**Novel Mediators of Diabetes-Associated Cardiovascular Disease**

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Novel Mediators of Diabetes-Associated Cardiovascular Disease

A dissertation presented

by

Mary Elizabeth Gearing

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

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Novel Mediators of Diabetes-Associated Cardiovascular Disease

Abstract

Individuals with diabetes have a two- to four-fold increased risk of cardiovascular disease (CVD) compared to non-diabetics, and over 65% of diabetics die from CVD. Current treatments do not normalize this increased risk, necessitating the investigation of new drug targets for diabetes-associated CVD.

Using the Liver Insulin Receptor Knockout (LIRKO) mouse model, we have characterized two novel mediators of diabetes-associated CVD. By examining microarray data from the LIRKO and other models of insulin resistance, we found that the cholesterol biosynthetic pathway is heavily downregulated in insulin resistant states. This decreased cholesterologenic gene expression can be attributed to an increased level of cholic acid in bile, which promotes cholesterol absorption in the gut. Knockdown of Cyp8b1, the rate-limiting enzyme in cholic acid synthesis, normalizes plasma total and LDL cholesterol in LIRKO mice on a Western diet. These data provide a novel role for bile acid metabolism in the pathophysiology of insulin resistance, and they suggest that lowering cholic acid levels could improve the metabolic health of diabetic patients.

Previous work by the Biddingger laboratory identified flavin-containing monooxygenase 3 (Fmo3) as an important regulator of lipid metabolism in insulin resistant states. Work from other groups has shown that an enzymatic product of FMO3, TMAO, is correlated with CVD in mice and humans, but its function in lipid
metabolism is only now being studied. We dissected the role of FoxO1 downstream of FMO3 using antisense oligonucleotides. Although many of FMO3’s effects on glucose and lipid metabolism are primarily mediated by FoxO1, we identified a novel, hepatic FoxO1-independent role for FMO3 in modulating adiposity. We also created an Fmo3 knockout mouse using CRISPR/Cas9 to examine the non-hepatic effects of FMO3. Studies using this model also suggest a role for Fmo3 in adipose tissue energy balance.

This dissertation improves our understanding of the mechanisms by which insulin regulates lipid metabolism. Our results validate CYP8B1 and FMO3 as molecules of great importance downstream of insulin signaling, and as potential drug targets for diabetes-associated CVD and other metabolic diseases.
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Glossary

18S: 18s ribosomal RNA
ACC: Acetyl-CoA carboxylase
ApoA1: Apolipoprotein A1
ApoB: Apolipoprotein B
ApoE: Apolipoprotein E
C57BL/6: common, insulin sensitive mouse strain
CA: Cholic acid
CDCA: Chenodeoxycholic acid
Cre: Cre recombinase
CVD: Cardiovascular disease
CYP27A1: Sterol 27-hydroxylase
CYP7A1: Cholesterol 7-alpha-monooxygenase
CYP7B1: 25-hydroxycholesterol 7-alpha-hydroxylase
CYP8B1: Sterol 12-alpha hydroxylase
CYP51A1: Lanosterol 14 alpha-demethylase
DCA: Deoxycholic acid
DCCT: Diabetic Control and Complications Trial
EDIC: Epidemiology of Diabetes Interventions and Complications
FASN: Fatty acid synthase
FDFT1: Farnesyl-disphosphate farnesyltransferase 1
FDPS: Farnesyl diphosphate synthase
Floxed: loxP sites flank one exon of a given gene
FMO3: Flavin-containing monooxygenase 3
FMO3 KO: Mice homozygous for a frameshift allele of Fmo3
FoxO1: Forkhead box protein O1
FXR: Farnesoid X receptor
GLP-1: Glucagon-like peptide-1
HbA1c: Hemoglobin A1c; measure of glycated hemoglobin
HDL: High density lipoprotein
HMCGR: 3-hydroxy-3methylglutaryl CoA reductase
I3C: Indole-3-carbinol
LCA: Lithocholic acid
LDKO: Liver Double Knockout (FoxO1lox/loxInsRlox/lox::alb-Cre+)
LDL: Low density lipoprotein
LDLR: LDL receptor
LFKO: Liver-specific FoxO1 Knockout (FoxO1lox/loxInsRwt/wt::alb-Cre+)
LIRKO: Liver Insulin Receptor Knockout (FoxO1wt/wtInsRlox/lox::alb-Cre+)
NTS: Nephrotoxic serum
ob/ob: Leptin-deficient mice (Lepob/ob)
SCD1: Stearoyl-CoA desaturase-1
SREBP: Sterol responsive element binding protein
TBP: TATA-box binding protein
VLDL: Very low density lipoprotein
WT: Wild-type
Acknowledgments

Science is not conducted in a vacuum, and I have many people to thank for the work in this thesis. First, my advisor Sudha Biddinger, for bringing her optimism and love of science to the laboratory. Former graduate students Mary Haas and Alisha Ling helped me through all the ins and outs of graduate school. Ji Miao was instrumental in helping me develop the FMO3 project and teaching me how to be a scientist. For the CYP8B1 story, I am very thankful to have collaborated with Alisha Ling and Ivana Semova. It has been wonderful to work side by side with all of the Biddinger laboratory members, and I hope our paths continue to cross in the future.

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Chapter 1 - Introduction
Diabetes-Associated Cardiovascular Disease

Cardiovascular disease (CVD) remains the leading cause of death in the United States. It is well known that diabetic patients have a much higher risk of CVD, estimated at about 2-4 fold (Fox 2010). About ~65% of diabetics ultimately die from CVD, mostly from atherosclerotic disease (rev. in Low Wang et al., 2016). Despite this well-established clinical correlation, we lack effective drug treatments for diabetes-associated CVD. Although rigorous blood glucose monitoring decreases CVD risk in diabetics, it does not normalize their risk to that of non-diabetics (Nathan et al., 2005).

Current treatments for cardiovascular disease aim to decrease levels of low density lipoprotein (LDL, commonly known as "bad cholesterol"). Through epidemiological studies like the Framingham Heart Study, high LDL levels have been shown to be associated with higher CVD risk (rev. in Fox 2010). Statins are the current standard of care for CVD primary and secondary prevention, and they lower LDL levels by inhibiting cholesterol synthesis and increasing receptor-mediated uptake of LDL from the blood (Brown & Goldstein, 1997). Although statins reduce LDL levels in diabetics, they unfortunately do not normalize CVD risk in this population (Collins et al., 2003; Colhoun et al., 2004).

As the prevalence of type 2 diabetes continues to increase, so also is the incidence of metabolic syndrome, previously referred to syndrome X and partially overlapping with prediabetes (Grundy 2008). Metabolic syndrome is defined by a complex constellation of traits: hypertriglyceridemia, high blood pressure, dyslipidemia, abdominal obesity, and fasting hyperglycemia (Grundy et al., 2005). Together, these
metabolic defects place individuals at a higher risk for CVD and other diseases, including non-alcoholic fatty liver disease/steatohepatitis, gallstones, and diabetes. While only about 10% of the US population suffers from diabetes, researchers estimate that more than 30% of the United States population suffers from metabolic syndrome, promoted by the Western diet and sedentary lifestyle (Grundy et al., 2016).

Although insulin was discovered in 1922, and this protein has since been extensively studied, it remains unclear how insulin absence (type 1 diabetes) and insulin resistance (type 2 diabetes/metabolic syndrome) increase the risk of cardiovascular disease. In humans and mice, hyperglycemia increases blood vessel stiffness and causes oxidative damage to the endothelium, changes that contribute to the pathology of atherosclerosis (Reaven et al., 1997; Perkins et al., 2015). It is clear that hepatic insulin resistance changes lipid metabolism, including cholesterol related processes such as very low density lipoprotein (VLDL) secretion and LDL uptake by the LDL receptor, but the precise molecular mechanisms have not been well characterized (Biddinger & Kahn, 2006).

Cholesterol and Bile Acid Metabolism

**Cholesterol Metabolism**

Lipoproteins transport triglycerides and cholesterol in the blood. Intestinally produced chylomicrons transport dietary triglycerides, whereas very low density lipoprotein (VLDL) particles from the liver transport endogenously produced triglycerides. Although chylomicron remnants are quickly and completely cleared from plasma, the half-life of the VLDL remnant low density lipoprotein (LDL) is 2-4 days (Cohen & Armstrong, 2011). Consequently, plasma cholesterol content is a product of
LDL levels, and can be defined by the combination of VLDL synthesis and secretion and LDL uptake and degradation levels, as well as the levels of high density lipoprotein (HDL)-mediated reverse cholesterol transport and bile acid formation.

As ApoB, the protein component of VLDL, is translated by ribosomes in the liver, triglycerides are added, forming a pre-VLDL particle. After elongation is complete, the particle moves to the Golgi, where it is further lipidated and subsequently secreted as mature VLDL. VLDL synthesis and secretion is highly regulated; for example, most ApoB is degraded before lipidation, and sortilin can direct VLDL to the lysosome for recycling (rev. in Haas et al., 2013).

In muscle and adipose tissue, triglycerides are hydrolyzed from VLDL, producing particles that are enriched in cholesterol. HDL transfers ApoE to the particles, now termed VLDL remnants or intermediate density lipoproteins (IDL). About 50% of IDL is cleared; the other 50% is converted to LDL, the lipoprotein most highly enriched in cholesterol. The LDL receptor (LDLR) clears LDL via receptor-mediated endocytosis, an inefficient process that contributes to the particles’ long half-life.

Upon lysosomal degradation of the LDL-LDLR complex, LDLR protein is recycled to the cell surface and unesterified cholesterol is released. The unesterified cholesterol signals cholesterol abundance in the cell, causing downregulation of HMGCR to lower cholesterol synthesis and downregulation of LDLR expression to decrease further cholesterol uptake (Brown & Goldstein, 1997; Radhakrishnan et al., 2008).

Formation of HDL, the mediator of reverse cholesterol transport, also takes place in the liver. Apolipoprotein AI (ApoAI) is secreted and interacts with ABCA1, which adds membrane phospholipids and unesterified cholesterol to ApoAI, producing pre-β-HDL.
In plasma, transfer proteins and scavenger receptors move cholesterol and phospholipids from peripheral tissues and other lipoproteins to pre-β-HDL, forming a mature, cholesterol-rich HDL molecule that then returns to the liver.

**Atherosclerosis**

Atherosclerosis, or hardening of the arteries, is a complex, multifactorial disease in which plaques containing mixtures of cholesterol and immune cells accumulate within blood vessels over time ([Fig. 1.1](#)) (rev. in Frostegård, 2013). When LDL cholesterol levels are too high in the blood, the LDL can move below the endothelium and become oxidized. This damage to the endothelium recruits monocytes, which are subsequently activated to become macrophages. Macrophages have some capacity to remove cholesterol, but if too much is present, they can rupture, attracting further immune system components and beginning the process of plaque formation. Large plaques can occlude arteries, and more commonly, unstable plaques can break off and block blood flow, causing a heart attack, stroke, or infarct.

**Figure 1.1. The progressive nature of atherosclerosis.**
The progression of atherosclerosis shown from left to right. Over time, fatty streaks in the arteries build up to form larger plaques. These plaque may narrow an artery, blocking blood flow, or break off and block blood flow in a different location. From Npatchett, licensed under a CC BY-SA 3.0 license.
The diabetic susceptibility to atherosclerosis is likely a combination of multiple factors. As noted above, hyperglycemia increases oxidative stress, leading the formation of advanced glycation end products (Reaven et al., 1997). These compounds increase vascular stiffness, LDL oxidation and immune cell recruitment, worsening blood vessel dynamics. Patients with obesity display increased inflammation, which helps promote atherosclerosis and may destabilize plaques (Libby, 2002). Diabetics often have low HDL and decreased reverse cholesterol transport, increasing the amount of cholesterol that remains in plaques (rev. in Goldberg, 2008). They often also display increased levels of small dense LDL, a subtype of LDL that passes more easily through the endothelium to become oxidized and is highly associated with atherosclerosis (Krauss, 1994).

**Bile Acid Metabolism**

Cholesterol delivered to the liver through reverse cholesterol transport is converted to bile acids, the main route for cholesterol loss. Bile acids play a key role in facilitating lipid absorption in the gut; however, they also as signaling molecules that influence metabolic homeostasis (Thomas et al., 2008). Bile acid synthesis proceeds through one of two pathways: the neutral or the acidic pathway (Fig. 1.2). The classical neutral pathway accounts for about 75% of bile acid synthesis and is initiated by the action of cholesterol 7 alpha-hydroxylase (CYP7A1) (Russell, 2003). Through a subsequent 12α-hydroxylation reaction catalyzed by CYP8B1, the neutral pathway produces cholic acid (CA) and its derivatives. The alternative acidic pathway accounts for the remaining 25% of bile acid synthesis through the action of enzymes CYP27A1 and CYP7B1. This pathway produces exclusively chenodeoxycholic acid (CDCA).
derivatives. The neutral pathway can also produce CDCA derivatives due to crossover before the CYP8B1-catalyzed 12α-hydroxylation step. CYP8B1 action represents a commitment to the formation of CA, a step that regulates the balance between CA and CDCA derivatives in bile (Bjorkhem, 1983).

Before leaving the liver, bile acids are conjugated to glycine or taurine to form bile acid salts. In the intestine, bile acids can be dehydroxylated by the gut bacteria to form secondary bile acids. These derivatives of CA and CDCA are known as deoxycholic acid (DCA) and lithocholic acid (LCA), respectively.

![A simplified schematic of bile acid synthesis.](image)

**Figure 1.2. A simplified schematic of bile acid synthesis.**
The majority of bile acid synthesis occurs through the neutral pathway, which produces both CA and CDCA derivatives, but about 25% proceeds through the acidic pathway and produces exclusively CDCA derivatives. Bile acids are glycine- or taurine-conjugated prior to secretion, and can be modified by intestinal bacteria to form secondary bile acids.
Bile acid formation is tightly controlled through the action of multiple signaling pathways. Particularly, bile acids activate farnesoid X receptor (FXR) signaling, and FXR effector SHP downregulates Cyp7a1 and Cyp8b1 expression through a negative feedback loop (Chiang et al., 2009). Since Cyp7a1 is the primary determinant of bile acid pool size, this mechanism maintains bile acid synthesis within a specified range (Chiang et al., 2009; Fiorucci et al., 2009). FXR also decreases CYP7A1 through activation of FGF15 in the ileum (Inagaki et al., 2005). When cholesterol and oxysterols accumulate, liver X receptor (LXR) signaling increases Cyp7a1 transcription (Gupta et al., 2002). Insulin also regulates bile acid synthesis by downregulating Cyp7a1 and Cyp8b1 expression (Chiang et al., 2009).

Although bile acids perform similar functions: promoting lipid absorption and signaling to receptor in the intestine, liver and other tissues, these species differ greatly in their signaling properties. As the most hydrophobic bile acids, cholic acid derivatives promote increased cholesterol absorption compared to CDCA derivatives. Bile acids also differ based on their ability to interact with various receptors (Table 1.1). CDCA is an especially potent ligand for the nuclear receptor FXR, which acts to decrease plasma triglyceride levels and increase insulin sensitivity (Watanabe et al., 2004). TGR5, the G-protein coupled bile acid receptor, is most potently activated by lithocholic acid species, followed by deoxycholic, chenodeoxycholic, and cholic acid species (Kawamata et al., 2003). TGR5 also improves insulin sensitivity by increasing secretion of the incretin GLP-1 (Thomas et al., 2009). TGR5 increases energy expenditure through its activation of iodothyronine deiodinase in skeletal muscle and brown adipose tissue (Watanabe et al., 2006).
We do not yet understand the minutiae of bile acid signaling, but it is clear that these molecules are metabolically significant. Bile acids have previously been manipulated to treat diabetes, hypertriglyceridemia, and gallstones. Bile acid sequestrants, resins that bind bile acids and increase their excretion, shunt more cholesterol into bile acid synthesis and have been shown to lower LDL cholesterol in diabetics (Staels, 2007). These resins also improve HbA1c levels, indicating that they improve glucose metabolism (Staels, 2007). Hypertriglyceridemia and gallstones have both been treated through direct administration of CDCA. In the case of hypertriglyceridemia, CDCA activates FXR signaling and promotes triglyceride lowering (Bateson et al., 1978; Watanabe et al., 2004). For patients with gallstones, CDCA lowers the hydrophobicity and relative cholesterol concentration of the bile, permitting gallstone regression (Heuman et al., 1989; Russell et al., 2003).

It is important to note that the composition of the bile acid pool differs between mice and humans. Although both animals synthesize bile acids through the neutral and acidic pathways, they have different dominant bile acid species. Human bile is

<table>
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<tr>
<th>Receptor</th>
<th>Location</th>
<th>Effectors</th>
<th>Metabolic Effects</th>
<th>Agonists</th>
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<tr>
<td>FXR</td>
<td>Nuclear</td>
<td>SHP, FGF15</td>
<td>Reduces plasma TG and adiposity; improves insulin sensitivity</td>
<td>CDCA &gt;&gt; LCA ≥ DCA &gt; CA Antagonists: α-MCA, β-MCA</td>
</tr>
<tr>
<td>TGR5</td>
<td>Surface (GPCR)</td>
<td>DIO, GLP-1</td>
<td>Increases energy expenditure in BAT and SKM; improves insulin sensitivity</td>
<td>LCA ≥ DCA &gt; CDCA &gt; CA</td>
</tr>
</tbody>
</table>

Table 1.1. Bile acid receptors
predominantly composed of CDCA, a strong FXR agonist (Table 1.1; Fig. 1.3) (Watanabe et al., 2004). In mice, CDCA is efficiently converted to \(\alpha\)- and \(\beta\)-muricholic acids, which do not activate FXR (Table 1.1; Fig. 1.3). In fact, tauro-conjugated \(\alpha\)- and \(\beta\)-MCA are FXR antagonists (Sayin et al. 2013). These differences are important to consider in modeling human bile acid metabolism using mice.

**Figure 1.3. Bile acid distribution in humans and mice.**
The distribution of bile acids differs between human and mice, and the relative amount of each bile acid is denoted by its text size at the bottom of the figure. Human bile is predominantly CA and CDCA. In mice, CDCA is converted to \(\alpha\)- and \(\beta\)-MCA. CA is also less abundant in mouse bile. Data adapted from de Aguiar Vallim et al., 2013.

**Insulin Regulation of Lipid Metabolism**

**LIRKO Model of Insulin Resistance**

Studies using mouse models have validated the role of hepatic insulin resistance in promoting cardiovascular disease (Fig. 1.4). The Liver Insulin Receptor Knockout (LIRKO) mouse, first characterized in 2000, has a >90% loss of hepatic insulin signaling, and displays glucose and insulin intolerance (Michael et al., 2000). Although
LIRKO mice display normal plasma cholesterol levels on a chow diet, they are uniquely susceptible to the effects of a Western or atherogenic diet. On the atherogenic Paigen diet (15% fat, 1% cholesterol, 0.5% cholic acid), LIRKO mice display plasma total cholesterol levels of ~500 mg/dl, compared to 100 mg/dl observed in their floxed controls (Biddinger et al., 2008a). This increased cholesterol comes mostly in the form of pro-atherogenic LDL, and 100% of LIRKO mice develop atherosclerosis after 12 weeks of diet, compared to 0% of control mice (Biddinger et al., 2008a). In addition to their susceptibility to atherosclerotic CVD, LIRKO mice also develop cholesterol gallstones on the Paigen diet (Biddinger et al., 2008b). On Paigen and Western diets, LIRKO mice also display increased lipid accumulation in the liver, as well as increased inflammation and a steatohepatitis-like phenotype. In sum, LIRKO mice recapitulate the cardiovascular complications of metabolic syndrome and diabetes.
Figure 1.4. The central role of insulin resistance in diabetes-associated CVD. We hypothesize that insulin resistance plays a central role in the development of diabetes-associated CVD and other diabetic sequelae. This hypothesis is supported by human data and studies conducted in the LIRKO mouse. Figure adapted from Biddinger & Kahn, 2006.

The LIRKO mouse model allows us to study how hepatic insulin signaling regulates lipid metabolism, which enables the discovery and characterization of novel drug targets for metabolic disease. LIRKO mice are a model of pure hepatic insulin resistance, in which all signaling downstream of the insulin receptor is blocked (Michael et al., 2000). Thus, in the LIRKO mouse, FoxO1 is overactive, promoting increased hepatic gluconeogenesis, but SREBP-1c is inactive, preventing lipogenesis.
This state contrasts with type 2 diabetes, a state of partial insulin resistance in which the sensitivity of lipogenesis to insulin signaling is usually maintained (Brown & Goldstein, 2008). Individuals with type 2 diabetes or metabolic syndrome often present with hypertriglyceridemia due to SREBP-1c hyperactivation (Fig. 1.5), but LIRKO mice do not display this defect. For this reason, we also use ob/ob and diet-induced obese (DIO) models of type 2 diabetes, in which lipogenesis is maintained. We also employ the streptozotocin (STZ) model of type 1 diabetes, which displays both hypercholesterolemia and hypertriglyceridemia (Biddinger & Kahn, 2006).

**FoxO1**

Forkhead box protein 1 (FoxO1) is a transcription factor involved in the regulation of many diverse processes, including metabolism, cellular survival and proliferation, and
longevity. FoxO1 is negatively regulated by insulin, as it is phosphorylated and thus excluded from the nucleus by Akt1/2. Phosphorylated FoxO1 is subsequently ubiquitinated and degraded. FoxO1 is also regulated by acetylation, methylation and glycosylation, but regulation by phosphorylation is the best studied mechanism of FoxO1 regulation (Cheng & White, 2011).

In the liver, FoxO1 is a dominant transcription factor in the fasted state and is well known to promote gluconeogenesis through transcriptional regulation of targets like glucose 6-phosphatase (G6pc) and phosphoenolpyruvate carboxykinase (Pck1) (Matsumoto et al., 2007). The reduction of FoxO1 and target gene expression is a key element of the fasting-to-feeding transition that is lost in mouse models of insulin resistance and diabetes (Cheng & White, 2011). In insulin resistant models such as the LIRKO, hepatic IRS1/IRS2 KO, and hepatic Akt1/Akt2 KO, nuclear FoxO1 and gluconeogenic target gene expression remain high even in the fed state. Similarly, diabetic humans show increased levels of gluconeogenesis due to insulin resistance (Biddinger & Kahn, 2006).

The hepatic knockout of FoxO1 in mouse models of insulin resistance rescues many of the negative effects of insulin resistance on metabolic homeostasis. For example, it normalizes plasma glucose and insulin levels, as well as gene expression of many genes involved in metabolism, with the exception of the lipogenic genes (Dong et al., 2008). Further studies have shown that FoxO1 ablation allows for the liver to regulate glucose production through an additional pathway that does not require hepatic insulin sensitivity (O-Sullivan et al., 2015; Titchenell et al., 2015). This extrahepatic signaling may take place in the brain or the adipose tissue.
In addition to its heavily studied role in controlling gluconeogenesis, FoxO1 regulates many other genes downstream of insulin signaling. FoxO1 induces expression of Mttp, increasing VLDL production in the fasted state (Kamagate et al., 2008). FoxO1 also activates expression of Abcg5 and Abcg8, which increase biliary cholesterol secretion (Biddinger et al., 2008b). FoxO1 also regulates the composition of the bile acid pool by promoting transcription of Cyp8b1 (Haeusler et al., 2012).

**CYP8B1**

CYP8B1 is required for the 12\(\alpha\)-hydroxylation reaction that forms cholic acid (Russell, 2003). Studies of Cyp8b1 knockout mice show that these mice have decreased levels of cholic acid derivatives, but they do not display a decreased bile acid pool size (Li-Hawkins et al., 2002). As cholic acid promotes increased cholesterol absorption, CYP8B1 knockout mice show decreased cholesterol absorption, as well as decreased gallstone formation and atherosclerosis (Table 1.2) (Li-Hawkins et al., 2002; Slatis et al., 2010). These mice also display improved glucose tolerance, linked to increased secretion of the incretin GLP-1 (Kaur et al., 2015).

Insulin inhibits CYP8B1 transcription by inactivating FoxO1 (Haeusler et al., 2012), and insulin resistant mouse models have increased levels of CA derivatives in their bile. Likewise, in humans, CA derivatives are positively correlated with insulin resistance (Haeusler et al., 2013). However, hepatic FoxO1 knockout (LFKO) mice, a low CA model, display lower fasting glucose but higher plasma triglycerides on a Western diet compared to control mice (Table 1.2) (Haeusler et al., 2013). These defects can be rescued by treatment with an FXR agonist. Although both LFKO mice
and CYP8B1 KO mice have decreased cholic acid levels, they have drastically different lipid phenotypes, further demonstrating the complexities underlying bile acid biology.

**Table 1.2. Cholic acid metabolism in mouse models**

<table>
<thead>
<tr>
<th>Model</th>
<th>CA Level</th>
<th>Metabolic Phenotype</th>
<th>Other Notes</th>
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</thead>
<tbody>
<tr>
<td>CYP8B1 KO</td>
<td>Low CA</td>
<td>Decreased cholesterol absorption, improved glucose tolerance</td>
<td>Decreased gallstone formation and atherosclerosis</td>
</tr>
<tr>
<td>LIRKO</td>
<td>High CA</td>
<td>Increased cholesterol absorption, increased LDL cholesterol</td>
<td>High susceptibility to gallstones and atherosclerosis</td>
</tr>
<tr>
<td>LFKO</td>
<td>Low CA</td>
<td>Increased LDL cholesterol, increased hepatic/plasma TG</td>
<td>TG phenotype rescued by treatment with an FXR agonist</td>
</tr>
</tbody>
</table>

**FMO3**

Flavin-containing monooxygenase 3 (FMO3) is one of five FMO genes in mice (Phillips & Shephard, 2008). The FMOs are analogous to the cytochrome P450 (CYP) family, and they were initially identified due to their role in xenobiotic metabolism (Lang et al., 1998; Parte & Kupfer, 2005). Unlike the CYPs, they are generally less sensitive to induction or inhibition by foreign chemicals. The yeast ancestor of the FMO family is necessary for maintaining cellular redox balance and the proper folding of secreted proteins (Suh et al., 2000), but it is not yet known if this function is conserved in mammalian FMOs.

FMOs require FAD as a prosthetic group and NADPH as a cofactor. Due to their
ability to activate oxygen prior to substrate binding, FMOs have wide, partially overlapping substrate ranges (Phillips & Shephard, 2008). Early research into FMO3 focused mostly on its ability to oxygenate the volatile compound trimethylamine (TMA) to the odorless trimethylamine N-oxide (TMAO). Humans with nonfunctional FMO3 are unable to convert TMA to TMAO and suffer from trimethylaminuria, a socially debilitating disease nicknamed fish-odor syndrome (Mitchell & Smith, 2001).

In 2011, TMAO was identified via metabolomics screen as a risk factor for cardiovascular disease (Wang et al., 2011). Subsequent studies have confirmed that betaine, choline and TMAO, all of which derive from phosphatidylcholine, are significantly associated with cardiovascular risk (Tang et al., 2013). However, the value of choline and betaine as predictors of CVD is heavily reliant on TMAO levels, underscoring the relevance of this metabolite (Wang et al., 2014). Carnitine, a component of red meat, was also found to be a source of TMAO (Koeth et al., 2013). It is important to note that conversion of choline derivatives to TMA requires the gut microbiome (Wang et al., 2011).

In additional to human studies, researchers have also examined the effects of TMAO in mouse models. Using the ApoE−/− mouse model, which is HDL-deficient and thus highly susceptible to atherosclerosis, these groups have shown that increased TMAO, as a result of choline or carnitine feeding, is positively correlated with atherosclerotic lesion size (Wang et al., 2011; Bennett et al., 2013; Koeth et al., 2013). They have also demonstrated that TMAO induces small changes in the level of scavenger receptors in macrophages responsible for cholesterol uptake, and in reverse cholesterol transport; however, they acknowledge that these changes likely do not fully
explain the contribution of TMAO to CVD risk. Although most studies have found a positive association between TMAO and atherosclerosis, a recent study found the opposite: that within the physiological range, TMAO is negatively associated with atherosclerosis risk in mice (Collins et al., 2016). Thus, the relationship and mechanisms connecting CVD and TMAO remain unclear and are an important topic of investigation. Multiple groups have explored the connection between TMAO and CVD using chemically modified antisense oligonucleotides (ASO) targeting Fmo3 (Shih et al., 2015; Warrier et al., 2015).

_Fmo3_ is exquisitely sensitive to insulin regulation, and the _Fmo3_ transcript is >1,000 fold increased in LIRKO mice, with TMAO levels increased three-fold in plasma compared to floxed mice (Miao et al., 2015). These findings led the Biddinger laboratory to investigate a role for FMO3 in diabetes-associated CVD, also using ASO-mediated knockdown. By seven weeks, FMO3 knockdown normalized glucose tolerance in LIRKO mice, such that it was indistinguishable from control floxed mice treated with the control ASO (Miao et al., 2015).

Moreover, after three months on the atherogenic Paigen diet, LIRKO mice treated with control ASO showed a four-fold increase in plasma cholesterol and a fifteen-fold increase in atherosclerosis compared to floxed mice, but FMO3 ASO treatment completely ameliorated these phenotypes in LIRKO mice (Miao et al, 2015). Thus, knockdown of FMO3 in LIRKO mice using ASO prevented hyperglycemia, hyperlipidemia and atherosclerosis. The putative mechanism behind the effects is FoxO1 downregulation, as FoxO1 protein is reduced to undetectable levels in LIRKO mice treated with FMO3 ASO (Miao et al., 2015).
Overview of the Dissertation

Although metabolic syndrome and diabetes-associated cardiovascular disease have been well-studied, we still lack effective treatments for these conditions. Previous research has identified multiple candidates, including CYP8B1 and FMO3, but further research must be completed to assess the suitability of these proteins as drug targets.

This dissertation aims to help delineate the molecular effects and mechanisms of CYP8B1 and FMO3 in the setting of insulin resistance. In chapter 2, the effects of Cyp8b1 knockdown in LIRKO and streptozotocin-treated type 1 diabetic mice are investigated, in addition to Cyp8b1 overexpression studies. In chapter 3, the differential effects of Fmo3 knockdown in LIRKO and LDKO mice are investigated to determine how FMO3 signaling relates to FoxO1 action. In chapter 4, FMO3 knockout mice are characterized to identify the effects of FMO3 in insulin sensitive mice, and to determine whether FMO3 plays an important role in metabolic tissues other than the liver. The data presented in this dissertation build upon previous research to provide a more thorough understanding of metabolic regulators CYP8B1 and FMO3 that will be valuable should either of these targets be pursued pharmaceutically.
Chapter 2 – Insulin Reprograms Whole Body Cholesterol Metabolism Through Cholic Acid
Abstract

Individuals with type 1 diabetes show an increase in cholesterol absorption coupled with a decrease in cholesterol synthesis; moreover, they are prone to the development of hypercholesterolemia, with increased amounts of Apolipoprotein B (ApoB)-associated cholesterol. Here, we used Liver Insulin Receptor Knockout (LIRKO) mice to dissect the molecular mechanisms underlying these pro-atherogenic changes. We find that insulin, in a manner dependent upon the transcription factor, FoxO1, reprograms whole body cholesterol metabolism by suppressing cholic acid. Cholic acid is a bile salt that promotes cholesterol absorption from the intestine, indirectly regulating hepatic and intestinal gene expression, and driving hyperlipidemia. Thus, the increase in biliary cholic acid, increase in intestinal cholesterol absorption, suppression of cholesterologenic genes, and hypercholesterolemia that we observed in LIRKO mice was normalized by hepatic deletion of FoxO1. Similar effects were observed by knocking down Cyp8b1, an enzyme required for cholic acid synthesis, in LIRKO mice.

Conversely, overexpression of Cyp8b1 led to an increase in the proportion of cholic acid in the bile and ApoB-containing cholesterol in the plasma of mice lacking both insulin receptor and FoxO1 in their livers. These data identify cholic acid as key mediator of insulin’s effects on cholesterol metabolism, and suggest that CYP8B1 may represent a novel target for the treatment of hypercholesterolemia, particularly in diabetic patients.
Introduction

In 1675, Sir Thomas Willis noted that the urine of diabetic patients was sweet (Ahmed et al., 2002), and thereby focused the diabetes narrative upon glucose. However, it is now clear that there are many metabolic perturbations associated with diabetes, besides hyperglycemia. For example, individuals with type 1 diabetes show a decrease in cholesterol synthesis, but an increase in cholesterol absorption (Miettinen et al., 2004). Individuals with poor control of their diabetes show increased levels of total cholesterol, LDL cholesterol and ApoB (Albers et al., 2008), and even individuals with good control can show increased levels of ApoB and increased amounts of small dense LDL cholesterol (Guy et al., 2009; Albers et al., 2008). Given the diligence and discipline required for tight glycemic control, it is not surprising that dyslipidemia is highly prevalent among diabetic patients (Keshnar et al., 2006) and patients with T1D can have a ten-fold increase in CVD risk (Libby et al., 2005; Krolewski et al, 1987).

At a molecular level, diabetes is caused by the failure of insulin to regulate its downstream targets. Normally, insulin binds to the insulin receptor, triggering its kinase activity (Biddinger & Kahn, 2006; Haas & Biddinger, 2009). The insulin receptor then phosphorylates the scaffold proteins IRS1 and IRS2. The IRS1/IRS2 proteins in turn initiate a branching cascade of signaling events. One of the most important of these is the activation of Akt, which suppresses the transcription factor FoxO1 by phosphorylating it. Phosphorylated FoxO1 is sequestered in the cytoplasm and unable to activate transcription of its target genes. How these proximal signaling events ultimately lead to physiological changes at the organismal level, however, remains an important question. This is particularly true with regard to cholesterol metabolism.
Though insulin appears to increase hepatic cholesterol synthesis, decrease cholesterol absorption, and prevent hypercholesterolemia in mice (Miao et al., 2015) as in humans (Gylling et al., 2004), the mechanisms by which it does so remain unclear. To some extent, the effects of insulin on cholesterol metabolism could be mediated by suppressing FoxO1 (Haas & Biddinger, 2009). FoxO1 drives expression of Mttp, an enzyme necessary for the synthesis and secretion of ApoB-containing lipoprotein particles from the liver (Kamagate et al., 2008); FoxO1 also appears to suppress the cholesterologenic enzymes, as mice with deletion of FoxO1 show an increase in cholesterologenic gene expression under certain conditions (Haeusler et al., 2010; Zhang et al., 2012) However, neither of these changes can fully account for the effects of insulin on cholesterol metabolism, particularly cholesterol absorption.

Here, we studied mice with hepatic deletion of insulin receptor, FoxO1, or both genes. As described previously, Liver Insulin Receptor Knockout (LIRKO) mice showed suppression of the cholesterologenic enzymes, increased cholesterol absorption, and hypercholesterolemia with an increase in ApoB-associated lipoprotein particles (Biddinger et al., 2008a; Biddinger et al., 2008b). Interestingly, deletion of FoxO1 normalized these changes by suppressing production of cholic acid, a bile acid species that promotes intestinal cholesterol absorption.

**Methods**

**Animals**

Generation of mice with floxed (flanked by loxP) insulin receptor and FoxO1 alleles (Paik et al., 2007) been described previously. To generate liver-specific knockout mice, floxed mice were crossed with Alb-Cre transgenic mice that express Cre recombinase
under the albumin promoter. LIRKO mice were previously described (Michael et al., 2000). All mice were maintained on a C57BL/6 background. Except where otherwise specified, we used male mice, fed a standard chow diet ad libitum, and sacrificed in the non-fasted state at 2 pm. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at Children’s Hospital Boston.

**ASO Experiments**

Leptin deficient ob/ob mice were purchased from Jackson Laboratories. A second generation control antisense oligonucleotide (ASO, IONIS-141923) or antisense oligonucleotide against Cyp8b1 (IONIS-431981) was diluted in normal saline, and injected intraperitoneally (50 mg/kg each week for five weeks). Mice were sacrificed at 2 pm in the non-fasted state, one day after their final dose of antisense oligonucleotide. Mice were maintained on a chow diet or a Western-type diet (Envigo TD.88137) as noted in the figure legends.

**Adenovirus Injection**

Adenoviruses encoding either Cyp8b1 (Pandak et al., 2001) or GFP (ViraQuest) were amplified by ViraQuest and administered to two- to three-month old mice via tail vein injection at a dose of 5-8x10⁸ PFU/mouse. Plasma was collected two days after injection after a four-hour fast, and the mice were sacrificed one week after injection.

**Streptozotocin Treatment**

4 week old male C57BL/6J mice were maintained on a Western diet for 5 weeks. One week prior to sacrifice, mice were injected with STZ (180 mg/kg body weight) or vehicle (0.1 M citric acid, pH=4.2).
**Gene Set Enrichment Analysis (GSEA)**

Microarray data were processed and analyzed using *limma* (Ritchie et al., 2015) and GenePattern (Reich et al., 2006). Gene set enrichment analysis was conducted using the GenePattern GSEA package with the CP:REACTOME gene set (v5.1 MSigDB), and enrichment was evaluated based on the normalized enrichment score (NES) and false discovery rate (FDR) values. Data used were previously published in Dong et al., 2008; Lu et al., 2012; and Miao et al., 2014.

**Blood Chemistry/Metabolic Analysis**

Plasma samples were collected from mice either after 4 hours of fasting or at the time of sacrifice in the non-fed state. Plasma lipids were measured using a colorimetric assay (Thermo Scientific) according to the manufacturer’s instructions.

**Glucose Tolerance Testing**

Mice were fasted for 14 hours overnight and then by intraperitoneal injection administered 1g glucose per kg body weight. Blood glucose levels were monitored via tail nick at 0, 15, 30, 60, 90 and 120 minutes after glucose injection. In Fig. S1.3B, two of the blood glucose measurements registered “HI” and were assigned a value of 600 mg/dl.

**Cholesterol Absorption**

Fractional cholesterol absorption measurements were performed as previously described (Temel et al., 2005). Thirteen-week old male mice were gavaged 0.025 µCi of [4-14C]cholesterol (Perkin Elmer) and 0.75µCi of [22,23-3H]sitosterol (American Radiolabeled Chemicals) dissolved in 50 µL of soybean oil. Mice were then individually housed in wire bottom cages, with free access to regular chow and water. After 72
hours, the feces were collected, desiccated in a vacuum oven overnight at 80°C, and then homogenized using a mortar and pestle. Duplicate aliquots of the feces were saponified in 2 mL 95% ethanol and 200 µL 50% KOH, while heated to 65°C for 1 hour with periodic vortexing. Neutral lipids were extracted by adding hexane and deionized water, vortexing, and centrifuging at 2000g for ten minutes. The upper hexane phase was transferred to a scintillation vial and dried overnight at room temperature. Ultima Gold Cocktail (Perkin Elmer) was added to the vials and the [14C]cholesterol and [3H]sitosterol counts were measured using a Liquid Scintillation Analyzer Tri-Carb 2900TR (Perkin Elmer). Percentage cholesterol absorption was calculated using the following equation:

\[
\frac{(14C/^{3}H \text{ dose ratio} - 14C/^{3}H \text{ feces ratio})}{14C/^{3}H \text{ dose ratio}} \times 100
\]

Fecal neutral sterol content was analyzed as previously described (Temel et al., 2005).

**HPLC Bile Acid Analysis**

Gallbladder bile was obtained at the time of sacrifice and analyzed by high-performance liquid chromatography. An octadecylsilane column (RP C-18, Beckman Instruments, Fullerton, CA) was used with isocratic elution at 0.75 mL/min. The eluting solution was composed of a mixture of methanol and 0.01 M KH2PO4 (67%, v/v), adjusted to an apparent pH of 5.3 with H3PO4. Bile acids were quantified by measuring their absorbance at 204 nm. Bile acid amidates (taurine and glycine) have similar extinction coefficients. Bile acids were tentatively identified by matching their relative retention times with those of known standards.

The ratio of cholic acid metabolites to chenodeoxycholic acid metabolites was calculated by dividing the sum of the percent abundances of CA metabolites (T-CA, T-
alloCA, T-DCA) by the sum of the percent abundances of CDCA metabolites (T-α-MCA, T-β-MCA, T-CDCA). The total bile hydrophobicity index was calculated by multiplying the percent abundance of each bile acid species by its hydrophobicity index as described previously (Heuman, 1989). Using the equations provided, the hydrophobicity index for T-allocholic acid was calculated as +0.06.

**Hepatic Lipids**

Livers were homogenized in 50 mM NaCl and lipid was extracted with chloroform and methanol (2:1). Lipid extract was then analyzed by a colorimetric assay per manufacturers’ instructions (Thermo Scientific).

**Primary Hepatocyte Isolation**

Primary mouse hepatocytes were isolated from 2-3 month old mice as previously described (Chandra et al., 2001). After isolation, cells were suspended in William’s E medium (Life Technologies) containing penicillin-streptomycin, 100nM glutamine, and 10% fetal bovine serum in wells coated with rat tail collagen I (BD Biosciences) for 3-6 hours. Cells were then washed twice with PBS and incubated overnight in M199 medium supplemented with 100nM dexamethasone, 100nM triiodothyronine, 1nM insulin, and penicillin-streptomycin. The next day, cells were washed twice with PBS and incubated for 6 hours in M199 medium supplemented with 100nM dexamethasone, 100nM triiodothyronine and penicillin-streptomycin, with or without the addition of 100nM insulin or 1µM GW4064 (Tocris). Cells were then harvested and RNA was prepared for qPCR analysis.
Gene Expression Analysis

RNA was isolated from frozen liver and ileum using the RNeasy kit (Qiagen). RNA from primary hepatocytes was isolated with Trizol (Life technologies). cDNA was synthesized using a reverse transcription kit (Applied Biosystems), then diluted ten-fold for real-time PCR analysis with SYBR green reagents (Thermo Scientific). Gene expression was normalized to TBP (*in vitro* and *in vivo* experiments) or 18S (*in vitro* experiments in Fig. 2.2C).

Table 2.1. Primers for gene expression analysis in Chapter 2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s</td>
<td>GTAACCCGTTGAACCCCATTT</td>
<td>CCATCCAATGGTACTGAGCG</td>
</tr>
<tr>
<td>Cyp27a1</td>
<td>GGAGGATTGGCAGAACTGGAG</td>
<td>TGCGGGGACAGACTTTACTT</td>
</tr>
<tr>
<td>Cyp51</td>
<td>AGCTGTACGACAGCTGGAT</td>
<td>ACGCCCCTTGATATGAG</td>
</tr>
<tr>
<td>Cyp7a1</td>
<td>GTCCGGATATTCAAGGATGCA</td>
<td>AGCAACTAAACACCTGCCAGTA</td>
</tr>
<tr>
<td>Cyp7b1</td>
<td>GAGTTTCTGAGCCTGCTGTC</td>
<td>TCCCTCCTGGAAAACGCT</td>
</tr>
<tr>
<td>Cyp8b1</td>
<td>GGACACCTATCCTCTGGTGGA</td>
<td>GGCCCCGATAGGGGAGTAGAC</td>
</tr>
<tr>
<td>Fdft1</td>
<td>CCAACTCAATGGGTTCGTTCCTCCT</td>
<td>TGGCTTAGCGAACACCTTTCAACT</td>
</tr>
<tr>
<td>Fg15</td>
<td>CAGTCTTTCCTCCGGATAGCG</td>
<td>TGAAGACGATGCCATGAC</td>
</tr>
<tr>
<td>Fxr</td>
<td>CACGGTTGTAATACAGACTAGTAG</td>
<td>TTGATTTAATTAGGCCAAAAGG</td>
</tr>
<tr>
<td>G6pc</td>
<td>TCTGTCGGGATCTACCTTG</td>
<td>GTAGAATCCAGGCCGAAAC</td>
</tr>
<tr>
<td>Hmgr</td>
<td>AGCCGAAGCAGACATGAGAT</td>
<td>CTTTGGGAATGCCCTTTGATTG</td>
</tr>
<tr>
<td>IncLSTR</td>
<td>TGTAGGAGCCCACAATGAA</td>
<td>CAACCTTAAGCCTCAGCAAA</td>
</tr>
<tr>
<td>Pck1</td>
<td>ATCCCAACTCGAGTTCTCGTC</td>
<td>CATGCTAAGCTGGAAAGGCGAA</td>
</tr>
<tr>
<td>Shp</td>
<td>CTGCTGGAGTCTTTCTGGGA</td>
<td>GTAGCCAGGGCCTCAAGAC</td>
</tr>
<tr>
<td>Smpd3</td>
<td>CCTGACCAGTGGCATTCTTTC</td>
<td>AGAAACGGGCTGCCGACT</td>
</tr>
<tr>
<td>Sg1e</td>
<td>CGCTGCTTGTCGATATTCT</td>
<td>AGCTGCTGCTGTTTAATGTGCT</td>
</tr>
<tr>
<td>Srebp-2</td>
<td>CTCTCTGGTGAGACCTACCA</td>
<td>ATGCTTGGTGTCTGACTG</td>
</tr>
<tr>
<td>Tbp</td>
<td>ACCCTCCACCAAATGACTTCTATG</td>
<td>TGAAGACGAAATACGCTTTG</td>
</tr>
</tbody>
</table>

Western Blotting

Livers were homogenized in RIPA buffer. 40 ug of lysate were loaded onto SDS-PAGE gels and transferred onto a PVDF membrane (Thermo Scientific). Blots were blocked in
SuperBlock buffer (Thermo Scientific), incubated overnight with primary antibody and detected with secondary antibody conjugated with HRP. Antibody complexes were visualized by enhanced chemiluminescence using X-ray film. Protein quantification was performed in ImageJ and normalized against GAPDH loading control.

Statistics

Mouse studies

Data are represented by the mean + SEM, unless otherwise indicated. Significance was assessed by a 2-tailed Student’s t-test with unequal variance, unless otherwise indicated. LFKO, LIRKO and LDKO mice were compared to their littermate Cre-controls; in addition, LDKO mice were compared to LFKO and LIRKO mice. Data are representative of two or three independent cohorts for phenotypic data and gene expression. Cholesterol analysis and fecal neutral sterol measurements were performed in only one cohort of mice. Similarly, LIRKO mice treated with Cyp8b1 ASO were compared to floxed mice treated with CON and LIRKO mice mice treated with CON ASO; results shown are representative of two cohorts.

In vitro studies

Primary hepatocyte studies were done in triplicate wells; results are representative of two to three independent experiments.

Results

FoxO1 is required for the defects in cholesterol metabolism produced by hepatic deletion of the insulin receptor

To dissect the role of FoxO1 in mediating the metabolic effects of insulin, we studied Liver FoxO1 Knockout (LFKO, Cre\textsuperscript{+/–}Foxo1\textsuperscript{loxP/loxP}IR\textsuperscript{wt/wt}) mice, Liver Insulin
Receptor Knockout (LIRKO, Cre<sup>−/−</sup>Foxo1<sup>wt/wt</sup>IR<sup>loxP/loxP</sup>) mice, and Liver Double Knockout (LDKO, Cre<sup>−/−</sup>Foxo1<sup>loxP/loxP</sup>IR<sup>loxP/loxP</sup>) mice. In addition, we studied the littermates of these mice that lacked the Cre recombinase (FoxO1-lox, Cre<sup>−/−</sup>Foxo1<sup>loxP/loxP</sup>IR<sup>wt/wt</sup>; IR-lox, Cre<sup>−/−</sup>Foxo1<sup>wt/wt</sup>IR<sup>loxP/loxP</sup>; Dlox, Cre<sup>−/−</sup>Foxo1<sup>loxP/loxP</sup>IR<sup>loxP/loxP</sup>), as controls. Western blotting revealed near complete knockout of FoxO1 in LFKO mice, the insulin receptor in LIRKO mice, and both FoxO1 and insulin receptor in LDKO mice (Fig. 2.1A). All mice were viable, fertile and of similar weight (Fig. S1.1A, and data not shown). In subsequent analyses, the three Cre<sup>−/−</sup> groups were pooled together as the control group.

LIRKO mice showed increased levels of the gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (Pck1) and glucose-6-phosphatase (G6pc), in their livers, as well as impaired glucose tolerance (Fig. 2.1B-C, Fig. S1.1B). LFKO and LDKO mice, on the other hand, showed normal levels of these genes and normal glucose tolerance, consistent with prior studies (Titchenell et al., 2015; O-Sullivan et al., 2015)
Figure 2.1. FoxO1 ablation restores cholesterologenic gene expression and cholesterol balance in insulin resistant mice.

(A-C, E-I) Male Liver FoxO1 Knockout (LFKO), Liver Insulin Receptor Knockout (LIRKO), Liver Double Knockout (LDKO) mice, and their Cre-negative littermate controls (FoxO1-lox, IR-lox and Dlox, or, when pooled together, CON) were maintained on a chow diet and sacrificed in the non-fasted state at 8-10 weeks of age. Hepatic gene expression was measured by (A, F) western blotting whole cell lysates and (B, E, G) quantitative RT-PCR. (C) Glucose tolerance testing was performed. (H) Hepatic cholesterol was measured in samples taken at the time of sacrifice. (I) Cholesterol absorption was measured as the percentage of an oral bolus of [14C]-cholesterol absorbed, as described in Methods. Error bars represent SEM; n = 5–12; *p<0.05 versus control mice; #p<0.05 LIRKO vs LDKO; $p<0.05 LFKO vs LDKO. (D) Lox and LIRKO microarray data from 2-3 month old male mice were subjected to GSEA analysis using the REACTOME pathway set. The enrichment plot and statistics for the cholesterol biosynthesis pathway are shown. n = 3 chips per genotype.
Figure 2.1 (Continued).

A. FOXO1 expression in different genotypes: IR, FOXO1lox, LFKO, IR-lox, LIRKO, Dlx, LDKO.

B. mRNA expression of G6pc and Pck1 in CON, LFKO, LIRKO, and LDKO genotypes.

C. Glucose Tolerance Test showing blood glucose levels over time in CON, LFKO, LIRKO, and LDKO genotypes.

D. Hepatic Deletion of Insulin Receptor showing enrichment plot for REACTOME_cholesterol BIOSYNTHESIS with NES = 2.6906, p < 0.001, FDR = 0.

E. mRNA expression of Hmgcr and Fdps in CON, LFKO, LIRKO, and LDKO genotypes.

F. Western blot analysis of HMGCR, FDPS, and ACTIN in CON, LFKO, LIRKO, and LDKO genotypes.

G. mRNA expression of Srebp-2 in CON, LFKO, LIRKO, and LDKO genotypes.

H. Hepatic cholesterol levels in mg/g liver for CON, LFKO, LIRKO, and LDKO genotypes.

I. Cholesterol absorption in fractional absorption (%) for CON, LFKO, LIRKO, and LDKO genotypes.
In addition to these defects in glucose metabolism phenotype, LIRKO mice have an equally profound perturbation in cholesterol metabolism. Gene set enrichment analysis (Subramanian et al., 2005) performed on microarray data from LIRKO livers revealed a highly significant change in the expression of the cholesterologenic enzymes (Fig. 2.1D). Interestingly, highly significant changes were also observed in mice harboring deletions of either the IRS (Dong et al., 2008) or the Akt (Lu et al., 2012) signaling nodes (Fig. S1.1D-E). Mice with deletion of the IRS node harbored floxed alleles of the two IRS isoforms present in liver, IRS1 and IRS2, as well as the Cre recombinase driven by the albumin promoter (Cre⁺; IRS-1\textsuperscript{Flox/Flox}; IRS2\textsuperscript{Flox/Flox}, or IRS DKO); these mice were compared to their floxed controls. Mice with deletion of the Akt node harbored floxed alleles of the two major Akt isoforms in liver, Akt1 and Akt2, and were injected with an adeno-associated virus (AAV) encoding the Cre recombinase under the TBG promoter (Cre⁺; Akt1\textsuperscript{Flox/Flox}; Akt2\textsuperscript{Flox/Flox}, or Akt DKO) to produce hepatocyte specific deletion of these genes; these mice were compared to their floxed controls injected with a control virus.

Heat map analysis revealed that IRS-DKO, as well as Akt-DKO, mice (Fig. S1.1F-G) showed the same profound and coordinate suppression of the cholesterologenic enzymes as LIRKO mice (Miao et al., 2014). Interestingly, the suppression of the cholesterologenic transcripts in the IRS-DKO and Akt-DKO livers was largely reversed by the additional deletion of hepatic FoxO1 by albumin-Cre-(Dong et al., 2008) or AAV-Cre-mediated recombination (Fig. S1.1F-G). In parallel, qRT-PCR analysis showed that mRNA levels of the cholesterologenic enzymes 3-hydroxy-3-methylglutaryl-CoA reductase (Hmgcr), farnesyl diphosphate synthase (Fdps), and
cytochrome P450, family 51 (Cyp51) were reduced in LIRKO livers, but normalized in LDKO livers, as were protein levels of HMGCR and FDPS (Fig. 2.1E-F).

The transcription factor Sterol Regulatory Element Binding Protein (SREBP)-2 is a master regulator of cholesterol synthesis and sufficient to drive the entire cholesterologenic gene program; as part of a feedback inhibition loop, SREBP-2 is suppressed by intracellular cholesterol levels (Horton, 2010). In LIRKO livers, SREBP-2 was reduced by 30%, whereas cholesterol levels were slightly but significantly increased (Fig. 2.1G-H). Interestingly, SREBP-2 was increased and hepatic cholesterol decreased in LDKO versus LIRKO mice.

Hepatic cholesterol levels are determined, in part, by cholesterol absorption from the gut. To assess cholesterol absorption, we administered a [14C]-cholesterol bolus via gavage to the mice, and calculated the percentage of labelled cholesterol that was absorbed (Fig. 2.1I). LIRKO mice showed a slight increase in cholesterol absorption, whereas LFKO and LKO mice showed a decrease in cholesterol absorption. We also assessed cholesterol absorption by measuring fecal neutral sterol content, which reflects the amount of cholesterol which is lost into the feces rather than being absorbed, and similarly found that the deletion of FoxO1, particularly in the absence of insulin signaling, decreased cholesterol absorption (Fig. S1.1C).

We have previously shown that LIRKO mice show diet-dependent hypercholesterolemia (Biddinger et al., 2008a; Miao et al., 2014; Miao et al., 2015). On a chow diet, they show a decrease in plasma cholesterol, due to a decreased HDL cholesterol. In the presence of atherogenic diets, however, LIRKO mice develop severe hypercholesterolemia, with a marked increase in the amount of cholesterol associated
with the atherogenic apoB-containing lipoprotein particles, VLDL and LDL compared to control mice on the same diet. Interestingly, the decrease in plasma cholesterol observed in LIRKO mice on the chow diet was also observed in LDKO mice (S1.2A). However, after feeding a Western-type diet (21% fat, 0.2% cholesterol by weight), LIRKO mice showed a two-fold increase in plasma cholesterol levels which was normalized in LDKO mice, with a marked reduction in the proportion of ApoB-containing lipoprotein particles (Fig. 2.2A-B; Fig. S1.2D). Plasma and hepatic triglyceride levels, on the other hand, were similar between LIRKO and LDKO mice on both diets (Fig. 2.2C; Fig. S1.2B-C).
Insulin and FoxO1 regulate the expression of multiple enzymes that participate in bile acid synthesis

The fact that insulin receptor and FoxO1 were deleted in the liver, but that cholesterol absorption was altered in the gut suggested the involvement of a factor secreted by the hepatocyte. We hypothesized that the secreted factor could be bile acids, based on the facts that bile acids are (1) synthesized by the liver and secreted into the gut; (2) known to modulate cholesterol absorption (Wang et al., 2003); and (3)
known to be regulated by insulin and FoxO1 (Biddinger et al., 2008b; Haeusler et al., 2012).

Bile acids vary in their ability to promote cholesterol absorption (Wang et al., 2003). There are two primary bile acids synthesized by the liver, cholic acid (CA) and chenodeoxycholic acid (CDCA); these are further modified by the liver and gut microbiota to form various metabolites (see Fig. 1.3). Importantly, in mice, the metabolites derived from cholic acid (tauro-cholic acid, tauro-allocholic acid, tauro-deoxycholic acid) are generally more hydrophobic and better at promoting cholesterol absorption than those derived from CDCA (tauro-α-muricholic acid, tauro-β-muricholic acid, tauro-chenodeoxycholic acid) (Heuman et al., 1989; Wang et al., 2003).

We found that bile isolated from the gallbladders of LFKO mice showed increased amounts of CDCA derived bile acids (coded in blue, tauro-cholic acid, tauro-allocholic acid, tauro-deoxycholic acid) and decreased amounts of CA derived bile acids (coded in red, tauro-α-muricholic acid, tauro-β-muricholic acid, tauro-chenodeoxycholic acid) (Fig. 2.3A). Consequently, the hydrophobicity index, a measure of the relative hydrophobicity of the bile based on the hydrophobicity of each bile acid as well as the relative amount present, decreased from -0.3 in control mice to -0.4 (Fig. 2.3B). This corresponds to a decrease in the hydrophobicity of the bile and is consistent with the decrease in cholesterol absorption noted in these mice (Fig. 2.1I).
Figure 2.3. FoxO1 is responsible for the altered CA:CDCA balance observed in insulin resistant states.

(A-C, F-G) Male LFKO, LIRKO, LDKO mice and their Cre-negative littermate controls (CON) were maintained on a chow diet and sacrificed in the non-fasted state at two to three months of age. (A) Bile was collected from the gallbladders of LFKO, LIRKO, LDKO and CON mice at sacrifice and the bile acid composition was determined using HPLC. The results are represented as relative proportions, with CA metabolites in red and CDCA metabolites in blue. Hepatic gene expression was measured using (C-F) quantitative RT-PCR and (G) Western blotting whole cell lysates. Error bars represent SEM; n = 5–12; *p<0.05 versus control mice; #p<0.05 LIRKO vs LDKO; $p<0.05 LFKO vs LDKO. (D-E) Primary hepatocytes were isolated from FoxO1-lox (Control) and LFKO (FoxO1 knockout) mice, serum starved overnight, and treated for 6 hours with or without (D) 100 nM insulin or (E) 1 uM GW4064. Gene expression was measured using quantitative RT-PCR. Error bars represent SEM; n=3 wells per condition; *p<0.05 FoxO1-lox versus LFKO hepatocytes with the same treatment, #p<0.05 +/- insulin or GW4064 treatment for the same genotype.
Figure 2.3 (Continued).

A  Bile Salt Profile

![Bile Salt Profile](image)

B  Bile Hydrophobicity

![Bile Hydrophobicity](image)

C  Cyp8b1, Cyp7a1, Cyp7b1, Cyp27a1

![Expression Graphs](image)

D  Cyp8b1, Pck1

![Expression Graphs](image)

E  Cyp8b1, Shp

![Expression Graphs](image)

F  IncLSTR

![Expression Graphs](image)

G  Western Blot

![Western Blot](image)
In contrast, LIRKO mice showed an extreme shift to CA derivatives and a reduction in the hydrophobicity index to -0.1, consistent with increased cholesterol absorption in these mice (Fig. 2.1I; Fig. 2.3A-B). Importantly, in LDKO mice, the bile salt profile was restored toward normal, with increased amounts of CDCA derivatives and decreased amounts of CA derivatives, and a reduction in the hydrophobic index to -0.3. (Fig. 2.3A-B).

The ratio of CA to CDCA produced is tightly regulated, potentially at two levels. First, bile acids can be synthesized via either the acidic or neutral pathways. Whereas the acidic pathway produces primarily CDCA, the neutral pathway produces both CA and CDCA. Thus, increased flux through the acidic pathway could decrease the proportion of cholic acid synthesized. Second, the synthesis of cholic acid requires the enzyme CYP8B1, which catalyzes the 12α-hydroxylation of bile acid precursors necessary for cholic acid formation. A decrease in CYP8B1 could therefore also decrease the proportion of cholic acid synthesized. Importantly, insulin is required for expression of Cyp27a1 and Cyp7b1 (Biddinger et al., 2008b), and FoxO1 has previously been shown by to be required for the expression of Cyp8b1 (Haeusler et al, 2012).

Consistent with prior studies, we found that LFKO mice show normal levels of Cyp27a1, Cyp7b1 and Cyp7a1 but a 50% reduction in Cyp8b1 (Fig. 2.3C) (Haeusler et al, 2012). On the other hand, LIRKO mice showed a 30% decrease in Cyp27a1 and a 90% reduction in Cyp7b1, but normal levels of Cyp7a1 and Cyp8b1. Finally, in LDKO mice, Cyp27a1 and Cyp7b1 were significantly increased compared to LIRKO mice, whereas Cyp8b1 was decreased by 50% (Fig. 2.3C).
To determine whether the effects of FoxO1 on these genes were cell autonomous, we studied primary hepatocytes with and without knockout of FoxO1. In vitro, as in vivo, Cyp8b1 expression showed remarkably exquisite regulation by insulin and FoxO1. Insulin suppressed Cyp8b1 by 80%, knockout of FoxO1 also suppressed Cyp8b1 by approximately 80%, and the effects of insulin and FoxO1 knockout were not additive (Fig. 2.3D). Interestingly, the effects of insulin and FoxO1 on Cyp8b1 were of similar magnitude to those observed for the well-known gluconeogenic target, Pck1 (Fig. 2.3D). On the other hand, under the same experimental conditions, the effects of insulin and FoxO1 on Cyp7a1, Cyp27a1 and Cyp7b1 in vitro were more modest in magnitude and variable (Fig. S1.3A).

Another key regulator of Cyp8b1 is the nuclear hormone receptor, farnesoid X receptor (FXR, or NR1H4), which inhibits Cyp8b1 expression. FXR, upon activation by its bile acid ligands, promotes expression of Shp, which in turn inhibits transcription of Cyp7a1 and Cyp8b1. This results in a feedback loop to prevent the excessive accumulation of bile acids. To determine how FoxO1 interacts with FXR in the regulation of Cyp8b1 we treated control and FoxO1 knockout primary hepatocytes with the FXR agonist, GW4064. As expected, the FXR agonist strongly suppressed Cyp8b1 in control cells; however, in FoxO1 knockout cells, Cyp8b1 was already low and the effects of the FXR agonist were markedly attenuated (Fig. 2.3E). Shp, on the other hand, was induced by the FXR agonist in the presence or absence of FoxO1 (Fig. 2.3E).

Given the profound effects of insulin and FoxO1 on Cyp8b1 found in vitro, and the fact that other targets of FoxO1, like G6pc, were increased in LIRKO livers (Fig.
it was somewhat surprising that Cyp8b1 mRNA levels were also not increased in LIRKO livers. We therefore examined expression of long noncoding RNA, IncLSTR. IncLSTR is expressed in the liver, is suppressed by fasting, and promotes Cyp8b1 transcription (Li et al., 2015). We found IncLSTR to be decreased in LIRKO livers and normalized in LDKO livers, suggesting that insulin exerts both positive (via IncLSTR) and negative effects (via FoxO1) at the Cyp8b1 promoter (Fig. 2.3F).

We further examined expression of the bile acid synthetic enzymes at the protein level (Fig. 2.3G). LIRKO mice showed markedly increased levels of CYP8B1 and reduced levels of CYP27A1 and CYP7B1. On the other hand, LDKO livers, compared to LIRKO mice, showed an increase in CYP27A1 and CYP7B1 as well as a slight reduction in CYP8B1.

Excess cholic acid is necessary and sufficient for the cholesterol metabolism phenotype of LIRKO mice

The reduction in CA derivatives observed in LDKO versus LIRKO bile is correlated with both the induction of the enzymes of the acidic pathway enzymes CYP27A1 and CYP7B1 and the suppression of CYP8B1. Since CYP8B1 is required for the synthesis of cholic acid, as it catalyzes the necessary 12α-hydroxylation reaction, we chose to manipulate bile acid synthesis by modulating Cyp8b1 expression. We began by treating LDKO mice, fed a diet enriched with 2% cholesterol, with adenovirus encoding either a control gene, green fluorescent protein (GFP), or Cyp8b1. Treatment with the adenovirus encoding Cyp8b1 (Ad-Cyp8b1) produced a fifteen-fold induction of Cyp8b1 mRNA, but only a modest induction of CYP8B1 protein, and no changes in the other bile acid synthetic genes (Fig. 2.4A-B; Fig S1.4A). Nonetheless, overexpression
of Cyp8b1 produced a marked increase in the proportion of cholic acid derivatives (Fig. 2.4C) and hydrophobicity (Fig. S1.4B) of the bile.
Figure 2.4. Cyp8b1 overexpression partially recapitulates the negative metabolic effects of FoxO1. 

(A-G) 7-12 week old LDKO mice were fed a chow diet supplemented with 2% cholesterol for four weeks and injected with adenovirus expressing GFP or Cyp8b1 one week before sacrifice in the non-fasted state. Hepatic gene expression was measured by (A, F, J) quantitative RT-PCR and (B) Western blotting in whole cell lysates. (C) Bile was collected from the gallbladders of these mice. Equal amounts of gallbladder bile from 4-5 mice per group, collected at the time of sacrifice, were pooled and subjected to HPLC. The results are represented as relative proportions (C), with CA metabolites in red and CDCA metabolites in blue. (D) Hepatic cholesterol and (E) triglycerides, as well as (G) plasma cholesterol and (H) triglycerides, were measured in samples taken at the time of sacrifice. (I) Equal amounts of plasma from 4-5 mice per group, collected at the time of sacrifice, were pooled and subjected to FPLC analysis to determine the distribution of cholesterol. Error bars represent SEM; n=4-6, *p<0.05.
Figure 2.4 (Continued).

A. **Cyp8b1**

B. Bile Salt Profile

C. **Hepatic Triglycerides**

D. **Hepatic Cholesterol**

E. **Plasma Triglycerides**

F. **Plasma Cholesterol**

G. **Cholesterol Distribution**

H. **Hmgcr**

I. **Srebp-2**

J. **Fdx1**

K. **Fgf15**

L. **Fdps**

M. **Sqle**

N. **Cyp51**
Hepatic triglycerides trended upwards, and hepatic cholesterol levels increased more than two-fold (Fig. 2.4D-E). Consistent with this, SREBP-2 and its targets were decreased by 20-50% (Fig. 2.4F). Though plasma triglyceride and cholesterol levels did not change (Fig. 2.4G-H), there was a marked redistribution of cholesterol in the plasma, such that the proportion of cholesterol associated with the ApoB containing lipoproteins, VLDL and LDL, was increased, and the proportion associated with HDL was markedly reduced (Fig. 2.4I; Fig S1.4C). We also noted that these changes in bile acid metabolism resulted in a transcriptional changes in the liver (Fig. S1.4D) and ileum (Fig. 2.4J) consistent with FXR activation: Shp was increased in both tissues and Fgf15 was increased in the ileum, though the results did not all reach significance.

We also knocked down Cyp8b1 using antisense oligonucleotides (ASO) to determine whether cholic acid and its derivatives were necessary for the changes in cholesterol metabolism observed in LIRKO mice. We studied IR-lox mice treated with a control ASO, LIRKO mice treated with a control ASO, and LIRKO mice treated with an ASO against Cyp8b1, fed a standard chow diet. The ASO produced a 70% decrease in Cyp8b1 mRNA (Fig. 2.5A), but no significant changes in Cyp7a1, Cyp27a1 or Cyp7b1 in LIRKO mice (Fig. S1.5A). Examination of the gallbladder bile revealed that the cholic acid enriched bile observed in LIRKO mice was restored towards normal by knockdown of Cyp8b1 (Fig. 2.5B), with a marked decrease in the proportion of cholic acid derivatives and a bile hydrophobicity index similar to IR-flox mice (Fig. S1.5B). This improvement in the bile salt profile was associated with a small, but statistically significant reduction in hepatic cholesterol (Fig. 2.5C), normalization of the cholesterologenic enzymes, and a slight decrease in plasma cholesterol (Fig. 2.5D-E).
Figure 2.5. Cyp8b1 ASO improves cholesterol metabolism in chow-fed mice.

4-5 week old male LIRKO mice and their floxed controls were maintained on a chow diet and treated with control (CON) or Cyp8b1 ASO (50 mg/kg body weight per week IP for seven weeks. (A, E) Gene expression was measured by real-time PCR in the liver. (C) Bile was collected from the gallbladders of these mice. Equal amounts of gallbladder bile from 4-5 mice per group, collected at the time of sacrifice, were pooled and subjected to HPLC. The results are represented as relative proportions (B), with CA metabolites in red and CDCA metabolites in blue. (C) Hepatic cholesterol and (D) triglycerides were measured in samples taken at the time of sacrifice. Error bars represent SEM; n=4-10; *p<0.05 Lox CON ASO vs LIRKO CON ASO, #p<0.05 LIRKO CON ASO vs LIRKO Cyp8b1 ASO.
Figure 2.5 (Continued).

A. **Cyp8b1**

B. **Bile Salt Profile**

C. **Hepatic Cholesterol**

D. **Plasma Cholesterol**

E. **Srebp-2**, **Hmgcr**, **Fdps**, **Fdf1**, **Sqle**, **Cyp51**
Interestingly, there was no increase in plasma or hepatic triglycerides (Fig. S1.5C-D), and no worsening of glucose tolerance (Fig. 2.6A, Fig. S1.5E). Indeed, there was a slight, but statistically significant improvement in glucose tolerance, a reduction in plasma insulin levels, and a decrease in the gluconeogenic enzyme Pck1, though not G6pc (Fig. 2.6B-C). On the other hand, body weight and plasma leptin levels were not changed (Fig. 2.6D-E).
Figure 2.6. Cyp8b1 ASO improves glucose metabolism in chow-fed mice.

4-5 week old male LIRKO mice and their floxed controls were maintained on a chow diet and treated with control (CON) or Cyp8b1 ASO (50 mg/kg body weight per week IP for seven weeks. (A) Glucose tolerance testing was performed after the fifth ASO injection. (B, E) Plasma hormone levels were measured via ELISA in samples taken at the time of sacrifice. (C) Gene expression was measured by real-time PCR in the liver. (D) Body weights were measured weekly. Error bars represent SEM; n=4-10; *p<0.05 Lox CON ASO vs LIRKO CON ASO, #p<0.05 LIRKO CON ASO vs LIRKO Cyp8b1 ASO.
To further dissect the role of $Cyp8b1$ in controlling plasma lipids, we repeated this experiment in the presence of a Western diet. The severe hypercholesterolemia observed in LIRKO mice was abolished by knockdown of $Cyp8b1$ (Fig. 2.7A) with no elevation in plasma triglycerides (Fig. 2.7B). Moreover, there was a marked alteration in the distribution of cholesterol, such that the proportion of atherogenic, ApoB containing lipoproteins was reduced from 41% in LIRKO mice treated with the control ASO to 26% in LIRKO mice treated with the ASO against $Cyp8b1$ (Fig. 2.7C; Fig S1.5G).
Importantly, similar changes in plasma cholesterol were observed in streptozotocin-treated mice, a mouse model of type 1 diabetes (Fig. S1.5F). We also noted that the knockdown of Cyp8b1 appeared to reduce FXR action, as intestinal Shp.
*Fgf15*, and *Smpd3* trended downwards or were reduced by knockdown of *Cyp8b1* (Fig. 2.7D).

**Discussion**

Here, we find that insulin programs whole-body cholesterol metabolism by suppressing cholic acid production. Consequently, LIRKO mice, like insulin deficient mice and humans, show increased cholesterol absorption, decreased cholesterol synthesis, and a susceptibility to hypercholesterolemia. These pro-atherogenic defects are due to dysregulation of bile acid production and reversed by deletion of either the transcription factor FoxO1, or *Cyp8b1*.

The regulation of cholic acid synthesis by insulin is exceedingly complex. At the transcriptional level, insulin both induces the acidic pathway enzymes, which synthesize predominantly CDCA, and suppresses the neutral pathway enzyme, *Cyp8b1*, which is required for the production of CA. The regulation of *Cyp8b1* transcription is mediated not only by FoxO1, but by other factors, including lnCLSTR. On top of this, there appears to be a significant post-transcriptional effect, such that CYP8B1 protein levels in LIRKO livers are three-fold elevated, despite normal *Cyp8b1* mRNA levels. The deletion of FoxO1 in LIRKO livers increases mRNA and protein levels of CYP27A1 and CYP7B1, and reduces levels of CYP8B1. In parallel, the proportion of cholic acid derivatives is reduced in LDKO versus LIRKO bile.

Cholic acid induces hypercholesterolemia, but only in the presence of a diet containing excess fat and cholesterol. On the chow diet, mice with hepatic deletion of insulin receptor, despite an excess of cholic acid derivatives in their bile, show only a slight reduction in plasma cholesterol levels that is not reversed by the reduction of
either FoxO1 or CYP8B1. These effects on the chow diet likely reflect changes in HDL metabolism. On the other hand, in the presence of excess fat and cholesterol on the Western diet, LIRKO mice become markedly hypercholesterolemic, with the accumulation of ApoB-containing lipoproteins. These changes are reversed by deletion of either FoxO1 or CYP8B1.

In addition to promoting cholesterol absorption and, in the presence of excess dietary cholesterol, hypercholesterolemia, cholic acid appears to regulate several transcriptional programs. In the liver, cholic acid leads promotes the deposition of excess cholesterol, thereby suppressing SREBP-2 and cholesterologenic gene expression. However, it should be noted that FoxO1 may also have a direct effect on the cholesterologenic enzymes, as the FoxO proteins, primarily FoxO3 and FoxO4—have been reported to suppress SREBP-2 and the cholesterologenic genes in a cell autonomous manner (Zhu et al., 2010; Tao et al., 2013). The use of both cell autonomous and non-autonomous mechanisms is therefore emerging as a common theme in FoxO1 action, as FoxO1 has recently been reported to promote hepatic gluconeogenesis not only by directly activating transcription of the gluconeogenic genes, but also indirectly, by promoting adipocyte lipolysis to fuel gluconeogenesis (Lu et al., 2012; Titchenell et al., 2015; Titchenell et al., 2016). In any case, the net result is that the absence of insulin, FoxO1 activation increases cholic acid production and reprograms cholesterol metabolism, favoring the absorption of cholesterol from the gut over than the energy consuming process of de novo cholesterologenesis in the liver. These changes could potentially increase the energy available to the liver for other processes like gluconeogenesis, which is simultaneously activated by FoxO1.
It also appears that overexpression or knockout of Cyp8b1 alters FXR action. While an increase in Cyp8b1 increases the expression of FXR target genes like Shp in the intestine and liver, the knockout of Cyp8b1 has the opposite effect. This is expected given that, in mice, the derivatives of cholic acid activate FXR, whereas the dominant derivatives of CDCA, the muricholates, are actually FXR antagonists. FXR agonists, in both mice and humans, appear to have numerous beneficial effects, in particular lowering hepatic triglycerides and improving glucose homeostasis. Thus, the fact that there was not a worsening of hyperglycemia or the development of steatosis and hypertriglycerideremia in mice with knockdown of Cyp8b1 may seem surprising. However, it is likely that knockdown of Cyp8b1 also had beneficial effects on glucose and triglyceride metabolism via FXR-independent mechanisms. Such mechanisms could potentially include the induction of GLP-1, which has been reported in Cyp8b1 null mice (Kaur et al., 2015), or alterations in other bile acid receptors or even the gut microbiome, which is in intimate contact with bile acids.

The remarkable effects of Cyp8b1 knockdown in our mice beg the question of whether inhibition of CYP8B1 would be beneficial in humans, particularly in patients with Type 1 diabetes, who also have a relative defect in hepatic insulin action, increased cholesterol absorption, and, particularly in patients with poor glycemic control, hypercholesterolemia. Indeed, prior studies have shown that Cyp8b1 null mice are not only protected against atherosclerosis and gallstones, but also show improved glucose tolerance (Slatis et al., 2010; Kaur et al., 2015).

Based on our current understanding of bile acids, we would expect that CYP8B1 inhibition would have multiple beneficial effects in diabetic patients. First, we expect
CYP8B1 inhibition to reduce cholesterol absorption, as feeding CDCA reduces and feeding CA increases cholesterol absorption, in humans as well as mice (Ponz del Leon, 1979; Wang et al., 2003; Woollett et al., 2004). Second, we expect that the accumulation of CDCA derivatives upon CYP8B1 inhibition would induce FXR: CDCA in humans is not further metabolized to muricholic acids and is a better agonist of FXR than cholic acid. Indeed, CDCA feeding in humans reduces plasma triglycerides, and the FXR agonist, obetcholic acid, is one of the few available treatments of nonalcoholic steatohepatitis. It would also be expected that a shift from cholic acid to CDCA would increase activation of TGR5, which reduces inflammation and atherosclerosis. Thus, we expect that CYP8B1 inhibition would reverse a fundamental defect in the bile salt profile of diabetic patients, and thereby simultaneously treat many aspects of the diabetic phenotype, including dyslipidemia, NASH, hyperglycemia and prevent atherosclerosis.

Acknowledgments/Attributions
The work in this chapter will be submitted as a manuscript with the author list as follows: Alisha V. Ling (co-first author), Mary E. Gearing (co-first author), Ivana Semova, Ji Miao, Dong-Ju Shin, Satyapal Chahar, Amanda L. Brown, Mark J. Graham, Rosanne M. Crooke, Lee R. Hagey, J. Mark Brown, and Sudha B. Biddinger. AVL, MEG, IS, JM, DJS and SC performed the experiments and/or analyzed the data; ALB and JMB performed the fecal neutral sterol experiment and analyzed the data; LRH performed the bile salt profiling; SBB oversaw the animal experiments, and wrote the manuscript. Figure panels generated by MEG are: Fig. 2.1D; Fig. 2.2; Fig. 2.3A-B; Fig. 2.4A, C, F-I; Fig. 2.5A-B, D-E; Fig. 2.6; Fig. 2.7A-C; and supplemental figures Fig.
S1.1D-G; Fig. S1.4A-B; Fig. S1.5A-B, D-E. MEG also coordinated the animal experiments shown in Fig. 2.2; Fig. 2.4-2.7. We thank Dr. William Pandak (VCU Health System) for the Cyp8b1 adenovirus. This work was funded by an American Heart Association Predoctoral Fellowship (AVL), DK100539 (JM) and DK094162 (SBB).
Chapter 3 – The Role of FoxO1 in Mediating the Metabolic Effects of FMO3
Abstract

FMO3 knockdown using antisense oligonucleotides (ASO) has wide-ranging and varied effects on gene expression and physiology. FoxO1 is known to regulate glucose and lipid metabolism, and FMO3 ASO treatment reduces FoxO1 protein levels. To determine which effects of FMO3 ASO are FoxO1-dependent, we treated liver insulin receptor knockout (LIRKO) and liver insulin receptor/FoxO1 double knockout (DKO) mice with FMO3 ASO. Using these mice, we dissected the role of FoxO1 in FMO3’s effects on bile, glucose and lipid metabolism. Although many of the positive effects of FMO3 ASO, notably improved bile acid profile and reduced gluconeogenic gene expression, are FoxO1-dependent, a number of FMO3 ASO’s effects are partially mediated by other effectors.

We further explored the relationship of FMO3 enzyme activity to its regulation of FoxO1 protein levels using the weak FMO3 inhibitor indole 3-carbinol (I3C). In both LIRKO and ob/ob mice, I3C treatment lowered FoxO1 protein levels. These results indicate that FMO3 enzymatic activity may increase FoxO1 levels.

Since FMO3 knockdown or enzymatic inhibition may not be feasible due to the role of this enzyme in detoxification processes, it is important to better characterize the signaling pathways downstream of this protein. FoxO1 knockdown might be an alternative strategy that would allow us to reap many of the benefits observed with FMO3 ASO treatment.

Introduction

Cardiovascular disease, the leading cause of death in the United States, is a complex, multifactorial disease with genetic and dietary risk factors. Using unbiased
metabolic profiling, researchers have identified trimethylamine N-oxide (TMAO) as a metabolite correlated with atherosclerosis risk in humans and mice (Wang et al., 2011; Bennett et al., 2013). TMAO is produced by the catalytic activity of flavin-containing monooxygenase 3 (FMO3), an enzyme traditionally known for its role in xenobiotic metabolism (Phillips and Shephard, 2008). Our laboratory has shown that FMO3 is exquisitely sensitive to insulin regulation; in male mice with hepatic insulin resistance, Fmo3 expression is increased more than 1,000 fold compared to floxed controls (Miao et al., 2015). Knockdown of Fmo3 using antisense oligonucleotides (ASO) normalizes cholesterol metabolism and atherosclerosis risk in mice fed an atherogenic Paigen diet (15% fat, 1% cholesterol, 0.5% cholic acid) (Miao et al., 2015).

Other groups have explored the effects of FMO3 ASO in models of cholesterol metabolism. In an atherosclerosis-prone LDLR knockout model on a Western diet, researchers observed decreased atherosclerosis rates in FMO3 ASO-treated mice, but also other lipid improvements, including decreased triglycerides and cholesterol in the liver and plasma, as well as decreased plasma glucose and insulin levels (Shih et al., 2015). They also identified changes in bile acid metabolism, including lower concentrations of bile acids and cholesterol in bile in mice treated with FMO3 ASO (Shih et al., 2015).

In wild-type mice on a chow diet, FMO3 ASO reduces dietary cholesterol absorption, thus increasing sterol loss in the feces (Warrier et al., 2015). These trends were also observed in mice fed a 2% cholesterol diet. FMO3 knockdown stimulated transintestinal cholesterol excretion (TICE), a method of reverse cholesterol transport that increases cholesterol loss into feces (Warrier et al., 2015).
Although FMO3 ASO treatment has numerous positive effects on systemic metabolism, it also produces some negative effects. In LDLR knockout mice, FMO3 ASO increases plasma hepatotoxicity markers AST and ALT, indicating liver damage (Shih et al., 2015). In wild-type mice, FMO3 ASO increases hepatic ER stress, inflammation, and immune cell infiltration, hypothesized to result from changes in LXR signaling (Warrier et al., 2015).

Looking forward, these results suggest that FMO3 ASO may not be the best method to downregulate the insulin-FMO3-TMAO axis in humans. For this reason, we must better understand the downstream effectors of FMO3 and which parts of the FMO3 ASO phenotype they modulate. Our group is especially interested in FoxO1, for which protein expression is reduced to undetectable levels by FMO3 ASO treatment (Miao et al., 2015). FoxO1 regulates hepatic gluconeogenesis and modulates bile acid enzyme expression, and we hypothesize that many of the positive effects of FMO3 ASO can be attributed to its downregulation of FoxO1 expression.

It also remains unclear how FMO3 enzymatic activity, including TMAO production, contributes to the effects of FMO3 knockdown. Studies combining FMO3 knockdown with TMAO rescue or antibiotic-mediated inhibition of TMA production have attempted to dissect these relationships (Warrier et al., 2015). Preliminary studies show that TMA/TMAO may not mediate the increased inflammation and decreased lipogenesis observed with FMO3 ASO treatment. Recent work using DMB, an inhibitor of microbial trimethylamine lyase, to disrupt TMA production has confirmed that blocking TMAO production lessens atherosclerosis in ApoE-/- mice, but this study did not explore the relationship between TMAO and FoxO1 (Wang et al., 2015).
Methods

Animals

Generation of mice with floxed (flanked by loxP) insulin receptor and FoxO1 alleles (Paik et al., 2007) been described previously. To generate liver-specific knockout mice, floxed mice were crossed with Alb-Cre transgenic mice that express Cre recombinase under the albumin promoter. LIRKO mice were previously described (Michael et al., 2000). All mice were maintained on a C57BL/6 background and sacrificed at 2 pm in the non-fasted state. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at Children’s Hospital Boston.

ASO Experiments

6-8 week old female liver insulin receptor knockout mice (LIRKO) and mice with hepatic knockout of both the insulin receptor and FoxO1 (liver double knockout, or LDKO) were maintained on a Western-type diet (Envigo TD88137; 21% fat and 0.2% cholesterol by weight). A second generation control antisense oligonucleotide (ASO, IONIS-141923) or antisense oligonucleotide against Fmo3 (IONIS-555847) was diluted in normal saline, and injected intraperitoneally at 50 mg/kg each week for 11 weeks. Mice were sacrificed at 2 pm in the non-fasted state, one day after their final dose of antisense oligonucleotide.

I3C Experiments

6-8 week old male LIRKO mice and their floxed controls were maintained on a chow diet with or without 0.25% I3C (TestDiet) for 5 weeks. In other experiments, 6 week old male ob/ob mice were maintained on a chow diet with or without 0.25% I3C for 5 weeks.
Blood Chemistry/Metabolic Analysis

Plasma samples were collected from mice either after 4 hours of fasting or at the time of sacrifice in the non-fed state. Plasma lipids were measured using a colorimetric assay (Thermo Scientific) according to the manufacturer’s instructions.

DEXA

Bone densitometry scanning was performed by Animal Resources at Children’s Hospital (ARCH). The following parameters were measured: total animal mass (g), fat mass (g), lean mass (g). Fat percentage was calculated by dividing fat mass by total mass.

Glucose tolerance testing

For FMO3 ASO experiments, mice were fasted 12-16 hours overnight and injected with glucose at a dose of 1 mg/kg body weight. Blood glucose levels were measured from the tail at 0, 15, 30, 60, 90, and 120 minutes post injection using a handheld glucometer. In Fig. 3.4A, one blood glucose measurement was determined to be an outlier using the Grubbs Outlier Test, and this data point was excluded. For I3C experiments, mice were fasted for 3 hours beginning at 7 am to permit overnight consumption of I3C.

HPLC Bile Acid Analysis

Gallbladder bile was obtained at the time of sacrifice and analyzed by high-performance liquid chromatography. An octadecylsilane column (RP C-18, Beckman Instruments, Fullerton, CA) was used with isocratic elution at 0.75 mL/min. The eluting solution was composed of a mixture of methanol and 0.01 M KH$_2$PO$_4$ (67%, v/v), adjusted to an apparent pH of 5.3 with H$_3$PO$_4$. Bile acids were quantified by measuring their absorbance at 204 nm. Bile acid amidates (taurine and glycine) have similar extinction coefficients.
Bile acids were tentatively identified by matching their relative retention times with those of known standards.

The ratio of cholic acid metabolites to chenodeoxycholic acid metabolites was calculated by dividing the sum of the percent abundances of CA metabolites (T-CA, T-alloCA, T-DCA) by the sum of the percent abundances of CDCA metabolites (T-α-MCA, T-β-MCA, T-CDCA). The total bile hydrophobicity index was calculated by multiplying the percent abundance of each bile acid species by its hydrophobicity index as described previously (Heuman, 1989). Using the equations provided, the hydrophobicity index for T-allocholic acid was calculated as +0.06.

**Hepatic Lipids**

Livers were homogenized in 50 mM NaCl and lipid was extracted with chloroform and methanol (2:1). Lipid extract was then analyzed by a colorimetric assay per manufacturers’ instructions (Thermo Scientific).

**Gene Expression Analysis**

RNA was isolated from liver using TRIzol (Ambion) or an RNeasy kit (Qiagen) and reverse transcribed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). cDNA was diluted ten-fold for analysis using SYBR Green (Invitrogen) on the QuantStudio® 6 Flex Real-Time PCR System (Applied Biosystems). Gene expression was normalized to the reference gene 18S (Fig. 3.1-3.5) or TBP (Fig. 3.6).
Western Blotting

Livers were homogenized in RIPA buffer. 40-80 micrograms of protein lysates were loaded onto sodium dodecyl sulfate–PAGE (SDS–PAGE) gels and transferred onto a PVDF membrane (Thermo Scientific). Blots were blocked in SuperBlock buffer (Thermo Scientific), incubated overnight with primary antibody and detected with secondary antibody conjugated with HRP. Antibody complexes were visualized by enhanced chemiluminescence using X-ray film.

### Table 3.1. Primers for gene expression analysis in Chapter 3.

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TMAO Measurements

LC/MS/MS studies of mouse liver were conducted in collaboration with the Andrew J. Morris lab at the University of Kentucky. TMAO was analyzed as previously described (Petriello et al., 2016).

Statistics

Data are represented by the mean + SEM, unless otherwise indicated. Significance was assessed by a 2-tailed Student’s t-test with unequal variance, unless otherwise indicated. For ASO studies, LIRKO or LDKO mice treated with control ASO were compared to mice of the same genotype receiving FMO3 ASO. LIRKO mice treated with CON ASO were also compared to LDKO mice treated with CON ASO. Results shown are representative of two cohorts. For I3C studies, LIRKO mice on an I3C-supplemented diet were compared to LIRKO mice on a chow diet. LIRKO results are representative of two cohorts, whereas studies in ob/ob mice were conducted once.

Results

Six- to eight-week-old female liver insulin receptor knockout mice (LIRKO) and mice with hepatic knockout of both the insulin receptor and FoxO1 (liver double knockout, or LDKO) were maintained on a Western-type diet (Envigo TD.88137). Mice were treated with antisense oligonucleotides (Ionis Pharmaceuticals) via intraperitoneal injection (50 mg/kg body weight weekly). Mice of both genotypes were treated with either control (IONIS-141923) or Fmo3-targeting ASO (IONIS-555847). Multiple physiological parameters were evaluated to determine how the presence of FoxO1 affected the metabolic response to FMO3 ASO. FMO3 ASO treatment significantly decreased Fmo3 expression in both LIRKO and LDKO mice, although LDKO mice
treated with CON ASO displayed significantly lower Fmo3 expression than LIRKO mice treated with CON ASO (Fig. 3.1). Fmo3 expression did not vary between LIRKO and LDKO mice treated with FMO3 ASO. Importantly, mRNA expression of other FMOs did not vary between mice of the same genotype treated with CON and FMO3 ASO. (Fig. 3.1).

**Figure 3.1. FMO3 ASO reduces Fmo3 expression in LIRKO and LDKO mice.**
6-8 week old LIRKO and LDKO mice were maintained on a Western diet and treated weekly with control (CON) or FMO3 ASO. Hepatic expression of the FMOs was measured via quantitative RT-qPCR. Error bars represent SEM; n=5; *p<0.05 LIRKO CON ASO vs LDKO CON ASO, #p<0.05 CON ASO vs FMO3 ASO of the same genotype.

**FMO3’s effects on bile are FoxO1-dependent**

Bile acid metabolism is coupled to cholesterol metabolism and thus affects an organism’s cholesterol balance. The liver synthesizes two primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), which are further modified by the liver and gut. 

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microbiota to form various metabolites (Fig. 3.1A). Bile acids can be synthesized via either the neutral or acidic pathways, which produce mostly CA and CDCA, respectively.

Prior studies have shown that in LIRKO mice, the acidic pathway is suppressed and cholic acid derivatives are increased (Biddinger et al., 2008b). In mice with hepatic deletion of FoxO1 (LFKO mice), Cyp8b1 is suppressed and cholic acid derivatives are decreased (Haeusler et al., 2012). The bile acid profiles of LIRKO and LDKO mice treated with control ASO in this study fit well with these previous data – LIRKO mice display a very high ratio of CA:CDCA derivatives, whereas LDKO mice have a lower ratio of CA:CDCA derivatives (Fig. 3.1B-C).
Figure 3.2. FMO3 ASO decreases biliary cholic acid levels.

(A) A simplified schematic of bile acid synthesis. (B-E) 6-8 week old LIRKO and LDKO mice were maintained on a Western diet and treated weekly with control (CON) or FMO3 ASO. (B-D) Bile was collected from the gallbladders of LFKO, LIRKO, LDKO and CON mice at sacrifice and the bile acid composition was determined using HPLC. The results are represented as (B) relative proportions, with CA metabolites in red and CDCA metabolites in blue, and (C) the CA:CDCA ratio. (D) The bile hydrophobicity index was calculated as previously described (Heuman et al., 1989). (E) Hepatic gene expression was measured via quantitative RT-qPCR. Error bars represent SEM; n=5; p<0.05 CON ASO vs FMO3 ASO of the same genotype.
FMO3 ASO’s effects on cholesterol metabolism are multifaceted

Previous studies conducted using FMO3 ASO in insulin-sensitive mice have shown differential effects on bile acid metabolism (Shih et al., 2015; Warrier et al., 2015). In this study, LIRKO mice treated with FMO3 ASO displayed a lower CA:CDCA ratio than their CON ASO counterparts, but no change in bile acid pool composition was observed between LDKO mice treated with CON or FMO3 ASO (Fig. 3.2B-C). FMO3 ASO decreased bile hydrophobicity in LIRKO, but not LDKO, mice (Fig. 3.2D).

We next examined if changes in bile acid pool composition were associated with changes in expression of bile acid synthetic enzymes. Surprisingly, we did not observe consistent changes in CYP8B1, CYP7A1 or CYP7B1 mRNA expression associated with FMO3 ASO in either LIRKO or LDKO mice (Fig. 3.2E).

Since bile acids vary in their ability to solubilize cholesterol, changes in the bile acid pool can alter cholesterol absorption in the gut. Cholic acid metabolites are generally more hydrophobic and better at promoting cholesterol absorption than CDCA metabolites (Heuman, 1989; Wang et al., 2003). As cholesterol absorption from the gut increases, hepatic cholesterol synthesis should decrease due to suppression of SREBP-2 and its cholesterologenic gene targets (Brown and Goldstein, 1997).

As expected, LIRKO mice treated with FMO3 ASO displayed decreased hepatic cholesterol levels compared to those treated with CON ASO (Fig. 3.3A). In fact, LIRKO mice treated with FMO3 ASO had hepatic cholesterol levels similar to LDKO mice treated with CON ASO, demonstrating that FMO3 ASO has effects on hepatic cholesterol of a similar magnitude to those of genetic FoxO1 ablation (Fig. 3.3A).
Plasma cholesterol levels followed the same pattern as hepatic cholesterol for LIRKO mice treated with CON and FMO3 ASO (Fig. 3.3B).

Figure 3.3. FMO3 ASO decreases cholesterol availability in LIRKO and LDKO mice. 6-8 week old LIRKO and LDKO mice were maintained on a Western diet and treated weekly with control (CON) or FMO3 ASO. (A) Hepatic cholesterol was measured in samples taken at sacrifice. (B) Plasma cholesterol was measured after a 4 hour fast. (C) Hepatic gene expression was measured via quantitative RT-qPCR. Fold changes between CON and FMO3 ASO treated mice for a given genotype are shown for comparison. Error bars represent SEM; n=5; *p<0.05 LIRKO CON ASO vs LDKO CON ASO; #p<0.05 CON ASO vs FMO3 ASO of the same genotype. In panel C, p<0.05 for any CON ASO vs FMO3 ASO comparison denoted by a fold change.

Surprisingly, we observed that FMO3 ASO also decreased plasma and hepatic cholesterol levels in LDKO mice, albeit by a small percentage (Fig. 3.3A-B). To determine if these changes were physiologically significant, we analyzed hepatic choleseterologenic gene expression in these four groups of mice (Fig. 3.3C). Interestingly, both LIRKO and LDKO mice treated with FMO3 ASO displayed increased
expression of SREBP-2 target genes compared to their CON ASO-treated controls. Although FMO3 ASO had much larger effects on plasma and hepatic cholesterol in the LIRKO mice compared to the LDKO mice, the effects on cholesterologenic gene expression were of a similar magnitude (Fig. 3.3A-C).

In our laboratory’s previous studies of LIRKO and ob/ob mice treated with FMO3 ASO, the improvement in glucose tolerance was very striking. As seen with plasma and hepatic cholesterol, FMO3 ASO normalized abnormal LIRKO glucose tolerance and gluconeogenic gene expression back to baseline. Due to the well-known role of FoxO1 in regulating gluconeogenic gene expression, we hypothesized that FMO3 ASO’s effects on glucose tolerance would be dependent on FoxO1. Glucose tolerance testing showed that LIRKO mice treated with FMO3 ASO were similarly glucose tolerant to LDKO mice treated with CON ASO (Fig. 3.4A-B). Again, as seen with cholesterol metabolism, the effect size of FMO3 ASO treatment in LIRKO mice is similar to that of genetic FoxO1 ablation (Fig. 3.3A-B; Fig. 3.4A-B). In LIRKO mice, FMO3 ASO lowered expression of gluconeogenic genes G6pc, Pck1, and Pgc1α to levels similar to those seen in LDKO CON ASO mice (Fig. 3.4C).
Surprisingly, FMO3 ASO also improved glucose tolerance in LDKO mice, again with a small effect size (Fig. 3.4A-B). Since these changes could not be mediated by hepatic FoxO1, we examined gene expression of other gluconeogenic genes in the liver. However, observed no significant differences in expression of canonical
gluconeogenic genes $G6pc$, $Pck1$, or $Pgc1\alpha$ between LDKO mice treated with CON or FMO3 ASO (Fig. 3.4C).

**FMO3 ASO treatment decreases adiposity independent of hepatic FoxO1**

As mice in these experiments were maintained on a Western-type diet for 11 weeks, we expected a substantial weight gain. Unexpectedly, we noticed that both LIRKO and LDKO mice treated with FMO3 ASO displayed significantly lower body weight after 3-7 weeks of ASO injection, a phenotype that persisted through the duration of the experiment (Fig. 3.5A). To understand if this effect was due to a change in lean mass or fat mass, the body fat percentages of all four groups of mice were quantified using DEXA. DEXA scanning revealed similar decreases in body fat percentages in both LIRKO and LDKO mice treated with FMO3 ASO (Fig. 3.5B).
Figure 3.5. FMO3 has FoxO1-independent effects on adiposity and hepatic triglycerides.
6-8 week old LIRKO and LDKO mice were maintained on a Western diet and treated weekly with control (CON) or FMO3 ASO. (A) Body weights were measured weekly at the time of ASO injection. (B) DEXA scanning was performed the week of the 10th ASO injection. (C) Hepatic triglycerides were measured in samples taken at the time of sacrifice. (D) Plasma cholesterol was measured after a 4 hour fast during the week of the fifth ASO injection. (E) Hepatic gene expression was measured via quantitative RT-qPCR. Error bars represent SEM; n=5; *p<0.05 LIRKO CON ASO vs LDKO CON ASO; #p<0.05 CON ASO vs FMO3 ASO of the same genotype. In panel A, brackets indicate that p<0.05 for any CON ASO vs FMO3 ASO time point contained within the brackets. Brackets comparing LDKO mice are at the top of the graph; brackets comparing LIRKO mice are at the bottom.
As adipose tissue is the primary storage depot for triglycerides, we hypothesized that FMO3 ASO-treated mice could have decreased whole-body triglyceride availability. To test this hypothesis, we examined hepatic and plasma triglyceride levels. In both LIRKO and LDKO mice, FMO3 ASO decreased hepatic triglycerides with a similar effect size (Fig. 3.5C). However, we did not observe consistent changes in plasma triglyceride levels (Fig. 3.5D). We also did not observe changes in hepatic lipogenic gene expression (Fig. 3.5E).

**Indole-3-carbinol treatment partially mimics FMO3 ASO treatment**

The powerful effects of FMO3 ASO in LIRKO mice described here and in our previous studies support FMO3 as a drug target for metabolic syndrome and diabetes-associated cardiovascular disease. To determine if enzymatic inhibition of FMO3 also decreases FoxO1 and improves the metabolic profile, we conducted studies using the compound indole-3-carbinol, previously shown to be a weak inhibitor of FMO3 (Cashman et al., 1999).

Six- to eight-week-old male LIRKO mice were maintained on a chow diet +/- 0.25% indole-3-carbinol (I3C) (Sigma, TestDiet) for five weeks. I3C treatment did not decrease hepatic Fmo3 expression (Fig. 3.6A). Hepatic TMAO levels were decreased in I3C-fed LIRKO mice compared to chow-fed, although this trend did not reach significance (Fig. 3.6B).

To characterize the effects of I3C supplementation on glucose metabolism, we performed glucose tolerance testing. LIRKO mice treated with I3C showed improved glucose tolerance compared to untreated mice, but the effect size was relatively small.
I3C supplementation also lowered plasma cholesterol and triglyceride levels in LIRKO mice, albeit with a small effect size due to the chow diet (Fig. 3.6D-E).

**Figure 3.6.** I3C treatment decreases FoxO1 expression in LIRKO and ob/ob mice. (A-F) 3 to 6 week old male LIRKO mice were maintained on a chow diet (CON) or a chow diet supplemented with 0.25% I3C for five weeks. Hepatic gene expression was measured by (A) quantitative RT-PCR or (F) Western blotting whole cell lysates. (B) Hepatic TMAO was measured using LC/MS/MS as described in the Methods. (C) Glucose tolerance testing was performed after three weeks of dietary treatment. Plasma (D) cholesterol and (E) triglycerides were measured in samples taken after a 4 hour fast. Error bars represent SEM; n=3-8; *p<0.05 (G) 6 week old male ob/ob mice were maintained on a chow diet (CON) or a chow diet supplemented with 0.25% I3C for five weeks. Hepatic gene expression was measured by Western blotting whole cell lysates.
Having seen that I3C treatment improved glucose tolerance, we examined whether I3C-treated mice had altered hepatic FoxO1 protein levels. Indeed, Western blotting revealed that I3C treatment lowered hepatic FoxO1 protein expression in LIRKO mice (Fig. 3.6F).

In previous studies, our laboratory observed that FMO3 ASO lowered FoxO1 levels in ob/ob mice, and we wondered if the same phenotype could be observed with I3C supplementation. To test this hypothesis, six-week-old male ob/ob mice were maintained on chow with or without 0.25% I3C. I3C treatment slightly decreased hepatic TMAO levels, indicating weak FMO3 enzymatic inhibition (data not shown). As seen in LIRKO mice, I3C treatment demonstrably decreased hepatic FoxO1 levels in ob/ob mice (Fig. 3.6G).

Discussion

The results obtained from these parallel studies confirm a key finding from previous work: that FMO3 has wide-ranging, complex, and overlapping effects on metabolism and physiology. These studies represent only the beginning of our mechanistic understanding of FMO3. Since direct FMO3 inhibition may not be feasible due to its role in xenobiotic metabolism, it is important to understand the pattern of effectors downstream of FMO3.

Based on previous results from our laboratory and others, we expected to find that FoxO1 is a key effector of FMO3, and that many of the beneficial effects of FMO3 ASO result from hepatic FoxO1 reduction. Indeed, the improvements in bile acid pool composition seen only in LIRKO mice, and the increased magnitude of changes in cholesterol, and glucose metabolism in LIRKO mice compared to LDKO mice partially
confirm this theory. It is striking that FMO3 ASO are similar in magnitude to those of liver-specific FoxO1 genetic ablation, and this finding demonstrates that FMO3 is an important, if not the most important, regulator of FoxO1 in insulin resistant states.

However, our results also compellingly show that FoxO1 is not the only effector acting downstream of FMO3. LDKO mice treated with FMO3 ASO show decreased adiposity and hepatic triglycerides comparable to that of LIRKO mice treated with FMO3 ASO (Fig. 3.5). This result, observed in addition to slight improvements in glucose and cholesterol metabolism in LDKO FMO3 ASO mice, indicates that FMO3 interacts with other signaling pathways (Fig. 3.3-3.4). Given the interest in FMO3 as a modulator of cholesterol metabolism, the increase in cholesterologenic gene expression in LDKO mice treated with FMO3 ASO is especially intriguing. Previous studies of mice on a 2% cholesterol diet found that FMO3 ASO lowered organismal cholesterol availability by upregulating transintestinal cholesterol excretion (TICE) (Warrier et al., 2015). If demonstrated to occur independently of FoxO1, TICE could explain the decreased cholesterol levels observed in FMO3 ASO-treated LDKO mice.

Although antisense oligonucleotides act primarily on the liver, it is possible that FMO3 ASO is modulating FMO3 expression in other tissues. We have convincingly demonstrated that FMO3 ASO decreases hepatic FoxO1 protein, but the potential of FMO3 to regulate FoxO1 expression in other tissues has not been explored. Since FoxO1 is a key regulator of adipocyte differentiation, it is possible that downregulating FMO3 in adipose tissue would decrease adipocyte formation and overall adiposity (Nakae et al., 2003). Such effects could be investigated in a whole-body FMO3
knockout mouse, such as the model described in Chapter 4, or in an adipose-specific FMO3 knockout.

**FMO3 enzymatic activity and hepatic FoxO1**

Previous studies have suggested multiple, overlapping mechanisms of FMO3 ASO, including effects that may be independent of FMO3 enzymatic activity. Using indole-3-carbinol to inhibit TMAO production, we found that decreased FMO3 enzymatic activity was correlated with decreased hepatic FoxO1 expression in both LIRKO and ob/ob mice. In comparing the effects of FMO3 ASO and I3C treatment, the effects of I3C are generally milder, as expected given that I3C is only a weak inhibitor of FMO3 activity (Fig. 3.6). Interestingly, I3C treatment reduces plasma triglycerides but does not appear to decrease adiposity.

One question regarding I3C is its specificity for FMO3. Nutritional biochemists have investigated the effects of I3C on mice fed a high-fat diet, finding that I3C modulates AMPK and PPARα signaling (H. Choi et al., 2014; Y. Choi et al., 2012). It will be important to determine if these effects are FMO3-dependent or -independent by examining the effects of I3C in FMO3 knockout mice.

Given that FMO3 activity appears to be necessary for FoxO1 expression in insulin resistant states, it will be important to understand which substrates of FMO3 interact with FoxO1. The Hazen laboratory has identified and characterized choline analog DMB, an inhibitor of bacterial TMA production (Wang et al., 2015). Mice treated with DMB show decreased foam cell production and atherosclerosis, but the effects of DMB on FoxO1 and hepatic lipid metabolism have not been examined. If mice treated with DMB showed similar reductions in FoxO1 compared to I3C-treated mice, it would
provide strong confirmation for TMAO as the key effector connecting FMO3 and FoxO1. If mice treated with DMB did not show improved glucose and lipid metabolism, it would instead suggest that a different enzymatic product of FMO3 regulates these processes.

In sum, these studies confirm the important role of FoxO1 downstream of hepatic FMO3 signaling, and suggest that enzymatic activity of FMO3 plays a role in regulating FoxO1 expression in the liver. Further mechanistic studies are needed to understand if this regulation also occurs in other tissues, namely adipose tissue, and to understand if TMAO directly regulates FoxO1 expression.

Acknowledgments/Attributions

Other contributors to this work include Lee R. Hagey, Satyapal Chahar, Michael C. Petriello, and Andrew J. Morris. LRH performed bile acid measurements shown in Fig. 3.2B. SC performed hepatic lipid measurements in Fig. 3.3A and Fig. 3.5C. MCP and AJM performed LC/MS/MS analysis of TMAO shown in Fig. 3.6B. DEXA scanning was performed by Animal Resources at Children’s Hospital (ARCH). MEG performed all data analysis and figure design.
Chapter 4 – The Effects of Global FMO3 Knockout in Insulin Sensitive Mice
Abstract

Multiple groups have identified a role for flavin-containing monooxygenase 3 in the regulation of glucose and lipid metabolism, but such studies have been conducted using temporal antisense oligonucleotide (ASO) knockdown rather than genetic ablation of *Fmo3*. Since the five FMO isoforms are closely related, and other FMOs have been shown to play a role in metabolic homeostasis, it is important to validate that FMO3 is indeed a key regulator of metabolic homeostasis. Moreover, we do not yet understand whether FMO3 acts alone or is dependent on other family members to exert its metabolic effects.

To examine the effects of *Fmo3* ablation, we created an *Fmo3* whole-body knockout mouse using CRISPR/Cas9. In addition to the advantages described above, this model allows us to understand if *Fmo3* ablation can prevent the development of insulin resistance in mice challenged with a high-fat diet. It also permits us to characterize FMO3’s effects in other metabolic tissues, as opposed to antisense oligonucleotides, which primarily target the liver.

In general, *Fmo3* knockout produced mild, sex-dependent effects on metabolic homeostasis and hepatic FoxO1 protein levels. Surprisingly, FMO3 KO mice were not resistant to diet-induced obesity or insulin resistance. In a streptozotocin (STZ) model of type 1 diabetes, FMO3 KO mice showed rapid deletion of triglycerides in adipose tissue, suggesting that FMO3 regulates adipose energy storage. These results illustrate the importance of examining *Fmo3* outside of the ASO model.
Introduction

In liver insulin receptor knockout (LIRKO) mice, Fmo3 mRNA and TMAO are among the most highly induced transcripts and metabolites; moreover, antisense oligonucleotide (ASO)-mediated knockdown of FMO3 in LIRKO mice has remarkable effects: it completely corrects glucose intolerance and dyslipidemia, and entirely prevents atherosclerosis on an atherogenic Paigen diet (Miao et al., 2015). Other groups have found similarly remarkable phenotypes with FMO3 ASO. In an atherosclerosis-prone LDLR knockout model on a Western diet, researchers observed decreased atherosclerosis rates in FMO3 ASO-treated mice, but also other lipid improvements, including decreased triglycerides and cholesterol in the liver and plasma, as well as decreased plasma glucose and insulin levels (Shih et al., 2015). They also identified changes in bile acid metabolism, including lower concentrations of bile acids and cholesterol in bile in mice treated with FMO3 ASO (Shih et al., 2015).

In wild-type mice on a chow diet, FMO3 ASO reduces dietary cholesterol absorption, thus increasing sterol loss in the feces (Warrier et al., 2015). These trends were also observed in mice fed a 2% cholesterol diet. FMO3 knockdown stimulated transintestinal cholesterol excretion (TICE), a method of reverse cholesterol transport that increases cholesterol loss into feces (Warrier et al., 2015).

Although studies using FMO3 ASO have provided great insight into this protein, it remains unclear how FMO3 ASO affects the levels of other FMOs, as these proteins share 40-60% sequence identity (Phillips & Shephard, 2008). FMO1, also expressed in liver, has been implicated as a regulator of energy balance (Veeravalli et al., 2014); if FMO3 ASO also targets this protein, it could be difficult to dissect the effects of FMO3
ablation alone.

The FMO3 ASO model has helped dissect FMO3’s role in hepatic metabolism; however, it remains unclear what role this protein plays in other tissues. The kidney appears to be especially sensitive to TMAO levels; plasma TMAO is elevated in chronic kidney disease patients, and TMAO induces renal fibrosis in mouse models (Wang et al., 2015). Given that FMO3 overexpression increases adiposity, it is also conceivable that this protein plays a role in adipose tissue signaling (Shih et al., 2015). The potential caveats of FMO3 ASO described above necessitate the development of a new model - a global Fmo3 knockout mouse.

Methods

Mice

The generation of whole-body FMO3 knockout mice using CRISPR/Cas9 genome editing is described in Chapter 6. Unless noted otherwise, mice were maintained on a chow diet and sacrificed in the non-fasted state. High fat (D12451 – 24% fat by weight) and Western (TD.18837 – 21% fat and 0.2% cholesterol by weight) diets were obtained from Research Diets and Envigo, respectively. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at Children’s Hospital Boston.

Streptozotocin Treatment

8 week old male FMO3 WT and KO mice were injected intraperitoneally with streptozotocin (STZ) (180 mg/kg body weight) or vehicle (0.1 M citric acid, pH=4.2), and sacrificed 7 days post-injection.
**Nephrotoxic Serum**

Nephrotoxic serum was used as previously described (Lloyd & Gutierrez-Ramos, 2000; Haas et al., 2016). 5-8 week old male mice were preimmunized with 100 μL sheep IgG:Complete Freund's Adjuvant subcutaneously and then injected with either 50 μL nephrotoxic serum (NTS) or normal sheep serum (vehicle) daily for three days. Mice were sacrificed in the non-fasted state three weeks after the first NTS or vehicle injection.

**TMAO Measurements**

Pilot LC/MS/MS studies of mouse plasma were conducted in collaboration with the Therapeutics Group at the Broad Institute. TMAO was analyzed as previously described (Wang et al., 2014). Further LC/MS/MS studies of mouse liver were conducted in collaboration with the Andrew J. Morris lab at the University of Kentucky. TMAO was analyzed as previously described (Petriello et al., 2016).

**Bile Acid Measurements**

Gallbladder bile was obtained at the time of sacrifice and analyzed by high-performance liquid chromatography. An octadecylsilane column (RP C-18, Beckman Instruments, Fullerton, CA) was used with isocratic elution at 0.75 mL/min. The eluting solution was composed of a mixture of methanol and 0.01 M KH$_2$PO$_4$ (67%, v/v), adjusted to an apparent pH of 5.3 with H$_3$PO$_4$. Bile acids were quantified by measuring their absorbance at 204 nm. Bile acid amidates (taurine and glycine) have similar extinction coefficients. Bile acids were tentatively identified by matching their relative retention times with those of known standards.
DEXA

Bone densitometry scanning was performed by Animal Resources at Children’s Hospital (ARCH). The following parameters were measured: total animal mass (g), fat mass (g), lean mass (g). Fat percentage was calculated by dividing fat mass by total mass.

Blood Chemistry/Metabolic Analysis

Plasma cholesterol (Thermo Scientific), triglycerides (Thermo Scientific), and free fatty acids (Wako) were measured via colorimetric assay according to the manufacturer’s instructions.

Glucose Tolerance Testing

Mice were fasted 12-16 hours overnight and injected with glucose at a dose of 1 mg/kg body weight. Blood glucose levels were measured from the tail at 0, 15, 30, 60, 90, and 120 minutes post injection using a handheld glucometer.

Gene Expression Analysis

RNA was isolated from liver using TRIzol (Ambion) or an RNeasy kit (Qiagen) and reverse transcribed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). cDNA was diluted ten-fold for analysis using SYBR Green (Invitrogen) on the QuantStudio® 6 Flex Real-Time PCR System (Applied Biosystems). Gene expression was normalized to the reference gene TBP. Primer sequences are listed in the Methods section of Chapter 3.

Western Blotting

Livers were homogenized in RIPA buffer. 40-80 ug of lysate were loaded onto SDS-PAGE gels and transferred onto a PVDF membrane (Thermo Scientific). Blots were blocked in SuperBlock buffer (Thermo Scientific), incubated overnight with primary
antibody and detected with secondary antibody conjugated with HRP. Antibody
complexes were visualized by enhanced chemiluminescence using X-ray film.

Statistics
Data are represented by the mean + SEM, unless otherwise indicated. Significance was
assessed by a 2-tailed Student’s t-test with unequal variance, unless otherwise indicated.
WT and KO mice of the same sex were compared. Results shown are pilot studies
c Conducted in a single cohort.

Results
As described in the Appendix, the FMO3 KO mice contain a 7 base pair deletion
in exon 3 of Fmo3. These animals are viable and fertile, with offspring that closely
resemble their WT counterparts (Fig. 4.1A, data not shown). To verify that Fmo3
expression was reduced, we measured Fmo3 abundance in WT and KO males and
females. Fmo3 mRNA was reduced 80-90% in female KO mice compared to WT mice
(Fig. 4.1B). In males, however, Fmo3 mRNA levels were not significantly different
between WT and KO animals, as males exhibit sex-dependent Fmo3 silencing (Li et al.,
2013). FMO3 protein is undetectable by Western blotting in male C57BL/6J mice, but
female KO mice showed decreased FMO3 protein expression (Fig. 4.1C).
To determine if \textit{Fmo3} knockout affected TMAO levels, plasma TMAO levels were measured in mice of both sexes and genotypes. Female KO mice showed significantly reduced plasma TMAO compared to WT mice (Fig. 4.1D). Male KO mice also showed...
lower plasma TMAO than their WT counterparts, but with a much smaller effect size (Fig. 4.1D). We subsequently measured hepatic TMAO in FMO3 KO females. As with hepatic Fmo3 expression, hepatic TMAO was decreased in KO females compared to WT (Fig. 4.1E). We also examined hepatic gene expression of the other FMO family members. For WT and KO mice of the same sex, expression of other FMOs did not differ (Fig. 4.1F-G).

**Fmo3 knockout has mild metabolic effects on young, chow-fed mice**

After validating that the FMO3 KO mice displayed reductions in FMO3 mRNA, protein and enzymatic activity, we compared WT and KO mice maintained on a chow diet. Male and female WT and KO mice (n=4-5 per group for the first cohort, and n=6-8 per group for the second cohort) were sacrificed at eight weeks of age. The first cohort was sacrificed in the non-fasted state, while the second cohort included non-fasted mice as well as mice that had been fasted for 24 hours prior to sacrifice. FoxO1 protein levels were modestly reduced in both sexes of KO mice, but not to the extent observed with ASO treatment.

In general, the phenotypic effects of Fmo3 knockout were milder and less consistent between the two sexes than those seen with ASO treatment. The bile acid pool composition was not altered by FMO3 knockout (Fig. 4.2A). DEXA scanning revealed a lower body fat percentage in male KO mice compared to WT, but did not find a difference in female mice (Fig. 4.2B). Similarly, glucose tolerance testing showed that Fmo3 knockout improved glucose tolerance in male mice, but not in female mice (Fig. 4.2C-D).
Some effects of Fmo3 knockout were also seen exclusively in female mice. Female mice showed modestly decreased plasma cholesterol in the fed state and decreased plasma triglycerides in the fasted state (Fig. 4.3A-B). In contrast, plasma triglyceride levels in male KO mice trended higher than in controls, and cholesterol levels were unchanged (Fig. 4.3A-B). However, no significant changes in gluconeogenic, lipogenic, or cholesterogenic gene expression were observed in male or
female FMO3 KO and WT mice (data not shown). Plasma free fatty acid levels trended higher in KO mice compared to WT, but these differences did not reach significance (Fig. 4.3C).

**Figure 4.3. Effects of FMO3 ablation on plasma lipids.**
FMO3 WT and KO mice of both sexes were sacrificed at 8 weeks of age in the non-fasted state (Fed) or after a 24 hour fast (Fasted). Plasma lipids were measured using colorimetric assays. Error bars represent SEM; n=7-9 per genotype; #p<0.05 FMO3 WT vs KO mice of a given sex.
**Fmo3 knockout does not prevent diet-induced obesity and insulin resistance**

Aberrantly high Fmo3 expression and TMAO levels are associated with insulin resistance, and ASO treatment decreases these parameters and improves the metabolic profile. We hypothesized that, since Fmo3 is necessary to maintain the insulin resistant phenotype in LIRKO mice, that its genetic ablation might prevent the onset of diet-induced obesity and insulin resistance.

To test this hypothesis, six-week-old male and female WT and KO mice were fed a high fat diet for twelve weeks. Surprisingly, WT and KO mice of the same sex gained similar amounts of weight during the experimental period (Fig. 4.4A). Fat percentage was measured via DEXA scanning and did not differ between WT and KO mice (Fig. 4.4B); glucose tolerance and 4 hour fasted glucose levels were also similar (Fig. 4.4C-D). No differences in lipogenic, cholesterogenic, or gluconeogenic gene expression were observed between WT and KO mice (data not shown). Plasma lipids did not differ, with the exception of free fatty acids, which were higher in the female WT compared to KO (Fig. 4.4E-G).
Figure 4.4. FMO3 KO mice are susceptible to diet-induced obesity. 3 to 5 week FMO3 WT and KO mice of both sexes were maintained on a high-fat diet for 4 months. (A) Body weights were recorded at sacrifice. (B) DEXA scanning was conducted after 2 months of dietary treatment. (C) Glucose tolerance testing was conducted two weeks prior to sacrifice. (D) Glucose was measured after a 4 hour fast after 2 months of dietary treatment. (E-G) Plasma lipids were measured in samples obtained at sacrifice. Error bars represent SEM; n=5 per genotype; #p<0.05 FMO3 WT vs KO mice of a given sex.

Fmo3 knockout alters the streptozotocin response

Streptozotocin treatment, a common model of type 1 diabetes, induces Fmo3 expression over 1,000 fold (Miao et al., 2015). To determine if Fmo3 ablation protects
against the negative effects of streptozotocin, 8-week-old male FMO3 WT and KO mice were injected with high-dose streptozotocin (STZ), or with vehicle as a control, and sacrificed one week post-injection in the fed state. Body weights at sacrifice were similar between FMO3 WT and KO mice (Fig. 4.5A). Surprisingly, blood glucose levels trended higher in the STZ-treated FMO3 KO mice for the duration of the experiment, but did not reach significance (Fig. 4.5B). Fasted plasma free fatty acids also trended higher in the KO mice in samples taken at day 4 (Fig. 4.5C). This trend then reversed in fed samples taken at sacrifice, where KO mice displayed a trend towards lower plasma free fatty acids (Fig. 4.5C). At sacrifice, plasma triglycerides were lower in KO mice, but plasma cholesterol was unchanged (Fig. 4.5D-E).
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**Figure 4.5. FMO3 KO mice are not protected against STZ-induced hyperlipidemia.** 9 to 10 week old male FMO3 WT and KO mice were treated with streptozotocin (STZ) or vehicle and sacrificed one week later in the non-fasted state. (A) Body weights were recorded at sacrifice. (B) Blood glucose was measured daily in the non-fasted state, except for the day 4 measurement which was conducted after a 4 hour fast (C) Plasma free fatty acids were measured after a 4 hour fast (day 4) and in samples taken at sacrifice (day 7) Plasma triglycerides (D) and cholesterol (E) were measured in samples obtained at sacrifice. Error bars represent SEM; n=5 per genotype; #p<0.05.

**Fmo3 knockout does not protect against NTS-induced hyperlipidemia**

TMAO is elevated in chronic kidney disease, and work in mouse models has shown that this molecule contributes to renal dysfunction (Tang et al., 2015). We hypothesized that FMO3 KO animals may be protected against this phenotype. Nephrotoxic serum (NTS), which contains anti-glomerular antibodies, was used to induce nephritis, a condition characterized by diffuse interstitial fibrosis throughout the
kidney.

Six- to eight-week-old Fmo3 WT and KO males were injected with NTS or a sheep serum (SS) control as previously described and sacrificed three weeks post-injection (Lloyd & Gutierrez-Ramos, 2000). On day 4 post-injection, NTS-treated mice were placed in metabolic cages for a 6 hour urine collection. In accordance with our hypothesis, KO NTS-treated mice had a significantly smaller urine volume than WT NTS-treated mice (Fig. 4.6A).

The period shortly following NTS injection, known as the nephrotic phase, is associated with hyperlipidemia (Haas et al., 2016). Although Fmo3 knockout reduced urine output, it did not prevent hyperlipidemia, as 4 hour fasted plasma cholesterol and triglyceride levels did not differ between NTS-treated WT and KO animals (Fig. 4.6B-C).

![Figure 4.6. FMO3 KO mice are not protected against NTS-induced hypercholesterolemia.](image)

7-8 week old male FMO3 WT and KO mice were injected with control sheep serum (SS) or nephrotoxic serum (NTS). (A) NTS-treated mice were subjected to a 6 hour urine collection on day 4 post-injection. (B-C) Plasma was collected after a 4 hour fast on day 5 post-injection. Plasma (B) cholesterol and (C) triglycerides were measured. Error bars represent SEM; n=5 per genotype; *p<0.05 SS- versus NTS-treated mice of a given genotype; #p<0.05 WT NTS-treated vs KO NTS-treated mice.

To determine if FMO3 KO mice were protected in the later, nephritic stages of
kidney disease, mice were subjected to a second urine collection at day 22 post-injection. We performed a comprehensive urinalysis on these samples using colorimetric urine dipsticks. We did not observe any visual differences in the following tested parameters: pH, specific density, ascorbic acid, bilirubin, glucose, ketones, leukocytes, and protein. In addition, Fmo3 knockout did not prevent NTS-induced fibrosis, as assessed via Masson Trichrome collagen staining (data not shown).

Discussion

In sum, these studies show that genetic ablation of Fmo3 in C57BL/6J mice has only mild effects on metabolism and physiology, compared to the stark effects observed with FMO3 ASO in LIRKO mice. This result was partially expected, as the knockout of genes that are of utmost importance in insulin resistant settings does not always result in a profound phenotype in insulin sensitive mice. For example, FoxO1 knockout in insulin sensitive mice has little effect on glucose tolerance, but its knockout in multiple insulin resistant models completely rescues multiple defects of insulin resistance (Dong et al., 2008; O-Sullivan et al., 2015; Titchenell et al; 2015).

We did observe a surprising difference between FMO3 KO mice and insulin sensitive mice treated with FMO3 ASO in our laboratory’s previous studies. Rather than the complete reduction in hepatic FoxO1 observed in FMO3 ASO treated mice, FMO3 KO mice show only a slight reduction in FoxO1 protein levels (Fig. 4.1C) (Miao et al., 2015). There are a few explanations as to why Fmo3 ablation failed to reduce FoxO1 protein to the same extent as FMO3 ASO. This difference could be attributed to off-target effects of the ASO on other FMO isoforms, although our laboratory’s previous work did not find that the ASO reduced expression of other FMOs (Miao et al., 2015)
One other theory is FMO compensation. In metabolism especially, isoforms with overlapping targets can often compensate for another when one is ablated; for example, SREBP-1c has been found to stimulate both lipogenic and cholesterologenic gene expression when cholesterologenic regulator SREBP-2 is knocked out (Horton et al., 2003).

Work in mice with triple knockout of Fmo1, Fmo2, and Fmo4 has implicated Fmo1 as a regulator of energy storage in white adipose tissue and skeletal muscle (Veeravalli et al., 2014). Interestingly, Fmo1 has the second highest specific activity for TMAO production, behind Fmo3, indicating that it could potentially substitute for Fmo3 (Bennett et al., 2013). Although we did not observe changes in Fmo1 mRNA resulting from Fmo3 knockout (Fig. 4.1F), it is possible that protein levels or enzymatic activity of FMO1 could have been altered.

Another potential explanation for the high FoxO1 protein levels seen in FMO3 KO animals is developmental effects. In working with our LIRKO model and mice treated with insulin receptor ASO, we have seen very different phenotypes, despite both these models displaying reduced hepatic insulin receptor expression. We attribute these differences to developmental effects, as the LIRKO model loses most insulin receptor expression before birth due to albumin-Cre activation, whereas ASO experiments typically begin at age 3-4 weeks (Weisend et al., 2009). The early loss of Fmo3 in the whole body knockout may induce rewiring of hepatic gene regulation such that other genes, potentially including fetally-expressed Fmo1, play a more important role in metabolic regulation.
It is also possible that our FMO3 KO mouse in fact retains enough residual \textit{Fmo3} that it cannot be considered a true knockout mouse. The 7 bp deletion in exon 3 should induce nonsense-mediated decay of \textit{Fmo3} mRNA, and our results show that this mutation lowers FMO3 mRNA and protein expression in female mice (\textbf{Fig. 4.1B-C}). If, however, FMO3 works through an RNA-dependent mechanism, the residual FMO3 mRNA might suffice to maintain a wild-type phenotype.

In order to dissect the potential contributions of developmental changes and other FMO isoforms, a different mouse model will be required. I propose using CRISPR/Cas9 to insert loxP sites into \textit{Fmo3} to create a conditional knockout allele. These mice could then be bred to mice expressing inducible Cre under the albumin promoter, allowing the researcher to control the knockout temporally. Comparing mice with temporal liver-specific FMO3 KO to mice treated with FMO3 ASO would reduce the potential for developmental effects and would help determine if 1. ablation of \textit{Fmo3} increases expression of other FMOs and 2. FMO3 ASO targets other FMOs.

Given that FMO3 ASO treatment rescues the defects of genetic insulin resistance, it is somewhat surprising that \textit{Fmo3} ablation does not prevent diet-induced obesity and insulin resistance (\textbf{Fig.4.4}). The potential development effects, ASO non-specificity, and RNA persistence discussed above may explain this phenotype. It is also possible that with \textit{Fmo3} induction in insulin resistance comes increased expression of a related mediator, both of which are necessary to maintain the insulin resistant state. However, insulin resistance can develop in the presence of either one of the two factors. Using the floxed model described above would help to tease out these competing hypotheses.
The floxed model would also enable researchers to directly compare the effects of Fmo3 loss in the liver or another single organ to those of whole-body ablation. Since Fmo3 is expressed in the liver, white adipose tissue, and kidney, its metabolic effects on the whole organism are a sum total of its effects on each tissue. Previous research has focused primarily on the liver because antisense oligonucleotides target this organ, and Fmo3 effects in other tissues have not been thoroughly studied. A whole-body knockout could potentially mask the effects of knockout in a single organ, but these effects would become more apparent using a model carrying Fmo3 floxed alleles and Cre under control of a tissue-specific promoter.

Despite the challenges of using a whole-body Fmo3 knockout mouse, we have uncovered a potentially novel role for Fmo3 in adipose tissue energy balance using the streptozotocin model of type 1 diabetes (Fig. 4.5). In this experiment, FMO3 KO mice displayed a trend towards increased fasted blood glucose and increased plasma FFA at day 4, but showed decreased plasma FFA at sacrifice. I hypothesize that energy from the white adipose tissue was mobilized more readily in KO mice, and that these triglycerides/fatty acids served as gluconeogenic substrates, explaining the higher blood glucose levels. Indeed, the KO mice had very little gonadal fat at sacrifice compared to controls, indicating that the energy stores in the white adipose tissue had been depleted. These data indicate that FMO3 plays a role in regulating adipose energy storage and fit well with the results from FMO3 ASO experiments in Chapter 3.

Acknowledgments/Attributions

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blots shown in Fig. 4.1C. VK assisted with the plasma TMAO measurements shown in Fig. 4.1D. MCP and AJM performed the hepatic TMAO measurements shown in Fig. 4.1E. LRH performed the bile acid measurements shown in Fig. 4.2A. DEXA scanning shown in Fig. 4.2B and Fig. 4.4B was conducted by Animal Resources at Children’s Hospital. MEH assisted with the NTS experiment shown in Fig. 4.6. MEG performed all data analysis and figure design.
Chapter 5 – Conclusions
The Enduring Problem of Diabetes-Associated Cardiovascular Disease

The eminent diabetes physician Elliott P. Joslin referred to modern-day diabetes research and management as the “era of complications” (Sanders, 2002). With insulin and sophisticated blood glucose monitoring systems available, a diabetes diagnosis is no longer a death sentence. However, it is clear that suboptimal insulin signaling leads to many comorbidities, notably retinopathy, neuropathy, nephropathy and cardiovascular disease, especially atherosclerosis (DCCT, 1993).

The Diabetes Control and Complications Study (DCCT), the landmark study of diabetes complications, took place from 1983-1993 under control of the National Institutes of Health. This study compared standard blood glucose monitoring to more rigorous “intensive” monitoring to see how more stringent blood glucose control affected diabetic complications. The study was an incredible success, lowering the risk of retinopathy by 76%, nephropathy by 50%, and neuropathy by 60% (DCCT, 1993). A follow-up study, Epidemiology of Diabetes Interventions and Complications (EDIC), a 42% reduced risk of CVD events and a 57% reduced risk of death from CVD in patients who underwent intensive monitoring (Nathan et al., 2005).

Despite the encouraging results from DCCT and EDIC, cardiovascular disease remains a major problem for diabetic individuals. Intensive glucose monitoring lowers CVD risk for diabetics, but it does not normalize it. For example, for patients with type 2 diabetes, CVD occurs on average 14.6 years earlier in patients with type 2 diabetes and is generally more severe than in non-diabetic individuals (Beckman et al., 2013).
Patients with type 1 diabetes can have up to a ten-fold increase in CVD risk (Libby et al., 2005; Krolewski et al., 1987).

Given that glucose monitoring only partially improves CVD risk, researchers have looked for other mechanisms to explain how changes in insulin signaling affect CVD risk. In particular, insulin’s regulation of lipid metabolism is an important and growing field. Lipid abnormalities occur in 60-70% of type 2 diabetics, and these changes can be identified years before the progression to clinical diabetes (Lorenzo et al., 2013). In general, the diabetic lipid profile consists of low HDL or “good” cholesterol, increased small dense LDL, the worst subtype of “bad” cholesterol, and high triglycerides. Studies published in 2008 showed that a mouse model of hepatic insulin resistance recapitulated many of the lipid abnormalities and complications of diabetes (Biddinger et al., 2008a; Biddinger et al., 2008b). These findings give credence to the idea that lipid alterations due to insulin resistance, not hyperglycemia, are at the heart of diabetes-associated CVD.

**Cyp8b1 and Diabetic Bile Acid Metabolism**

The work in this dissertation expands our understanding of insulin’s role in lipid metabolism by examining previously uncharacterized signaling pathways. In Chapter 2, we dissect the role of bile acid synthetic enzyme CYP8B1 using antisense oligonucleotide-mediated knockdown in the LIRKO mouse model. CYP8B1 catalyzes the 12α hydroxylation reaction that is the rate-limiting step in cholic acid synthesis. Thus, this enzyme is a key regulator of the balance between bile cholic acid and CDCA levels (*Fig. 1.2*) (Russell, 2003).
Insulin resistant mice and humans are known to have an increased proportion of cholic acid in bile, which is thought to promote cholesterol absorption (Biddinger et al., 2008b; Haeusler et al., 2013). Through microarray analysis of three insulin resistant mouse models, we found that the cholesterol biosynthetic pathway was the most downregulated in a set of 674 REACTOME gene sets (Fig. 2.1D; Fig. S1.1D-E). We hypothesized that this change was due to increased cholesterol absorption mediated by cholic acid, and we found that the increased plasma total and LDL cholesterol seen in LIRKO mice on a Western diet could be rescued by Cyp8b1 knockdown (Fig. 2.7A; Fig. 2.7C). In addition, Cyp8b1 knockdown also improved other aspects of the metabolic profile, including glucose tolerance (Fig. 2.6A-B; Fig. S1.5E). Conversely, Cyp8b1 overexpression increased hepatic cholesterol and plasma LDL in mice with normal cholesterol metabolism (Fig. 2.4E, 2.4I).

Previous data also suggest that targeting bile acid metabolism improves the diabetic lipid profile. Bile acid sequestrants, or resins that increase bile excretion to promote the conversion of cholesterol into bile, lower LDL cholesterol by about 15-20% and also reduce HbA1c (rev. in Staels, 2010). When given in combination with statins, bile acid sequestrants further lower plasma LDL cholesterol. Other studies have shown positive effects of direct CDCA administration, which activates FXR signaling to lower plasma triglycerides and downregulate lipogenesis (Bateson et al., 1978; Watanabe et al., 2004).

Given that mouse and human bile acid metabolism differ, it is important to consider how our exploratory results in mice would translate to humans. In humans, the dominant bile acid species is CDCA, which in mice is efficiently converted to alpha and
beta-muricholic acid (Fig. 1.3). As mice treated with Cyp8b1 ASO have increased levels of CDCA-derived muricholic acids, we would expect that humans treated with Cyp8b1 ASO would have bile enriched in CDCA (Fig. 1.3). Given that CDCA is a potent agonist for FXR (Table 1.1), we would expect additional beneficial effects of Cyp8b1 ASO on triglycerides and adiposity in addition to the improvements in glucose and cholesterol metabolism observed in mice. If so, then Cyp8b1 reduction would represent a way to improve two negative aspects of the diabetic lipid profile: increased ApoB-containing particles VLDL and LDL and increased triglycerides.

While CYP8B1 is a promising target for diabetes-associated cardiovascular disease, the mechanisms underlying its effects on lipid metabolism are unclear. The role of cholic acid in promoting cholesterol absorption clearly is important to hypercholesterolemia, and increased FXR signaling would also be metabolically beneficial. However, researchers have also noted that Cyp8b1 knockout increases levels of GLP-1, an incretin hormone that increases insulin secretion, suppresses glucagon secretion, and promotes satiety (Kaur et al., 2015). Dissecting signaling downstream of CYP8B1 and cholic acid production will help us to understand the multifaceted nature of CYP8B1’s effects.

More generally, our findings bring bile acids, a set of molecules often neglected by metabolism researchers, to the forefront. It is clear that these compounds play a key role in systemic metabolism, and their individual signaling properties should be investigated. Our work implicates 12α-hydroxylation in negative metabolic effects, but it remains unclear what function other bile acid modifications, such as hydroxylations, conjugations, and sulfonations play in determining bile acid properties. With over 30 bile
acid species present in mice and humans (Alnouti et al., 2008), we must begin to
dissect the bile acid signaling code.

FMO3 Links the Diet and Gut Microbiome to Organismal Health

Since TMAO was identified as a risk factor for CVD in 2011, interest in this
cube has skyrocketed. Since TMAO precursor TMA is
produced exclusively by gut microbiota, this axis has the potential to connect an
individual’s diet and gut microbiome to his/her cardiovascular disease risk. Rather than
focus solely on FMO3 and cholesterol metabolism, we chose to examine its role in
insulin resistant models like the LIRKO mouse, where it represents one of the most
highly induced transcripts (Miao et al., 2015).

In Chapter 3, we explored the relationship of FMO3 to key insulin target FoxO1.
Having previously found that FMO3 ASO treatment ablated FoxO1 protein expression in
LIRKO mice, we sought to understand which effects of FMO3 could be attributed to
hepatic FoxO1 action. We found that FoxO1 is a key mediator in FMO3 action on
glucose, bile acid, and cholesterol metabolism in the liver, but we also observed
smaller, FoxO1-independent effects on glucose and cholesterol metabolism (Fig. 3.3;
Fig. 3.4). In addition, we identified a hepatic FoxO1-independent effect of FMO3 on
body weight and adiposity (Fig. 3.5A-B). Using the weak FMO3 inhibitor indole-3-
carbinol, we showed for the first time that FMO3 enzymatic activity is tied to its
regulation of FoxO1 protein level in insulin resistant settings (Fig. 3.6F-G).

Although TMAO is perhaps the most well-known of FMO3 enzymatic products, it
is by no means the only one. FMO3, like its family members, can bind molecular oxygen
prior to binding a substrate, and this interaction is thought to lend it a broad substrate
specificity (Phillips & Shephard, 2008). Large-scale, unbiased metabolomic studies may be the best strategy to understand which metabolites are produced by FMO3, and if these compounds vary under different physiological conditions, such as insulin resistance or hypercholesterolemia.

To determine if TMA/TMAO play a direct role in influencing FoxO1, studies using FMO3 ASO in tandem with TMA/TMAO supplementation would be enlightening. For a more general look at the contribution of the gut microbiota, researchers have previously used antibiotics with great success (Koeth et al., 2013; Warrier et al., 2015).

In Chapter 4, we examined the effects of global Fmo3 ablation on systemic metabolism. We found that Fmo3 loss had only minimal effects on glucose and lipid metabolism in insulin sensitive mice, a finding that parallels the minor effects of FoxO1 loss in wild-type mice (Cheng & White, 2011). We were more surprised to see that Fmo3 ablation did not prevent mice from developing insulin resistance and obesity on a high fat diet, given that ASO-mediated Fmo3 knockdown rescues established insulin resistance (Fig. 4.4). It is unclear if this result indicates that other FMOs play a role in the development of insulin resistance, or if the development and maintenance of insulin resistance occur through distinct mechanisms. It is also possible that our model, which contains a frameshift in Fmo3, retains enough residual Fmo3 to promote low-level FMO3 expression, or that developmental effects from prenatal Fmo3 ablation have altered transcriptional circuitry.

Answering these questions will require additional models, including the development of Fmo3 floxed mice to generate conditional knockout models in various tissues. It might also be desirable to use adeno-associated virus (AAV) encoding Cas9
with guide RNAs (gRNAs) flanking *Fmo3* to create a clean deletion of the gene in
postnatal mice (Ran et al., 2015). This model would completely ablate *Fmo3*
transcription, which would prevent any miRNA- or mRNA-mediated signaling from this
gene. CRISPR/Cas9 could help researchers ascertain if other FMOs can compensate
for FMO3, as multiple gRNAs targeting multiple FMOS could be packaged into a single
vector construct (Ran et al., 2015).

**FMO3 in Adipose and Kidney Physiology**

Since previous studies have focused primarily on FMO3’s role in hepatic
metabolism, we used our global knockout mouse to begin to dissect FMO3 action in
other tissues. We found that *Fmo3* ablation alter adipose energy storage in both insulin-
sensitive and STZ type 1 diabetic male mice (*Fig. 4.2B; Fig. 4.5C-D*). We are currently
exploring the mechanism behind this phenotype. If FMO3 regulates FoxO1 expression
in adipose tissue, it is possible that decreased FoxO1 is preventing adipocyte
maturation and thus decreasing the size of fat depots (Nakae et al., 2003). FMO3 may
also affect lipolysis rates or white/brown adipose energy utilization, as was observed for
FMO1 (Veeravalli et al., 2014).

We also attempted to explore FMO3 action in the kidney using the NTS model of
nephritis, which produces hyperlipidemia in the early stages of the disease (Haas et al.,
2016). Loss of *Fmo3* did not protect mice against hyperlipidemia (*Fig. 4.6B-C*).
However, FMO3 KO mice did show lower urine output in the early stages of the
disease, which could indicate that *Fmo3* or TMAO play a role in kidney dysfunction (Fig.
4.6A). A kidney-specific *Fmo3* knockout mouse would allow researchers to further
explore this important topic, given that TMAO appears to be both a biomarker of kidney injury and a renal toxin (Fogelman et al., 2015).

**Sexual Dimorphism and FMO3 Signaling**

One challenge in the study of FMO3 is the extreme sexual dimorphism of this gene in laboratory mice. In male *Mus musculus*, hepatic *Fmo3* expression is silenced by six weeks of age, but *Fmo3* expression in the kidney is maintained (Li et al., 2013). Humans do not exhibit sex-dependent *Fmo3* silencing and display *Fmo3* levels more similar to female mice (Bennett et al., 2013). Despite not observing differences in hepatic *Fmo3* expression between insulin-sensitive FMO3 WT and KO males, we did find positive effects of *Fmo3* ablation on glucose tolerance and adiposity in male mice (Fig. 4.2B-D). It will be important to dissect if these effects are the result of *Fmo3* loss in the liver or in other tissues, again using tissue-specific *Fmo3* knockouts.

Despite the sexual dimorphism of *Fmo3* in mice, it is clear that both male and female insulin resistant mice benefit from FMO3 ASO treatment. *Fmo3* knockdown rescues glucose intolerance and hyperlipidemia in both sexes of mice, and this phenotype is robust and reproducible. However, given the challenges of extrapolating studies in insulin sensitive male mice to humans, a rat model may be a better system for detangling the mechanisms of FMO3 signaling. Rats have traditionally been difficult to modify genetically, but the advent of CRISPR/Cas9 has improved our ability to conduct gene targeting in this species (Ma et al., 2014). Studies in rats would more closely mimic the physiology of humans and, if the results were similar to those obtained in male and female mice, would help validate the FMO3 signaling pathway as a drug target for diabetes-associated cardiovascular disease and general insulin resistance.
Summary of Findings

Although we know that insulin signaling regulates lipid metabolism, our molecular understanding of these pathways remains unclear. Here, we show that insulin targets Cyp8b1 and FMO3 are novel mediators of diabetes-associated cardiovascular disease. Cyp8b1 knockdown rescues hypercholesterolemia in insulin resistant mice and also improves glucose metabolism, suggesting that decreasing cholic acid levels could benefit diabetic patients. Our studies of FMO3 reveal a close relationship between this protein and FoxO1, but suggest that we have only scratched the surface of its complex, multiorgan signaling. These findings further illustrate the complexity and translational relevance of lipid metabolism in insulin resistance.
References


Supplement 1 - Material Related to Chapter 2
Figure S1.1. Further characterization of insulin resistant models.

(A-C) Male Liver FoxO1 Knockout (LFKO), Liver Insulin Receptor Knockout (LIRKO), Liver Double Knockout (LDKO) mice, and their Cre-negative littermate controls (FoxO1-lox, IR-lox and Dlox, or, when pooled together, CON) were maintained on a chow diet and sacrificed in the non-fasted state at 8-10 weeks of age. (A) Body weights of male LFKO, LIRKO, LDKO mice and their Cre-negative controls were measured at 6-8 weeks of age. (B) The area under the curve for the glucose tolerance test shown in Fig. 2.1C. (C) Fecal neutral sterol loss was measured by gas chromatography. Error bars represent SEM; n = 5–12; *p<0.05 versus control mice; #p<0.05 LIRKO vs LDKO; $p<0.05 LFKO vs LDKO. (D-G) Previously published microarray data (IRS1/2 deletion: Dong et al., 2008) and (Akt1/2 deletion: Lu et al., 2012) were subjected to GSEA analysis using the REACTOME pathway set. (D-E) The enrichment plot and statistics for the cholesterol biosynthetic pathway for the two group comparison (floxed vs insulin resistant) are shown. (F-G) Heat maps comparing expression of the genes in the REACTOME cholesterol biosynthesis pathway for the three groups of mice (floxed, insulin resistant, insulin resistant + hepatic FoxO1 deletion.) n = 2-4 chips per genotype.
Figure S1.1 (Continued).

A. Body Weight

B. GTT AUC

C. Fecal Neutral Sterols

D. Hepatic Deletion of IRS1 and IRS2

E. Hepatic Deletion of Akt1 and Akt2

F. IRS- flox IRS-DKO TKO

G. Akt-flox Akt-DKO Akt-TKO

- SQLE
- MVD
- ID1
- FDP5
- CYP51A1
- HMGS1
- HMGCR
- NSDHL
- FDFT1
- LSS
- PMVK
- DHCR7
- TM7SF2
- MVK
- SC5DL
- HSD17B7
- DHCR24
- EBP
- GGPS1
- LBR

- SQLE
- MVD
- ID1
- FDP5
- CYP51A1
- HMGS1
- HMGCR
- NSDHL
- FDFT1
- LSS
- PMVK
- DHCR7
- TM7SF2
- MVK
- SC5DL
- HSD17B7
- DHCR24
- EBP
- GGPS1
- LBR
Figure S1.2 Further characterization of CON, LFKO, LIRKO, and LDKO mice. (A-B) Male Liver FoxO1 Knockout (LFKO), Liver Insulin Receptor Knockout (LIRKO), Liver Double Knockout (LDKO) mice, and their Cre-negative littermate controls (FoxO1-lox, IR-lox and Dlox, or, when pooled together, CON) were maintained on a chow diet and sacrificed in the non-fasted state at 8-10 weeks of age. (A) Plasma cholesterol and (B) triglycerides were measured in samples taken at the time of sacrifice. (C) Hepatic cholesterol was measured in samples taken at the time of sacrifice. (D) Male Liver FoxO1 Knockout (LFKO), Liver Insulin Receptor Knockout (LIRKO), Liver Double Knockout (LDKO) mice, and their Cre-negative littermate controls (FoxO1-lox, IR-lox and Dlox, or, when pooled together, CON) were maintained on a Western diet for 6 weeks and sacrificed at 2-3 months of age. (D) Raw FPLC traces from lipoprotein data shown in Fig. 2.2B. Error bars represent SEM; n = 3–7; *p<0.05 versus control mice; #p<0.05 LIRKO vs LDKO.
Figure S1.3. Further analysis of insulin regulation of bile acid synthetic enzyme expression.

Primary hepatocytes were isolated from FoxO1-lox (Control) and LFKO (FoxO1 knockout) mice, serum starved overnight, and treated for 6 hours with or without 100 nM insulin. Gene expression was measured using quantitative RT-PCR. Error bars represent SEM; n=3 wells per condition; *p<0.05 FoxO1-lox versus LFKO hepatocytes with the same treatment, #p<0.05 +/- insulin treatment for the same genotype.
Figure S1.4. Phenotypic characterization of Ad-Cyp8b1 treated mice.
7-12 week old LDKO mice were fed a chow diet supplemented with 2% cholesterol for four weeks and injected with adenovirus expressing GFP or Cyp8b1 one week before sacrifice in the non-fasted state. (A, C) Hepatic gene expression was measured using quantitative RT-PCR. (B) Bile was collected from the gallbladders of these mice; equal amounts of gallbladder bile from 5 mice per group, collected at the time of sacrifice, were pooled and subjected to HPLC. The bile hydrophobicity index was calculated as previously described (Heuman et al., 1989). (C) Raw traces from FPLC curves shown in Fig. 2.4I. The Ad-GFP trace is shown at left, and the Ad-Cyp8b1 trace at right. Error bars represent SEM; n=4-5 per genotype; *p<0.05.
Figure S1.5. Phenotypic characterization of Cyp8b1 ASO-treated mice.
(A-J) 4-5 week old male LIRKO mice and their floxed controls were maintained on a chow diet and treated with control (CON) or Cyp8b1 ASO (50 mg/kg body weight per week IP for seven weeks. (A) Hepatic gene expression was measured using quantitative RT-PCR. (B) Bile was collected from the gallbladders of these mice; equal amounts of gallbladder bile from 5 mice per group, collected at the time of sacrifice, were pooled and subjected to HPLC. The bile hydrophobicity index was calculated as previously described (Heuman et al., 1989). (C) Hepatic and (D) plasma triglycerides were measured in samples taken at the time of sacrifice. (E) The area under the curve for the glucose tolerance test shown in Fig. 2.6A. (F) 4 week old male C57BL/6J mice were maintained on a Western diet and treated with CON or Cyp8b1 ASO for four mice and subsequently injected with high-dose streptozotocin (180 mg/kg body weight) or vehicle. Plasma cholesterol was measured after a 4 hour fast. (G) 5-9 week old male LIRKO mice and their floxed controls were maintained on a Western diet and treated with CON or Cyp8b1 ASO for five weeks. Raw traces from FPLC curves shown in Fig. 2.7C.
Figure S1.5 (Continued).

A. mRNA levels of Cyp27a1, Cyp7b1, and Cyp7a1.

B. Bile hydrophobicity.

C. Hepatic triglycerides.

D. Plasma triglycerides.

E. GTT AUC.

F. Plasma cholesterol.

G. Lox CON ASO, LIRKO CON ASO, LIRKO Cyp8b1 ASO.
Supplement 2 - Material Related to Chapter 4
We chose to create a global knockout of FMO3 in mice using the recently developed CRISPR/Cas9 genome engineering method (Wang et al., 2013). This method employs a guide RNA (gRNA) to target the nuclease Cas9 to a defined locus, where Cas9 can induce a double stranded break (DSB). DSBs are repaired via the error-prone non-homologous endjoining, which produces insertions and deletions that can result in frameshifts and early termination. We designed 4 gRNAs that spanned the length of Fmo3 and cloned them into a Cas9-containing vector obtained from Addgene. We then PCR amplified Cas9 and the 4 gRNAs and used them as templates for in vitro transcription as described in Wang et al., 2013. RNA was analyzed for size and purity using a Bioanalyzer. We selected a gRNA with the sequence 5’
TGGGAAAGTCATCGGGATAGGGG 3’. This gRNA binds nucleotides 215-238 of Fmo3 (AA 72-80). Since this gRNA binds upstream of the Fmo3 NADPH binding site, a frameshift in this region would result in a protein unable to bind NADPH, rendering it nonfunctional (Fig. S2.1). Multiple trimethylaminuria-causing mutations have been identified near the site of gRNA binding, indicating that this region of the protein is functionally significant (Fig S2.1).
RNA encoding Cas9 and the gRNA was injected into C57BL/6J embryos by the Boston Children’s Hospital Gene Manipulation Core. These embryos were then placed into pseudopregnant foster mothers. 12 pups survived and were genotyped at one week of age. The region surrounding the targeted locus was PCR amplified and sent for sequencing to identify potential mutants. PCR products from three potential mutants were then subcloned into pGEM-T Easy (Promega) and six colonies from each mouse were sequenced and aligned to the wild-type PCR sequence to determine mutations carried by the mice.

Each of the three founder mice carried at least one frameshift mutation at the specified locus. At 5 weeks of age, these founder mice were mated to C57BL6 mice to determine which, if any, mutations were found in the germ line and to begin the process of backcrossing. These breedings revealed that 1, 6, and 7 base pair deletions were present in the germline of the founder mice. We elected to move forward with the mice carrying the 7 base pair deletion because this deletion resulted in a frameshift and prematurely truncated FMO3 protein with a stop codon at residue 98 (Fig. S2.2).

**Figure S2.1. FMO3 protein structure and known mutations.**
The 532 amino acid FMO3 protein contains an FAD binding site (green) at the extreme N-terminus and an NAPDH binding site (blue) in the first half of the sequence. A subset of the mutations known to cause trimethylaminuria are indicated in red (Phillips & Shephard, 2008). We chose a gRNA that would target upstream of the NAPDH binding site, in a region where other mutations have been shown to reduce FMO3 activity.

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We developed a genotyping method to easily distinguish offspring carrying the 7 base pair deletion (from here described as FMO3 KO) from FMO3 WT mice. Mice were backcrossed for three generations to C57BL/6J mice (Jackson Laboratories) to reduce the likelihood of other mutations resulting from off-target Cas9 editing.

To verify that the FMO3 WT and KO mice were congeneric strains, tail samples were sent to Jackson Laboratories for SNP genotyping. These tests verified that both mouse lines were 100% C57BL/6J. Litters were subsequently generated from WT x WT and KO x KO breedings. This breeding scheme did not generate littermate controls, but it did ensure that mice were age-matched and exposed to a similar environment, while permitting us to generate significant mice for each experiment (n=5-10 per group).

**Figure S2.2. Partial translation of WT and mutant FMO3.**

The 7 bp deletion in the mutant Fmo3 allele leads to a frameshift at amino acid residue 74 and premature termination at amino acid residue 98.