Effect of Flavin-Containing Monooxygenase (FMO) Genotype, Mouse Strain and Gender on Trimethylamine N-oxide Production, Plasma Cholesterol Concentration and an Index of Atherosclerosis

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Running Title: TMAO is not correlated with Plasma Cholesterol Concentration or Als

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Abbreviations: Als, index of atherosclerosis; ABCB 11, ATP-binding cassette sub-family B, member 11; ABCG 5, ATP-binding cassette sub-family G, member 5; ABCG 8, ATP-binding cassette sub-family G, member 8; CYP7A1, cholesterol 7α hydroxylase; CYP27A1, sterol 27-hydroxylase; FMO, flavin-containing monooxygenase; HDL, high-density lipoprotein cholesterol; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase; LDL, low-density lipoprotein cholesterol; SR-B1, scavenger receptor class B, member 1; SS, squalene synthase; SREBP-2, sterol regulatory element-binding protein 2; TMA, trimethylamine; TMAO, trimethylamine N-oxide; UCL, University College London; WT, wild type.
ABSTRACT

The objectives of the study were to determine the contribution, in mice, of members of the flavin-containing monooxygenase (FMO) family to the production of trimethylamine N-oxide (TMAO), a potential proatherogenic molecule, and whether, under normal dietary conditions, differences in TMAO production were associated with changes in plasma cholesterol concentration or with an index of atherosclerosis (Als). Concentrations of urinary trimethylamine (TMA) and TMAO and of plasma cholesterol were measured in 10-week-old male and female C57BL/6J and CD-1 mice and in mouse lines deficient in various Fmo genes (Fmo1−/−, 2−/−, 4−/− and Fmo5−/−). In females most TMA N-oxygenation was catalyzed by FMO3, but in both genders 11-12% of TMA was converted to TMAO by FMO1. Gender-, Fmo genotype- and strain-related differences in TMAO production were accompanied by opposite effects on plasma cholesterol concentration. Plasma cholesterol was negatively, but weakly, correlated with TMAO production and with urinary TMAO concentration. Fmo genotype had no effect on Als. There was no correlation between Als and either TMAO production or urinary TMAO concentration. Our results indicate that under normal dietary conditions TMAO does not increase plasma cholesterol or act as a proatherogenic molecule.
Introduction

A correlation between plasma trimethylamine N-oxide (TMAO) concentrations and atherosclerotic plaque size has been reported in atherosclerosis-prone mice fed on diets supplemented with dietary precursors of trimethylamine (TMA) (Wang et al., 2011; Koeth et al., 2013).

TMAO production is the result of a two-step process, requiring interplay between commensal gut bacteria and the host (Fennema et al., 2016). This process involves the liberation of TMA from dietary precursors, such as choline, carnitine and TMAO itself (Mitchell et al., 2002; Fennema et al., 2016), and the subsequent host-dependent hepatic N-oxygenation of TMA to TMAO (Ayesh et al., 1993). In humans, this oxygenation reaction is catalyzed by flavin-containing monooxygenase 3 (FMO3) (Dolphin et al., 1997; Lang et al., 1998). Healthy individuals excrete in their urine ~95% of total TMA (TMA + TMAO) as TMAO and ~5% as TMA (Al-Waiz et al., 1987a). Individuals homozygous or compound heterozygous for mutations that severely affect FMO3 activity have impaired N-oxygenation of TMA and suffer from the inherited metabolic disorder primary trimethylaminuria (Dolphin et al., 1997; Shephard et al., 2015; Phillips and Shephard, 2015).

There are five functional FMOs in humans: FMO1, 2, 3, 4, and 5 (Phillips et al., 1995; Hernandez et al., 2004). Of these, FMO2 is not expressed in the majority of humans (Dolphin et al., 1998; Veeramah et al., 2008). FMO4 is expressed in very low amounts and little is known of the role of this FMO (Dolphin et al., 1996; Zhang and Cashman, 2006). In human liver, at the time of birth, there is a switch in the FMO genes expressed: FMO3 is switched on and FMO1 is switched off (Dolphin et al., 1996; Koukouritaki et al., 2002). The FMO1 gene continues to be expressed in humans in the kidney (Dolphin et al., 1996) through the use of an alternative promoter (Shephard et al., 2007). Therefore, after birth,
humans primarily express two FMO genes in their liver, FMO3 and FMO5 (Dolphin et al., 1996; Overby et al., 1997; Koukouritaki et al., 2002; Zhang and Cashman, 2006). However, all other mammals studied continue to express FMO1 in adult liver (Phillips et al., 2007).

In mice, there is an age-related gender difference in the expression of Fmo genes in the liver: male mice, post 5-6 weeks of age, switch off the expression of Fmo3 and, thus, represent natural liver-specific knockouts for FMO3 (Falls et al., 1995; Janmohamed et al., 2004). Adult male mice consequently express in liver two Fmo genes, Fmo1 and Fmo5. In contrast, adult female mice, in addition to expressing Fmo1 and Fmo5, continue to express Fmo3 in liver. The gender-related differences in Fmo gene expression in mice are due to the influence of sex steroids (Falls et al., 1997).

Reports associating TMAO with atherosclerosis and increased risk of cardiovascular disease (Wang et al., 2011; Koeth et al., 2013) are largely based on studies involving concentrations of precursors of TMA or of TMAO itself far in excess of those found under normal dietary conditions and it is not clear whether atherosclerosis risk factors are influenced by physiologically relevant concentrations of TMAO.

Here, we report the use of male and female mice of two different strains and of knockout mouse lines deficient in various FMOs (Fmo1<sup>-/-</sup>, 2<sup>-/-</sup>, 4<sup>-/-</sup> and Fmo5<sup>-/-</sup>) to determine the contribution of particular FMOs to the production of TMAO in vivo and to investigate whether, under normal dietary conditions, differences in TMAO production are associated with changes in plasma cholesterol concentration or with an index of atherosclerosis (AIs). We found that in females conversion of TMA to TMAO is catalyzed mainly by FMO3, an enzyme that is absent from the liver of adult males (Falls et al., 1995; Janmohamed et al., 2004), but in both genders FMO1 contributes to TMAO production. Differences in TMAO production were accompanied by opposite effects on plasma cholesterol concentration and
were not correlated with ALS. Our results indicate that at physiologically relevant concentrations TMAO does not act as a proatherogenic molecule in mice.

Materials and Methods

Animal Maintenance

C57BL/6J wild-type (WT) mice and two knockout mouse lines (Fmo1−/−, 2−/−, 4−/− and Fmo5−/−, each of which was back-crossed for eight generations on the C57BL/6J background) were bred at University College London. Generation of the knockout mouse lines has been described previously (Hernandez et al., 2009; Gonzalez Malagon et al., 2015). CD-1 mice were purchased from Charles River (Margate, Kent, UK) and allowed to acclimatise for 14 days before sample collection. All mice were housed in the same room and given free access to water and fed ad libitum with a standard chow diet (Teklad Global 18% Protein Rodent Diet (2018), Harlan Laboratories, Inc., Madison, WI), which contained a choline content of 1.2g/kg. Blood and urine were collected from 10-week-old male and female mice between 10:00 AM and 12:00 PM (noon). Animal procedures were carried out in accordance with the UK Animal Scientific Procedures Act and with local ethics committee approval (Animal Welfare and Ethical Review Body) and appropriate Home Office Licenses.

Urine analyses

Concentrations of TMA, TMAO and creatinine in urine samples were determined by capillary liquid chromatography electrospray ionization mass spectrometry as described previously (Veeravalli et al., 2017).
Production of TMAO was assessed by determining the percentage of total TMA excreted as TMAO, using the formula: \( \frac{\text{TMAO}}{\text{TMA + TMAO}} \times 100 \) (Al-Waiz et al., 1989). To control for differences in urine concentration, urinary TMAO and TMA concentrations were expressed relative to urinary creatinine concentration.

**Plasma Metabolites**

Blood samples were collected and plasma isolated as described (Hough et al., 2002). Concentrations of total cholesterol, high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL) were determined via an autoanalyzer at the Medical Research Council Mammalian Genomics Unit (Harwell, Oxfordshire, UK) as described (Hough et al., 2002). Als was calculated as (total plasma cholesterol – HDL)/HDL (Gao et al., 2014).

**Quantitative Real-Time (qRT)-PCR**

RNA was isolated from liver and individual mRNAs were quantified, by qRT-PCR, according to the \( \Delta \Delta CT \) method (Liu and Saint, 2002), as described previously (Veeravalli et al., 2014). Primers for sterol regulatory element-binding protein 2 (SREBP2), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), cholesterol 7\( \alpha \) hydroxylase (CYP7A1), sterol 27-hydroxylase (CYP27A1), scavenger receptor class B, member 1 (SRB1), ATP-binding cassette sub-family G, member 5 (ABCG5), ATP-binding cassette sub-family G, member 8 (ABCG8), and ATP-binding cassette sub-family B, member 11 (ABCB11) were as described previously (Gonzalez Malagon et al., 2015). Additional primer sequences, forward (F) and reverse (R), for 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1) and squalene synthase (SS) were: HMGCS1F 5'GTGGCACCAGATGTTTTG3' and HMGCS1R 5'ACTCTGACCAGATACCACGT3'; SSF
5’ATGGAGTTCGTCAAGTGCTAGG3’ and SSR 5’CGTGCCGTATGTCCCCATC3’.
The use of a geNormTM kit and geNorm software (Primer Design Ltd), as described
previously, identified GAPDH as the most suitable housekeeping gene for liver (Veeravalli
et al., 2014).

Statistical Analyses
Values are given as means ± S.E.M. Statistical significance was determined using an
unpaired two-tailed student’s t-test. Statistical significance is represented as *P < 0.05, **P
< 0.01, and ***P < 0.001. Correlations were assessed using Pearson’s r.

Results
Mouse Gender, Fmo Genotype and TMAO Production
Urine from male and female C57BL/6J WT mice and from two Fmo-knockout lines, Fmo1
/, 2^{+/-}, 4^{+/-} and Fmo5^{+/-}, was analysed for TMAO and TMA content. The FMO isoforms
expressed in the livers of these animals at 10 weeks of age are given in Table 1. Irrespective
of Fmo genotype, the percentage of total TMA (TMA + TMAO) excreted as TMAO was
far less in male than in female mice (Fig. 1A and B).

In both male and female Fmo1^{+/-}, 2^{+/-}, 4^{+/-} mice the proportion of total TMA excreted
as TMAO was less than in gender-matched C57BL/6J WT mice (Fig. 1A and B). In males
the proportion of total TMA excreted as TMAO by Fmo1^{+/-}, 2^{+/-}, 4^{+/-} mice (8.4 ± 1.6%) was
56% less than by WT mice (19.5 ± 2.6%), whereas in females TMAO excretion by Fmo1^{+/-},
2^{+/-}, 4^{+/-} mice (70.7 ± 0.1%) was 14% less than by WT mice (83.2 ± 1.3%). There is no
increase in expression of the Fmo3 gene in the liver of Fmo1^{+/-}, 2^{+/-}, 4^{+/-} mice, in response to
deletion of Fmo genes (Hernandez et al., 2009). The results, therefore, indicate that in mice
FMOs 1, 2 or 4 contribute to the N-oxygenation of TMA. It is most likely to be FMO1
because this enzyme is expressed in adult mouse liver, the site of TMA N-oxygenation, whereas FMOs 2 and 4 are not expressed in this tissue (Janmohamed et al., 2004). Thus, in both male and female mice, 11-12% of TMA is converted to TMAO by FMO1. In Fmo5−/− mice the proportion of total TMA excreted as TMAO was the same as that in WT mice, in both males and females, indicating that FMO5 plays no role in the conversion of TMA to TMAO in vivo.

**Mouse Gender, Fmo Genotype and Plasma Cholesterol Concentration**

Increased concentration of TMAO has been linked to the formation of proatherogenic plaques (Wang et al., 2011), of which cholesterol is an important component. The gender- and Fmo genotype-related differences in TMAO production (Fig. 1) lead us to investigate the effect of gender and Fmo genotype on plasma cholesterol concentrations. Plasma concentrations of total cholesterol, HDL and LDL were determined in male and female C57BL/6J WT, Fmo1−/−, 2−/−, 4−/− and Fmo5−/− mice (Table 2). In WT mice and in both knockout mouse lines females had significantly lower total plasma concentrations of cholesterol and HDL than did their male counterparts, but the plasma concentration of LDL was similar in male and female mice. Therefore, the greater production of TMAO in female mice was accompanied by significantly lower plasma concentrations of total cholesterol and HDL.

Both male and female Fmo1−/−, 2−/−, 4−/− mice had higher plasma concentrations of total cholesterol and HDL than did their WT counterparts, and in females the plasma concentration of LDL was also higher than in WT mice (Table 2). Based on the tissue-specific expression patterns of FMOs in mice (Janmohamed et al., 2004), the higher plasma cholesterol concentration observed in Fmo1−/−, 2−/−, 4−/− mice is likely to be due to disruption of the Fmo1 gene, which is expressed in liver, and not to disruption of Fmo2 or Fmo4.
which are not expressed in liver. Consequently, our results indicate that in mice FMO1 plays a role in the regulation of plasma cholesterol concentration.

Comparison of Fmo5/− and WT mice revealed that plasma concentrations of total cholesterol and of HDL were similar in females and lower in males (Table 2), indicating, in males, a role for FMO5 in promoting plasma cholesterol concentration.

Mouse Gender, Fmo Genotype and Atherosclerosis Index

We next investigated the effect of Fmo genotype on Als. There was no significant difference in Als between C57BL/6J WT mice and either of the two knockout lines, in either male or female animals (Fig. 2). Similarly, there was no difference in Als between males and females of either WT or Fmo5/− mice, despite production of TMAO being 4-fold greater in females (Fig.1). However, in Fmo1/−, 2/−, 4/− mice, Als was ~26% lower in males than in females (Fig. 2).

Mouse Strain, TMAO, Plasma Cholesterol and Als

We also investigated the relationship between TMAO production, plasma total cholesterol and Als in another strain of mouse, CD-1. In males, the urinary concentration of TMAO (mM/mM creatinine) was 0.18 ± 0.02 (n = 5) for CD-1 mice and 0.70 ± 0.03 (n = 4) for C57BL/6J WT mice. For females the values were 0.66 ± 0.06 (CD-1, n = 6) and 2.73 ± 0.20 (C57BL/6J WT, n = 4). Therefore, for both genders the urinary concentration of TMAO of CD-1 mice was ~75% less than that of C57BL/6J WT mice (P < 0.0001) and, as was the case for C57BL/6J WT mice, was higher in female than in male animals (P < 0.0001).

The plasma concentration of total cholesterol of CD-1 mice was significantly higher than that of C57BL/6J WT mice in both genders (Table 2). In males, Als was significantly higher in CD-1 (0.68 ± 0.08, n = 5) than in C57BL/6J WT (0.38 ± 0.02, n = 4) (P < 0.05),
but not in females (CD-1: 0.51 ± 0.04, n = 6; C57BL/6J WT 0.44 ± 0.02, n = 4). Therefore, although CD-1 mice had substantially lower urinary concentrations of TMAO than did C57BL/6J mice, they had higher plasma concentration of total cholesterol, in both genders, and in males had higher Als.

**Correlations of TMAO with Plasma Cholesterol or Als**

Our results indicate an inverse relationship between TMAO production and the plasma concentration of total cholesterol. Therefore, we investigated whether there was a correlation between total plasma cholesterol concentration and either TMAO production, which is measured by the percentage of total TMA excreted as TMAO, or the urinary concentration of TMAO. Analysis of male and female C57BL/6J WT, CD-1 WT, Fmo1<sup>−/−</sup>, 2<sup>−/−</sup>, 4<sup>−/−</sup> and Fmo5<sup>−/−</sup> mice (a total of 34 animals) showed a weak negative correlation between plasma total cholesterol concentration and both TMAO production and urinary TMAO concentration (Table 3, Supplemental Fig. S1 A and B). There was no correlation between Als and either TMAO production or urinary TMAO concentration (Table 3, Supplemental Fig. S1 C and D).

**Quantification of mRNAs Encoding Proteins Involved in Cholesterol Synthesis, Uptake, Transport or Disposition**

Fmo1<sup>−/−</sup>, 2<sup>−/−</sup>, 4<sup>−/−</sup> mice, both male and female, had higher plasma concentrations of total cholesterol and HDL, compared with their WT and Fmo5<sup>−/−</sup> counterparts (Table 2). To attempt to identify a potential basis for the elevated plasma concentrations of cholesterol and HDL in Fmo1<sup>−/−</sup>, 2<sup>−/−</sup>, 4<sup>−/−</sup> mice, mRNAs encoding proteins involved in cholesterol biosynthesis, cholesterol uptake and transport and in bile acid synthesis and secretion were
quantified, by qRT-PCR, in livers of male Fmo1<sup>−/−</sup>, 2<sup>−/−</sup>, 4<sup>−/−</sup> and C57BL/6J WT mice (Table 4).

The mRNA for SREBP-2, a transcription factor that upregulates expression of genes involved in cholesterol synthesis, was more abundant in Fmo1<sup>−/−</sup>, 2<sup>−/−</sup>, 4<sup>−/−</sup> than in C57BL/6J WT animals (Table 4). Although relatively small, the increased abundance of the mRNA for this transcription factor may contribute to the elevated plasma cholesterol observed in Fmo1<sup>−/−</sup>, 2<sup>−/−</sup>, 4<sup>−/−</sup> mice. However, the abundance of mRNAs encoding proteins involved in cholesterol biosynthesis was not increased (Table 4).

SR-B1 mRNA was more abundant in the Fmo1<sup>−/−</sup>, 2<sup>−/−</sup>, 4<sup>−/−</sup> mice (Table 4). Because of the role of SR-B1 in promoting cholesterol uptake (Varban et al., 1998; Ji et al., 1999) the increase in the abundance of its mRNA may be an attempt to reduce the elevated concentration of plasma HDL cholesterol in Fmo1<sup>−/−</sup>, 2<sup>−/−</sup>, 4<sup>−/−</sup> mice. The mRNA for ABCB11, which encodes the bile salt export pump located on the canalicular membrane of hepatocytes, a protein that controls the rate-limiting step in hepatic bile secretion (Henkel et al., 2013), was more abundant in the Fmo1<sup>−/−</sup>, 2<sup>−/−</sup>, 4<sup>−/−</sup> mice (Table 4). The increase in ABCB11 mRNA may be in response to increased SR-B1-mediated cholesterol uptake and suggests that bile flow from the liver to the intestine may be greater in Fmo1<sup>−/−</sup>, 2<sup>−/−</sup>, 4<sup>−/−</sup> than in WT mice. However, there was no difference between Fmo1<sup>−/−</sup>, 2<sup>−/−</sup>, 4<sup>−/−</sup> and WT mice in the abundance of mRNAs encoding either CYP7A1 or CYP27A1 (Table 4), two enzymes important in bile acid synthesis (Norlin and Wikvall, 2007).

Discussion

Our results show that female mice produced far more TMAO than did male animals. This marked gender difference is consistent with previous findings (Li et al., 2013) and can be explained by the fact that at 5-6 weeks of age the expression of the gene encoding FMO3,
the major enzyme involved in conversion of TMA to TMAO in both human and mouse (Dolphin et al., 1997; Lang et al., 1998; Zhang et al., 2007), is switched off in the liver of male, but not of female mice (Falls et al., 1995; Janmohamed et al., 2004).

In both male and female C57BL/6J WT mice FMO1, but not FMO5, contributed to TMAO production, with 11-12% of TMA being converted to TMAO by the action of FMO1. Consequently, in females the majority of TMAO was produced by hepatic FMO3, but in males, which lack hepatic FMO3, most of the much lower production of TMAO was derived from the action of FMO1. Our results in vivo are consistent with those found in vitro (Bennett et al., 2013).

Results from Fmo1−/−, 2−/−, 4−/− mice indicate that, despite the disruption of genes encoding FMO1, FMO2 and FMO4 and the lack of hepatic FMO3, males still produced a small amount of TMAO. The reason for this is unclear, but could be due to the action of FMO3 in tissues other than liver, for instance, adult males continue to express FMO3 in Clara cells of the lung (Janmohamed et al., 2004). Other possibilities include the action of non-FMO enzymes or the production of TMAO from TMA by gut bacteria (reviewed by Fennema et al., 2016).

Humans, in contrast to mice and other mammals investigated, do not express FMO1 in liver after birth (Dolphin et al., 1996; Koukouritaki et al., 2002). Thus, in adult humans FMO1 cannot contribute to hepatic TMAO production. Although FMO1 is expressed in human kidney (Dolphin et al., 1996) and small intestine (Yeung et al., 2000), conversion of TMA to TMAO was not detected in microsomes isolated from these tissues (Lang et al., 1998). Recombinant human FMO1 can catalyze the conversion of TMA to TMAO but only at high (5 mM) concentrations of TMA (Lang et al., 1998). In humans, the concentration of TMA never reaches the mM range, even in individuals with severe trimethylaminuria (Al-
Waiz et al., 1987b), in contrast to mice, which excrete TMA in the mM range (Li et al., 2013). Consequently, FMO1 is unlikely to contribute to TMAO production in humans.

Gender-, Fmo genotype- and strain-related differences in TMAO production were in all cases accompanied by opposite effects on plasma total cholesterol concentration. Plasma total cholesterol concentration was negatively, but weakly, correlated with both TMAO production and urinary TMAO concentration.

Our results indicate that both FMO1 and FMO5 are involved in the regulation of plasma cholesterol concentration, but with opposing effects: FMO1 acting to decrease plasma cholesterol concentration in both male and female animals, whereas FMO5 promotes an increase in plasma cholesterol concentration in males. The latter is consistent with our previous finding that FMO5 promotes an age-related increase in plasma cholesterol concentration, which by 30 weeks of age is evident in both genders (Gonzalez Malagon et al., 2015).

Als was not influenced by Fmo genotype and, despite relatively large gender-related differences in production of TMAO, in only one of the three mouse lines, $Fmo1^{-/-}$, $2^{-/-}$, $4^{-/-}$, was Als influenced by gender, and then to a relatively small extent. There was no correlation between Als and either TMAO production or urinary TMAO concentration.

Several studies of mice have implicated TMAO as a proatherogenic molecule. Most of these have been done on mice carrying an ApoB transgene or on ApoE$^{-/-}$ knockout animals (Wang et al., 2011; Bennett et al., 2013; Koeth et al., 2013; Yang et al., 2014), in an attempt to better mimic the situation in humans, a species in which plasma LDL cholesterol constitutes a higher proportion of total cholesterol than is found in mice. Many of these studies involved feeding the animals with precursors of TMA or with TMAO itself in amounts far in excess of normal dietary levels; for instance, in some cases diets were supplemented with concentrations of choline as high as 13g/kg, more than ten times that
present in the standard chow diet used in our study. In contrast, a study by Mayr et al. (2005) found no significant difference in TMAO concentration in the aortas of 18-month-old ApoE−/− and ApoE+/+ mice, despite the former having severe aortic atherosclerotic lesions. The lesion formation in ApoE−/− mice was ascribed not to increased TMAO, but to an increase in oxidative stress (Mayr et al., 2005).

A role for TMAO in predisposition to cardiovascular disease is counter-intuitive given that the richest dietary source of TMAO is marine fish, the consumption of which has been shown to exert beneficial effects on the circulatory system (Zhang et al., 1999; Takata et al., 2013). Consistent with this, a study of C57BL/6J WT mice found that the increase in Als associated with a high-fat diet was prevented by dietary supplementation with TMAO, suggesting a protective effect of TMAO with regard to atherosclerosis (Gao et al., 2014).

In our study we used C57BL/6J and CD-1 mice and two Fmo gene knockout mouse lines (Fmo1−/−, 2−/−, 4−/− and Fmo5−/−) generated on a C57BL/6J background to investigate, under normal dietary conditions, the effect of mouse gender and strain and of Fmo genotype on TMAO production, plasma cholesterol concentration and an index of atherosclerosis. We elected not to complicate the experimental design or data interpretation by the use of an additional genotype change, such as the disruption of ApoE or the presence of an ApoB transgene, or by supplementation of diet with TMA precursors.

Our results indicate an inverse relationship between TMAO production and the plasma concentration of total cholesterol and that neither plasma cholesterol concentration nor Als is positively correlated with either TMAO production or urinary TMAO concentration. Indeed, there is a negative, albeit it weak, correlation between plasma cholesterol concentration and both TMAO production and urinary TMAO concentration. Our results, therefore, indicate that under normal dietary conditions TMAO does not act as a proatherogenic molecule.
Authorship contributions:

**Participated in research design:** Veeravalli, Karu, Phillips, and Shephard

**Conducted experiments:** Veeravalli, Karu, Scott, and Shephard

**Performed data analysis:** Veeravalli, Phillips, and Shephard

**Wrote or contributed to the writing of the manuscript:** Veeravalli, Scott, Fennema, Karu, Phillips and Shephard
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Figure Legends

Fig. 1. Effect of Fmo genotype and gender on TMAO production. Urinary excretion of TMAO as a percentage of total TMA (TMA + TMAO) in male (A) and female (B) mice. n = 3 to 5. *P <0.05, ***P <0.001.

Fig. 2. Effect of Fmo genotype, gender and mouse strain on Als. n = 3 to 5. *P <0.05.
Table 1. FMO isoforms expressed in the livers of 10-week-old WT and Fmo-knockout mouse lines

| Mouse line          | Male          | Female          |
|---------------------|---------------|-----------------|
| C57BL/6J WT         | FMO1, FMO5    | FMO1, FMO3, FMO5|
| Fmo1<sup>−/−</sup>, 2<sup>−/−</sup>, 4<sup>−/−</sup> | FMO5          | FMO3, FMO5      |
| Fmo5<sup>−/−</sup>  | FMO1          | FMO1, FMO3      |
Table 2. Effect of *Fmo* genotype and gender on plasma concentrations of total cholesterol, HDL, and LDL

| Mouse line/strain | Gender       | Total Cholesterol (mmol/L) | HDL (mmol/L) | LDL (mmol/L) |
|-------------------|--------------|-----------------------------|--------------|--------------|
| C57BL/6J WT       | Male         | 2.88 ± 0.09                 | 2.09 ± 0.07  | 0.54 ± 0.02  |
|                   | Female       | 2.41 ± 0.13                 | 1.67 ± 0.08  | 0.48 ± 0.03  |
| Fmo1−/−, 2−/−, 4−/− | Male       | 4.03 ± 0.12***              | 3.05 ± 0.09***| 0.61 ± 0.03  |
|                   | Female       | 3.41 ± 0.13**               | 2.39 ± 0.11**| 0.69 ± 0.01**|
| Fmo5−/−           | Male         | 2.63 ± 0.05*                | 1.88 ± 0.03* | 0.52 ± 0.01  |
|                   | Female       | 2.21 ± 0.11                 | 1.53 ± 0.08  | 0.48 ± 0.03  |
| CD-1              | Male         | 3.76 ± 0.26*                | 2.28 ± 0.21  | 0.66 ± 0.05  |
|                   | Female       | 3.21 ± 0.17*                | 2.14 ± 0.15* | 0.67 ± 0.05* |

*n = 3 to 5; P values are for comparisons of values for each mouse line or strain versus those for C57BL/6J WT mice of the same gender: *P < 0.05, **P < 0.01, ***P < 0.001.*
Table 3. Correlation of plasma total cholesterol concentration or Als with TMAO

|                                         | r     | R^2  | P-value |
|-----------------------------------------|-------|------|---------|
| Plasma total cholesterol vs [TMAO/(TMA+TMAO)]x100 | -0.357 | 0.127 | 0.038   |
| Plasma total cholesterol vs TMAO/creatinine | -0.464 | 0.215 | 0.006   |
| Als vs [TMAO/(TMA+TMAO)]x100             | 0.136  | 0.019 | 0.442   |
| Als vs TMAO/creatinine                   | -0.151 | 0.023 | 0.395   |

Each correlation analysis was performed on a total of 34 animals. Results from individual animals are shown in supplemental Fig. S1
Table 4. Relative difference in abundance of mRNAs in liver of C57BL/6J WT and Fmo1<sup>−/−</sup>, 2<sup>−/−</sup>, 4<sup>−/−</sup> mice

| mRNA                                              | Relative Expression, KO vs WT |
|---------------------------------------------------|-------------------------------|
| **Cholesterol Biosynthesis**                      |                               |
| HMG CoA synthase (HMGCS1)                         | 0.92 ± 0.22                   |
| HMG CoA reductase (HMGR)                          | 0.94 ± 0.25                   |
| Squalene synthase (SS)                            | 1.01 ± 0.12                   |
| Sterol regulatory element-binding protein 2 (SREBP-2) | 2.41 ± 0.36*                 |
| **Cholesterol uptake and transport**              |                               |
| Scavenger receptor class B, member 1 (SR-B1)      | 2.53 ± 0.36**                 |
| ATP-binding cassette sub-family G, member 5 (ABCG 5) | 1.06 ± 0.35                  |
| ATP-binding cassette sub-family G, member 8 (ABCG 8) | 1.24 ± 0.26                  |
| **Bile acid synthesis**                           |                               |
| Cholesterol 7α hydroxylase (CYP7A1)               | 3.12 ± 1.91                   |
| Sterol 27-hydroxylase (CYP27A1)                   | 1.67 ± 0.79                   |
| **Canalicular lipid transport**                   |                               |
| ATP-binding cassette sub-family B, member 11 (ABCB 11) | 4.40 ± 0.95*                  |
Figure 1
Figure 2

- **C57BL/6J WT**
- **Fmo6<sup>1<sup>-,2<sup>-,4<sup>-</sup></sup></sup>
- **Fmo5<sup>-</sup></sup>