The TMAO-Generating Enzyme Flavin Monooxygenase 3 Is a Central Regulator of Cholesterol Balance

Highlights
- Unbiased transcriptional profiling links FMO3 to reverse cholesterol transport
- FMO3 knockdown substantially impacts whole-body cholesterol balance
- FMO3 is a negative regulator of nonbiliary reverse cholesterol transport
- FMO3 knockdown results in diminished LXR activity, promoting hepatic inflammation

In Brief
Reverse cholesterol transport (RCT) can be mediated by either the classic biliary route or the nonbiliary transintestinal cholesterol excretion (TICE) pathway. Warrier et al. now identify a gut microbial-driven pathway that balances the amount of cholesterol entering the biliary and nonbiliary pathways. Inhibition of the enzyme flavin monooxygenase 3 (FMO3) diverts cholesterol away from biliary excretion into the nonbiliary TICE pathway, reorganizing total body cholesterol balance.

Accession Numbers
GSE64326
The TMAO-Generating Enzyme Flavin Monooxygenase 3 Is a Central Regulator of Cholesterol Balance

Manya Warrier,1 Diana M. Shih,6 Amy C. Burrows,1 Daniel Ferguson,1 Anthony D. Gromovsky,1 Amanda L. Brown,1 Stephanie Marshall,1 Allison McDaniel,9 Rebecca C. Schugar,7 Zeneng Wang,1 Jessica Sacks,1 Xin Rong,10 Thomas de Aguiar Vallim,2 Jeff Zhou,4 Pavlina T. Ivanova,1 David S. Myers,4 H. Alex Brown,4 Richard G. Lee,5 Rosanne M. Crooke,5 Mark J. Graham,5 Xiuli Liu,6 Paolo Parini,7 Peter Tontonoz,9,10 Aldon J. Lusis,6 Stanley L. Hazen,1 Ryan E. Temel,2 and J. Mark Brown1,*

1Department of Cellular and Molecular Medicine, Cleveland Clinic Lerner Research Institute, Cleveland, OH 44195, USA
2Saha Cardiovascular Research Center, University of Kentucky, Lexington, KY 40536-0509, USA
3Departments of Pathology and Biostatistics, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA
4Departments of Pharmacology and Biochemistry, The Vanderbilt Institute of Chemical Biology, Nashville, TN 37232, USA
5Cardiovascular Group, Antisense Drug Discovery, Isis Pharmaceuticals, Inc., Carlsbad, CA 92010, USA
6Department of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA
7Clinical Chemistry, Department of Laboratory Medicine, Karolinska Institutet at Huddinge University Hospital, 141 86 Stockholm, Sweden
8Department of Anatomical Pathology, Cleveland Clinic, Cleveland, OH 44195, USA
9Howard Hughes Medical Institute
10Department of Pathology and Laboratory Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA
*Correspondence: brownm5@ccf.org
http://dx.doi.org/10.1016/j.celrep.2014.12.036
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

SUMMARY

Circulating levels of the gut microbe-derived metabolite trimethylamine-N-oxide (TMAO) have recently been linked to cardiovascular disease (CVD) risk. Here, we performed transcriptional profiling in mouse models of altered reverse cholesterol transport (RCT) and serendipitously identified the TMAO-generating enzyme flavin monooxygenase 3 (FMO3) as a powerful modifier of cholesterol metabolism and RCT. Knockdown of FMO3 in cholesterol-fed mice alters biliary lipid secretion, blunts intestinal cholesterol absorption, and limits the production of hepatic oxysterols and cholesteryl esters. Furthermore, FMO3 knockdown stimulates basal and liver X receptor (LXR)-stimulated macrophage RCT, thereby improving cholesterol balance. Conversely, FMO3 knockdown exacerbates hepatic endoplasmic reticulum (ER) stress and inflammation in part by decreasing hepatic oxysterol levels and subsequent LXR activation. FMO3 is thus identified as a central integrator of hepatic cholesterol and triacylglycerol metabolism, inflammation, and ER stress. These studies suggest that the gut microbiota-driven TMA/FMO3/TMAO pathway is a key regulator of lipid metabolism and inflammation.

INTRODUCTION

Atherosclerosis and associated cardiovascular disease (CVD) remain the largest causes of mortality in developed countries (Go et al., 2013). Despite widespread use of statins, CVD-associated mortality and morbidity have been reduced by only ~30% (Go et al., 2013), demonstrating a clear need for better therapeutic strategies. Elevation of high-density lipoprotein (HDL) function is thought to be an attractive therapeutic strategy (Rader and Tall, 2012). However, recent clinical trials (Boden et al., 2011; Schwartz et al., 2012) and Mendelian randomization studies (Voight et al., 2012) have failed to show clinical benefits of HDL cholesterol elevation, calling into question the role of HDL cholesterol as a surrogate marker of protection from atherosclerosis (Rader and Tall, 2012). Both proponents and critics alike of the “HDL hypothesis” agree on one thing: further studies are needed to understand the mechanism regulating the fundamental process of HDL-driven reverse cholesterol transport (RCT). The prevailing model for RCT is that cholesterol from the artery wall is delivered to the liver via HDL, from where it is then secreted into bile before leaving the body through the feces (Dietschy and Turley, 2002; Rader et al., 2009; Rosenson et al., 2012). However, we and others have recently demonstrated that RCT can also proceed through a nonbiliary pathway known as transintestinal cholesterol excretion (TICE), which persists in both the surgical or genetic absence of biliary cholesterol secretion (Temel et al., 2010; Temel and Brown, 2012; Brown et al., 2008; Le May et al., 2013; van der Velde et al., 2007; van der Veen et al., 2009). Under normal physiologic conditions the hepatobiliary route predominates and TICE is a minor pathway, only contributing ~30% of the total cholesterol lost through the feces in mice (Temel and Brown, 2012). However, pharmacologic activation of liver X receptor (LXR) can preferentially stimulate the nonbiliary pathway to where TICE contributes greater than 60% of the total cholesterol lost through the feces (van der Veen et al., 2009). Although mechanisms regulating the classic hepatobiliary pathway of RCT have been well defined (Yu et al., 2002; Graf et al., 2003; Groen et al., 2008; Wiersma...
et al., 2009; Temel et al., 2007), almost no mechanistic information exists for the nonbiliary TICE pathway (Temel and Brown, 2012; Brufau et al., 2011).

We have previously described several mouse models where the nonbiliary TICE pathway is either chronically (Temel et al., 2010) or acutely stimulated (Marshall et al., 2014). Here, we have taken advantage of these mouse models as hypothesis-generating tools. To identify regulators of TICE and RCT, we performed transcriptional profiling in NPC1L1-liver-transgenic mice (which exhibit chronic TICE stimulation; Temel et al., 2010) and second generation acyl-coenzyme A: cholesterol acyltransferase 2 (ACAT2) antisense oligonucleotide (ASO)-treated mice (which exhibit acute TICE stimulation; Marshall et al., 2014). From this screening, we found that the hepatic expression of flavin-containing monoxygenase 3 (FMO3) was coordinately downregulated in mouse models of stimulated TICE. In parallel, independent studies have shown that plasma levels of FMO3’s product trimethylamine-N-oxide (TMAO) are highly predictive of atherosclerosis in humans, and TMAO is proatherogenic in mice (Wang et al., 2011; Wang et al., 2014; Tang et al., 2013; Koeth et al., 2013; Bennett et al., 2013; Brown and Hazen, 2014). Additionally, we have recently shown that dietary supplementation with the FMO3 product TMAO inhibits macrophage RCT in vivo (Koeth et al., 2013). Here we follow up on these collective observations, and demonstrate that FMO3 knockdown stimulates non-biliary macrophage RCT and reorganizes whole body cholesterol balance. The current studies have also uncovered a previously unknown role for FMO3 in regulating hepatic LXR signaling, which controls endoplasmic reticulum (ER) stress and inflammation. Collectively, these observations identify the gut microbiota-driven TMA/FMO3/TMAO pathway as a key integrator of lipid metabolism and immune cell function and specifically identify FMO3 as a key regulator of sterol balance and RCT, independent of its role in TMAO production. Given that this pathway has now been identified in two separate screens (work described here and metabolomics screening by Wang et al., 2011), we believe this pathway represents a central regulatory node for CVD pathogenesis.

RESULTS

Transcriptional Profiling Identifies FMO3 as a Regulator of RCT

To identify potential regulators of macrophage RCT, we performed microarray analysis in liver from two independent mouse models where TICE was either chronically (NPC1L1-liver-transgenic mice; Temel et al., 2010) or acutely (ACAT2 ASO treatment; Marshall et al., 2014) stimulated. With a stringent threshold (p < 0.001) set for differentially expressed genes (DEGs), there were less than 100 DEGs within each array data set (Figures 1A and 1B), and the vast majority of DEGs were not coordinately regulated in both cohorts (Tables S1, S2, S3, and S4). In fact, there were only two genes that were coordinately regulated in both models of TICE augmentation (Figures 1C and 1D). The sole gene that was upregulated in both acute and chronic TICE mouse models was Gm10567, which is predicted to be noncoding RNA of unknown biological function (Figure 1C). The only gene that was coordinately downregulated in both TICE models was FMO3 (Figure 1D; Tables S1 and S2). We subsequently utilized real-time PCR to confirm that FMO3 mRNA expression was significantly reduced in both acute (ACAT2 ASO-treated) and chronic (NPC1L1-liver-transgenic mice) TICE models (Figures 1E and 1F). Interestingly, FMO3 mRNA expression closely reflects the concentration of cholesterol in bile, given that FMO3 expression is significantly reduced in both NPC1L1-liver transgenic mice and in mice with diminished expression of the biliary cholesterol half transporter ABCG8 (data not shown). Given that the FMO3 enzymatic product TMAO has recently been associated with increased CVD risk in humans and shown to both promote atherosclerosis and inhibit RCT in mice (Wang et al., 2011; Tang et al., 2013; Koeth et al., 2013; Bennett et al., 2013; Brown and Hazen, 2014), we decided to further interrogate the primary role of FMO3 in cholesterol balance and RCT.

ASO-Mediated Knockdown of FMO3 Reorganizes Whole-Body Cholesterol Balance

To examine the role of FMO3 in cholesterol balance, we utilized second-generation ASO targeting (Crooke, 1997) in mice fed different levels of dietary cholesterol. FMO3 ASO treatment very effectively reduced hepatic FMO3 mRNA (Figure 2A) and protein (Figure 2B) expression, when compared to mice treated with a nontargeting control ASO. As expected, FMO3 knockdown significantly increased circulating levels of FMO3’s substrate trimethylamine (TMA) (Figure 2C), while it reciprocally decreased circulating levels of FMO3’s product TMAO (Figure 2D). In parallel, FMO3 knockdown resulted in significant elevations of TMA and reciprocal decreases in TMAO in the liver (data not shown). FMO3 knockdown did not alter the overall health or growth of mice during the period of investigation (data not shown), but it did result in a striking reorganization of cholesterol balance. First, FMO3 knockdown significantly reduced intestinal cholesterol absorption in mice fed a low-cholesterol diet, and it more modestly decreased cholesterol absorption in mice fed a high-cholesterol diet (Figure 2E). Corresponding trends were seen in the mass amount of cholesterol lost in feces, where FMO3 knockdown significantly increased fecal neutral sterol loss in mice fed a low-cholesterol diet, but the trend for increased fecal sterol loss failed to reach significance in FMO3 ASO-treated mice fed a high-cholesterol diet (Figure 2F). In both dietary conditions, FMO3 knockdown significantly reduced hepatic cholesteryl ester levels (Figure 2G). However, hepatic free cholesterol levels were not altered by diet or ASO treatment (Figure 2H). FMO3 knockdown also reduced the biliary secretion of cholesterol (Figures 2I, S1A, and S1B) and phospholipids (Figures S1E and S1F), without dramatically altering biliary bile acid levels (Figures S1C and S1D). FMO3 ASO-driven reductions in biliary phospholipid were accompanied by significant reductions in the hepatic expression of mdr2 (P-glycoprotein) (data not shown). Finally, FMO3 knockdown did not dramatically alter plasma lipid levels in low-cholesterol-fed mice, but it did cause a modest shift in plasma cholesterol distribution in high-cholesterol-fed mice (Figures 2J–2P). In mice fed a high-cholesterol diet, FMO3 knockdown significantly reduced very low-density lipoprotein (VLDL) cholesterol levels (Figures 2L and 2M) while causing an elevation
Figure 1. Transcriptional Profiling Defines FMO3 as a Regulator of RCT

(A) Microarray analysis in the acute TICE mouse model. Female C57BL/6 mice were treated with a nontargeting control ASO (Con. ASO) or an ASO targeting ACAT2 mRNA (A2 ASO) as previously described for 1 week (Marshall et al., 2014). Differentially expressed genes (DEGs) are shown as a heatmap (all genes shown have p < 0.001, n = 4).

(B) Microarray analysis in chronic TICE mouse model. Female wild-type (WT) or NPC1L1-liver transgenic mice (NPC1L1Tg; Temel et al., 2010) were maintained on a high-cholesterol diet (0.2%, w/w) for 8 weeks. Differentially expressed genes (DEGs) are shown as a heatmap (all genes shown have p < 0.001, n = 5).

(legend continued on next page)
of low-density lipoprotein (LDL) cholesterol levels (Figures 2L and 2N). In contrast, FMO3 knockdown did not alter levels of HDL cholesterol (Figure 2O) or plasma triglycerides (TG) (Figure 2P). Detergent block experiments demonstrated that FMO3 knockdown does not alter VLDL-TG secretion but does blunt VLDL-CE secretion significantly (data not shown). Finally, FMO3 knockdown resulted in a significant reduction of the total bile acid pool size (Figure S2A), which is likely driven by diminished expression of the major bile acid synthetic enzymes Cyp7a1 and Cyp8b1 (Figures S2B and S2C). Collectively, these results show that FMO3 knockdown reorganizes cholesterol balance in a diet-specific manner, indicating a mechanistic link between FMO3 and cholesterol and bile acid metabolism.

FMO3 Coordinates Sterol-Sensing Transcription Factor Activation in the Liver

Cellular cholesterol levels are carefully sensed and regulated by at least two major transcriptional mechanisms involving sterol regulatory element-binding proteins (SREBPs) and liver X receptors (LXRs) (Tontonoz and Mangelsdorf, 2003; Brown and Goldstein, 1999). Interestingly, FMO3 knockdown results in reciprocal regulation of the SREBP and LXR pathways in mouse liver (Figure 3) in a diet-specific manner. In mice fed a low cholesterol diet, where endogenous cholesterol synthesis rates are high, FMO3 knockdown caused significant elevation in SREBP2 target genes including 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Figure 3J), HMG-CoA synthase (Figure 3K), and squalene synthase (Figure 3L). In contrast, in mice fed a high-cholesterol diet, where the liver is burdened with excess cholesterol, FMO3 knockdown caused a marked reduction in LXR target genes including ATP binding cassette transporters G5 (Figure 3A) and G8 (Figure 3B), lysophosphatidylcholine acyltransferase 3 (LPCAT3) (Figure 3D), SREBP1c (Figure 3F), fatty acid synthase (Figure 3G), and stearoyl-CoA desaturase 1 (Figure 3H). However, LXR alpha and ATP binding cassette transporter A1 (ABCA1) mRNA expression was not altered by FMO3 knockdown (Figures 3C and 3E). FMO3 ASO-driven alteration in LXR signaling can likely be explained by reduced expression of oxysterol synthetic enzymes Cyp7a1 and Cyp46a1 (data not shown), resulting in decreased availability of endogenous oxysterol ligands in the liver (Figures 3M–3R). Interestingly, circulating oxysterol levels were not similarly altered by FMO3 knockdown, with the exception of the minor oxysterol 25-hydroxycholesterol being slightly elevated (data not shown). In agreement with dampened LXR signaling, FMO3 knockdown strikingly decreased the expression of LXR target genes involved in de novo lipogenesis, and it blunted hepatic steatosis (data not shown). Collectively, these data suggest that FMO3 knockdown depletes hepatic cholesterol stores to a level where SREBP2-driven transcription is activated (Figures 3I–3L) and LXR signaling is reciprocally repressed (Figures 3A–3H) due to diminished oxysterol abundance (Figures 3M–3R).

FM03 Knockdown Stimulates Nonbiliary Macrophage RCT

Given that FMO3 was coordinately downregulated in both acute and chronic models of TICE (Figure 1), and because prior studies showed TMAO-fed mice had significant reductions in macrophage RCT (Koeth et al., 2013), we wondered whether FMO3 might be linked mechanistically to biliary or nonbiliary RCT. In our macrophage RCT studies, a subset of mice were treated with the LXR agonist T0901317, since it is known that LXR activation promotes macrophage RCT primarily by augmenting the nonbiliary TICE pathway (van der Veen et al., 2009). FMO3 knockdown very modestly decreased total plasma cholesterol levels (Figure 4A). When treated with the LXR agonist T0901317, both control ASO- and FMO3 ASO-treated mice exhibited mild hypercholesterolemia, with the majority of the cholesterol elevation seen in large HDL particles (data not shown), as has been previously described (Grefhorst et al., 2002). Following [3H]-cholesterol-labeled macrophage injection into the peritoneal cavity, the plasma [3H]-cholesterol accumulation was significantly lower in FMO3-inhibited mice, both in the vehicle- and T0901317-treated groups (Figure 4B). This reduction in [3H]-cholesterol was largely due to a decrease in large HDL particles (Figure 4C), which tracked closely with the cholesterol mass distribution (data not shown). Importantly, in control ASO-treated mice, there was an expected increase in LXR agonist-inducible biliary [3H]-cholesterol secretion (Figure 4G). In contrast, FMO3 ASO-treated mice had lower biliary [3H]-cholesterol recovery, both in the vehicle- and T0901317-treated groups, and lacked the expected LXR agonist-inducible response for biliary cholesterol secretion (Figure 4G). Interestingly, FMO3 ASO-treated mice also lack T0901317-induced biliary [3H]-bile acid secretion (Figure 4H). Despite having reduced levels of biliary [3H]-cholesterol (Figure 4G), FMO3 knockdown mice had increased basal and LXR agonist-stimulated disposal of cholesterol into the feces, as measured by both [3H]-cholesterol recovery (Figure 4D) and mass fecal neutral sterol loss (Figure 4F). FMO3 knockdown also modestly increased fecal [3H]-bile acid recovery in both basal and LXRxstimulated conditions (Figure 4E). At the time of necropsy, the hepatic recovery of macrophage-derived [3H]-cholesterol in the form of [3H]-cholesterol (Figure 4I) and [3H]-bile acid (Figure 4J) was not altered by FMO3 knockdown. Likewise, intestinal recovery of macrophage derived [3H]-cholesterol in the form of [3H]-cholesterol (Figure 4K) and [3H]-bile acid (Figure 4L) was not altered by FMO3 knockdown, but there was a slight increase in intestinal [3H]-cholesterol in mice treated with T0901317 (Figure 4K). Interestingly, LXR activation significantly represses hepatic FMO3 mRNA (Figure 4M) and protein (Figure 4N). However, FMO3 knockdown did not dramatically alter canonical LXR-driven target gene expression in the liver and small intestine in these mice maintained on a low-cholesterol diet (data not shown). Of particular relevance to RCT, FMO3 knockdown significantly reduced intestinal NPC1L1 expression (Figure 4O),
Figure 2. ASO-Mediated Knockdown of FMO3 Reorganizes Whole-Body Cholesterol Balance

Female C57BL/6 mice were fed either a low- (0.02%, w/w) or high-cholesterol (0.2%, w/w) diet and treated with either a control (nontargeting) ASO or an ASO targeting FMO3 mRNA for 6 weeks.

(A) qPCR quantification of hepatic FMO3 mRNA levels.
(B) Western blot determination of FMO3 protein levels.
(C) Circulating levels of the FMO3 substrate trimethylamine (TMA).
(D) Circulating levels of the FMO3 product trimethylamine-N-oxide (TMAO).
(E) Fractional cholesterol absorption was determined using the dual fecal isotope method.
(F) Fecal neutral sterol excretion was determined by gas liquid chromatography.
(G) Hepatic cholesteryl ester (CE) levels.
(H) Hepatic free cholesterol (FC) levels.
(I) Biliary cholesterol secretion rate was measured following common bile duct cannulation.
(J) Total plasma cholesterol (TPC) levels.
(K) Cholesterol distribution of pooled plasma in mice fed a low-cholesterol diet (n = 5 per pool).
(L) Cholesterol distribution of pooled plasma in mice fed a high-cholesterol diet (n = 5 per pool).

(legend continued on next page)
which is in agreement with alterations in intestinal cholesterol absorption (Figure 2E). Collectively, these results demonstrate that FMO3 knockdown promotes both basal and LXR agonist-stimulated macrophage RCT (Figure 4D and 4F) while repressing biliary cholesterol levels (Figures 4G, S1A, and S1B) and intestinal cholesterol absorption (Figure 2E).

**FMO3 Knockdown Promotes Hepatic ER Stress and Inflammation by Dampening LXR Activation**

LXR activation has previously been shown to dampen both inflammatory responses and ER stress by upregulating direct target genes, such as LPCAT3 (Rong et al., 2013), and transrepressing proinflammatory genes, such as iNOS and COX-2 (Joseph et al., 2003). Given that oxysterol ligand availability is lower in FMO3 knockout mice, we hypothesized that this may promote hepatic inflammation and ER stress. Indeed, FMO3 knockdown caused marked infiltration of macrophages into the liver (Figures 5A and 5B) and increased the expression of macrophage-derived proinflammatory cytokines and chemokines (Figures 5C and 5D). Furthermore, FMO3 knockdown increased the expression of several genes linked to ER stress (ATF3, CHOP) (Figures 5C and 5D) and increased CHOP protein expression (Figure 5E). FMO3 knockdown also increased the activation of c-Src (Figure 5E), which has also recently been linked to saturated fatty acid-induced ER stress and inflammatory signaling (Holzer et al., 2011). Importantly, either providing endogenous LXR agonists (i.e., feeding 0.2% cholesterol in the diet) or providing an exogenous LXR agonist (T0901317) blunted FMO3 ASO-driven hepatic inflammation (Figures 5B–5D), c-Src activation (Figure 5E), and ER stress (Figures 5C–5E). Collectively, these results suggest that FMO3 knockdown promotes hepatic inflammation and ER stress in part by diminishing LXR activity. Collectively, our results are consistent with hepatic FMO3 serving as a critical determinant of the well-known ability of LXR to reciprocally regulate lipid metabolism and inflammation (Joseph et al., 2003; Rong et al., 2013).

**FMO3 Gain-of-Function Reciprocally Reorganizes Cholesterol Balance and Hepatic Inflammation**

To confirm the specificity of our results with ASO-mediated knockdown of FMO3, we performed parallel gain-of-function experiments by means of adenoviral-mediated gene delivery (Figure S3). Administration of FMO3 adenovirus resulted in an 18-fold increase in hepatic FMO3 mRNA levels (Figure S3A). Hepatic overexpression of FMO3 resulted in modest but significant reductions in fecal neutral sterol loss (Figure S3B) while more substantially increasing biliary cholesterol loss (Figure S3C). Furthermore, FMO3 overexpression resulted in a ~20% increase in total plasma cholesterol levels (Figure S3D). In further agreement with loss-of-function data, FMO3 overexpression significantly reduced the hepatic expression of genes involved in inflammation (CD68, F4/80) and ER stress (ATF3) (Figures S3E–S3G). FMO3 overexpression also increased expression of the LXR target genes ABCA1 (Figure S3H) and LPCAT3 (Figure S3I). These data further support the notion that FMO3 is a central regulator of cholesterol balance and hepatic inflammatory responses, given that both gain and loss of function impacts the same biochemical pathways.

**FMO3 Knockdown Regulates Intestinal Cholesterol Balance in a Gut Microbe-Dependent Fashion**

FMO3 has many potential endogenous and xenobiotic substrates (Cashman and Zhang, 2006; Cashman 2008) that could be involved in its ability to regulate cholesterol balance and inflammation. However, we know that the FMO3 substrate TMA and product TMAO are generated solely from gut microbe-dependent metabolism of dietary methylamine nutrients (Wang et al., 2011; Koeth et al., 2013). To determine the involvement of gut microbial metabolites in the ability of FMO3 ASOs to reorganize cholesterol balance and hepatic inflammation, we suppressed gut microbial communities using a poorly absorbed cocktail of antibiotics. After only 1 week of treatment, levels of both the FMO3 substrate TMA and its product TMAO were barely detectable in antibiotic treated mice (Figures S4A and S4B). Interestingly, antibiotic-mediated suppression of gut microbes completely blocked the ability of FMO3 ASO treatment to reduce intestinal cholesterol absorption (Figure S4C) and elevate fecal neutral sterol loss (Figure S4D). However, antibiotic treatment did not significantly alter the activity of FMO3 ASO treatment to increase hepatic CD68 expression (Figure S4E), decrease hepatic FAS expression (Figure S4G), or decrease hepatic Cyp8b1 expression (Figure S4H). Collectively, these results suggest that the ability of FMO3 ASO treatment to alter intestinal cholesterol absorption and fecal neutral sterol excretion likely relies on gut microbial metabolites, of which TMA is a strong candidate, but FMO3 ASO-driven effects on hepatic inflammation, fatty acid synthesis, and bile acid synthesis likely arise from another non-microbial source.

**FMO3 Knockdown Regulates Intestinal Cholesterol Absorption**

As expected, FMO3 knockdown reduced circulating TMAO concentrations in FMO3-inhibited mice (Figure S4I), likely arising from bacterial conversion of TMAO to TMA (Ansaldi et al., 2007).

(M) Very low-density lipoprotein cholesterol (VLDLc) levels.
(N) Low-density lipoprotein cholesterol (LDLc) levels.
(O) High-density lipoprotein cholesterol (HDLc) levels.
(P) Total plasma triglyceride (TG) levels.
All data represent the mean ± SEM from five to ten mice per group. *Significantly different from the control ASO group within each diet group (p < 0.05).
Figure 3. FMO3 Coordinates Sterol-Sensing Transcription Factor Activation in the Liver

Female C57BL/6 mice were fed either a low- (0.02%, w/w) or high-cholesterol (0.2%, w/w) diet and treated with either a control (nontargeting) ASO or an ASO targeting FMO3 mRNA for 6 weeks.

(A–H) Expression of LXR and downstream target genes in the liver.

(I–L) Expression of SREBP2 and downstream target genes in the liver.

(M–R) Hepatic levels of oxysterols

All data represent the mean ± SEM from four to five mice per group. *Significantly different from the control ASO group within each diet group (p < 0.05).
coupled with diminished hepatic conversion of TMA into TMAO by FMO3. Despite the ability of dietary TMAO to rescue circulating plasma TMAO levels in FMO3 ASO-treated mice (Figure S4J), this did not alter the ability of FMO3 ASO treatment to alter cholesterol balance (Figures S4K and S4L) or hepatic gene expression (Figures S4M–S4P). Interestingly, in this TMAO add-back experiment, the levels of circulating TMA are positively correlated ($R^2 = 0.45$, $p < 0.001$) with the amount of fecal neutral sterol loss (data not shown), indicating a potential role for TMA as a gut microbe-derived signal regulating cholesterol balance. Collectively, these data strongly suggest that although chronic elevation of TMAO can be proatherogenic (Wang et al., 2011, Koeth et al., 2013), TMAO is not likely involved in the ability of FMO3 inhibitors to reorganize cholesterol balance and hepatic inflammation.

**DISCUSSION**

Now identified in two independent screens of altered RCT or CVD risk (Figure 1; Wang et al., 2011), the gut microbe-driven TMA/FMO3/TMAO pathway is increasingly being pursued for the treatment or prevention of CVD. However, this pathway exhibits many levels of complexity including dietary inputs (Wang et al., 2011; Koeth et al., 2013), requirement of bacterial metabolism (Brown and Hazen, 2014), and complex hormonal regulation of the FMO enzyme family (Bennett et al., 2013).

Furthermore, TMAO feeding (Wang et al., 2011; Koeth et al., 2013) and FMO3 knockdown (data shown here) exert some consistent but many divergent effects on cholesterol and bile acid metabolism across multiple tissues (Figure S5). Although FMO3 and TMAO are directly biochemically connected, our studies suggest that the FMO3 enzyme and the TMAO product likely impact lipid metabolism and inflammation through distinct mechanisms. Therefore it will become increasingly important to differentiate the ability of FMO3 itself versus TMAO to alter the pathogenesis of CVD. The major findings of the current study include the following: (1) transcriptional profiling links FMO3 to RCT, (2) FMO3 knockdown reorganizes multiple processes determining cholesterol balance, (3) FMO3 is a negative regulator of nonbiliary reverse cholesterol transport, (4) FMO3 regulates LXR activity to impact hepatic inflammation and ER stress, (5) FMO3 gain of function reciprocally reorganizes intestinal cholesterol balance and dampens hepatic inflammation, (6) the reorganization of cholesterol balance seen with FMO3 knockdown does not involve TMAO yet can be blocked by antibiotic-mediated suppression of gut microbes, and (7) the hepatic inflammation seen with FMO3 knockdown does not likely involve either TMA or TMAO because it is not suppressed by antibiotics or dietary TMAO. Collectively, these observations identify transcriptional control of FMO3 as an important regulatory switch by which cholesterol balance and hepatic inflammatory responses are integrated.

Given that normalization of circulating TMAO levels in FMO3-inhibited mice does not rescue the abnormal lipid and inflammatory phenotype, it is likely that other FMO3 substrates or products are involved. An obvious candidate would be the accumulation of the FMO3 substrate TMA in FMO3 ASO-treated mice. Two pieces of evidence support that TMA may be involved in the ability of FMO3 to regulate cholesterol balance: (1) antibiotic treatment, a condition that causes disappearance of circulating TMA, blocks the ability of FMO3 ASO treatment to blunt intestinal cholesterol absorption and increase fecal cholesterol loss (Figures S4C and S4D); and (2) circulating TMA levels significantly correlate with the level of fecal neutral sterol loss (data not shown). Although these data suggest a potential role for TMA in regulating cholesterol balance, there is no similar support for TMA being involved in the hepatic inflammation seen in FMO3-inhibited mice given that antibiotic treatment does not reverse FMO3 ASO-driven hepatic inflammation. Furthermore, the ability of FMO3 knockdown to suppress genes involved in hepatic lipogenesis (FAS, ACC1, SCD1) does not involve the TMA/TMAO axis, given that antibiotic treatment or dietary TMAO supplementation do not reverse FMO3 ASO-driven repression of these genes (Figure S4). Collectively, these data suggest that the ability of FMO3 inhibitors to alter cholesterol balance, inflammation, and ER stress likely involves several molecular mechanisms including gut microbial metabolism (alterations in cholesterol absorption and fecal neutral sterol loss) and gut microbe-independent mechanisms (alterations in hepatic inflammation, ER stress, and lipogenesis). Therefore, it will become increasingly important to identify and consider all potential substrates and products (both natural products and xenobiotics) of FMO3 that have the potential to reorganize these diverse phenotypes. Identification of such FMO3-regulated substrates and products could create untapped therapeutic opportunities for lipid- or inflammation-driven diseases.

Our discovery of FMO3 as a coordinately downregulated gene in acute and chronic TICE models (Figure 1) lead us to hypothesize that FMO3 may be a key integrator of biliary and nonbiliary RCT. To our surprise, FMO3 knockdown actually phenocopies our chronic TICE mouse model (NPC1L1-liver-transgenic mice), which displays severely reduced biliary cholesterol levels with normal fecal cholesterol loss (Temel et al., 2010). Much like NPC1L1-liver-transgenic mice (Temel et al., 2010), FMO3-inhibited mice seem to preferentially utilize the nonbiliary TICE pathway for fecal disposal of cholesterol (Figure 4). In particular, FMO3 ASO treatment strongly enhances LXR agonist-driven macrophage RCT into the feces (Figure 4D) while at the same time preventing LXR agonist-driven increases in biliary cholesterol (Figure 4G). This observation is important because LXR agonists preferentially stimulate the nonbiliary TICE pathway (van der Veen et al., 2009), further supporting FMO3 as a negative regulator of TICE. It is interesting to note that treatment with the LXR agonist T0901317 in control ASO-treated mice significantly reduces hepatic FMO3 mRNA (Figure 4M) and FMO3 protein (Figure 4N). These data demonstrate that now in three independent conditions where TICE is stimulated (van der Veen et al., 2009; Temel et al., 2010; Marshall et al., 2014), FMO3 is transcriptionally repressed (Figures 1, 4M, and 4N). Collectively, these findings demonstrate that FMO3 gene expression is coordinately repressed in several mouse models of augmented TICE and that FMO3 knockdown promotes basal and LXR agonist-stimulated nonbiliary RCT (Figure 4). However, it is important to point out that since multiple steps of cholesterol balance are altered by FMO3 knockdown, it is difficult to interpret which step predominates to promote RCT and whether the
reorganization of cholesterol balance seen in FMO3 knockdown mice is derived simply from alterations in TICE. For instance, by inhibiting cholesterol absorption (Figure 2E), FMO3 knockdown could indirectly impact both biliary and nonbiliary macrophage RCT rates (Sehayek and Hazen, 2008; Jakulj et al., 2010). Therefore, although FMO3 knockdown does clearly reorganize the biliary and nonbiliary RCT pathways toward TICE predominance, it also alters multiple steps of forward and reverse cholesterol transport, culminating in a net-negative cholesterol balance. It has long been known that a complex reciprocal relationship exists between many lipid metabolic and inflammatory pathways. This antagonism, collectively known as transrepression, is orchestrated by crosstalk between several nuclear hormone receptors (GR, PPARα, PPARγ, PPARδ, LXRα, NURR1, etc.)

Figure 4. FMO3 Knockdown Stimulates Nonbiliary Macrophage RCT
Female C57BL/6 mice were fed a low-cholesterol (0.02%, w/w) diet and treated with either a control (nontargeting) ASO or an ASO targeting FMO3 mRNA for 6 weeks. During the last week of treatment, mice were also orally gavaged with either a vehicle or an exogenous LXR agonist (T0901317) as described in the macrophage RCT experiments in Experimental Procedures.

(A) Total plasma cholesterol (TPC) levels.
(B) Time course of [3H]cholesterol accumulation in plasma.
(C) [3H]Cholesterol distribution of pooled plasma (n = 5 per pool).
(D) [3H]Cholesterol recovery in the feces.
(E) Mass fecal neutral sterol excretion.
(F) [3H]Bile acids recovery in gall bladder bile.
(G) [3H]Bile acids recovery in gall bladder bile.
(H) [3H]Cholesterol recovery in the liver.
(I) Mass fecal neutral sterol excretion.
(J) [3H]Bile acids recovery in the liver.
(K) [3H]Bile acids recovery in the small intestine (SI) wall.
(L) [3H]Bile acids recovery in the small intestine (SI) wall.
(M) qPCR quantification of hepatic FMO3 mRNA levels.
(N) Western blot analysis of hepatic FMO3 protein levels.
(O) qPCR quantification of NPC1L1 mRNA levels in the duodenum.

All data represent the mean ± SEM from six to ten mice per group, and means not sharing a common superscript differ significantly (p < 0.05).

Figure 5. FMO3 Knockdown Promotes Hepatic Inflammation and ER Stress by Dampening LXR Activation
For dietary cholesterol-induced LXR activation (A–C), female C57BL/6 mice were fed either a low- (0.02%, w/w) or high-cholesterol (0.2%, w/w) diet and treated with either a control (nontargeting) ASO or an ASO targeting knockdown of FMO3 for 6 weeks. For exogenous ligand-induced LXR activation (D and E), female C57BL/6 mice were fed a low-cholesterol (0.02%, w/w) diet and treated with either a control (nontargeting) ASO or an ASO targeting FMO3 mRNA for 6 weeks. During the last week of treatment, mice also were orally gavaged with either a vehicle or an exogenous LXR agonist (T0901317) as described in the macrophage RCT experiments in Experimental Procedures.

(A) H&E-stained liver sections (200× magnification) from female C57BL/6 mice fed a low-cholesterol (0.02%, w/w) diet and treated with either a control ASO or an FMO3 ASO for 6 weeks. Arrows indicate areas of localized immune cell infiltration.
(B) Hepatic lobular inflammation score from pathological report. Data represent the mean ± SEM from four mice per group; *significantly different from the control ASO group within each diet group (p < 0.05).
(C) qPCR quantification of genes involved in inflammation and ER stress in the liver of mice fed a low-cholesterol (L) or high-cholesterol (H) diet.
(D) qPCR quantification of genes involved in inflammation and ER stress in the liver of vehicle (V) or T0901317-treated (T) mice.
(E) Western blot analysis of FMO3, CHOP, total Src, and activated Src (p-Src promotes Src; n = 3 individual animals shown per group. Data represent the mean ± SEM from four mice per group, and means not sharing a common superscript differ significantly (p < 0.05). *Significantly different from the control ASO group within each diet group (p < 0.05).
and the proinflammatory master transcription factor NF-κB (Glass and Sajo, 2010; Joseph et al., 2003; Khovidhunkit et al., 2003). Activation of diverse nuclear hormone receptors (GR, PPARα, PPARγ, LXRα, NURR1, etc.) can transrepress T cell- and macrophage-driven inflammatory responses orchestrated by NF-κB, while in a reciprocal manner, activation of NF-κB-driven proinflammatory signaling effectively blunts nuclear hormone receptor signaling (Glass and Sajo, 2010; Joseph et al., 2003; Khovidhunkit et al., 2003). Relevant to our work, LXR activation can strongly suppress NF-κB-driven cytokine and chemokine responses to a multitude of inflammatory stimuli (Joseph et al., 2003; Glass and Sajo, 2010). This transrepression pathway likely underlies the hepatic inflammation and ER stress seen with FMO3 knockdown (Figure 5). Recently, it was demonstrated that a direct transcriptional target of LXR (LPCAT3) mediates a large part of this transrepressive pathway (Rong et al., 2013). Here, we show that FMO3 activity is a major determinant of both LXR activation (Figure 3) and downstream anti-inflammatory responses in the liver. Our data suggest that the TMA/FMO3/TMAO pathway is a previously underappreciated regulator of LXR activity, which has broad implications in sterol balance and inflammatory processes.

Given that the link between the metaorganismal TMAO pathway and CVD has only been established in the last 3 years, many additional studies are required to gain insights into where therapeutic interventions should be targeted. The studies described here, along with previous reports linking TMAO to CVD risk in humans (Wang et al., 2011, 2014; Tang et al., 2013; Koeth et al., 2013), provide compelling evidence that the TMA/FMO3/TMAO pathway is the central regulatory pathway that deserves further exploration. However, the ability of TMAO to promote atherosclerosis (Wang et al., 2011, 2014; Tang et al., 2013; Koeth et al., 2013) may be mutually exclusive from the ability of FMO3 inhibitors to reorganize cholesterol balance and hepatic inflammation. Our studies highlight the necessity to understand the repertoire of substrates that can be utilized by FMO3 and also open the possibility that FMO3 may have regulatory functions distinct from its enzymatic activity. Given that FMO3 knockdown and gain of function reciprocally reorganize cholesterol balance, inflammation, and ER stress, FMO3 is uniquely positioned among the FMO family of enzymes to impact human disease. However, further studies are warranted to determine whether this pathway can be exploited pharmacologically in lipid- or inflammatory-driven disease. In particular identification of FMO3 substrates and products that play a primary role in promoting nonbiliary macrophage RCT, without increasing hepatic inflammation, would be an attractive strategy for innovative cholesterol lowering drugs in the poststatin era. Advancement in our understanding of the enzymology of FMO3 is thus not only predicted to be informative in xenobiotic toxicology studies but also may provide insights into therapeutic strategies for the treatment or prevention of atherosclerosis.

**EXPERIMENTAL PROCEDURES**

**Animal Studies**

The acute and chronic TICE mouse models used for screening purposes have been previously described (Marshall et al., 2014; Temel et al., 2010). For ASO-mediated knockdown of FMO3, female C57BL/6 mice were maintained on either low- (0.02%) or high-cholesterol (0.2%) diets for a period of 6 weeks and simultaneously injected with control (nontargeting) or FMO3-targeting ASOs biweekly (25 mg/kg body weight) as previously described (Marshall et al., 2014). Macrophage RCT experiments were done as previously described (Temel et al., 2010). All methods used for lipid biochemistry (quantification of cholesterol, oxysterols, bile acids, and phospholipids) have been previously described (Marshall et al., 2014; Koeth et al., 2013; Thomas et al., 2014). All mice were maintained in an American Association for Accreditation of Laboratory Animal Care-approved animal facility, and all experimental protocols were approved by the Institutional Animal Care and Use Committee at Wake Forest University School of Medicine and the Cleveland Clinic Lerner Research Institute.

**RNA and Protein Methods**

Tissue RNA extraction, real-time PCR, and microarray analyses were performed as previously described (Thomas et al., 2013; Marshall et al., 2014). Immunoblotting was conducted as previously described (Thomas et al., 2013). A detailed description of RNA and protein methods is available in Supplemental Experimental Procedures.

**Statistical Analysis**

Most data are expressed as the mean ± SEM and were analyzed using either a one-way or a two-way ANOVA followed by Student’s t tests for post hoc analysis using JMP version 5.0.12 software (SAS Institute). For microarray analysis, we used empirical Bayes method implemented in R package limma (Smyth 2004).

**ACCESSION NUMBERS**

The GEO accession number for the data reported in this paper is GSE64326.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.12.036.

**AUTHOR CONTRIBUTIONS**

J.M.B. planned the project, designed experiments, analyzed data, and wrote the manuscript; J.M.B., R.E.T., and S.L.H. designed experiments and provided useful discussion directing the project; M.W., D.M.S., A.C.B., D.F., A.D.G., A.L.B., S.M., A.M., R.C.S., Z.W., J.S., and J.C. conducted mouse experiments, analyzed data, and aided in manuscript preparation; R.G.L., R.M.C., and M.J.G. provided antisense oligonucleotides and valuable discussion; I.M., V.L., C.H., U.T., and R.Z. performed all in vitro enzyme assays and provided valuable discussion; X.L. provided histopathological reports; X.R., T.d.A.V., D.M.S., P.T., and A.J.L. performed adenoviral overexpression studies in mice; Z.W., S.L.H., P.P., P.T.L., D.S.M., and H.A.B. performed mass spectrometric analyses and provided critical insights for these studies; and all authors were involved in the editing of the final manuscript.

**ACKNOWLEDGMENTS**

This work was supported by NIH and Office of Dietary Supplements grants R00 HL096166 (J.M.B.), R01 HL122283 (J.M.B.), R01 HL103866 (S.L.H.), P20 HL113452 (S.L.H.), and U54 GM069338 (H.A.B.). Additional support was provided by American Heart Association grants 14POST18700001 (M.W.) and 14SDG18440015 (T.d.A.V.). Further support was provided by the Cleveland Clinic Foundation General Clinical Research Center of the Cleveland Clinic/Case Western Reserve University CTSA (1UL1RR024989). S.L.H. is also partially supported by a gift from the Leonardi Krieger Fund. The authors thank Stephen Milne for assistance with the glycerophospholipid mass spectrometry analysis. Some of the mass spectrometry studies were performed in the Lerner...
REFERENCES


The TMAO-Generating Enzyme Flavin Monooxygenase 3 Is a Central Regulator of Cholesterol Balance

Supplemental File

Report

The Trimethylamine-N-Oxide Generating Enzyme Flavin Monooxygenase 3 is a Central Regulator of Cholesterol Balance.


Extended Experimental Procedures:

Mice, Diets, and Treatments
Antisense oligonucleotide (ASO)-mediated knockdown was accomplished using 20-mer phosphorothioate ASOs designed to contain 2'-0-methoxyethyl groups at positions 1 to 5 and 15 to 20. All ASOs used in this work were synthesized, screened, and purified as described previously (Crooke et al., 2005) by ISIS Pharmaceuticals, Inc. (Carlsbad, CA). Creation of NPC1L1\(^{-}\)LiverTg mice has been described previously (Temel et al., 2007), and we used the high overexpressing line (L1-Tg112) that had been backcrossed onto the C57BL/6N background for > 6 generations here. For microarray analysis in WT vs. NPC1L1\(^{-}\)LiverTg, female mice were fed a diet containing 0.2% cholesterol (wt/wt) along with receiving a control (non-targeting) ASO for 8 weeks in order to compare to subsequent studies. For microarray analysis in control vs. ACAT2 antisense oligonucleotide (ASO)-treated mice, we purchased female C57BL/6N mice (6-8 weeks old) from Harlan (Indianapolis, IN, USA), and then placed them on a semisynthetic low-fat, high-cholesterol diet (10% of energy as palm oil-enriched fat, 0.2% cholesterol w/w) and maintained on this diet for the remainder of the study. After 6 weeks of high-cholesterol diet feeding, mice were injected intraperitoneally biweekly with 25 mg/kg of either non-targeting control ASO (5’-TCCCATTTCAGGAGACCTGG-3’) or and ASO directed against murine ACAT2 (5’-TTCGGAAATGTTCACCTCC-3’) as previously
described (Marshall et al., 2014). After one week of treatment, mice were sacrificed for subsequent microarray analysis. For FMO3 knockdown studies, young (6-8 week old) female C57BL/6 mice were purchased from Harlan (Indianapolis, IN, USA), and started on either a low (0.02%, wt/wt) or high (0.2%, wt/wt) cholesterol diet and injected intraperitoneally biweekly with 25 mg/kg of either non-targeting control ASO (5'-TCCCATTTCAGGAGACCTGG-3') or and ASO directed against murine FMO3 (5'-TGGAAGCATTTCCTTTAAA-3') for 8 consecutive weeks. For LXR agonist studies, T0901317 was suspended in a vehicle containing 1.0% carboxymethylcellulose (CMC) and 0.1% Tween 80. Mice were gavaged with either vehicle or 25 mg/kg T0901317 once daily for a period of seven days as previously described (Temel et al., 2010). All mice were maintained in an American Association for Accreditation of Laboratory Animal Care-approved pathogen-free animal facility, and all experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic and Wake Forest University School of Medicine.

**Adenoviral Overexpression Studies**

For FMO3 overexpression studies control adenovirus or adenovirus driving expression murine FMO3 were prepared as previously described (Bennett et al., 2013) using the AdEasy system (Agilent). All viruses were purified by cesium chloride gradient ultracentrifugation. For *in vivo* overexpression, 10⁹ plaque-forming units (PFUs) were injected into the tail vein of recipient mice. For FMO3 adenoviral-mediated overexpression studies male C57BL/6 mice were fed a low (0.02%, wt/wt) cholesterol diet for a total of 3 weeks. Following two weeks of dietary induction, mice received a tail vein injection of either a control adenovirus (pAdTrack-CMV) or an adenovirus driving overexpression of murine FMO3 (pAdTrack-CMV-mFMO3). Thereafter, plasma was collected 3 days post adenoviral administration, and tissues and gall bladder bile were collected 7 days post adenoviral administration. Feces were quantitatively collected during days 4-7 post adenoviral administration.

**Antibiotic Treatment Studies**

To examine the role of gut microbial metabolism in FMO3 ASO-mediated alteration in cholesterol balance and hepatic inflammation female C57BL/6 mice were started on a low (0.02%, wt/wt) cholesterol diet at 6-8 weeks of age and injected intraperitoneally with either a control ASO or FMO3 ASO (50 mg/kg body weight per week) for a total of 5
weeks. Following four weeks of dietary induction and ASO treatment, mice either continued to receive normal drinking water or were switched to water supplemented with a poorly absorbed antibiotic cocktail as previously described (Wang et al., 2011) for 7 consecutive days. After only 4 days of antibiotic treatment all mice were gavaged with 50 µl soybean oil containing trace amounts of $^{14}$C-cholesterol and $^3$H-sitosterol and place on wire bottoms for 3 additional days for quantitative fecal collection. After 7 days of antibiotic treatment mice were necropsied for tissue and blood collection.

**Dietary TMAO Addback Studies**

To determine whether adding back the FMO3 product TMAO could rescue FMO3 ASO-mediated alteration in cholesterol balance and hepatic inflammation female C57BL/6 mice were started on a low (0.02%, wt/wt) cholesterol diet at 6-8 weeks of age and injected intraperitoneally with either a control ASO or FMO3 ASO (50 mg/kg body weight per week) for a total of 5 weeks. Following four weeks of dietary induction and ASO treatment, mice either continued to receive the same (0.02%, wt/wt) cholesterol diet or were switched to a diet containing 0.02%, wt/wt cholesterol with supplemental TMAO (0.3% wt/wt) for 7 consecutive days. After only 4 days of diet switch all mice were gavaged with 50 µl soybean oil containing trace amounts of $^{14}$C-cholesterol and $^3$H-sitosterol and place on wire bottoms for 3 additional days for quantitative fecal collection. After 7 days of dietary TMAO addback mice were necropsied for tissue and blood collection.

**Necropsy Conditions**

To keep results consistent all experimental mice were fasted for 4 hours (from 9:00 a.m. to 1:00 p.m.) prior to necropsy. At necropsy, all mice were terminally anesthetized with ketamine/xylazine (100-160mg/kg ketamine-20-32mg/kg xylazine), and a midline laparotomy was performed. Blood was collected by heart puncture. Following blood collection, a whole body perfusion was conducted by puncturing the inferior vena cava and slowly delivering 10 ml of saline into the heart to remove blood from tissues. Tissues were collected and immediately snap frozen in liquid nitrogen for subsequent biochemical analyses.
Plasma Lipid and Lipoprotein Analyses
Plasma triacylglycerol levels were quantified enzymatically (L-Type TG M, Wako Diagnostics, Richmond, VA, USA). Total plasma cholesterol levels were quantified enzymatically (Pointe Scientific, Canton, MI, USA). The distribution of cholesterol across lipoprotein classes was performed by size exclusion chromatography (FPLC using a superose-6 column) coupled with an online enzymatic cholesterol quantification as previously described (Brown et al., 2008a; Brown et al., 2008b; Brown, et al., 2010).

Quantification of Plasma and Liver Trimethylamine and Trimethylamine-N-Oxide
Quantification of TMA and TMAO in mouse plasma was performed using stable isotope dilution HPLC with online electrospray ionization tandem mass spectrometry on an API 365 triple quadrupole mass spectrometer (Applied Biosystems, Foster, CA) with upgraded source (Ionics, Bolton, ON, Canada) interfaced with a Cohesive HPLC (Franklin, MA) equipped with phenyl column (4.6 × 250 mm, 5 µm Rexchrom Phenyl; Regis, Morton Grove, IL), and the separation was performed as reported previously (Wang et al., 2011). TMAO and TMA were monitored in positive MRM MS mode using characteristic precursor-product ion transitions: m/z 76 → 58, and m/z 60 → 44, respectively. The internal standards TMAO-trimethyl-d9 (d9-TMAO) and TMA-d9 (d9-TMA) were added to plasma samples before sample processing and were similarly monitored in MRM mode at m/z 85 → 68, and m/z 69 → 49. Various concentrations of TMAO and TMA standards and a fixed amount of internal standards were spiked into control plasma to prepare the calibration curves for quantification of plasma TMAO and TMA. For d9-TMA and d9-TMAO quantification in FMO activity analyses, 1,1,2,2-d4 choline (Sigma) was used as an internal standard followed by a 0.5 ml 3K cutoff centrifugal filter (Millipore) of sample prior to LC-MS/MS analysis. The characteristic precursor-product ion transition for 1,1,2,2-d4 choline is m/z 108 → 60 monitored in positive MRM MS mode.

Biliary Cannulations and Biliary Lipid Analyses
For biliary cannulation studies, mice were anesthetized with isofluane (4% induction, 2% maintenance), and common bile duct cannulations were performed to collect newly secreted bile for a 15-minute period (Temel et al., 2010; Brown et al., 2008b). Briefly, once fully anesthetized, the peritoneal cavity was opened to the rib cage, and the duodenum was exposed. Through an incision in the duodenum, a cannula (polyethylene
10 tubing; ID 0.28 mm; OD 0.61 mm) filled with sterile saline was inserted through the sphincter of Oddi into the common bile duct. The cannula was tied to the bile duct, and newly secreted bile was allowed to fill the cannula. Following successful surgery, the exposed viscera were wetted with saline and covered with a saline-soaked gauze pad. To maintain physiological body temperature, the mice were placed on a heating pad, and bile was collected for a brief 15-minute period. In separate cohort of mice, gall bladder bile was collected for comparison purposes. For analysis of biliary lipid concentrations, a measured volume (5–10 µl) of gall bladder or newly secreted (collected during common bile duct cannulation) was extracted with 2:1 chloroform/methanol in the presence of 10 µg 5α-cholestanate. The organic phase was analyzed for cholesterol content by gas-liquid chromatography, and for PL content using Phospholipids C enzymatic assay kit (Wako Diagnostics, Richmond, VA, USA) using a detergent solubilization method (Temel et al., 2010; Brown et al., 2008b). The aqueous phase was analyzed for BA content using an enzymatic assay employing hydroxysteroid dehydrogenase (Turley and Dietschy, 1978).

**Intestinal Cholesterol Absorption and Fecal Neutral Sterol Loss Measurements**

Intestinal cholesterol absorption was measured using the dual fecal isotope assay, and fecal neutral sterol loss was measured by gas chromatography as previously described (Temel et al., 2005).

**Bile Acid Pool Size and Composition**

Pool size was determined as the total bile acid content of the small intestine, gallbladder, and liver as previously described (Koeth et al., 2013). Briefly, these tissues were pooled and extracted together in ethanol with nordeoxycholate (Steraloids) added as an internal standard. The extracts were filtered (Whatman paper #2), dried and resuspended in water. The samples were then passed through a C18 column (Sigma Catalog # 52615-U) and eluted with methanol. The eluted samples were again dried down and resuspended in methanol. A portion of this was subjected to HPLC using Waters Symmetry C18 column (4.6 × 250 mm No. WAT054275, Waters Corp., Milford, MA) and a mobile phase consisting of methanol: acetonitrile: water (53:23:24) with 30mM ammonium acetate, pH 4.91, at a flow rate of 0.7 ml/min. Bile acids were detected by evaporative light scattering detector (Alltech ELSD 800, nitrogen at 3 bar, drift tube temperature 40°C) and identified by comparing their respective retention times to those
of valid standards (Taurocholate and Tauro-β-muricholate from Steraloids; Taurodeoxycholate and Taurochenodeoxycholate from Sigma; Tauroursodeoxycholate from Calbiochem). For quantitation, peak areas were integrated using software Chromperfect Spirit (Justice laboratory software) and bile acid pool size was expressed as µmol/100 g body weight (bw) after correcting for procedural losses with nordeoxycholate.

**Immunoblotting**

Whole tissue homogenates were made from tissues in a modified RIPA buffer as previously described (Brown et al., 2004), and protein was quantified using the BCA assay (Pierce). Proteins were separated by 4–12% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and proteins were detected after incubation with specific antibodies as previously described (Brown et al., 2004; Brown et al., 2008a; Brown et al., 2008b; Brown et al., 2010). Antibodies used include: anti-FMO3 rabbit polyclonal (ABCAM #Ab126790), anti-β actin rabbit monoclonal (Cell Signaling Technologies #4970), anti-CHOP mouse monoclonal (Cell Signaling Technologies #2895), anti-Src rabbit monoclonal (Cell Signaling Technologies #2123), and anti-phospho-Src<sup>Tyr416</sup> rabbit monoclonal (Cell Signaling Technologies # 6943).

**Microarray and Quantitative Real-Time PCR Analysis of Hepatic Gene Expression**

Tissue RNA extraction was performed as previously described for all mRNA analyses (Brown et al., 2008a; Brown et al., 2008b; Brown, et al., 2010; Lord et al., 2012). For microarray analyses, hepatic total RNA samples were further purified using RNeasy MinElute Cleanup Kit (Qiagen # 74204) followed by quality assessment using an Agilent 2100 bioanalyzer. Samples with RIN values > 8.0 were carried forward for cRNA synthesis and hybridization to GeneAtlas MG-430 PM Array Strips (Affymetrix, Santa Clara, CA) following the manufacturer’s recommended protocol. Briefly, approximately 250 ng of purified total RNA was reverse transcribed and biotin labeled to produce biotinylated cRNA targets according to the standard Affymetrix GeneAtlas 3’-IVT Express labeling protocol (GeneAtlas 3’ IVT Expression Kit User Manual, P/N 702833 Rev. 4, Affymetrix). Following fragmentation, 6 µg of biotinylated cRNA was hybridized for 16 hr at 45 °C on the Affymetrix GeneAtlas Mouse MG-430 PM Array Strip. Strips were washed and stained using the GeneAtlas Fluidics Station according to standard Affymetrix operating procedures (GeneAtlas™ System User’s Guide (P/N 08-0306
Strips were subsequently scanned using the GeneAtlas Imager system according to the standard Affymetrix protocol. Fluidics Control, Scan control and data collection was performed using the GeneAtlas Instrument Control Software version 1.0.5.267. The raw data generated were normalized using the robust multi-array average (RMA) method (Irizarry et al., 2003), and functional annotation to gene ontology was performed using Ingenuity-IPA software (Ingenuity Systems, Inc., Redwood City, CA). All microarray analyses were performed by the Wake Forest School of Medicine Microarray Shared Resource Core. Quantitative real time PCR (Q-PCR) analyses were conducted as previously described (Brown et al., 2008a; Brown et al., 2008b; Brown, et al., 2010; Lord et al., 2011). mRNA expression levels were calculated based on the ΔΔ-CT method. Q-PCR was conducted using the Applied Biosystems 7500 Real-Time PCR System. Primers used for Q-PCR are available on request.

Hepatic Cholesterol, Triglyceride, and Phospholipid Analyses

Extraction of liver lipids and quantification of molecular species by either mass spectrometric or enzymatic methods was performed as previously described (Lord et al., 2011; Ivanova et al., 2007; Myers et al., 2011). Briefly, total cholesterol (TC), cholesteryl ester (CE), free cholesterol (FC), triglyceride (TG) mass was measured using enzymatic assays as described previously (Temel et al., 2010; Brown et al., 2008a; Brown et al., 2008b). For glycerophospholipid analyses, liver tissue was extracted using a modified Bligh and Dyer procedure (Bligh and Dyer 1959). Approximately 10 mg of frozen mouse liver was homogenized in 800 µl of ice-cold 0.1 N HCl:CH₃OH (1:1) using a tight-fit glass homogenizer (Kimble/Kontes Glass Co, Vineland, NJ) for about 1 min on ice. Suspension was then transferred to cold 1.5 ml Eppendorf tubes and vortexed with 400 µl of cold CHCl₃ for 1 min. The extraction proceeded with centrifugation (5 min, 4°C, 18,000 x g) to separate the two phases. Lower organic layer was collected and solvent evaporated. The resulting lipid film was dissolved in 100 µl of isopropanol:hexane:100 mM NH₄COOH(aq) 58:40:2 (mobile phase A). Quantification of glycerophospholipids was achieved by the use of an LC-MS technique employing synthetic odd-carbon diacyl and lysophospholipid standards. Typically, 200 ng of each odd-carbon standard was added per 10-20 mg tissue. Glycerophospholipids were analyzed on an Applied Biosystems/MDS SCIEX 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) and a Shimadzu high pressure liquid chromatography system with a Phenomenex Luna Silica column (2 × 250
mm, 5-µm particle size) using a gradient elution as previously described (Ivanova et al., 2007; Myers et al. 2011). The identification of the individual species, achieved by LC-MS/MS, was based on their chromatographic and mass spectral characteristics. This analysis allows identification of the two fatty acid moieties but does not determine their position on the glycerol backbone (sn-1 versus sn-2).

**Quantification of Plasma and Liver Oxysterol Levels**

22(R)-hydroxycholesterol, 25-hydroxycholesterol and 25(R),26-hydroxycholesterol (“27”-hydroxycholesterol) were purchased from Steraloids (Newport, RI, USA), 24(S)-hydroxycholesterol and 24(S)-25-epoxycholesterol were purchased from Biomol International (Plymouth Meeting, PA, USA) and the deuterated internal standard (IS) 25-hydroxycholesterol-D₆ was from CDN Isotopes (Point-Claire, Quebec, Canada). Pyridine was from Sigma-Aldrich (St. Louis, MO, USA), N,O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was from Pierce (Rockford, IL), and all other chemicals were from Carlo Erba Reagenti (Rodano, Milan, Italy). Oxysterols from mouse liver and plasma were extracted and purified as described (Lund and Diczfalusy 2003) using Strata Si-1 columns (500 mg/6 ml) (Phenomenex, Torrance, CA, USA) in the solid phase extraction step. Dried extracted samples were converted to trimethylsilyl ethers by adding 250 µl pyridine/BSTFA (1:1) and incubating for 30 minutes at 60°C. After drying under a nitrogen flow, samples were resuspended with 50 µl hexane and analyzed by GC-MS using an Agilent 6890/5973 system equipped with the same column and using the same conditions described (Lund and Diczfalusy 2003). Analyses were performed in selected ion monitoring (SIM) mode using the following target ions: m/z 173 for 22(R)-hydroxycholesterol, m/z 145 for 24(S)-hydroxycholesterol, m/z 131 for 25-hydroxycholesterol, m/z 129 for both 25(R),26-hydroxycholesterol and 24,25-epoxycholesterol, and m/z 137 for the IS (25-hydroxycholesterol-D₆). Different retention times in the column allowed separation of oxysterols with the same target ion.

**J774 Cell Culture and [³H]-Cholesterol Loading for Macrophage RCT Studies**

J774 mouse macrophages were a generous gift from Dr. George Rothblat (The Children’s Hospital of Philadelphia). Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. [³H]-Cholesterol loading was initiated by incubating cells for 48 hours with 5 µCi/ml [³H]-cholesterol and 100 µg/ml acetylated LDL in 10% FBS containing RPMI-
1640. The resulting foam cells were washed twice with phosphate buffered saline and equilibrated for an additional 12-hour period in serum free RPMI-1640 supplemented with 0.2% bovine serum albumin (BSA). Cells were then harvested and resuspended in serum-free RPMI-1640 immediately before injection. An aliquot of cells was extracted using the method of Bligh and Dyer (1959), and lipids were separated by thin layer chromatography (TLC) using a 70:30:1 (hexane:diethyl ether:acetic acid) solvent system. Following TLC separation J774 foam cells were found to have ~62% [3H]-cholesteryl ester and ~38% [3H]-free cholesterol. On average, the cell suspension contained ~10x10^6 cells/ml at ~3x10^6 dpm/ml. All cell suspensions were analyzed microscopically in order to count and to ensure viability before injection, and all mice were injected within 5 minutes of resuspension of freshly isolated foam cells.

**In Vivo Macrophage RCT Studies in FMO3 ASO-Treated Mice**

*In vivo* measurement of macrophage RCT was conducted essentially as we have described previously (Temel et al., 2010), with minor modifications. In studies described here, female C57BL/6 mice were fed a low (0.02%, wt/wt) cholesterol diet and treated with either a control (non-targeting) ASO or an ASO targeting knockdown of FMO3 for 6 weeks. During the last week of treatment, mice also were orally gavaged with either a vehicle or an exogenous LXR agonist (T0901317; 25 mg/kg once daily). 48 hours before necropsy, mice were injected intraperitoneally with ~500 µl [3H]-cholesterol labeled foam cell suspension containing ~10x10^6 cells/ml at ~3x10^6 dpm/ml. To allow quantitative fecal collection, mice were housed individually on wire bottom cages for 48 hours with *ad libitum* access to food and water. At 24, and 48 hours post injection, blood was collected via the submandibular vein, and 20 µl of isolated plasma was used to determine [3H]cholesterol recovery. The [3H]cholesterol distribution across plasma lipoproteins was determined for the 48-hour time point following size exclusion chromatography of plasma as previously described (Temel et al., 2010). At 48 hours post macrophage injection, mice were anesthetized with ketamine/xylazine (100-160mg/kg ketamine-20-32mg/kg xylazine), and a midline laparotomy was performed for tissue and bile collection. To determine recovery of macrophage-derived [3H]cholesterol as [3H]cholesterol or [3H]bile acids in each tissue compartment, we used the following tissue-specific extraction protocols. For bile samples, 10 µl of gall bladder bile was extracted by adding 1 ml diH2O and 3 ml 2:1 chloroform:methanol and then vortexing. The phases were split by adding 2 ml chloroform, vortexing, and centrifuging at 1000 x g for 10 minutes. The bottom phase
containing the [³H]-cholesterol was carefully removed into a 7 ml scintillation vial, and dried under a N₂ stream. The remaining top phase containing the [³H]-bile acids was subsequently re-extracted with 3 additional volumes (5 ml) of chloroform to completely remove [³H]-cholesterol. All chloroform phases were pooled in the same scintillation vial and dried under a N₂ stream. The remaining cholesterol-depleted top phase containing [³H]-bile acids was then also transferred to a 7 ml scintillation vial and completely dried under N₂. All dried samples were resuspended in 5 ml of scintillation cocktail and subjected to liquid scintillation counting to determine [³H] recovery. Biliary lipid mass was analyzed using enzymatic methods as previously described (Brown et al., 2007, Temel et al. 2007).

To simultaneously determine [³H]cholesterol and [³H]bile acid recovery and sterol mass in feces, feces were quantitatively collected for 48 hours and then dried in a vacuum oven at 70°C overnight. Feces were ground into a fine powder, and a measured amount of powdered feces (~100 mg) was extracted as follows. All samples received 100 µg of 5-alpha-cholestane as an internal standard for subsequent sterol mass determinations. Thereafter, samples were saponified by adding 2 ml 95% ethanol and 200 µl 50% KOH (w/v in diH₂O) and then incubating for 3 hours at 70°C with periodic vortexing. The saponified samples were extracted by adding 2 ml hexane and 2 ml diH₂O with vortexing after each addition. The samples were then centrifuged at 2700 rpm at room temperature for 10 minutes to split the phases. The upper hexane phase (containing [³H]-cholesterol) was removed, and the remaining lower phase was re-extracted 3 times with 2 ml hexane. All [³H]-cholesterol hexane phases were pooled, and completely dried down under N₂, in a 7 ml scintillation vial. A small aliquot of the remaining bottom phase (containing [³H]-bile acids) was transferred to another 7 ml scintillation vial and completely dried under N₂. Dried samples were then resuspended in 5 ml of scintillation cocktail and subjected to scintillation counting for [³H] recovery determination. Mass fecal neutral sterol analysis was performed by gas liquid chromatography (GLC) as previously described (Brown et al., 2008; Temel et al., 2007).

For tissue (i.e. liver & small intestine wall) [³H]cholesterol and [³H]bile acid recovery, a piece of the liver (~100 mg) or entire intestinal segments were extracted for a minimum of 24h at room temperature in 3.75 ml 2:1 chloroform:methanol with frequent vortexing. Following extraction, 1.25 ml of chloroform was added to each sample, and vigorously vortexed. Thereafter, 1.25 ml of diH₂O was added to each sample, followed by vigorous vortexing. The phases (top phase containing and [³H]bile acid and bottom phase
containing and \([3^\text{H}]\text{cholesterol}\) were split by centrifuging samples at 1000 x g for 10 minutes. The bottom phase containing the \([3^\text{H}]\text{-cholesterol}\) was carefully removed into a 7 ml scintillation vial, and dried under a N\(_2\) stream. The remaining top phase containing the \([3^\text{H}]\text{-bile acids}\) was subsequently re-extracted with 3 additional volumes (3 ml) of chloroform to completely remove \([3^\text{H}]\text{-cholesterol}\). All chloroform phases were pooled in the same scintillation vial and dried under a N\(_2\) stream. The remaining cholesterol-depleted top phase containing \([3^\text{H}]\text{-bile acids}\) was then also transferred to a 7 ml scintillation vial and completely dried under N\(_2\). All dried samples were resuspended in 5 ml of scintillation cocktail and subjected to liquid scintillation counting to determine \([3^\text{H}]\) recovery. Total tissue recovery is calculated per total organ weight, and all macrophage RCT data are expressed at the % of dose injected recovered in each respective tissue.

**In Vivo Determination of Very Low Density Lipoprotein (VLDL) Secretion**

Female C57BL/6 mice were injected with control ASO or FMO3 ASO and maintained on a high cholesterol diet (0.2%; wt/wt) for a period of 6 weeks prior to experiment. After a 4 hour fast, mice were anesthetized with isoflurane (4% for induction, 2% for maintenance) and Triton WR 1339 (500 mg/kg body weight; Sigma) was delivered via retro-orbital injection, to block lipolysis. Thereafter, blood samples were collected from anesthetized mice by retro-orbital bleeding at 0, 0.5, 1, 2, and 3 h after injection. Plasma was harvested from the blood samples and used to quantify total cholesterol (TC), cholesteryl ester (CE), free cholesterol (FC), triglyceride (TG) mass using enzymatic assays as described previously (Temel et al., 2010; Brown et al., 2008a; Brown et al., 2008b).

**Liver Histopathology**

Formalin-fixed livers were paraffin embedded, sectioned, and stained with hematoxylin and eosin. Lobular inflammation was scored in a blinded fashion by a board certified pathologist (Xiuli Liu – Cleveland Clinic).

**Statistical Analysis**

Data are expressed as the mean ± S.E.M., and were analyzed using either a one-way or two-way analysis of variance (ANOVA) followed by Student’s t tests for post hoc analysis using JMP version 5.0.12 software (SAS Institute, Cary, NC). For microarray analysis we used empirical Bayes method implemented in R package limma (Smyth 2004).
Extended Experimental Procedures References:


Table S1 (Included as Excel Spreadsheet). Differentially Expressed Genes (DEGs) in Acute and Chronic Mouse Models of TICE Stimulation, Related to Figure 1. For microarray analyses in the acute TICE model, female C57BL/6 mice were treated with a non-targeting control ASO (Con. ASO) or an ASO targeting the knockdown of ACAT2 (A2 ASO) as previously described for 1 week (Marshall et al., 2014). For Microarray analysed in the chronic TICE mouse model, female wild type (WT) or NPC1L1-liver transgenic mice (NPC1L1\textsuperscript{Tg}; Temel et al., 2010) were maintained on a high cholesterol diet (0.2%, wt/wt) for 8 weeks. Differentially expressed genes (DEGs) are shown for both array datasets, with a p-value threshold set at either p<0.005 or p<0.001 (n = 4-5).

Table S2 (Included as Excel Spreadsheet). Differentially Expressed Genes (DEGs) Shared in both Acute and Chronic Mouse Models of TICE Stimulation, Related to Figure 1. For microarray analyses in the acute TICE model, female C57BL/6 mice were treated with a non-targeting control ASO (Con. ASO) or an ASO targeting the knockdown of ACAT2 (A2 ASO) as previously described for 1 week (Marshall et al., 2014). For Microarray analysed in the chronic TICE mouse model, female wild type (WT) or NPC1L1-liver transgenic mice (NPC1L1\textsuperscript{Tg}; Temel et al., 2010) were maintained on a high cholesterol diet (0.2%, wt/wt) for 8 weeks. Differentially expressed genes (DEGs) shared in both array datasets are shown here, with a p-value threshold set at either p<0.005 (n = 4-5).

Table S3 (Included as Excel Spreadsheet). Complete Microarray Dataset from the Acute Mouse Model of TICE Stimulation (Control ASO vs. ACAT2 ASO), Related to Figure 1. Female C57BL/6 mice were treated with a non-targeting control ASO (Con. ASO) or an ASO targeting the knockdown of ACAT2 (A2 ASO) as previously described for 1 week (Marshall et al., 2014), and data here show all probesets represented in the resulting microarray analyses using mouse liver as an RNA source.

Table S4 (Included as Excel Spreadsheet). Complete Microarray Dataset from the Chronic Mouse Model of TICE Stimulation (WT vs NPC1L1-Liver-Transgenic), Related to Figure 1. Female wild type (WT) or NPC1L1-liver transgenic mice (NPC1L1\textsuperscript{Tg}; Temel et al., 2010) were maintained on a high cholesterol diet (0.2%, wt/wt) for 8 weeks, and data here show all probesets represented in the resulting microarray analyses using mouse liver as an RNA source.
Figure S1. FMO3 Reorganizes Biliary Lipid Secretion, Related to Figure 2.

Female C57BL/6 mice were fed either a low (0.02%, wt/wt) or high (0.2%, wt/wt) cholesterol diet and treated with either a control (non-targeting) ASO or an ASO targeting knockdown of FMO3 for 6 weeks. Bile was collected from the gall bladder in one set of mice (Panels B, D, and F), and in another set of mice the common bile duct was cannulated to collect newly secreted hepatic bile (Panels A, C, and E).

(A and B) Biliary cholesterol levels.
(C and D) Biliary bile acid levels.
(E and F) Biliary phospholipid levels.

Data represent the mean ± S.E.M. from 4-10 mice per group. * = significantly different that the control ASO group within each diet group (p<0.05).
Figure S2. FMO3 Inhibition Reduces Bile Acid Pool Size, Related to Figure 2 and Figure 4. Female C57BL/6 mice were fed a high (0.2%, wt/wt) cholesterol diet and treated with either a control (non-targeting) ASO or an ASO targeting knockdown of FMO3 for 6 weeks. Thereafter, the total bile acid pool size was determined and associated gene expression examined.

(A) Bile acid pool size and composition is altered by FMO3 inhibition.
(B) qPCR quantification of Cyp7a1 mRNA levels in the liver.
(C) qPCR quantification of Cyp8b1 mRNA levels in the liver.

Data represent the mean ± S.E.M. from 5 mice per group. * = significantly different that the control ASO group within each time.
Figure S3. FMO3 Gain of Function Reorganizes Cholesterol Balance and Dampens Hepatic Inflammation and ER Stress, Related to Figures 2-5.

Male C57BL/6 mice were fed a low (0.02%, wt/wt) cholesterol diet for a total of 3 weeks. Following two weeks of dietary induction, mice received intravenous injection of either a control adenovirus (Control Adeno.) or an adenovirus driving overexpression of murine FMO3 (FMO3 Adeno.). Plasma was collected 3 days post adenoviral administration. Tissues and gall bladder bile were collected 7 days post adenoviral administration. Feces were quantitatively collected during days 4-7 post adenoviral administration.

(A) qPCR quantification of FMO3 mRNA levels in the liver.
(B) Fecal neutral sterol excretion was determined by gas liquid chromatography.
(C) Cholesterol levels in gall bladder bile.
(D) Total plasma cholesterol (TPC) levels 3 days post adenovirus administration.
(E) qPCR quantification of CD68 mRNA levels in the liver.
(F) qPCR quantification of F4/80 mRNA levels in the liver.
(G) qPCR quantification of ATF3 mRNA levels in the liver.
(H) qPCR quantification of ABCA1 mRNA levels in the liver.
(I) qPCR quantification of LPCAT3 mRNA levels in the liver.

Data represent the mean ± S.E.M. from 6-8 mice per group. * = significantly different that the control adenovirus group (p<0.05).
Figure S4. FMO3 Knockdown Regulates Intestinal Cholesterol Balance in a Gut Microbe-Dependent, but TMAO-Independent, Manner. Related to Figure 2-5. For antibiotic suppression studies (panels A-H) female C57BL/6 mice were started on a low (0.02%, wt/wt) cholesterol diet at 6-8 weeks of age and injected intraperitoneally with either a control ASO or FMO3 ASO for a total of 5 weeks. Following four weeks of dietary induction and ASO treatment, mice either continued to receive normal drinking water or were switched to water supplemented with a poorly absorbed antibiotic cocktail as previously described (Wang et al., 2011) for 7 consecutive days. For dietary TMAO add back studies (panels I-P) female C57BL/6 mice were started on a low (0.02%, wt/wt) cholesterol diet at 6-8 weeks of age and injected intraperitoneally with either a control ASO or FMO3 ASO (50 mg/kg body weight per week) for a total of 5 weeks. Following four weeks of dietary induction and ASO treatment, mice either continued to receive the same (0.02%, wt/wt) cholesterol diet or were switched to a diet containing 0.02%, wt/wt cholesterol with supplemental TMAO (0.3% wt/wt) for 7 consecutive days.

(A & I) Circulating levels of the FMO3 substrate trimethylamine (TMA).
(B & J) Circulating levels of the FMO3 product trimethylamine-N-oxide (TMAO).
(C & K) Fractional cholesterol absorption was determined using the dual fecal isotope method.
(D & L) Fecal neutral sterol excretion was determined by gas liquid chromatography.
(E & M) qPCR quantification of hepatic FMO3 mRNA levels.
(F & N) qPCR quantification of hepatic CD68 mRNA levels.
(G & O) qPCR quantification of hepatic FAS mRNA levels.
(H & P) qPCR quantification of hepatic Cyp8b1 mRNA levels.
Figure S5. Working Model Summarizing Mechanisms by Which FMO3 Knockdown Impacts Whole Body Cholesterol Balance, Related to Figures 1-5.

Shown is the reallocation of cholesterol balance in the liver and small intestine in FMO3 ASO treated mice. Abbreviations: ABCA1, ATP-binding cassette transporter A1; Abcg5/g8, ATP-binding cassette transporters g5 and g8; ASO, antisense oligonucleotide; ACC1, acetyl-CoA carboxylase 1; antisense oligonucleotide; CE, cholesteryl ester; Cyp7a1, cytochrome P450 – family 7 – subfamily B – polypeptide 1; Cyp8b1, cytochrome P450 – family 8 – subfamily B – polypeptide 1; FAS, fatty acid synthase; FMO3, flavin monooxygenase 3; LPCAT3, lysophosphatidylcholine acyltransferase 3; LXR, liver X receptor; MΦ, macrophage; Mdr2, multidrug resistance protein 2; NPC1L1, Niemann Pick C1-Like 1; PL, phospholipid; RXR, retinoid X receptor; SCD1, steroyl-CoA desaturase 1; TICE, transintestinal cholesterol excretion, TMA, trimethylamine; TMAO, trimethylamine-N-oxide; TG, triglyceride.