Flavin containing monooxygenase 3 exerts broad effects on glucose and lipid metabolism and atherosclerosis

Running title: FMO3 modulates TMAO, glucose, lipid metabolism and atherosclerosis

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ABBREVIATIONS

ALT – alanine aminotransferase
ASO – antisense oligonucleotides
AST – aspartate aminotransferase
CAD – coronary artery disease
E3L – Apo-E Leiden
FMO3 – flavin containing monooxygenase 3
LDLRKO – LDL receptor knockout
Tg – transgenic
TMA – trimethylamine
TMAO – trimethylamine-N-oxide
ABSTRACT

We performed silencing and overexpression studies of flavin containing monooxygenase 3 (FMO3) in hyperlipidemic mouse models to examine its effects on trimethylamine N-oxide (TMAO) levels and atherosclerosis. Knockdown of hepatic FMO3 in LDL receptor null (LDLRKO) mice using an antisense oligonucleotide resulted in decreased circulating TMAO levels and atherosclerosis. Surprisingly, we also observed significant decreases in hepatic lipids and in levels of plasma lipids, ketone bodies, glucose and insulin. FMO3 over-expression in transgenic mice, on the other hand, increased hepatic and plasma lipids. Global gene expression analyses suggested that these effects of FMO3 on lipogenesis and gluconeogenesis may be mediated through the PPARα and KLF15 pathways. In vivo and in vitro results were consistent with the concept that the effects were mediated directly by FMO3 rather than TMA/TMAO; in particular, over-expression of FMO3 in the human hepatoma cell line, Hep3B, resulted in significantly increased glucose secretion and lipogenesis. Our results indicate a major role for FMO3 in modulating glucose and lipid homeostasis in vivo, and they suggest that pharmacologic inhibition of FMO3 to reduce TMAO levels would be confounded by metabolic interactions.
INTRODUCTION

Flavin containing monooxygenase 3 (FMO3) is an enzyme expressed primarily in the liver that catalyzes the oxidation of trimethylamine (TMA) to trimethylamine N-oxide (TMAO) (1). TMA is a gas that has a characteristic “fishy” odor that is produced entirely from the catabolism of dietary choline or carnitine by gut microbiota and then absorbed into the circulation (2, 3). Rare, loss of function mutations of FMO3 have been shown to be a cause of “fish malodor syndrome” or trimethylaminuria, characterized by high levels of TMA (4-6). The incidence of trimethylaminuria caused by FMO3 mutations in the Caucasian populations is estimated to be 1 in 10,000 (4). Some but not all trimethylaminuria patients have hypertension (6). However, common polymorphisms of FMO3 gene are not associated with hypertension in a Caucasian population (7). To the best of our knowledge, whether loss of function FMO3 gene mutations influence metabolic traits such as plasma lipid, glucose, or insulin levels has not been reported.

Recently, TMAO was shown to be significantly associated with coronary artery disease in a large cohort of human subjects (3). A follow-up study confirmed those original findings, showing that increased TMAO levels are associated with major adverse cardiovascular events, with a hazard ratio for the highest versus lowest quartile of 2.54 (8). The increased risk of CAD remained after adjustment for traditional risk factors. Furthermore, TMAO appears to be causal for the disease since in mice TMAO supplementation of the diet promoted atherosclerosis and antibiotic treatment, resulting in a block in TMA production, reduced atherosclerosis (2, 3). The mechanism by which TMAO contributes to disease is unknown, but some evidence points to altered cholesterol metabolism by macrophages (3) and diminished reverse cholesterol transport (2). Supporting the human results are genetic studies of natural variations of TMA and TMAO metabolism in populations of mice. Thus, in genetic crosses (3) or in surveys of inbred strains (1) of mice on hyperlipidemic backgrounds, TMAO levels were positively associated with atherosclerosis. A fraction of this variation was explained by genetic differences in FMO3 expression (1) and variations in gut microbiota composition also appear to contribute (9).
These results raise the possibility that inhibition of FMO3 would lead to reduced levels of TMAO and retard the development of atherosclerosis.

We had previously shown in mice that second generation anti-sense oligonucleotide (ASO) mediated knockdown of hepatic FMO3 decreased TMAO levels and that overexpression of FMO3 using adenoviral transduction or transgenic mice increased TMAO levels (1). In order to further examine how alterations in FMO3 contribute to atherosclerosis, we now report studies with mouse models of atherosclerosis, including the low density lipoprotein receptor null (LDLRKO) mouse (10) and the Apo-E Leiden (E3L) transgenic mice, another mouse model of hyperlipidemia (11). Our results confirm that knockdown of FMO3 decreased TMAO levels and atherosclerosis, but they also reveal unexpected effects on glucose and lipid metabolism. Our findings suggest that either FMO3 or TMA/TMAO has novel effects on metabolic functions. We show that FMO3 deficiency leads to decreased glucose and lipid levels in the circulation and in the liver, whereas FMO3 overexpression has opposite effects, indicating a role of FMO3 in modulating glucose and lipid homeostasis \textit{in vivo}. Global gene expression and hepatic metabolite analyses suggest that these effects of FMO3 on glucose and lipid metabolism may be mediated through the PPAR\(\alpha\) and KLF15 pathways. Furthermore, studies of a hepatoma cell line in tissue culture suggest that the metabolic effects are mediated directly by FMO3 expression rather than by TMA/TMAO.
MATERIALS AND METHODS

ASO synthesis

A series of uniform chimeric 20-mer phosphorothioate oligonucleotides containing 2’-O-methoxyethyl (2’ MOE) groups at positions 1–5 and 15–20 targeted to murine FMO3, as well as a control antisense oligonucleotide (ASO), were synthesized and purified on an automated DNA synthesizer using phosphoramidite chemistry as previously described (12).

Animals, diets, and study design

All animal experiments were approved by the UCLA Animal Care and Use Committee, in accordance with PHS guidelines. Eight week old female LDLRKO mice on a C57BL/6J background were purchased from the Jackson Laboratory. Beginning at 9 weeks of age, they were treated with saline, a control ASO (ISIS 141923, 5’-CCTTCCCTGAAGG TTCCTCC-3’, 75 mg/kg body weight per week) or a FMO3 specific ASO (ISIS 555847, 5’-TGGAAGCATTGCCTTTAAA-3’, 50 mg/kg body weight per week) through weekly i.p. injection for a total of 13 injections. The mice were switched to a Western diet (Harlan TD.88137) during the last 8 weeks of the saline/ASO treatment to induce hyperlipidemia, a prerequisite for the development of significant atherosclerosis. At the end of the treatment period, the mice were fasted for 4 hr before blood and tissues were collected for further analysis. For FMO3 ASO studies in C57BL/6J mice, eight-week-old female mice purchased from the Jackson Laboratory were treated with control ASO (ISIS 141923, 50 mg/kg body weight per week) or FMO3 ASO (ISIS 555847, 50 mg/kg body weight per week) through weekly i.p. injection for a total of 13 (VLDL secretion study) or 5 (liver free fatty acid level determination study) injections, respectively. Five days after the last ASO injection, the mice were examined for VLDL-triglyceride secretion rates or liver free fatty acid determination as described below.
Apo E-Leiden (E3L) transgenic (Tg) mice on a C57BL/6J background were a generous gift of Dr. L. Havekes (Leiden University Medical Center). FMO3 transgenic mice (1) were crossed with the E3L transgenic mice (11) to generate FMO3 Tg/E3L Tg and E3L Tg mice for the study. Since the ratio of human FMO3 mRNA level to mouse FMO3 mRNA level was much greater in male than female FMO3 transgenic mice (data not shown), we chose to study the male FMO3 transgenic mice so that the difference in FMO3 expression is greater between the transgenic and non-transgenic mice. Four month old male FMO3 Tg/E3L Tg and E3L Tg mice fed a low fat chow diet were fasted for 4 hr before collection of blood for plasma lipid analysis. The mice were then fed a high fat (16% fat)/high cholesterol (1% cholesterol) (HF/HC) diet (D10042101, Research Diets, New Brunswick, NJ) for 16 weeks, fasted for 16 hr before blood and tissues were collected for further analysis.

**Lipid, glucose, and bile acid analyses, and liver toxicity markers**

For plasma lipid, lipoprotein, and glucose level determinations, mice were fasted for 4 or 16 hr before bleeding. Total cholesterol, HDL cholesterol, unesterified/free cholesterol, triglycerides, free fatty acid, and glucose levels were determined by enzymatic colorimetric assays (13). Phosphatidylcholine levels were assayed using an enzymatic colorimetric assay from WAKO (Richmond, VA). Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using a clinical analyzer with reagents purchased from Beckman Coulter (Brea, CA). Total bile acids levels were assayed using a kit from Diazyme Laboratories (Poway, CA) according to the manufacturer’s protocol. For lipid extraction, 50 mg of liver were homogenized in PBS and the lipids then extracted using the Folch method (14). The extracted lipids were dried and resuspended in 1% Triton X-100 before lipid assays were performed as described above. Plasma samples were fractionated by FPLC as described (15). Plasma concentrations of glucagon, IL-6, and insulin were determined by ELISA using kits from R&D.
systems (Minneapolis, MN), eBioscience (San Diego, CA), and ALPCO (Salem, NH), respectively.

**Quantification of TMA, TMAO, bile acids, and free fatty acids by mass spectrometry**

Quantification of TMAO and TMA in plasma samples was performed using stable isotope dilution HPLC with on line electrospray ionization tandem mass spectrometry on an API 365 triple quadrupole mass spectrometer (Applied Biosystems, Foster, CA) interfaced with a Cohesive HPLC (Franklin, MA) equipped with phenyl column (4.6 × 2505mm, 5 μm Rexchrom Phenyl; Regis, Morton Grove, IL) and the separation was performed as reported previously (3).

Bile acid was extracted from small intestine and liver/gallbladder with ethanol as described by Argmann et al. (16). Bile acid internal standard mixture containing deoxycholic-2,2,4,4-d4 acid (C/D/N Isotopes cat. no. D-2941); cholic-2,2,4,4-d4 acid (C/D/N Isotopes cat. no. D-2452); glycochenodeoxycholic-2,2,4,4-d4 acid (C/D/N Isotopes cat. no. D-5673) and glycocholic-2,2,4,4-d4 acid (C/D/N Isotopes cat. no. D-3878) was added to the ethanolic extract at a final concentration of 10 μM each. Supernatants (20 μl) were analyzed by injection onto a Prodigy 5 u ODS (2) column (2.0 × 150 mm, 5 μm Luna silica; Cat# 00F-3300-B0, Phenomenex, Torrance, CA) at a flow rate of 0.2 ml min⁻¹. Separation was performed using a discontinuous gradient generated by mixing solvent A (0.2% formic acid in water) with solvent B (0.2% formic acid in acetonitrile) at different ratios starting from 25% B for 5 minutes, then linearly to 100% B over 15 min, followed by 100%B for 5 min and then back to 25%B over 1 minute. The HPLC column effluent was introduced into an API 365 triple quadrupole mass spectrometer with Ionics EP 10+ upgrade (Concord, Ontario, CA) interfaced to a Cohesive Technologies Aria LX Series HPLC multiplexing system (Franklin, MA). Analyses were performed using electrospray ionization in negative-ion mode with multiple reaction monitoring of parent and characteristic daughter ions specific for components monitored. The transitions monitored were mass-to-charge ratio (m/z): m/z 391→345 for deoxycholic acid; m/z 395→349 for deoxycholic-2,2,4,4-d4 acid;
m/z 407→371 for α-muricholic acid and β-muricholic acid; m/z 407→343 for cholic; m/z 411→347 for cholic-2,2,4,4-d4 acid; m/z 432→74 for glycolithocholic acid; m/z 448→74 for glycochenodeoxycholic acid, glycodeoxycholic acid, glycohyodeoxycholic acid and glycoursodeoxycholic acid; m/z 452→74 for glycochenodeoxycholic-2,2,4,4-d4 acid; m/z 464→74 for glycocholic acid; m/z 468→74 for glycocholic-2,2,4,4-d4 acid; m/z 482→124 for tauroliothocholic acid; m/z 498→124 for taurochenodeoxycholic acid, taurodeoxycholic acid, taurohyodeoxycholic acid and tauroursodeoxycholic acid; m/z 514→124 for tauro-α-muricholic acid, tauro-β-muricholic acid and taurocholic acid. Various concentrations of non-isotopically labeled bile acid standard mix were spiked into internal standard mix to prepare the calibration curves for quantification of individual bile acids. Deoxycholic-2,2,4,4-d4 acid was used as internal standard for deoxycholic acid; cholic-2,2,4,4-d4 acid for α-muricholic acid, β-muricholic acid and cholic; glycochenodeoxycholic-2,2,4,4-d4 acid for glycochenodeoxycholic acid, glycodeoxycholic acid, glycohyodeoxycholic acid and glycoursodeoxycholic acid; and glycocholic-2,2,4,4-d4 acid for all the other bile acid species.

Liver free fatty acids levels were quantified using mass spectrometry as previously described (17).

Quantification of enzymatic activity of flavin monooxygenases (FMOs) in the liver

Quantification of enzymatic activity of hepatic FMOs was conducted in 250 μl reaction mix containing 1 mg liver protein homogenate, 100 μM d9-TMA and 100 μM reduced nicotinamideadeninedinucleotide phosphate (NADPH) in 10 mM Hepes, pH 7.4. The reaction was stopped after 8 h with 0.2 N formic acid followed by filtering through a 3 K cut-off spin filter. The filtrate was injected onto an HPLC column with on-line tandem mass spectrometer to measure the oxidized product d9-TMAO (3).
VLDL-TG secretion assay

After 16 hr fasting, plasma samples were collected from mice at baseline (Time 0). The mice were then injected with poloxamer 407 (Sigma) intraperitoneally at a dose of 1g/kg body weight. After two hours, plasma samples were collected and the mice were euthanized for tissue collection. Plasma triglyceride levels at times 0 and 2 hr were determined as described above. The rate of VLDL-TG secretion was then calculated as described (18).

In vitro studies: Adenovirus transduction, glucose secretion, and lipogenesis

Hep3B cells were cultured in MEM (Life Technology) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 1 mM sodium pyruvate. For the determination of glucose production, Hep3B cells were plated in 6-well plate, at a density of 5 x 10^5 cells/well. They were then infected with adenoviruses encoding mouse FMO3 (Ad-FMO3) or an empty vector (Ad-CMV) at multiplicity of infection (MOI) of 50 and cultured for 2 days. Afterwards, the cells were washed twice with phosphate buffered saline (PBS), then incubated with a glucose production media containing DMEM (no glucose), 4 mM L-glutamine, 20 mM sodium lactate, 2 mM sodium pyruvate, 100 units/mL penicillin, and 100 µg/mL streptomycin for 20 hr before the conditioned media were collected for determination of glucose concentration by a PicoProbe™ Glucose Fluorometric Assay kit (BioVision, Milpitas, CA) according to the manufacturer’s protocol. For the lipogenesis study, Hep3B cells were plated in 24-well plate, at a density of 2 x 10^5 cells/well, and then infected with Ad-FMO3 or Ad-CMV at MOI of 50 and cultured for 2 days. Afterwards, the cells were washed twice with phosphate buffered saline (PBS), incubated in 0.5 ml of growth media containing 2 µCi of ^14C-acetate (50 mCi/mmol, American Radiolabeled Chemicals, St. Louis, MO) per well for 3 hr before conditioned media and cell lysate were collected for lipid extraction by the Folch method (14). The extracted lipids were spiked with unlabeled lipids (62.5 µg each of phospholipid, cholesterol, free fatty acid,
triglyceride, and cholesterol ester), spotted on TLC plates (Silica Gel G plate, Alltech), and
developed by petroleum ether/ethyl ether/acetic acid (84:15:1). Afterwards, the lipid bands were
stained with 2,7-dichloroflourescin (Sigma) and visualized using a UV lamp. The $^{14}$C
radioactivity associated with various lipid bands were scraped and counted by liquid scintillation
counting (19). The lipid- associated radioactivity in cell lysate and condition media samples were
normalized by the protein concentration of the cell lysate before comparison.

RNA isolation and quantitative RT-PCR analyses

Total RNA samples from tissues were isolated using Trizol reagent ( Life Technologies,
Carlsbad, CA) according to the manufacturer’s protocol. The cDNA was synthesized using the
High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was
performed using gene-specific primers (Supplemental Table I) and the Roche SYBR green master
mix in a Roche Lightcycler 480 system (Roche). The mRNA levels of specific genes were
normalized to the mRNA levels of the housekeeping gene, Rpl13a, of the same sample.

Expression microarray

Total RNA samples from the livers of LDLRKO mice treated with control or
FMO3 ASO (n = 4 for each group) were prepared for use in Illumina MouseRef-8 v2.0
Expression BeadChips according to manufacturer’s recommendations at the
Neuroscience Genomics Core at UCLA. Array data were processed using the limma R
package; any probes which were not expressed (detection p-value < 0.05) in any of the
arrays were excluded. Differential expression ratios were then calculated for the two
groups (Control ASO vs FMO3 ASO) and filtered at a 5% FDR cutoff. Fisher’s exact test
was performed to determine whether there is an enrichment of PPAR$\alpha$ target genes (20,
21) in the differentially expressed gene set.
**Atherosclerosis lesion analyses**

Atherosclerotic lesion size at the aortic root and proximal aorta was determined as described (13). Immunohistochemistry was performed, as previously described (22), using primary antibodies against cluster of differentiation 68 (CD68) (AbD Serotec, Raleigh, NC) to determine macrophage content of the aortic root lesion.

**Statistical analyses**

Student’s t-test was utilized for comparison of means between two groups. In addition, analysis of variance (ANOVA) was used to compare means among 3 treatment groups. Values shown in bar graphs and tables are means and standard errors of the treatment groups. Biweight midcorrelation analysis was performed to examine the pair-wise relationships between plasma TMAO, HDL-cholesterol, VLDL/IDL/LDL-cholesterol levels, and atherosclerotic lesion size among 133 female apoB Tg/HMDP mice (1, 23). Stepwise linear regression analysis was performed to examine the relative importance of TMAO, HDL-cholesterol and VLDL/IDL/LDL-cholesterol in influencing atherosclerotic lesion size among 133 female apoB Tg/HMDP mice (1, 23).
RESULTS

FMO3 knockdown in vivo in LDLRKO mice: effects on TMA/TMAO and atherosclerosis

To examine how FMO3 expression influences atherogenesis, we administered saline, a control antisense oligonucleotide (ASO), or an FMO3 ASO to LDL receptor knockout (LDLRKO) mice for 13 weeks. During the last 8 weeks of the ASO treatment, the mice received a Western diet to enhance plasma cholesterol levels and atherosclerosis development. At the end of the study, the mice were sacrificed and examined for plasma TMA/TMAO levels and atherosclerosis. We observed over 90% reduction in FMO3 protein expression in the livers of the FMO3 ASO treated mice as compared to the saline and control ASO treated mice (Figure 1A). Liver FMO activity as measured by conversion of TMA to TMAO was decreased by 87% in the liver samples of FMO3 ASO treated mice as compared to those of the control ASO group (Figure 1B). Circulating TMAO levels in the FMO3 ASO treated mice were decreased by about 50% as compared to the control ASO treated mice (Figure 1C), whereas circulating TMA levels were significantly increased in the FMO3 ASO-treated mice (Figure 1D). While the FMO3 ASO treatment did not discernibly affect body weight as compared to the control groups (Figure 1E), it did result in a small but significant reduction in weight of 4 major fat pads (Figure 1F).

We observed a significant decrease in atherosclerotic lesion size at the aortic root of the FMO3 ASO group as compared to both the saline and the control ASO groups (Figure 2A), whereas no difference in atherosclerotic lesion size was observed between the saline and control ASO groups (Figure 2A). The macrophage content of lesions, as assessed by the relative area of CD68 staining, was similar (about 70%) in all three groups (Figure 2B).

FMO3 knockdown results in altered levels of plasma lipoproteins and insulin in LDLRKO mice fed a Western diet

Mice injected with the FMO3 ASO exhibited dramatically decreased levels of plasma lipids, including triglyceride, HDL, VLDLIDL/LDL, unesterified cholesterol and free fatty acids,
as compared to mice treated with the control ASO (Table 1). The control ASO had a slight effect on plasma triglyceride levels and HDL cholesterol levels but did not affect the other lipids (Table 1). These results were consistent with FPLC analyses which revealed decreased levels of triglyceride, total cholesterol, and phosphatidylcholine in the VLDL and IDL/LDL fractions of the FMO3 ASO plasma samples as compared to those of the control ASO samples (Figure 3A-3C). Decreased levels of total cholesterol and phosphatidylcholine were also observed in the HDL fractions of the FMO3 ASO plasma samples (Figure 3B, 3C). Bile acids were elevated about two-fold in the FMO3 ASO injected mice (Figure 4C). In addition to these effects, the FMO3 ASO treated mice also exhibited significant decreases in both glucose and insulin levels as compared to the control ASO treated mice (Table 1).

To evaluate the potential toxic effects of FMO3 ASO treatment, we measured plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels as biomarkers for normal liver function. Though levels were elevated about two-fold in the FMO3 ASO treated mice as compared to the control ASO treated mice (Figure 4A and 4B) they did not exceed the normal ranges previously reported for untreated LDLRKO (15) and C57BL/6 mice (24). Further, circulating levels of a pro-inflammatory cytokine, IL-6, were similar between the FMO3 ASO treated mice and the saline and control ASO treated mice (Figure 4E). Circulating levels of urea, a metabolite mainly produced by the liver, were significantly decreased in the FMO3 ASO injected mice as compared to the saline and control ASO treated mice (Figure 4D). Thus, FMO3 knockdown in mice is associated with changes in the levels of plasma lipoproteins, glucose, and insulin, without significant impairment in liver function.

**FMO3 knockdown alters hepatic lipid and glucose metabolism**

We observed significantly decreased levels of triglycerides, total cholesterol, and phosphatidylcholine in the livers of FMO3 ASO injected mice as compared to those of the saline and control ASO treated mice (Figure 5A to 5C), whereas control ASO injected mice were
unaffected in hepatic lipids except for a small decrease in liver triglyceride content as compared to the saline group (Figure 5A). Liver glucose levels in the FMO3 ASO injected mice were significantly lower as compared to the control ASO group (Figure 5D), while liver glycogen levels of the FMO3 ASO and control ASO groups were both slightly elevated as compared to the saline group (Figure 5E).

To examine the effects of the ASO on hepatic gene expression, we performed quantitative RT-PCR. We observed significantly decreased expression of several genes involved in lipogenesis, including sterol regulatory element-binding protein 1c (Srebp-1c), fatty acid synthase (Fasn), malic enzyme (Me), stearoyl-CoA desaturase-1 (Scd-1), and ELOVL family member 6, elongation of long chain fatty acids (Elovl6) in the livers of FMO3 ASO treated mice as compared to those of the control ASO group (Figure 5F). These data provided a potential mechanism for reduced hepatic triglyceride and phosphatidylcholine levels found in the FMO3 ASO treated group as compared to the control ASO treated group. The mRNA level for microsomal triglyceride transfer protein (Mttp) was significantly decreased in the FMO3 ASO treated group as well (Figure 5F), providing an explanation for decreased VLDL levels seen in these mice (Figure 3). Significant decreases in the expression of genes involved in gluconeogenesis or its regulation, such as Alt, fibroblast growth factor 21 (Fgfr21), peroxisome proliferator activated receptor γ coactivator protein-1α (PGC-1α), forkhead box O1 (Foxo1), glucose-6-phosphatase, catalytic subunit (G6pc), pyruvate dehydrogenase kinase, isozyme 4 (Pdk4), and phosphoenolpyruvate carboxykinase (Pepck) were also observed in FMO3 ASO group as compared to the control ASO group (Figure 5F). These transcriptional changes may explain, in part, the decreased plasma and hepatic glucose levels observed in the FMO3 ASO treated group. The mRNA levels of genes involved in fatty acid oxidation, such as acyl-coenzyme A dehydrogenase, long-chain (Acadl) and enoyl-coenzyme A,
hydratase/3-hydroxyacyl coenzyme A dehydrogenase (Ehhadh) were also significantly decreased in the FMO3 ASO treated group (Figure 5F).

**PPARα and KLF15 pathways perturbed by FMO3 knockdown**

Since substantial decreases in mRNA levels of many genes involved in glucose and lipid metabolism were observed in the FMO3 ASO treated group, we asked whether the expression levels of transcription factors regulating these processes, including LXRα, LXRβ, and PPARα, were altered in these mice. Our data showed that the expression of LXRα and LXRβ was not changed in the FMO3 ASO treated group, whereas PPARα mRNA levels were significantly decreased in the FMO3 ASO treated group (Figure 5F). Global gene expression profiling analysis was performed to identify differentially expressed liver genes between the control ASO and FMO3 ASO treated groups. Overall, 27% of the liver transcripts (4223 out of 15552) were differentially expressed (DE) between the two groups with about 50% of the DE transcripts up-regulated and the other 50% down-regulated. In contrast, out of 331 PPARα target genes, 156 of those (47%, 136 down-regulated, 20 up-regulated) were differentially expressed between FMO3 ASO and control ASO treated groups (Supplemental Table II). Thus, we observed an enrichment of PPARα target genes in the differentially expressed gene set (Fisher’s exact test, p = 3.6 x 10^-15). Furthermore, 87% (136 out of 156) of the DE PPARα target genes vs. 50% of transcripts of the overall DE gene set were down-regulated in the FMO3 ASO groups (Fisher’s exact test, p = 2.7 x 10^-23). Therefore, our data show that FMO3 knockdown preferentially decreased PPARα target gene
expression in the liver. Our results suggest that FMO3 may influence lipid and glucose metabolism through modulating PPARα expression and activity.

To further examine why FMO3 knockdown leads to decreased levels of circulating and hepatic glucose in LDLRKO mice, we examined the circulating levels of 12 amino acids that are glucogenic and/or ketogenic in a second set of mice that received either control or FMO3 ASO treatment. Interestingly, the levels of 7 out of these 12 amino acids examined, namely, Glu, Ile, Leu, Pro, Trp, Tyr, and Val, were significantly elevated, by 14% to 38%, as compared to those of the control ASO treated mice (Figure 5G). Plasma glucose and β-hydroxybutyrate (a ketone body produced by the liver) in these FMO3 ASO treated mice were also significantly lower as compared to the control ASO treated mice (Figure 5H and 5I). We noticed that plasma glucose levels (Figure 5H) of this second set of mice, housed at UCLA, were generally lower than those of the first set of mice, housed at ISIS, (Table 1), although both experiments used similar study protocols (LDLRKO females, Western diet, same ASOs, same duration of fasting, 4 hr). However, differences in the environment, animal handling, and stress levels of the mice could all contribute to the differences in plasma glucose levels observed between these two cohorts of mice. Our data suggest decreased hepatic gluconeogenesis and ketone body production in the FMO3 ASO treated mice as compared to the control ASO treated mice. However, circulating glucagon levels were similar between the FMO3 ASO and control ASO treated mice (Figure 5J), indicating the decreased hepatic expression of gluconeogenic genes in the livers of FMO3 ASO treated mice (Figure 5F) was not due to decreased glucagon stimulation. On the other hand, Fgf21 and Pgc-1α mRNA levels were significantly decreased in the FMO3 ASO treated group (Figure 5F). FGF21 is a PPARα target gene that induces PGC-1α expression, leading to increased gluconeogenesis and ketogenesis (25). Therefore, decreased FGF21 and PGC-1α expression in the liver may be one of causes leading to decreased plasma glucose and ketone levels observed in the FMO3 ASO treated mice.
The Kruppel-like factor 15 (Klf15) is a transcription factor that regulates gluconeogenesis, amino acid degradation, and urea cycle in the liver (26-28). Our liver microarray data revealed significant decreases in the mRNA levels of Klf15 and some of its target genes including G6c, Pck1, Otc, Prodh, and Hpd in the FMO3 ASO treated group (Supplemental Table III). The decreased expression of Klf15 and its target genes could also explain, at least in part, the decreased, plasma and liver glucose levels, decreased plasma urea level, and increased plasma gluconeogenic amino acid levels seen in the FMO3 ASO treated mice.

**FMO3 knockdown alters bile acid metabolism, fecal lipid content, and intestinal gene expression**

Since the substrate for FMO3, TMA, is produced by gut microbiota, we asked whether the lower plasma and hepatic lipid levels might be mediated by bile acid metabolism, intestinal cholesterol absorption, or triglyceride digestion. Bile samples collected from the gall bladders of FMO3 ASO treated mice exhibited significantly lower levels of cholesterol, bile acids, and phosphatidylcholine as compared to those of the saline and control ASO treated mice (Figure 6A). FMO3 ASO treated mice exhibited significantly decreased bile acid pool size as compared to the control ASO group (Figure 6B, left panel). The levels of various bile acids, including cholate, taurocholate, taurochenodeoxycholate, tauro-α-muricholate, taurodeoxycholate, and tauroursodeoxycholate, were significantly decreased by more than 50% in the liver/gallbladder of FMO3 ASO treated mice (Figure 6B, middle panel). The levels of taurochenodeoxycholate, tauro-α-muricholate, taurodeoxycholate, and tauroursodeoxycholate were significantly decreased in the small intestine of the FMO3 ASO treated mice as well (Figure 6B, right panel). Interestingly, two major bile acids, tauro-cholic acid and tauro-β-muricholic acid, were increased in both the control ASO and FMO3 ASO groups as compared to the saline group in the small intestine (Figure 6B, right panel). This might be caused by a non-
specific effect of the ASO in general. Hepatic mRNA levels of enzymes involved in bile acid synthesis, including cytochrome P450, family 7, subfamily A, polypeptide 1 (Cyp7a1), cytochrome P450, family 8, subfamily b, polypeptide 1 (Cyp8b1), and cytochrome P450, family 27, subfamily A, polypeptide 1 (Cyp27a1), were all significantly reduced in the FMO3 ASO treated mice (Figure 6C), suggesting lower hepatic bile acid synthesis may account for the lower bile acid concentrations found in the bile, liver, and small intestine of these mice. Hepatic mRNA levels of the bile acid transporter, Na/taurocholate co-transporting polypeptide 1 (Ntcp), were also lower in the FMO3 ASO treated mice (Figure 6C). This might also explain the higher circulating bile acid levels observed in these mice. We also observed increased inflammation in the livers of FMO3 ASO treated mice as evidenced by increased mRNA levels for Cd68, a marker for macrophages, and tumor necrosis factor α (Tnfα) (Figure 6C). The cholesterol content of the fecal samples collected from the FMO3 ASO treated mice were significantly higher as compared to those of saline and control ASO treated mice (Figure 6D), indicating either lower intestinal cholesterol absorption or increased cholesterol excretion in the FMO3 ASO treated mice. Fecal triglyceride contents, on the other hand, were not affected by the FMO3 ASO treatment (Figure 6D). We further examined gene expression in the small intestine of these mice. Interestingly, the mRNA levels of Niemann-Pick C1-like 1 (Npc1l1), a key player in intestinal cholesterol absorption, were significantly lower in the FMO3 ASO treated mice (Figure 6E), which may explain, in part, the increased fecal cholesterol levels seen in the FMO3 ASO treated mice. The mRNA levels of the cholesterol transporters, ATP-binding cassette, sub-family G (WHITE), member 5 (Abcg5) and ATP-binding cassette, sub-family G, member 8 (Abcg8), were also decreased in the FMO3 ASO treated mice as compared to the saline and control ASO treated mice (Figure 6E). The mRNA levels of two farnesoid X receptor (Fxr) target genes, fibroblast growth factor 15 (Fgf15) and small heterodimer partner (Shp), were also significantly decreased in the FMO3 ASO treated mice (Figure 6E), suggesting decreased intestinal bile acid levels in the
FMO3 ASO treated mice. The mRNA levels of genes involved in bile acid transport, including apical sodium dependent bile acid transporter (Asbt), intestinal bile acid-binding protein (Ibabp), multidrug resistance-associated protein 2 (Mrp2), and organic solute transporter β (Ostβ), were similar between the FMO3 ASO and control ASO groups (Figure 6E), providing indirect evidence that FMO3 knockdown did not directly affect bile acid transport in the intestine.

**FMO3 knockdown results in altered plasma lipoprotein, glucose, and insulin levels, and decreased hepatic VLDL-triglyceride secretion in non-hyperlipidemic genetic background**

We examined the effects of FMO3 knockdown in a separate cohort of female C57BL/6J (B6) mice injected with either control or FMO3 ASO for 13 weeks while maintained on a low fat chow diet. B6 mice injected with the FMO3 ASO showed significant decreases in plasma triglycerides, HDL, and unesterified cholesterol levels as compared the control ASO treated mice (Table 2). The FMO3 ASO treated mice also exhibited significant decreases in both glucose and insulin levels as compared to the control ASO treated mice (Table 2). Hepatic triglyceride levels were significantly decreased in the FMO3 ASO treated group (Figure 7A), and this was accompanied by a small but significant increase in total cholesterol level (Figure 7A). No differences in phosphatidylcholine levels were observed between the two groups of mice (Figure 7A). FMO3 ASO treated mice exhibited a significant 20% decrease in triglyceride secretion rate as compared to the control mice (Figure 7B), suggesting that the decreased plasma triglyceride levels observed in the FMO3 ASO treated mice (Table 2) were caused, in part, by decreased triglyceride secretion from the liver.

In a second study, saturated and unsaturated fatty acids in the liver samples of female C57BL/6J mice injected with either control or FMO3 ASO for 5 weeks were quantified by mass spectrometry. We observed no significant differences in most of the saturated fatty acids measured, except for decreased caproate and myristate levels in the FMO3 ASO group (Figure...
In contrast, hepatic levels of most of the unsaturated fatty acids, including palmitoleate, oleate, α-/γ-linolenate, and eicosapentaenoic acid (EPA), were significantly decreased in the FMO3 ASO treated group as compared to the control ASO group (Figure 7D). Since many unsaturated fatty acids are known PPARα ligands (21), the decreased levels of unsaturated fatty acids observed in the livers of FMO3 ASO treated mice may explain the decreased hepatic expression of PPARα target genes associated with FMO3 knockdown (Supplemental Table II).

**FMO3 knockdown using a second FMO3 ASO results in altered plasma lipoprotein, glucose, and insulin levels**

To rule out the possibility that the lipid and glucose lowering effect of the FMO3 ASO used in the LDLRKO and B6 mouse studies described above was due to a non-specific effect, we examined the effect of a second FMO3 ASO (FMO3 ASO#2, ISIS 555926) on plasma lipid and glucose levels in B6 mice. After six weekly ASO injections, male B6 mice treated with the FMO3 ASO#2 showed significant decreases in plasma triglycerides, total, HDL, and unesterified cholesterol levels as compared to the control ASO treated mice (Supplemental Table IV). The FMO3 ASO#2 treated mice also exhibited significant decreases in both glucose and insulin levels as compared to the control mice (Supplemental Table IV). Thus, FMO3 knockdown using two independent FMO3 ASOs *in vivo* yielded reproducible lipid and glucose lowering effects, indicating a role for FMO3 in lipid and glucose homeostasis.

**Overexpression of human FMO3 in transgenic mice results in altered plasma lipoprotein, glucose, and insulin levels, altered hepatic lipid contents, and increased adiposity**

We examined FMO3 transgenic mice to determine whether FMO3 overexpression also has an impact on plasma lipids and glucose metabolism. We previously reported the generation of
FMO3 transgenic mice overexpressing human FMO3 from a liver-specific albumin promoter (1). The male FMO3 transgenic (Tg) mice exhibited a significant 55% increase in liver FMO activity (Supplemental Figure I) and a significant 75% increase in plasma TMAO levels as compared to the male non-transgenic littermates when the mice were supplemented with water containing 1.3% choline chloride (Supplemental Figure I).

We introduced the FMO3 transgene onto a hyperlipidemic mouse model, the E3L Tg mouse (11) for further analysis. The E3L Tg was chosen because it shows a dominant effect with respect to hypercholesterolemia, in contrast to the LDLRKO model and thus reduces the breeding required for generation of the FMO3 transgenic mice on a hyperlipidemic background. When fed either low fat chow or high fat/high cholesterol (HF/HC) diets, the FMO3 Tg/E3L Tg mice exhibited significantly increased plasma triglyceride, VLDL/IDL/LDL, and unesterified cholesterol as compared to the E3L Tg mice (Table 3), consistent with the ASO experiments which had the opposite effects. We also observed increased glucose, and insulin levels in the FMO3 Tg mice (Table 3, p < 0.1). No significant changes in HDL cholesterol or free fatty acid levels were observed between these two groups of mice fed the same diet (Table 3). FPLC analysis of plasma lipoproteins revealed increased levels of triglyceride, total cholesterol, and phosphatidylcholine in the VLDL fractions of the FMO3 Tg plasma samples as compared to those of the E3L Tg samples (Supplemental Figure IIA-IIC) from mice fed the HF/HC diet. Increased levels of total cholesterol and phosphatidylcholine were also observed in the IDL/LDL fractions of the FMO3 Tg plasma samples (Supplemental Figure IIB, IIC). No changes in total cholesterol or phosphatidylcholine levels were observed between the HDL fractions of the two groups (Supplemental Figure IIB, IIC).

When fed a HF/HC diet, the FMO3 Tg/E3L Tg mice exhibited a 40% increase in liver FMO activity (p = 0.08) as compared to the E3L Tg mice (Figure 8A, left panel). There was a trend of increased circulating TMAO levels (p = 0.09) in the FMO3 Tg/E3L Tg mice as compared to the E3L Tg mice (Figure 8A, right panel), whereas circulating TMA levels were
similar between the 2 groups (Figure 8A, right panel). We also observed increased triglyceride accumulation in the livers of FMO3 Tg mice (Figure 8B), although hepatic total cholesterol and phosphatidylcholine levels were similar between the two groups (Figure 8B). There was a significant 10% increase in overall bile acid pool size in the FMO3 Tg/E3L Tg mice as compared to the E3L Tg mice (Figure 8C, left panel). The hepatic concentrations of various bile acids were similar between the FMO3 Tg/E3L Tg and E3L Tg mice except for a small but significant decrease in tauroursodeoxycholate level in the FMO3 Tg/E3L Tg mice (Figure 8C, middle panel). There were significant increases in tauro-chenodeoxycholate (54% increase) and tauro-β-muricholate (31% increase) levels in the small intestine of FMO3 Tg/E3L Tg mice (Figure 8C, right panel). Hepatic gene expression analysis revealed no significant differences in mRNA levels of genes involved in lipid, glucose, bile acid, or cholesterol metabolism between the FMO3/E3L Tg and E3L Tg mice (Supplemental Figure IIIA). Gene expression analysis of the small intestine revealed a significant decrease in Asbt mRNA level in the FMO3 Tg/E3L Tg mice (Supplemental Figure IIIB), whereas no other changes in the expression of genes involved in cholesterol and bile acid transport were observed. Asbt is the major bile salt uptake protein in the small intestine. FXR is shown to inhibit the expression of mouse Asbt via the FXR-SHP-LRH-1 cascade (29). Since the FMO3 Tg/E3L Tg mice had a significant 54% increase in tauro-chenodeoxycholate, a potent FXR ligand, in the small intestine, this may explain the decreased Asbt expression in these mice.

In addition, FMO3 Tg mice exhibited significantly increased body weight (Figure 8D) and adiposity (Figure 8E) after the HF/HC diet feeding. There was a small increase (20%) in atherosclerotic lesion size in the FMO3 Tg/E3L Tg mice (vs. E3L Tg, p = 0.23, Figure 8F). Therefore, data from the FMO3 transgenic mice support a role for FMO3 in glucose, lipid homeostasis, and adiposity.

**Overexpression of FMO3 in Hep3B cells enhances lipogenesis and glucose production**
To further examine the effect of FMO3 on lipid and glucose metabolism, we infected the human Hep3B hepatoma cells with a control (Ad-CMV) or a mouse FMO3-encoding adenoviral vectors (1). Forty-eight hr after infection, we observed high levels of FMO3 protein being expressed in the Ad-FMO3 infected cells as compared to the Ad-CMV infected cells (Figure 9A, left panel). The FMO activity was increased by 14-fold in the Ad-FMO3 infected cells as well (Figure 9A, right panel). The glucose secretion rate of the Ad-FMO3 infected cells was increased by approximately 80% as compared to that of the Ad-CMV infected cells (Figure 9B) when the cells were incubated with a glucose free media that promotes gluconeogenesis. In addition, the Ad-FMO3 infected cells exhibited a 43% increase in lipogenesis as determined by incorporation of $^{14}$C-acetate into cellular lipids including free fatty acid, phospholipid, triglyceride, cholesterol, and cholesterol ester, as compared to the Ad-CMV infected cells (Figure 9C). Interestingly, the mRNA levels of genes involved in gluconeogenesis, such as ALT, G6PC, and PEPCK, were significantly elevated in the Ad-FMO3 infected cells (Figure 9D). The expression of ornithine transcarbamylase (OTC), a gene involved in the urea cycle, was also significantly increased in the Ad-FMO3 infected cells as compared to the Ad-CMV infected cells (Figure 9D). Our data suggest that FMO3 overexpression is associated with increased expression of genes involved in gluconeogenesis. The mRNA levels of several genes involved in lipogenesis, including SREBP-1c, a transcription factor that controls the expression of lipogenesis genes, fatty acid synthase (FASN), and stearoyl-CoA desaturase (SCD) were similar between the Ad-FMO3- and Ad-CMV-infected cells (data not shown). Therefore, FMO3 overexpression in cell culture did not directly increase the expression of genes involved in lipogenesis. The increased lipogenesis we observed in Ad-FMO3 infected cells (Figure 9C) might have been caused by increased availability of glucose in these cells, leading to elevated acetyl CoA, the precursor for fatty acid synthesis.
Associations between plasma TMAO levels, lipid levels, and atherosclerosis resulting from natural variation among inbred strains of mice

Our FMO3 knockdown and overexpression studies suggest a role for FMO3 in modulating plasma lipoprotein levels in mice. We have, therefore, explored whether common functional variations of FMO3 in mice might also influence plasma lipid traits. We previously reported a significant cis-expression quantitative trait locus (cis-eQTL, p = 1.78 x 10^{-6}) located in the Fmo3 gene (SNP rs30760034, located in Chr. 1, 164896447 bp) that controls the hepatic FMO3 mRNA levels among a panel of about 100 different inbred strains of mice (30). Indeed, in this same panel, significant associations were observed between plasma lipid traits (including VLDL/IDL/LDL-cholesterol, total, HDL-cholesterol, and unesterified cholesterol) and single nucleotide polymorphisms (SNPs) located in a region of mouse chromosome 1 within 1 Mb of Fmo3 locus (Supplemental Table V).

Furthermore, we previously observed that atherosclerotic lesion size was significantly correlated with both plasma lipid and TMAO levels among 22 inbred strains of mice that were bred onto a hyperlipidemic apoB transgenic mouse background (apoB Tg/HMDP) (1, 23). We performed biweight midcorrelation analyses of these data to examine the pair-wise relationships between plasma TMAO, HDL-cholesterol, and VLDL/IDL/LDL-cholesterol levels, and atherosclerotic lesion size among the 22 strains of apoB Tg/HMDP mice (133 female mice) (Supplemental Table VIA). We observed a moderate but significant positive correlation between TMAO and VLDL/IDL/LDL levels, and an inverse correlation between TMAO and HDL levels (Supplemental Table VIA). The positive association between TMAO and VLDL/IDL/LDL levels is consistent with our results, since FMO3 knockdown led to decreases in both TMAO and VLDL/IDL/LDL-cholesterol levels, whereas FMO3 overexpression increased both. The inverse correlation between TMAO and HDL-cholesterol levels suggests that there are additional factors, other than FMO3, which have effects on HDL-cholesterol levels in the set of mouse strains. Moreover, we observed significant associations between atherosclerotic lesion size and plasma
levels of TMAO, HDL-cholesterol, and VLDL/IDL/LDL-cholesterol (Supplemental Table VIA), with the order of association: VLDL/IDL/LDL cholesterol > HDL cholesterol > TMAO. To estimate the relative importance of these factors in atherosclerosis development in mice, we performed stepwise linear regression analysis (Supplemental Table VIB). The analysis showed that among these 3 factors, VLDL/IDL/LDL-cholesterol was the most important predictor of lesion size, followed by HDL-cholesterol and then TMAO (Supplemental Table VIB).

We conclude that common natural variations perturbing expression of FMO3 in mice influence plasma lipid levels as well as TMA/TMAO levels.
DISCUSSION

We began this study to test whether inhibition of FMO3 would reduce atherosclerosis in hyperlipidemic mouse models, providing a possible approach to human therapy for the disease. We were surprised to discover that perturbation of FMO3 expression has profound effects on lipid and glucose metabolism, as well as potential pro-inflammatory effects. Hepatic gene expression analysis revealed significantly decreased expression of genes involved in gluconeogenesis and lipogenesis in the FMO3 ASO treated mice as compared to the control mice, providing a molecular basis for the lower lipid and glucose levels seen in these mice. These results reveal a novel role of FMO3, and possibly TMA/TMAO, in lipid and glucose metabolism. Clearly, efforts to modulate TMAO levels for therapeutic purposes by targeting FMO3 will have to consider these metabolic effects.

We observed that FMO3-specific ASO knockdown in LDLRKO mice led to 90% decreases in hepatic FMO3 protein and FMO3 activity levels and resulted in significant decreases in plasma levels of TMAO and atherosclerosis. In addition, we observed decreased levels of plasma lipids, glucose, insulin, and ketone bodies, as well as concomitant increases in plasma levels of glucogenic and ketogenic amino acids, suggesting decreased hepatic gluconeogenesis and ketogenesis. Hepatic glucose and lipid levels were also significantly decreased in the FMO3 ASO treated mice. Treatment of female and male B6 mice by two independent FMO3-specific ASOs led to similar decreases in plasma lipids, glucose, and insulin levels, suggesting the observed metabolic changes associated with the FMO3 ASO treatment were caused by FMO3 deficiency rather than a non-specific effect. Using a complementary approach, we found that FMO3 overexpression in transgenic mice led to a moderate but significant increases in liver FMO activity and significant increases in plasma lipid levels, hepatic triglyceride content, and adiposity. There was a trend of increased plasma glucose and insulin levels and increased atherosclerosis in FMO3 Tg mice as well.
The lipid and glucose changes observed in our studies in response to perturbations of FMO3 are not likely to be mediated by TMAO since elevated TMAO levels caused by dietary supplementation of TMAO, L-carnitine, or choline, did not lead to significant changes in plasma triglyceride, cholesterol, lipoprotein, or glucose levels, or hepatic triglyceride or cholesterol levels in apolipoprotein E null (ApoEKO) mice (2, 3). On the other hand, in a study using male C57BL/6J mice, TMAO supplementation was associated with significantly decreased plasma triglyceride and total cholesterol levels, the opposite of our findings, and increased insulin levels (31). Moreover, overexpression of FMO3 in Hep3B cells by adenovirus mediated gene transduction led to significantly increased glucose production and lipogenesis, suggesting a direct role for FMO3 in modulating lipid and glucose homeostasis. While TMA/TMAO may explain some of the effects on lipids and glucose metabolism, we feel the data are most consistent with the concept that FMO3 modulates the hepatic level(s) of (an) unknown physiological substrate(s) which, in turn, influence(s) lipid and glucose metabolism in the liver.

The decreased lipogenesis we observed in the livers of FMO3 ASO treated mice could be the result of decreased insulin levels. Insulin regulates liver lipogenesis through a series of signaling steps that activate SREBP-1c (32–37), which in turn increases the expression of lipogenic genes such as Fasn and Scd1. We observed decreased hepatic expression of SREBP-1c and lipogenesis genes in the FMO3 ASO treated mice, consistent with decreased insulin levels.

Our data revealed decreased hepatic gluconeogenesis in the FMO3 ASO treated mice. Hepatic gluconeogenesis is closely regulated by the opposing actions of insulin and glucagon. In a fed state, insulin inhibits gluconeogenesis through AKT mediated phosphorylation of the transcription factor FOXO1, leading to its nuclear exclusion and degradation (38). In the fasting state, glucagon stimulates gluconeogenesis by multiple pathways, leading to increased expression of FOXO1, G6PC, and PEPCK (39, 40). We observed decreased levels of FOXO1 in the FMO3 ASO-treated mice. However, we did not observe significant differences in circulating glucagon levels between the FMO3 ASO treated and the control mice.
In an attempt to understand the mechanism by which FMO3 expression is linked to lipid and glucose metabolism, we carried out global microarray expression analyses. The studies revealed that FMO3 knockdown is associated with a preferentially decreased expression of PPARα and its target genes in the liver, possibly due to reduced hepatic concentrations of PPARα ligands, including palmitoleate, oleate, α- or γ-linolenate and EPA (Figure 7D). Both linolenate and EPA are essential fatty acids derived entirely from dietary sources. Since there was no difference in the daily fecal triglyceride output between the mice treated with FMO3 or control ASO (Figure 6D), it is unlikely that FMO3 knockdown impaired the intestinal absorption of essential fatty acids. However, FMO3 may modulate the catabolism of these unsaturated fatty acids, and indirectly influence the activation of PPARα. PPARα is activated during fasting and stimulates fatty acid oxidation, ketogenesis, and gluconeogenesis through transactivation of its target genes (21), and thus could explain many of the observed effects of the FMO3 perturbation. PPARα also regulates genes involved in bile acid synthesis, including Cyp7a1 and Cyp8b1 (21, 41) and plays a role in modulating inflammatory responses in the artery wall and liver by interference with the NF-κB pathway through direct binding to p65 (42) and other mechanisms (43, 44).

The microarray data also revealed decreased expression of Klf15 and some of its target genes in the livers of FMO3 ASO treated mice. Recent studies have shown the importance of KLF15 in modulating gluconeogenesis, amino acid degradation, and urea cycle (26-28). Moreover, KLF15 regulates inflammation by altering the acetylation status and activity of NF-κB through direct interaction with p300 (45). Thus, the increased hepatic inflammation we observed in the FMO3 ASO treated mice might have been caused by impaired PPARα and KLF15 activities.

Previously, dietary TMAO supplementation was shown to decrease bile acid pool size in ApoEKO mice (2). In contrast, we observed decreased TMAO levels and decreased bile acid pool
sizes in FMO3 ASO treated LDLRK0 mice. Furthermore, FMO3 overexpression led to increased bile acid pool size (Figure 8C) in the FMO3 transgenic mice. These results suggest that FMO3 influences bile acid homeostasis through pathways other than TMAO. The observed changes in bile acid metabolism could be linked to the inflammatory effects in liver. Specifically, TNFα has been previously demonstrated to decrease the expression of Cyp7a1 through activation of the mitogen-activated protein kinase (MAPK) pathway (46). Moreover, decreased Cyp7a1 and Cyp8b1 expression due to reduced transactivation by PPARα in the livers of FMO3 ASO treated mice probably contributed to the decreased bile acid pool size as well.

Our study suggests a role for FMO3 in modulating adiposity as well, since FMO3 knockdown and over-expression lead to significantly decreased and increased adiposity, respectively. This could be modulated in part by the effects of FMO3 on plasma triglyceride and glucose levels. Thus, lower plasma triglyceride and glucose levels in the FMO3 ASO treated LDLRK0 mice lead to lower triglyceride accumulation and decreased fat pad weight/adiposity, whereas the opposite is true in the FMO3 transgenic mice.

Our results in mice suggest that naturally-occurring subtle variations in FMO3 expression can significantly perturb lipid metabolism as well as TMA/TMAO levels. Thus, we observed that among different inbred strains of mice, plasma lipid levels mapped to the region of the Fmo3 gene and that TMAO levels were significantly correlated with plasma lipid levels. This raises the possibility that common variations of the FMO3 gene in human populations may also affect lipid or glucose metabolism and that these could explain, in part, the observed correlations between TMAO level and atherosclerosis. This does not appear to be the case, however, since plasma lipids were not correlated with TMAO in human studies and neither TMAO levels nor lipid levels were associated with the FMO3 gene in a genome-wide association study (9). Perhaps common variations in FMO3 expression or activity do not occur in the human populations that were studied. Alternatively, there may be differences between mice and humans in the metabolic
interactions of FMO3. The recessive disorder trimethylaminuria, due to loss of function mutations of \textit{FMO3} gene, occurs at a frequency of about 1 in 10,000 in Caucasian populations (4). It will be of interest to examine whether these individuals or obligate heterozygous individuals exhibit alterations in lipid and glucose metabolism.

In summary, we uncovered a novel role for FMO3 in modulating glucose and lipid homeostasis \textit{in vivo}. Our findings suggest that FMO3 modulates lipid and glucose in a dose-dependent manner and that these effects are most likely independent of TMA/TMAO metabolism. Whether the underlying mechanism, involves unknown catalytic activities of FMO3 or regulatory functions distinct from its catalytic activity, is unknown. However, our data revealed that FMO3 influences the activities of two master regulators of hepatic metabolite homeostasis and inflammation, namely PPAR\textsubscript{\alpha} and KLF15.
ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

Figure 1. FMO3 knockdown in LDLRKO mice leads to decreased hepatic FMO3 protein and activity levels, altered plasma TMAO and TMA levels and decreased adiposity. (A) Liver FMO3 protein levels of saline (S), control ASO (control), or FMO3 ASO (FMO3) treated mice were determined by immunoblotting. (B) Liver FMO activity (n = 6 for each group) was determined using TMA as the substrate. (C) Plasma TMAO and (D) TMA levels were determined by mass spectrometry (n = 12 for each group). Body weight (E), and percent weight of 4 fat pads (gonadal, retroperitoneal, subcutaneous, and mesentery fat pads) (F) were normalized by the body weight (n = 12 for each group). Symbols for Student’s t test: #: p < 0.05 as compared to the saline group. *: p < 0.05 as compared to the control ASO group. Symbol for ANOVA: &: p < 0.05 between the means of the 3 treatment groups.

Figure 2. FMO3 knockdown in LDLRKO mice leads to decreased atherosclerotic lesion development. (A) Atherosclerotic lesion size at the aortic root region of mice treated with saline, control ASO, or FMO3 ASO and fed the Western diet are shown. N = 11 for each group. Symbols are the same as Figure 1. (B) Macrophage content in the atherosclerotic lesions of the aortic root region was determined by immunohistochemistry using antibodies against CD68. Four mice from each group with 3 lesion sections per mouse were used in the comparison.

Figure 3. Altered plasma lipoprotein profile in FMO3 ASO treated LDLRKO mice fed a Western diet. FPLC was used to fractionate pooled plasma samples (5 individual samples per pool) from LDLRKO mice treated with the control ASO or the FMO3 ASO and fed the Western diet. (A) Triglyceride, (B) Total cholesterol, and (C) Phosphatidylcholine concentrations of each FPLC fraction were measured and shown.
Figure 4. FMO3 knockdown in LDLRKO mice is associated with increased levels of hepatotoxicity markers and bile acids and decreased levels of urea in the blood. (A) plasma ALT, (B) plasma AST, (C) plasma total bile acid, (D) plasma urea, and (E) plasma IL-6 concentrations are shown. For (A) to (E), n = 10 to 12 per group. Symbols are the same as in Figure 1.

Figure 5. FMO3 knockdown in LDLRKO mice leads to altered hepatic glucose and lipid levels, altered hepatic gene expression, and changes in plasma metabolites. Liver triglyceride (A), total cholesterol (B), phosphatidylcholine (C), glucose (D), and glycogen (E) levels were determined in LDLRKO mice treated with saline, control ASO, or FMO3 ASO and fed the Western diet (n = 8 for each group). (F) Liver mRNA levels (n = 6 for each group) of genes involved in lipid and glucose metabolism were quantified by qPCR and normalized by the mRNA level of the housekeeping gene, Rpl13a. In a separate set of mice, circulating levels of (G) amino acids, (H) glucose, (I) β-hydroxybutyrate, and (J) glucagon were determined (n = 10 for each group). Symbols are the same as in Figure 1.

Figure 6. FMO3 knockdown in LDLRKO mice leads to altered bile composition, bile acid pool size, fecal lipid output and intestinal gene expression. (A) Bile acid, cholesterol and phosphatidylcholine (PC) levels of bile collected from the gall bladder, (B) bile acid pool size and composition [for bile acid composition, only the major (≥ 0.5% of total amount) bile acid species are plotted], (C) hepatic mRNA levels, (D) daily fecal output of cholesterol and triglyceride, and (E) mRNA levels in the small intestine of LDLRKO mice treated with saline, control ASO, or FMO3 ASO were determined (n = 6 for each group). Symbols are the same as in Figure 1.

Figure 7. FMO3 knockdown in C57BL/6J mice fed a chow diet leads to decreased liver triglyceride and fatty acid concentrations and a decreased rate of secretion of VLDL-
**triglyceride.** Female C57BL/6J mice were treated with control or FMO3 ASO for 13 weeks before (A) liver concentrations of triglyceride (TG), total cholesterol (TC), and phosphatidylcholine (PC), and (B) the rate of VLDL-triglyceride secretion were determined (n = 8 for each group). In a separate study, the levels of saturated fatty acids (C) and unsaturated fatty acids (D) in the livers of C57BL/6J mice treated with 5 weekly injection of control or FMO3 ASO were determined by mass spectrometry (n = 4 for each group). Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Symbol: Student’s t test, *: p < 0.05 as compared to the control ASO group.

**Figure 8.** FMO3 overexpression in Tg mice influences liver FMO activity, plasma TMAO, hepatic lipid content, bile acid pool size and composition, body weight, adiposity, and atherosclerotic lesion size. FMO3 Tg/E3L Tg and E3L Tg mice were fed the HF/HC diet for 16 week prior to tissue collection. (A) Liver FMO activity (n = 7 for each group) and Plasma TMA and TMAO levels (n = 8 to 11 for each group), (B) hepatic levels (n = 10 for each group) of triglyceride (TG), total cholesterol (TC), and phosphatidylcholine (PC), and (C) bile acid pool size and composition (n = 7 to 10 for each group) were measured. For bile acid composition, only the major (> 0.5% of total amount) bile acid species are plotted. Body weight (D), and percent weight (E) of 4 fat pads (gonadal, retroperitoneal, subcutaneous, and mesentery fat pads), normalized by the body weight, were determined (n = 9 to 12 for each group). (F), Mean atherosclerotic lesion sizes at the aortic root region were quantified (n = 15 for E3L Tg, n = 31 for FMO3 Tg/E3L Tg). The lesion data were logarithmically transformed for the t-test. Symbol: Student’s t test, *: p < 0.05 as compared to the E3L Tg mice.

**Figure 9.** FMO3 overexpression in Hep3B cells leads to increased glucose production and lipogenesis. Hep3B cells were infected with a control adenovirus (Ad-CMV) or an adenovirus
encoding the mouse FMO3 (Ad-FMO3) for 2 days and (A) FMO3 protein levels (n = 4 for each group), (B) FMO activity (n = 3 for each group), (C) glucose secretion rates, (D) lipogenesis rates, and (E) mRNA levels were measured. For (C) to (E), n = 4 to 6 for each group. Symbols: Student’s t test, *: p < 0.05, **: p < 0.01, and ***: p < 0.0001 as compared to the Ad-CMV group, respectively.
Table 1. Plasma lipid, lipoprotein, glucose, and insulin levels of female LDLRKO mice maintained on a Western diet with various treatments

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<th>HDL Cholesterol</th>
<th>VLDL/IDL/LDL Cholesterol</th>
<th>Unesterified Cholesterol</th>
<th>Free Fatty Acids</th>
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<td>FMO3 ASO</td>
<td>12</td>
<td>66 (5)#,*</td>
<td>332 (23)#,*</td>
<td>43 (3)#,*</td>
<td>289 (21)#,*</td>
<td>102 (7)#,*</td>
<td>24 (1)#,*</td>
<td>267 (12)*</td>
<td>146 (25)#,*</td>
</tr>
</tbody>
</table>

Mice were fasted for 4 hr. Values shown are means (standard errors) of the groups. Units are mg/dL except insulin. For insulin, unit is pg/ml. Symbols for Student’s t test: #: p < 0.05 vs. saline. *: p < 0.05 vs. control ASO. ANOVA was also performed to analyze the differences between the 3 group means with all traits exhibiting p values <0.05.
Table 2. Plasma lipid, lipoprotein, glucose, and insulin levels of female B6 mice injected with control or FMO3 ASO and maintained on a chow diet

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Triglyceride</th>
<th>Total Cholesterol</th>
<th>HDL Cholesterol</th>
<th>VLDL/IDL/LDL Cholesterol</th>
<th>Unesterified Cholesterol</th>
<th>Free Fatty Acid</th>
<th>Glucose</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ASO</td>
<td>8</td>
<td>103 (24)</td>
<td>93 (4)</td>
<td>64 (3)</td>
<td>28 (2)</td>
<td>14 (1)</td>
<td>55 (7)</td>
<td>130 (8)</td>
<td>260 (49)</td>
</tr>
<tr>
<td>FMO3 ASO</td>
<td>8</td>
<td>50 (11)*</td>
<td>70 (5)*</td>
<td>50 (5)*</td>
<td>21 (2)</td>
<td>11 (1)*</td>
<td>50 (5)</td>
<td>111 (6)*</td>
<td>136 (13)*</td>
</tr>
</tbody>
</table>

Mice were fasted for 16 hr. Values shown are means (standard errors) of the groups. Units are mg/dL except insulin. For insulin, unit is pg/ml. *: p < 0.05 vs. control ASO
Table 3. Plasma lipid, lipoprotein, glucose and insulin levels of FMO3 Tg/ E3L Tg and E3L Tg mice maintained on chow or high fat/high cholesterol (HF/HC) diets

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>n</th>
<th>Triglyceride</th>
<th>Total Cholesterol</th>
<th>HDL Cholesterol</th>
<th>VLDL/IDL/LDL Cholesterol</th>
<th>Unesterified Cholesterol</th>
<th>Free Fatty Acid</th>
<th>Glucose</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3L Tg</td>
<td>Chow</td>
<td>8</td>
<td>104 (13)</td>
<td>93 (9)</td>
<td>48 (10)</td>
<td>45 (5)</td>
<td>29 (3)</td>
<td>36 (2)</td>
<td>182 (13)</td>
<td>852 (172)</td>
</tr>
<tr>
<td>FMO3 Tg/E3L Tg</td>
<td>Chow</td>
<td>12</td>
<td>148 (8)*</td>
<td>115 (6)*</td>
<td>51 (4)</td>
<td>64 (5)*</td>
<td>37 (2)*</td>
<td>38 (2)</td>
<td>205 (4)*</td>
<td>1454 (222)*</td>
</tr>
<tr>
<td>E3L Tg</td>
<td>HF/HC</td>
<td>9</td>
<td>438 (71)</td>
<td>702 (92)</td>
<td>87 (3)</td>
<td>616 (94)</td>
<td>243 (43)</td>
<td>44 (2)</td>
<td>139 (15)</td>
<td>912 (240)</td>
</tr>
<tr>
<td>FMO3 Tg/E3L Tg</td>
<td>HF/HC</td>
<td>13</td>
<td>619 (53)*</td>
<td>916 (44)*</td>
<td>95 (4)</td>
<td>821 (44)*</td>
<td>335 (19)*</td>
<td>37 (3)</td>
<td>164 (7)*</td>
<td>981 (65)*</td>
</tr>
</tbody>
</table>

Mice fed the chow diet were fasted for 4 hr. Mice fed the HF/HC diet were fasted for 16 hr. Values shown are means (standard errors) of the groups. Units are mg/dL except insulin. For insulin, unit is pg/ml. *: p ≤ 0.05 vs. E3L Tg of the same diet. #: p < 0.1 vs. E3L Tg of the same diet. †: Natural log transformed data were used for comparison, p = 0.1 vs. E3L Tg of the same diet.
Figure 1

A: Western blot analysis of FMO3 and β-actin expression levels in control and FMO3 ASO-treated groups.

B: Graph showing liver FMO activity in response to Saline, Control ASO, and FMO3 ASO treatments.

C: Graph depicting plasma TMAO levels in response to Saline, Control ASO, and FMO3 ASO treatments.

D: Graph showing plasma TMA levels in response to Saline, Control ASO, and FMO3 ASO treatments.

E: Graph illustrating body weight changes in response to Saline, Control ASO, and FMO3 ASO treatments.

F: Graph depicting weight of fat pads in relation to body weight percentage in response to Saline, Control ASO, and FMO3 ASO treatments.
Figure 2

A

Mean Lesion Area
(μm² x 10⁻³/Section)

Saline  Control ASO  FMO3 ASO

B

% CD68/Lesion Area

Saline  Control ASO  FMO3 ASO

Figure 2
Figure 3
Figure 4
Figure 5

A. Plasma Amino Acid Level (μM)

B. Glucagon Level (pg/ml)

C. β-Hydroxybutyrate Level (mM)

D. Glucose Level (mg/dL)

E. Triglyceride (mg/g liver)

F. Total Cholesterol (mg/g liver)

G. Phosphatidylcholine (mg/g liver)

H. Glycogen Content (mg/g liver)

I. Glucose Content (mg/g liver)

J. Acyl CoA Content (mg/g liver)

Relative mRNA Level

Acadl, Ehhad, Elovl6, FASN, FGF21, FOXO1, G6PC, LXRα, LXRβ, Me, MTP, PDK4, PEPC, PGC-1α, PPARα, SREBP-1c

Saline, Control ASO, FMO3 ASO

* indicates significant differences compared to baseline.

& indicates significant differences between groups.
A

Concentration in Bile (mM)

- Saline
- Control ASO
- FMO3 ASO

B

BA Pool Size

Liver/Bile Bladder

Small Intestine

C

Relative mRNA Level

Liver

D

Daily Fecal Lipid Output (mg)

E

Relative mRNA Level

Small Intestine

Figure 6
Figure 7
Figure 8
Figure 9

B

Glucose Secretion Rate (nmol/mg protein/hr)

C

Incorporation of 14C-Acetate into Lipids (x1000 cpm/mg protein)

D

Relative mRNA Level

ALT         G6PC        OCT        PEPCK

FMO3
β-actin

Ad-CMV Ad-FMO3

FMO Activity (pmol d9-TMAO/mg protein/hr)

* 52

Figure 9