf) were measured in plasma and urine over a 4 hour period. Compared to healthy children, patients with NASH had increased APAP-gluc in plasma and urine. Previous reports demonstrate that APAP glucuronidation is not disrupted in NASH, implicating cellular transport as primary mechanism for its disposition in the disease. Further investigation reveals an induction of hepatic MRP3 as well as altered canalicular localization of MRP2 in NASH. Given that APAP-gluc is a substrate for both transporters, this pattern in the molecular regulation of these transporters suggests a potential mechanism by which sinusoidal efflux is favored over biliary excretion in NASH. This shift in the disposition of APAP-gluc was demonstrated previously in rodent models, but this is the first account identifying these effects in the clinical setting. Fortunately, APAP-gluc is pharmacologically and toxicologically inactive; however, future caution is warranted in the setting by which a toxic metabolite utilizes this route of excretion, leading to the potential for adverse drug reactions in patients with NASH.

To extend our knowledge on the effects of NASH on xenobiotic disposition, a comprehensive analysis on membrane transporter expression in the kidneys was performed and forms the basis for the studies outlined in Chapter 4. Using the same rodent models described in Chapter 1, the results demonstrate coordinated responses in renal transporter expression in NASH that are similar to what was observed in the liver. The representative models mentioned previously (MCD, ob/ob and db/db) have global inductions of apical efflux transporters in the kidney whereas basolateral uptake transporters are generally
unchanged or up-regulated. The \textit{fa/fa} rat, in contrast, did not have extensive changes in transporter regulation. Together, these results suggest a coordinated, compensatory mechanism in NASH that promotes renal secretion of endogenous and exogenous substrates that may otherwise accumulate systemically during times of hepatic dysfunction. Increased systemic exposure of xenobiotics, particularly pharmaceuticals is a common observation in NASH (Lickteig et al., 2007a; Hardwick et al., 2012) (Appendix A) and is a result of reduced basolateral uptake of substrates into hepatocytes as well as enhanced sinusoidal efflux (Clarke et al., 2014a). Therefore, the kidneys serve as a secondary route for excretion during these times and may compensate by enhancing secretion and suppressing reabsorptive mechanisms.

Direct hepatic injury is closely associated with transporter gene and protein expression dysregulation in the liver. Interestingly, this does not appear to hold true in the case of renal membrane transporters. Although NASH severity appears to correlate with membrane transporter expression in the kidney, renal injury does not appear to be a primary factor in kidney transporter regulation in NASH. The MCD, \textit{ob/ob} and \textit{db/db} models do not develop extensive kidney injury, whereas the \textit{fa/fa} rat, which failed to manifest advanced disease, developed significant renal damage. This suggests an inverse relationship, in which the development of kidney injury is not the primary mediator in regulating renal transporters in the disease. Instead, the severity and extent of hepatic injury sustained by NASH is more closely associated with the adaptive changes occurring in the kidney, implicating the liver in possibly orchestrating these extra-hepatic compensatory
effects in the kidney, possibly through the induction of inflammatory responses and secretion of cytokines, such as TNF-α.

To provide functional significance for the findings reported, Chapter 5 aimed to investigate the effects of NASH on the disposition of the environmental contaminant, arsenic. This is the first report of this disease impacting the disposition and excretion of an environmental toxicant and given the increasing prevalence of NASH, these findings have significant implications to general public health. Using the MCD model, a single bolus dose of either pentavalent arsenic or arsenic trioxide, given orally, resulted in increased total arsenic excretion into the urine within a 24 h period. One of the most interesting findings that resulted from this study was the preferential excretion of trivalent inorganic arsenic species into the urine, whereas pentavalent species were retained in the liver of mice with NASH. This shift in increased excretion of trivalent species is presumably due to Mrp1 induction in the liver, which favors the transport of trivalent arsenic conjugates. Given the differential toxicity of pentavalent and trivalent arsenic oxidation states, these findings have significant implications to the dynamics of arsenic-mediated toxicity, which can be altered as a consequence of membrane transporter dysregulation in NASH.

In general, no change in the relative amounts of methylated arsenic metabolites was observed in the liver and urine of NASH mice. Furthermore, no change in the expression of arsenic-3-methyltransferase was detected in the liver, suggesting that arsenic metabolism is not perturbed in NASH. However, a significant decrease in the relative amounts of dimethylated arsenic was detected in kidney and liver tissue of mice with disease. At the time, this result was difficult to explain; however, a recent study conducted
by an independent group has uncovered a novel mechanism that may offer an explanation for these results. The group identified MRP4 having the capacity to transport arsenic and in particular, having a high affinity for dimethylated arsenic species (Banerjee et al., 2014). Mrp4 is significantly up-regulated in the kidney and liver of mice fed an MCD diet and offers a potential mechanism for the reduction of dimethylated species found in these tissues. More importantly, this links both liver and kidney dysfunction in tissue accumulation of arsenic metabolites in NASH. A working model that summarizes the work described in this dissertation is presented in Figure 6.1.

The results highlighted in this dissertation provide novel platforms for future studies investigating the effects of NASH on xenobiotic disposition. Importantly, the identification of coordinated hepatic and renal regulation of membrane transporters during the progression of the disease should increase awareness that the pharmacokinetic changes resulting from liver dysfunction are not solely a result of changes in hepatic disposition, but a contribution by global alterations in tissue adaptations in disease.
Figure 6.1: Working Model Summarizing Dissertation Studies. A model highlighting the overall perspective and significance of this dissertation work. Chapter 2 demonstrates that NASH causes a coordinated up-regulation of efflux transporters and a down-regulation of uptake transporters in the liver. Chapter 3 identifies a novel antioxidant response element within the eight intron of the ABCC3 gene, which functionally links Nrf2 activation and MRP3 up-regulation in human NASH. By mechanisms that are not completely understood, the results highlighted in Chapter 4 suggest an adaptive response in renal membrane transporters leading to a shift towards renal secretion mechanisms. Together, these adaptations lead to increased urinary excretion of xenobiotics and decreased tissue exposure as evidenced by the results obtained from Chapter 5 (arsenic disposition).
Future Studies

The aims and studies of the current dissertation have significantly enhanced our understanding of global responses in hepatic and renal membrane transporter dysregulation during disease states such as NASH. Moreover, several physiologically and biochemically relevant rodent models were characterized, which set the foundation for future studies to be conducted that will expand on the conclusions drawn herein.

Study 1: Investigate the Role of the Pro-Inflammatory Cytokine, TNF-α, in Mediating Liver and Kidney Transporter Regulation in NASH.

Inflammation is a critical component of NASH pathogenesis; however, the causal relationship between inflammation and NASH remains poorly understood. It is increasingly recognized that the activation and secretion of inflammatory mediators propagates the progression of disease, leading to increased oxidative stress and hepatocellular injury (Fujii & Kawada, 2012). Recently, a complex interplay between adipose tissue and the liver is proposed by which “lipotoxicity,” resulting from excess free-fatty acids secreted by insulin-resistant adipocytes, causes hepatic injury by accumulating excess free fatty acids. This, in turn, activates resident hepatic Kupffer cells, which secrete pro-inflammatory mediators, particularly TNF-α, that further propagate a chronic state of hepatic inflammation (Fujii & Kawada, 2012; Feldstein, 2010).

The pro-inflammatory cytokine, TNF-α, is central in NAFLD progression and its levels are readily induced systemically in both patients and animal models of the disease (Alaaeddine et al., 2012; Larter & Yeh, 2008). Moreover, systemic levels of TNF-α were found to correlate with hepatic fibrosis stage and overall NASH activity scores (Jarrar et
al., 2008; Manco et al., 2007). Interestingly, disease severity is also associated with the degree of gene expression changes in the disease, suggesting that the concomitant induction of pro-inflammatory cytokines, such as TNF-α, may be partly responsible for the disrupted patterns of gene expression profiles in the disease. Indeed, TNF-α activates several intracellular signaling cascades that result in the activation or repression of gene transcription, which include genes encoding for membrane transporters (Malekshah et al., 2012; Donner et al., 2007).

The induction of TNF-α into systemic circulation in NASH may regulate gene transcription in distal tissue sites in a paracrine fashion. The results gathered in Chapter 3 further support this suggestion. Specifically, altered transporter regulation in the kidney is associated with NASH severity whereas renal injury does not appear to influence renal gene expression. These findings implicate mediators that originate outside the kidney, possibly TNF-α and other pro-inflammatory cytokines, as primary factors contributing to deregulated transporter expression in the kidney. The current study aims to explore the role of TNF-α in renal and hepatic transporter expression in NASH. Furthermore, determining the contribution of hepatically-derived mediators in renal membrane transporter regulation in NASH will provide a more accurate assessment of the cause and effect relationship between liver and kidney transporter dysregulation in NASH.

Experimental Design:

*Treatment of NASH Rodents with Anti-TNF Antibody* - To investigate the role of TNF-α on membrane transporter expression in NASH, ob/ob and MCD rodent models will be administered doses of anti-TNF antibody (Centocor, Malvern, PA) in an attempt to
normalize renal and hepatic gene expression to control levels. Briefly, Sprague Dawley rats or "ob/ob" mice will be fed an MCD (or control) diet for 8 and 4 weeks, respectively to induce NASH. Experimental and control groups within each model cohort will be created as follows: Group 1 - control diet, treatment with normal saline; Group 2 - control diet, treatment with 1 mg of anti-TNF antibody; Group 3 - MCD diet, treatment with normal saline; Group 4 - MCD diet, treatment with anti-TNF antibody. Dose and frequency of treatment will be 1 mg/weekly via intraperitoneal injection, which will begin at diet onset as determined and validated previously (Li et al., 2003). Plasma will be collected weekly at time of dosing for routine testing of TNF-α levels to verify treatment effectiveness. At the conclusion of the dietary regimen, all animals will be euthanized and tissues (kidney and liver) will be harvested immediately. A small cross-section of each tissue will be placed in 10% neutral-buffered formalin for paraffin embedding and subsequent histological analyses. The remaining tissue will be snap-frozen and stored at -80° C for future analyses.

**Histological Analyses** - Hematoxylin and eosin (H & E) staining will be performed on formalin-fixed, paraffin-embedded (FFPE) sections of liver and kidney tissue. Full pathology evaluations will be conducted as described in Chapters 1 and 3.

**mRNA and Protein Expression in Liver and Kidney** - Total RNA will be extracted from tissue as described in Chapter 1. mRNA analyses will be conducted using bDNA analysis to determine the mRNA expression of membrane transporters outlined in Chapter 1 (Mrp2, Mrp3, Mrp4, P-gp, Bcrp, Oatp1a1, Oatp1a2, Oatp1b2, Oatp2b1) for liver and Chapter 3 (Mrp2, Mrp4, P-gp, Bcrp, Oat1, Oat3, Oct2, Oatp1a1) for kidney. Whole cell
and crude membrane protein preparations will be made for immunoblot analyses of transporters as described previously.

**Supplemental Approach** - To determine a more direct role of TNF-α on transporter regulation, an alternative approach proposed would be to include Sprague Dawley rats and C57bl/6 mice that are treated with TNF-α directly, via intraperitoneal injection, followed by measurement of transporter expression in the kidney and liver. Recombinant TNFα, which is commercially available, will be injected to the above mentioned rodents at a dose of 0.166 mg/kg as described previously (Endo et al., 2007). A separate group will be dosed with normal saline as a control. Blood will be collected at 3, 6, and 12 hours to measure serum TNF-α levels. The animals will be euthanized 24 hours post-dosing and the liver and kidneys will be harvested and analyzed as described above in this study. mRNA and protein analyses will be conducted on membrane transporters mentioned above. Dosing and the timing-post of animal euthanasia will be determined empirically. The advantage of including this study is that it will be a more direct way to investigate the role of systemic TNF-α on membrane transporter in the liver and kidney of mice and rats.

**Study 2: Investigate the effect of NASH on Intestinal Membrane Transporter Expression and Function.**

The small intestine serves as an important site for the absorption of orally-derived substances that include nutrients, environmental contaminants, and pharmaceuticals. Membrane transporters localized to the basolateral and apical membranes within enterocytes, which form the epithelium lining of the gut wall, are major determinants of the oral bioavailability of substrates administered orally. It was shown previously that
patients and rodents with obstructive cholestasis have decreased expression of the membrane transporters MRP2 and BCRP and increased expression of BCRP in the proximal portion of the small intestine (Dietrich et al., 2004; Zimmermann et al., 2006). This causes a functional increase in the absorption of the MRP2 substrate, PhIP—a food-derived carcinogen (Dietrich et al., 2004). Moreover, humans with cholestasis were shown to have decreased expression levels of the bile acid transporter, ASBT, which localized to the distal segment of the small intestine and responsible for bile acid uptake from the gut (Hruz et al., 2006). This limits further bile acid absorption from the gut and more importantly, functions as a protective mechanism to limit bile acid-induced liver injury.

This study aims to investigate adaptive changes in intestinal membrane transporter expression in NASH. The oral route is the most common route of exposure for pharmaceuticals and toxicants so identifying NASH-mediated transporter alterations in this tissue site will be relevant to clinical pharmacotherapy in disease states.

Experimental Design:

Animals and Histological Analyses - Sprague Dawley rats and ob/ob mice will be placed on an MCD diet for 8 and 4 weeks, respectively to induce NASH. In addition to the MCD diet, a control diet will be fed to Sprague Dawley rats and C57bl/6 mice, which will represent control, healthy animals. At the conclusion of the dietary regimens, all animals will be euthanized and the liver and small intestine will be immediately harvested. A small representative section of the liver will be placed in 10% neutral buffered formalin for tissue processing an H & E staining for histopathological evaluations as outlined in Chapter 1. The small intestine (excised from the base of the stomach to the base of the caecum) will
be placed on a dissection tray and shaped into a ‘Z.’ The tissue will be subsequently cut into thirds (at the two joints of the Z structure). The proximal segment will represent the duodenum, middle portion the jejunum whereas the distal segment (excised at the caecum) will represent the ileum. Representative slices will be placed into 10% neutral buffered formalin for histological analyses described above. All remaining tissue will be snap frozen and stored at -80°C for future analyses.

*mRNA and Protein Analyses of Transporters in Small Intestine* - Total RNA will be extracted from duodenum, jejunum and ileum tissue as described in the methods section of Chapter 1. mRNA of the following transporter genes: P-gp, Mrp2, Mrp3, Mrp4, and Oatp2b1 will be determined by bDNA analyses as described in the methods section of Chapters 1 and 3. Whole cell extracts will be made that will be used for immunoblot analyses for protein quantification of transporters mentioned above.

*Determine the Functional Outcome of Intestinal Absorption of Xenobiotics in NASH* - To determine transport function in the small intestine following the manifestation of NASH, a single-pass intestinal perfusion will be performed with select, probe substrates, according to the methods of Zakeri-Milani, *et al.* (Zakeri-Milani *et al.*, 2007). Mrp2, Mrp4, and P-gp activity will be measured using the respective probe substrates: 5(and 6)-carboxy-2',7'-dichlorofluorescein (CDF) (Mrp2), [H]³ digoxin (P-gp), and adefovir dipivoxil (Mrp4). Sprague-Dawley rats and *ob/ob* mice will be placed on the MCD diet for 8 or 4 weeks to induce NASH, respectively. Also, control diets will be fed to Sprague Dawley rats and C57bl/6 mice, which will represent the control, healthy group. After conclusion of the dietary regimens, the animals will be anesthetized and the duodenum and ileum will be
surgically exposed and small incisions will be made to insert intestinal cannulas which will be subsequently ligated using silk sutures. The inlet cannula (in the duodenum) will be attached to a peristaltic pump to control flow rate of the solution containing individual probe substrates. The exit cannula will collect the perfusate into pre-weighed glass vials. The inclusion of particular probe substrates will depend on the mRNA and protein expression of their respective transporters. Perfusion of each probe will occur for 90 minutes. Analysis of CDF, and adefovir in the perfusate will be carried out using previously published methods whereas digoxin will be measured using liquid scintillation counting (Maeng et al., 2011).
APPENDIX A: ALTERED REGULATION OF HEPATIC EFFLUX TRANSPORTERS DISRUPTS ACETAMINOPHEN DISPOSITION IN PEDIATRIC NONALCOHOLIC STEATOHEPATITIS

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in the US and many other industrialized nations (Wieckowska & Feldstein, 2008; Lomonaco et al., 2013). Although initially described almost 30 years ago, the true scope of the disease has only recently been understood. NAFLD encompasses a number of progressive disease stages, linked by the presence of hepatocellular lipid accumulation. The full spectrum of the disorder ranges from simple steatosis to non-alcoholic
steatohepatitis (NASH), which may further progress to end stage liver diseases such as cirrhosis, and hepatocellular carcinoma (Starley et al., 2010; Lomonaco et al., 2013).

Given the disease’s close association with metabolic morbidities such as obesity and insulin resistance (IR), NAFLD is often regarded as the hepatic manifestation of the metabolic syndrome. The number of Americans with these three conditions (NAFLD, IR, obesity) has increased dramatically in the last few decades, and is still on the rise. In 2008, the prevalence of obese adults [body mass index (BMI) > 30 kg/m²] in the US was approximately 32% and is projected to reach more than 50% by 2030 (Flegal et al., 2010; Wang et al., 2008). Similarly, the prevalence of diabetes in the U.S. is estimated to increase from 14% in 2007 up to 33% by 2050 and therefore concomitantly increasing the incidence of metabolic comorbidities such as NAFLD (Boyle et al., 2010).

While NAFLD cases are largely benign and present as simple steatosis, the progression to NASH significantly increases the risk for hepatic morbidity and mortality. NASH is the most common cause of cryptogenic cirrhosis, and was reported to be the most likely etiologic risk factor in developing hepatocellular carcinoma (Preiss & Sattar, 2008; Rahimi & Landaverde, 2013). The prevalence of NAFLD is estimated to be 6-30% worldwide, whereas NASH is reported to affect as high as 12.2% of the general population (Rahimi & Landaverde, 2013; Lomonaco et al., 2013). Moreover, once thought of as primarily an adult disease, NAFLD is now known to afflict children as well.

It is now recognized that NAFLD is an important pediatric liver disorder (Patton et al., 2006; Lavine et al., 2010). Like adults, as the prevalence rates for obesity are on the rise among pediatric patients, so does the prevalence of NAFLD. It is estimated that 17%
of children in Western society are overweight and among them, up to 80% may also have NAFLD (Giorgio et al., 2013). In pediatric patients, the prevalence of NAFLD is estimated to be 9.6%, and the rate is higher among adolescents (17.3%) than infants (0.7%) (Patton et al., 2006; Schwimmer et al., 2006; Bozic et al., 2013).

It is well documented that NAFLD and especially NASH alter the expression of proteins involved in drug metabolism and disposition, particularly membrane transporters and biotransformation enzymes (Hardwick et al., 2011; Fisher et al., 2009c; Lake et al., 2011; Gomez-Lechon et al., 2009). We have previously reported that experimental NASH in a rat model alters drug transporter expression, resulting in a significant shift in the disposition of the acetaminophen (APAP) metabolite acetaminophen-glucuronide (APAP-gluc), from bile to blood and urine (Lickteig et al., 2007a). Moreover, a recent study has reported altered APAP-gluc disposition in children; however, a mechanism for these observations was left to speculation (Barshop et al., 2011).

The purpose of the current study was to determine the effects of pediatric NASH on the disposition of APAP and its primary metabolites, APAP-sulfate (APAP-sulf) and APAP-gluc. In addition, the regulation of two hepatic membrane transporters, MRP2 and MRP3, were investigated in NASH as a potential mechanism for altered APAP disposition. Using a pilot study design, this investigation contributes to the current knowledge of NASH affecting drug disposition and offers an incentive for exploring these findings in a larger randomized-controlled setting.

Materials and Methods
Materials

Tris-HCL, EDTA, NaCl, glycerol, and nonidet P-40 were obtained from Sigma-Aldrich (St. Louis, MO).

Clinical subjects

Pediatric subjects (n=12) between the ages of 12 and 18 were recruited from a pool of NAFLD patients. All patients had undergone a prior liver biopsy as part of routine patient care. These biopsies were used to establish two distinct groups of patients (simple steatosis and NASH) based on the severity of three characteristics of NAFLD, including steatosis, fibrosis, and inflammation. In addition, pediatric subjects (n=12) between the ages of 12 and 18 were recruited from a panel of non-NAFLD patients. Many of these subjects were patients with constipation or abdominal pain without NAFLD. All patients and their legal guardians were approached during regular office visits, and informed consent received. To participate in the current study, each subject passed a screening evaluation based on medical history and physical examination. Subjects also had to meet basic inclusion/exclusion criteria highlighted below:

Inclusion criteria:

- Liver biopsy indicating either simple steatosis or NASH
- Age 12-18
- Informed consent and assent

Exclusion criteria:

- History of significant alcohol consumption (> 20 g/d)
- Clinical or histological evidence of cirrhosis
- Evidence of other chronic liver disease (i.e. Dubin-Johnson syndrome)
- Presence of the hepatitis B virus surface antigen or hepatitis C virus antibodies
- Use of drugs historically associated with NAFLD
- Use of anti-NAFLD drugs in the three months prior to enrollment in this study
- Pregnancy or breastfeeding
- History of renal dysfunction
- Other disease or conditions considered by the physician to be significant

Inclusion and exclusion criteria were applied to non-NAFLD participants as well, with the obvious exception of NAFLD diagnosis. Non-NAFLD patients were lean, and had no significant medical history. The study and all study procedures were approved by the University of Arizona Institutional Review Board (#00004218) prior to study commencement.

Visit procedures
Subjects arrived at the Clinical and Translational Science Center at the University of Arizona Medical Center on the morning of the study following an overnight fast. Subjects were advised to avoid any product containing APAP for at least three days prior to study participation. Approximately 7cc of blood were collected in 2 separate tubes, one of which
was immediately sent for serum biochemistry analysis. The second blood sample and an initial urine sample were used as blank samples in the quantification of serum APAP levels by HPLC. Following these baseline collections of blood and urine, subjects were given one oral dose of 1000 mg APAP (McNeil Consumer Healthcare; Fort Washington, PA). Subjects were allowed access to food and water during the four hour study. Blood and urine were collected at 1, 2, and 4 hours by the attending nursing staff. Approximately 7 cc of blood were collected at each time point into a serum-separator vacutainer tube (BD; Franklin Lakes, NJ). Samples were allowed to clot, and centrifuged to obtain the serum fraction. Each sample was subdivided into multiple aliquots to avoid excessive freeze/thaw cycles. The serum and urine samples for HPLC analysis were stored at -80° C for future analyses.

**Human Liver Samples**

Frozen and formalin-fixed, paraffin-embedded adult human liver tissue was obtained from the Liver Tissue Cell Distribution System (LTCDS) coordinated through the University of Minnesota, Virginia Commonwealth University and the University of Pittsburgh as described previously (Hardwick RN, 2011). Briefly, all samples were scored and categorized by a medical pathologist within the Liver Tissue Cell Distribution System according to a previously validated scoring rubric developed by Kleiner, et al. and pathology was then confirmed at the University of Arizona (Kleiner et al., 2005). Donor information, including age and gender, was published previously (Fisher et al., 2009d). The samples were diagnosed as either normal (n=20), steatotic (n=12), NASH with fatty liver
(NASH fatty, n=11), and NASH without fatty liver (NASH not fatty/cirrhosis, n=11). For the purposes of this study, NASH fatty and NASH without fat were combined as one experimental group diagnosed as “NASH.” Those samples exhibiting >10% fatty infiltration of hepatocytes were staged as steatotic. Samples were diagnosed as NASH when >5% fatty infiltration of hepatocytes occurred with significant inflammation and fibrosis.

**Tissue Preparations**

Whole cell lysate preparations of human liver tissue were prepared from tissue homogenized in NP-40 buffer (20 mM Tris HCl, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, and 2 mM EDTA with 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) per 25mL) at 4°C. Homogenized tissue was then agitated at 4°C for 2 hours, centrifuged at 10,000 x g for 30 minutes, and the supernatant transferred to a clean collection tube. Protein concentrations were determined using the Pierce BCA Protein Quantitation Assay (Thermo Scientific, Rockford, IL) per the manufacturer’s recommendations.

**MRP3 Immunoblot Analysis**

Whole cell lysate proteins (50 µg/well) were prepared in Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA) in non-reducing conditions (without β-mercaptoethanol or boiling) and separated by SDS-PAGE using 7.5% Tris-Glycine gels followed by transfer to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked for one hour at room temperature using a 5% nonfat dry milk solution dissolved in phosphate buffered
saline-tween (PBST). Following the membrane block, the membrane was incubated overnight in a primary antibody incubation (in 5% milk solution) using the following mouse monoclonal antibody raised against MRP3 protein: M3II-9, Abcam, Inc. (Cambridge, MA). An anti-mouse HRP-conjugated secondary (sc-2005, Santa Cruz Biotechnology, Santa Cruz, CA.) was used for detection and incubated for 1 h at room temperature. Quantification of relative protein expression was determined using image processing and analysis with Image J software (NIH, Bethesda, MD) and normalized to total pan-Cadherin (1:7000, Abcam, Inc., Cambridge, MA).

**MRP2 Liver Immunohistochemistry**

Immunohistochemical staining for all proteins was performed on formalin-fixed, paraffin-embedded (FFPE) human liver samples as described previously (Hardwick et al., 2011). Briefly, tissue sections were de-paraffinized in xylene and re-hydrated in ethanol, followed by antigen retrieval in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 0.3% (v/v) H2O2 in methanol for 20 minutes. Immunohistochemical staining for MRP2 was performed with the MACH3 staining kit (Biocare Medical, Concord, CA) per the manufacturer’s protocol. Samples were incubated in an MRP2 primary antibody (M3II-5, Kamiya Biomedical Company, Seattle, WA) solution overnight at 4°C. All slides were counterstained with hematoxylin (Sigma Aldrich, St. Louis, MO) following color development with Betazoid DAB (Biocare Medical, Concord, CA). All slides were imaged with a Nikon Eclipse E4000 microscope and a Sony Exwave DXC-390 camera.
**APAP and APAP Metabolite Quantification**

Acetaminophen (APAP) and its primary metabolites (APAP-glucuronide and APAP-sulfate) were analyzed in serum and urine under high performance liquid chromatography conditions followed by UV detection as previously described (Lickteig et al., 2007a).

**Microarray Expression Analysis of Sulfur Activation Genes**

Individual Affymetrix GeneChip Human 1.0 ST Arrays (Affymetrix, La Jolla, CA) were generated from purified mRNA for each liver sample as previously described (Lake et al., 2011). The expression of 33,252 annotated and unannotated genes among three diagnosis groups (normal, steatosis, and NASH) is available in the array data set which is accessible at the ArrayExpress public repository for microarray data (accession number E-MEXP-3291) ([http://www.webcitation.org/5zyojNu7T](http://www.webcitation.org/5zyojNu7T)).

**Statistical Analysis**

Statistical differences between patient groups at each time point were determined using a one-way analysis of variance followed by a Bonferroni post-hoc test. Immunoblot data were analyzed by a non-parametric trend analysis and described as box and whisker plots. The level of significance was set at $p \leq 0.05$ for all analyses using Stata9 statistical software (Stata, College Station, TX).

**Results**
Patient Demographics and Serum Chemistry

Table A.1 displays study participant demographics and the results of serum biochemistry tests taken at the time of the study. Male patients were more predominant than females, particularly within the simple steatosis and NASH patient groups. The majority of NAFLD patients recruited for the study (steatosis and NASH) were male and of a Hispanic racial background indicative of the local population (data not shown). Median ages were also similar across groups. Analysis of hepatic function was measured, including total serum protein, serum albumin, conjugated (direct) and total bilirubin, alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT). No statistical difference was observed in all analytes measured except AST and ALT, which were significantly elevated in patients with NASH (Table A.1).
### Table A.1: Study Participant Demographics and Blood Chemistry

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<th>NASH (N=3)</th>
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<td>13 (12-16)</td>
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<td>4.6 ± 0.1</td>
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<td>ALP (IU/L)</td>
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*p <0.05 from NASH compared to healthy and simple steatosis.

nm: not measured
Serum and Urine APAP and APAP Metabolites in Pediatric NAFLD Patients

To determine the effects of NAFLD on APAP disposition, all study participants were given a single oral dose of APAP (1000 mg) followed by measurement of parent and APAP-gluc and APAP-sulf in serum and urine at 1, 2, and 4 hours post-dose. The results of the analysis are described in Figure A.1. No difference was observed in APAP serum or urine levels between the three groups at any time point. The levels of APAP-sulf in serum tended to decrease at all time points measured in patients with NASH, but these data did not reach statistical significance (Figure A.1A). In contrast, serum APAP-gluc levels in children with NASH were significantly increased above normal patients at one hour following administration, and remained elevated compared to patients in both the normal and steatosis groups over all time points measured (Figure A.1A). The increase in serum APAP-gluc in NASH subjects was accompanied by a significant increase in urine excretion of APAP-gluc at 4 hrs. compared to patients with simple steatosis (Figure A.1B). No change in APAP-sulf was detected in the urine across all subjects.
Figure A.1: Plasma and Urine APAP, APAP-gluc, and APAP-sulf in Pediatric NAFLD. Plasma (A) and urine (B) concentrations of APAP, APAP-gluc, and APAP-sulf following a single oral administration of 1000 APAP. Samples were collected at 1, 2, and 4h post-dosing and measured by HPLC. * p < 0.05 healthy patients compared to NASH patients; † p < 0.05 patients with simple steatosis compared to NASH.
Hepatic MRP3 and MRP2 Regulation in Human NASH Patients

To investigate a potential molecular mechanism responsible for the observed effects of NASH on APAP-gluc disposition, the regulation of hepatic membrane transporters, MRP3 and MRP2, was determined via Western blot analysis and immunohistochemistry, respectively. The expression of the basolateral efflux transporter, MRP3, is significantly elevated in the livers of patients with NASH (Figure A.2). In contrast, the apical efflux transporter responsible for biliary efflux of APAP-gluc, MRP2, appears to be improperly localized away from the canalicular membrane of hepatocytes in NASH patients (Figure A.3). This is evidenced by the lack of sharp, localized staining on the apical membrane (Figure A.3, circled), which is in contrast to what is observed in healthy and steatosis subjects.
Figure A.2: Hepatic MRP3 Protein Induction in NASH. MRP3 protein was measured via immunoblot analysis in human liver samples that were diagnosed as being normal, steatotic (NAFLD), or NASH. * p < 0.05 healthy patients compared to NASH patients; † p < 0.05 patients with simple steatosis compared to NASH.
Figure A.3: Hepatic MRP2 Localization in Patients with NASH.

Immunohistochemistry was used to detect and visualize MRP2 protein localization within normal, steatosis, and NASH liver. The red circles indicate regions of perturbed localization of MRP2 on the canalicular membrane. All images were taken at 100x magnification.
To further determine the potential for altered SULT activity in vivo we used results from a previously validated and published microarray experiment in these same human samples to determine the expression of key players in the sulfur activation and utilization pathway (Lake et al., 2011). Results of the gene expression analysis are shown in Figure A.4. Expression of the sulfur uptake transporter, SLC26A1, is significantly reduced in NASH liver compared to normal; however, expression of SLC26A2 was unchanged. In addition to extracellular uptake, sulfur may also be liberated from cysteine pools in the cell through the action of cysteine dioxygenase type 1 (CDO1) resulting in sulfite which is then converted to sulfate by sulfite oxidase (SUOX) (Wilkinson & Waring, 2002; Markovich, 2001; Feng et al., 2007). CDO1 and SUOX were both significantly down-regulated in NASH samples compared to normal suggesting that there is a decreased potential for sulfate activation from intracellular sources.
Figure A.4: Normalized Gene Expression of Sulfur Activation and Utilization Pathways. Hepatic gene expression of enzymes and transporters involved in the activation and utilization of sulfur are shown in human liver samples diagnosed as normal, steatotic, and NASH. Gene expression data was mined from a previously validated and published microarray experiment performed in a subset of aforementioned human liver samples (Lake et al., 2011). Data was normalized to the median of the normal diagnostic category and presented as normalized gene expression. * p < 0.05 healthy patients compared to NASH patients.
Discussion

The purpose of the current study was to investigate the effects of NASH on the disposition of APAP and its two primary metabolites, APAP-gluc and APAP-sulf, in a cohort of pediatric patients. Using a small, pilot study design, our results indicate that children with NASH tend to have increased retention of the metabolite, APAP-gluc, in systemic circulation along with increased excretion into the urine. Moreover, we identify the dysregulation of the hepatic membrane transporters, MRP2 and MRP3, as potential mechanism for these observations. However, a significant limitation of this study is the low number of NASH patients that were recruited on study, as well as the lack of ethnic, racial and sexual diversity within our patient pool, which was primarily male and of Hispanic background. Therefore, the validation and significance of these findings as a potential diagnostic to distinguish patients with NASH from those with milder disease will benefit from the inclusion of a larger randomized trial in the future. Nonetheless, the results gathered from this study warrant further consideration when administering pharmaceuticals to children with NAFLD as they may be at higher risk for developing adverse drug reactions as a result of aberrant drug disposition.

The metabolism and disposition of APAP has been heavily investigated and well characterized. Glucuronidation and sulfonation of APAP in the liver are the predominant metabolic pathways, and account for 50-70% and 25-35% of acetaminophen, respectively (McGill & Jaeschke, 2013). Unlike APAP parent, APAP-gluc and APAP-sulf are considerably more polar and require the aid of membrane transporters for proper efflux and excretion from the body. It is important to emphasize the distinctive location of the
transporters within hepatocytes, as well as their respective APAP metabolite substrates. In healthy livers, biliary excretion of the sulfate and glucuronide conjugates of APAP is predominantly mediated by MRP2, which is localized to the apical, or canalicular membrane of hepatocytes (Xiong et al., 2000). Sinusoidal excretion of the APAP-gluc metabolite from hepatocytes is predominantly mediated by MRP3 (Manautou et al., 2005) while MRP4, which is also expressed on the sinusoidal membrane, appears to mediate excretion of APAP-sulf metabolites (Zamek-Gliszczynski et al., 2006).

Despite the limited number of NASH patients, our findings are consistent with previous investigations that have cited increased plasma APAP-gluc levels following a dose of APAP in NAFLD. Specifically, we have previously reported the effects of experimental NASH on the disposition of APAP-gluc (Lickteig et al., 2007a). In that previous study, dietary rat models of steatosis and NASH were administered APAP and levels of APAP metabolites in the blood, bile, and urine were measured over a 90 minute time frame. Similar to our observations in pediatric patients, NASH rodents demonstrated an increase in plasma and urine levels of APAP-gluc (Lickteig et al., 2007a). Moreover, these findings were coupled to decreased biliary excretion of APAP-gluc. This shift from bile to plasma disposition was presumably due to an induction of the hepatic sinusoidal membrane transporter, MRP3, which we also found to be induced in human NASH herein. However, it is interesting to note that although biliary excretion of APAP-gluc was decreased, ABCC2 protein was induced in experimental NASH (Lickteig et al., 2007a). This anomaly was later confirmed in an independent study investigating the effects of NASH on ezetimibe disposition. Using a rat model, it was noted that Mrp2 localization in
NASH was altered, which subsequently lead to altered ezetimibe disposition (Hardwick RN, 2012). These effects were suggested to be due to MRP2 being internalized in rodent NASH, which would consequently diminish MRP2-mediated excretion into the bile. In a similar fashion, our data confirm these original findings in the rodent model and suggest that MRP2 localization is altered in human NASH, which would potentially explain the functional outcome of increased APAP-gluc in the serum of pediatric NASH patients.

Barshop et al., previously reported that acetaminophen disposition is altered in pediatric patients afflicted with NAFLD. In this study, APAP-gluc was found to be slightly increased in both the plasma and urine of pediatric patients with NAFLD (Barshop et al., 2011). Our results are in agreement with these previous findings and demonstrate that the mechanistic features of transport function in pediatric NASH causes a functional disruption in APAP-gluc disposition leading to increased systemic exposure to this metabolite. However, in contrast to our conclusions, the previous investigators allude to altered glucuronidation, presumably via disruption of enzymatic activity, as a mechanism for the observed increase in systemic APAP-gluc levels (Barshop et al., 2011). Although we did not investigate glucuronidation capacity in these patients, more recent findings have demonstrated that APAP glucuronidation is not altered in NASH subjects, suggesting that the observed effects of NASH on APAP-gluc disposition is less likely mediated by altered glucuronidation (Hardwick et al., 2013). In contrast, we argue that decreased MRP2 function (due to altered membrane localization) coupled to induction of MRP3 is the primary mechanism resulting in increased systemic exposure to APAP-gluc in these patients. However, glucuronidation capacity is known to be variable among children and
therefore further characterization of APAP glucuronidation in children with NASH is needed for a more comprehensive conclusion.

In spite of the induction in MRP3 protein expression, we do not observe a concomitant increase in serum APAP-sulf levels following APAP administration in these NASH subjects. In contrast, we report a decreasing trend in serum APAP-sulf in NASH, which is consistent with previous findings in a rodent model dosed with APAP (Lickteig et al., 2007a). These results are also in parallel with previous data that demonstrate decreased pan-sulfotransferase activity in NASH despite the protein induction of several sulfotransferase isoforms in the disease (Hardwick et al., 2013). Moreover, decreased total sulfotransferase activity in humans with alcoholic and nonalcoholic liver disease was identified in an independent study (Yalcin et al., 2013). Together, these results are suggestive of disrupted cellular sulfur activation and utilization in NASH, which would ultimately limit the intracellular concentrations of the sulfonation cofactor, 3’-phosphoadenosine-5’-phosphosulfate (PAPS). Indeed, by using data that was generated from a previously validated and published gene microarray, we demonstrate altered expression of genes critically involved in sulfur utilization. Specifically, we identify decreased expression of the sulfur uptake transporter, SLC26A1, as well as deceased expression of CDO1 and SUOX, which are important in liberating intracellular sulfur pools from the amino acid cysteine (Wilkinson & Waring, 2002; Feng et al., 2007). Together, these results demonstrate decreased hepatic capacity to synthesize PAPS in NASH, which may partially explain the decrease in serum APAP-sulf observed in this study.
It is worth noting that in addition to APAP-gluc being elevated when compared to normal, healthy clinical subjects in NASH, it is also elevated over patients with simple steatosis due to the mechanistic features of altered transporter function only present at the later stage of the disease. This raises the possibility of using serum APAP-gluc levels as a potential biomarker to be used as a non-invasive tool for diagnosing NASH from “not NASH”. More generally, given the extensive knowledge and characterization of normal APAP disposition, the administration of a single oral dose of APAP may be a useful measure of hepatic transporter function. Histological analysis of a liver biopsy still remains the gold standard in diagnosing NAFLD, as it is able to assess steatosis, fibrosis, and inflammation, as well as changes in overall liver architecture (Wieckowska & Feldstein, 2008). However, this is an invasive procedure and impractical to use per standard of care. Moreover, current methods of distinguishing patients with steatosis from those with NASH lack the specificity and sensitivity to replace liver biopsy. While the majority of NAFLD diagnoses are currently made on the basis of elevated aminotransferase levels, normal serum aminotransferase tests can be seen in patients with both steatosis and NASH (Mofrad et al., 2003; Ipekci et al., 2003), which is consistent with our observations (Table A.1). Furthermore, several investigators have reported that two-thirds of NASH patients may have normal aminotransferase levels at any given time (Oh et al., 2008; Wieckowska & Feldstein, 2008; Delgado, 2008), highlighting the need for a more effective, non-invasive means of diagnosing NASH. The clinical development of an APAP-gluc disposition test for NASH may help indicate at risk patients for diagnostic liver biopsies, or serve as a noninvasive means of tracking progression or treatment of the disease.
In conclusion, the results obtained from this preliminary pilot study demonstrate the potential for NAFLD to disrupt drug pharmacokinetics in children. Specifically, altered MRP2 localization and MRP3 induction appear to represent a primary mechanism for the increase in APAP-gluc in pediatric NAFLD patients. Although APAP-gluc is pharmacologically and/or toxicologically inactive, serious health risks may be imposed in the event of increased systemic exposure of a highly active metabolite in patients with NAFLD. Further studies utilizing a larger sample size in a randomized controlled setting is warranted for further verification of these findings and their clinical implications.
APPENDIX B: SUPPLEMENTAL DATA FOR CHAPTER 2

Supplemental Table B.1: Glass’s Δ in mRNA Expression of Human NASH and Rat NASH Models. Effect size of human transporter mRNA expression compared to effect sizes tabulated for mRNA expression of rat NASH models. Positive effect changes reflect induction of mRNA expression whereas negative effect changes reflect repression in mRNA expression. The magnitude of the values calculated represents the power of that particular model in detecting a difference in gene expression over control.

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<th>Rat G Δ</th>
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Supplemental Table B.2: Glass’s Δ in mRNA Expression of Human NASH and Mouse NASH Models. Effect size of human transporter mRNA expression compared to effect sizes tabulated for mRNA expression of mouse NASH models. Positive effect changes reflect induction of mRNA expression whereas negative effect changes reflect repression in mRNA expression. The magnitude of the values calculated represents the power of that particular model in detecting a difference in gene expression over control.

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Supplemental Table B.3: Glass’s $\Delta$ in Protein Expression of Human NASH and Rat NASH Models. Effect size of human transporter protein expression compared to effect sizes tabulated for protein expression of rat NASH models. Positive effect changes reflect induction of protein expression whereas negative effect changes reflect repression in protein expression. The magnitude of the values calculated represents the power of that particular model in detecting a difference in protein expression over control.

<table>
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<tr>
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<th>Rat G $\Delta$</th>
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**Supplemental Table B.4: Glass’s Δ in Protein Expression of Human NASH and Mouse NASH Models.** Effect size of human transporter protein expression compared to effect sizes tabulated for protein expression of mouse NASH models. Positive effect changes reflect induction of protein expression whereas negative effect changes reflect repression in protein expression. The magnitude of the values calculated represents the power of that particular model in detecting a difference in protein expression over control.

<table>
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<th>Mice G Δ</th>
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APPENDIX C: SUPPLEMENTAL DATA FOR CHAPTER 5

Supplemental Figure C.1: Chemical Structure of Inorganic and Methylated Arsenic Species. The chemical structure of inorganic arsenic and methylated metabolites biotransformed in vivo. MMA\textsuperscript{V}, methylarsonic acid; MMA\textsuperscript{III}, methylarsonous acid; DMA\textsuperscript{V}, dimethylarsinic acid; DMA\textsuperscript{III}, dimethylarsinous acid.
APPENDIX D: FUNDING ACKNOWLEDGEMENTS

Funding for this work was supported in part by the National Institutes of Health Grants [DK068039, ES006694, AI083927, HD062489], the National Institute of Environmental Health Science Toxicology Training Grant [ES007091], The Superfund Research Program [ES04940], and the Science Foundation of Arizona Fellowship Program. The Liver Tissue Cell Distribution System (human liver tissue) was sponsored by the National Institutes of Health Contract [NO1-DK-7-0004/HHSN267200700004C].
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