Comparative Genome-Wide Association Studies in Mice and Humans for Trimethylamine N-Oxide, a Proatherogenic Metabolite of Choline and 1-Carnitine


Objective—Elevated levels of plasma trimethylamine N-oxide (TMAO), the product of gut microbiome and hepatic-mediated metabolism of dietary choline and 1-carnitine, have recently been identified as a novel risk factor for the development of atherosclerosis in mice and humans. The goal of this study was to identify the genetic factors associated with plasma TMAO levels.

Approach and Results—We used comparative genome-wide association study approaches to discover loci for plasma TMAO levels in mice and humans. A genome-wide association study in the hybrid mouse diversity panel identified a locus for TMAO levels on chromosome 3 ($P=2.37\times10^{-6}$) that colocalized with a highly significant ($P=1.07\times10^{-8}$) cis-expression quantitative trait locus for solute carrier family 30 member 30 member 7. This zinc transporter could thus represent 1 positional candidate gene responsible for the association signal at this locus in mice. A genome-wide association study for plasma TMAO levels in 1973 humans identified 2 loci with suggestive evidence of association ($P=3.0\times10^{-5}$) on chromosomes 1q23.3 and 2p12. However, genotyping of the lead variants at these loci in 1892 additional subjects failed to replicate their association with plasma TMAO levels.

Conclusions—The results of these limited observational studies indicate that, at least in humans, genes play a marginal role in determining TMAO levels and that any genetic effects are relatively weak and complex. Variation in diet or the repertoire of gut microbiota may be more important determinants of plasma TMAO levels in mice and humans, which should be investigated in future studies. (Arterioscler Thromb Vasc Biol. 2014;34:1307-1313.)

Key Words: atherosclerosis ■ genetics ■ humans ■ mice ■ trimethylamine N-oxide

Choline is a key nutrient with various metabolic roles in lipid metabolism and cell membrane structure, and it serves as a precursor for the synthesis of the neurotransmitter acetylcholine. Dietary choline is also an important source of methyl groups that are required for proper metabolism of certain amino acids, such as homocysteine and methionine.

A variety of animal studies have shown that choline deficiency adversely affects brain and cognitive development during fetal and neonatal life, which has led to specific nutritional guidelines recommending adequate intake of choline for infants and pregnant or lactating women.

One route for the initial catabolism of dietary choline (in the form of phosphatidylcholine) is mediated by intestinal microbes and leads to the formation of trimethylamine (TMA). TMA is efficiently absorbed from the gastrointestinal tract and subsequently oxidized by the liver to form TMA N-oxide (TMAO). This latter reaction is catalyzed by one or more of the flavin monoxygenase (FMO) enzymes, of which there are 6 gene family members in higher mammals. Interestingly, mutations of FMO3 that result in deficiency of this enzyme are the cause of trimethylaminuria, otherwise known as fish malodor syndrome. This relatively rare recessive disorder is characterized by the near absence of plasma TMAO levels and highly elevated TMA levels, depending on the functional severity of the mutation in FMO3. The pungent odor of rotting fish that characterizes trimethylaminuria is because of the release of the volatile gas TMA through the breath, skin, and urine.

Recently, we uncovered a novel mechanism through which gut microbiota and hepatic-mediated metabolism of dietary choline promote atherosclerosis and increase the risk of...
coronary artery disease (CAD).\textsuperscript{11,12} These studies demonstrated that plasma TMAO levels in humans were positively associated with the presence of multiple CAD phenotypes, including atherosclerotic plaque burden and future risk of myocardial infarction, stroke, or death in a dose-dependent fashion. A similar relationship was observed between plasma TMAO levels and aortic lesion development among various inbred mouse strains.\textsuperscript{13} More recently, we also demonstrated that l-carnitine, a trimethylamine abundant in red meat, is also metabolized by intestinal microbiota to produce TMAO in mice and humans and that l-carnitine supplementation accelerated atherosclerosis in mice.\textsuperscript{14} Notably, short-term administration of broad-spectrum antibiotics eliminated the production of TMAO in both mice and humans and decreased atherosclerosis in mice. Furthermore, TMAO supplementation in mice, or dietary supplementation of either choline or l-carnitine, in the presence of intact gut microbiota led to alterations in cholesterol and sterol metabolism in multiple distinct compartments, including reduction in reverse cholesterol transport, providing a mechanistic rationale for the association between TMAO levels and atherosclerotic cardiovascular phenotypes.\textsuperscript{14} Taken together, these studies provide evidence consistent with the proatherogenic role of TMAO in mammals and support the notion that gut microbiota plays an obligatory role in the formation TMAO from dietary choline and l-carnitine.

It is reasonable to assume that variation in plasma TMAO levels could also be affected by intrinsic genetic factors of the host. However, with the exception of \textit{FMO3}, the genes that control plasma TMAO levels are not known. Therefore, the aim of the present study was to use comparative genome-wide association study (GWAS) approaches in mice and humans to identify novel genetic determinants associated with plasma TMAO levels.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

#### Association of the \textit{FMO} Cluster With \textit{FMO3} Gene Expression, Plasma TMAO Levels, and CAD in Humans

In previous studies, we reported that \textit{Fmo3} expression varied significantly among inbred strains from the hybrid mouse diversity panel (HMDP) and that a major locus regulating its expression mapped directly over \textit{Fmo3}, suggesting \textit{cis}-acting regulation in mice.\textsuperscript{15} Furthermore, \textit{Fmo3} expression was positively correlated with both plasma TMAO levels and atherosclerosis in mice. Based on these observations, we first used a targeted approach to evaluate whether genetic associations could specifically be observed with the human \textit{FMO} locus on chromosome 1q24.3. To evaluate the association of the \textit{FMO} cluster with hepatic \textit{FMO3} mRNA levels, we used a previously published liver gene expression data set.\textsuperscript{15} These analyses were performed in a subset of 151 white subjects for whom complete gene expression and genotype data were publicly available. Fifty-seven single nucleotide polymorphisms (SNPs) were available for analysis in a specified \(\approx 451\)-kb region containing \textit{FMO3}, \textit{FMO6P}, \textit{FMO2}, \textit{FMO1}, and \textit{FMO4}, including 200 kb of flanking sequence (100 kb from each end). As shown in Figure 1A, 1 SNP (rs2075988) yielded age- and sex-adjusted association with \textit{FMO3} mRNA levels \((P=4.5\times10^{-4})\) that remained significant after correction for multiple testing \((0.05/57; \text{Bonferroni-corrected} P=8.8\times10^{-5})\). \textit{Cis}-expression quantitative trait loci (QTL) were not observed for any other members of the \textit{FMO} gene family at this locus (data not shown).

We next determined whether variation in the \textit{FMO} cluster influenced plasma TMAO levels using the GWAS results from the GeneBank study, a cohort of patients undergoing elective cardiac evaluation at the Cleveland Clinic. Table 1 describes the clinical characteristics of the 3865 individuals used in the present study. As expected for a patient population undergoing coronary angiography as part of their clinical evaluation, a majority of these subjects were men, had prevalent CAD, and were taking lipid-lowering medication (Table 1). In this analysis, 471 SNPs were available, but none were significantly associated with plasma TMAO levels (Figure 1B). Finally, we evaluated whether the \textit{FMO} locus was associated with risk of CAD in the Coronary Artery Disease Genome-wide Replication And Meta-Analysis (CARDIoGRAM) consortium, which represents a meta-analysis of GWAS data from a discovery set of \(\approx 22\,000\) CAD cases and \(\approx 65\,000\) controls.\textsuperscript{16} In CARDIoGRAM, 388 SNPs were available for analyses, of which 21 yielded values of \(P<0.05\) for association with CAD (Figure 1C). However, none of these associations were significant at the Bonferroni-corrected significance threshold \((P=1.3\times10^{-4}; 0.05/388)\). Furthermore, the SNP that exhibited the strongest association with \textit{FMO3} mRNA levels (rs2075988) did not demonstrate evidence for association with either plasma TMAO levels or risk of CAD (Figure 1).

#### GWAS for Plasma TMAO Levels in Mice

To identify novel genetic factors associated with plasma TMAO levels in mammals, we next used the HMDP to perform an unbiased GWAS in mice. This newly developed genetic platform consists of \(\approx 100\) classic inbred and recombinant inbred mouse strains that are maximally informative for association analysis and have been used to perform GWAS for other quantitative traits relevant to human diseases, including atherosclerosis, metabolites, and hepatic mRNA levels.\textsuperscript{17-20} For the present study, we performed a GWAS for plasma TMAO levels in male mice on a chow diet and identified 1 locus on mouse chromosome 3 between 110 and...
highly significant-expression QTL (cis QTL) on chromosome 3 contained several genes and exhibited a significant association with plasma TMAO levels (P = 1.07 × 10−20) for the region centered around the lead SNP (rs2075988; Figure 2C). The colocalization of QTLs for plasma TMAO and Slc30a7 mRNA levels suggests that this zinc transporter could represent 1 positional candidate gene responsible for the association signal at this locus. Suggestive evidence for association of plasma TMAO levels (P = 7.62 × 10−6) was also observed with a region on mouse chromosome 1 at 184 Mb (Figure 2A), although this locus did not achieve genome-wide significance. The lead SNP on chromosome 1 maps to within 40 kb of the lamin β-receptor gene but ≈20 Mb distal from the Fmo3 gene cluster (162–163 Mb).

**Table 1. Clinical Characteristics of the Study Population**

<table>
<thead>
<tr>
<th>Trait</th>
<th>n=3865</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>64±11</td>
</tr>
<tr>
<td>Male/female</td>
<td>6372/2789</td>
</tr>
<tr>
<td>Number with CAD at baseline, %</td>
<td>6776 (76)</td>
</tr>
<tr>
<td>CAD severity</td>
<td></td>
</tr>
<tr>
<td>0 vessels, %</td>
<td>2766 (30)</td>
</tr>
<tr>
<td>1 or 2 vessels, %</td>
<td>3392 (37)</td>
</tr>
<tr>
<td>≥3 vessels, %</td>
<td>3003 (33)</td>
</tr>
<tr>
<td>No. of MACE, %</td>
<td>1285 (14)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>29.6±6.2</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>170±41.1</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>40.0±13.5</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>99.0±33.5</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>151.5±110.1</td>
</tr>
<tr>
<td>TMAO, μmol/L</td>
<td>6.2±13.0</td>
</tr>
<tr>
<td>Taking lipid-lowering medication (%)</td>
<td>5751 (63)</td>
</tr>
</tbody>
</table>

Data are shown as mean±SD or numbers of individuals (%). BMI indicates body mass index; CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MACE, major adverse cardiac events; and TMAO, trimethylamine N-oxide.

**GWAS for Plasma TMAO Levels in Humans**

To complement the mouse studies, we performed a 2-stage GWAS in GeneBank. In the first stage, ≈2.4 million genotyped and imputed autosomal SNPs were evaluated for association with plasma TMAO levels in 1973 subjects with adjustment for age and sex. The quantile-quantile plot for these analyses is shown in Figure 3A, and the observed genomic inflation factor (λ) was 1.007, indicating that the GWAS results are not confounded by underlying population stratification. As shown by the Manhattan plot in Figure 3B, 2 loci with suggestive evidence of association were identified on chromosomes 1q23.3 and 2p12. The lead SNP at the chromosome 1 locus (rs17359359; P = 2.8 × 10−7) is located ≈47 kb telomeric of NUFS2, which is a component of the kinetochore complex that is required for chromosome segregation but, to our knowledge, has no known relationship to TMAO metabolism. This locus is also located ≈8 Mb telomeric to the FMO gene cluster and is clearly distinct because there is no apparent long-range linkage disequilibrium between these 2 loci. By comparison, the lead SNP at the chromosome 2p12 locus (rs885187; P = 2.8 × 10−7) does not map near any known gene. Based on previously observed sex differences in plasma TMAO levels, we also performed a GWAS in men and women separately. However, these analyses did not reveal sex-specific effects on chromosomes 1q23.3 and 2p12 or identify other loci (Figure I in the online-only Data Supplement).

In stage 2, we evaluated the chromosome 1 locus further by genotyping rs17359359 in 1892 additional GeneBank subjects for whom plasma TMAO levels were available. These analyses failed to replicate the association of rs17359359 with plasma TMAO levels in stage 2 (P=0.85), and a combined analysis with all subjects attenuated the overall association (P=1.8×10−4; Table 2). Based on the chromosome 3 locus identified in the
HMDP (Figure 2A and 2B), we used synteny mapping and evaluated the association of plasma TMAO levels with SNPs in the 1-Mb genomic region centered on the human SLC30A7 ortholog located on chromosome 1p21.2. In the GWAS data set (n=1973), 1 SNP located ≈225 kb telomeric to SLC30A7 (rs12402441) demonstrated nominal association (P=0.006) with plasma TMAO levels (Table 2). However, the association of rs12402441 with plasma TMAO levels did not replicate in the stage 2 samples, and a combined analysis with all subjects was not significant (Table 2). In the combined data set, there was also no evidence for an interaction between sex and either SNP on plasma TMAO levels (rs17359359, P_int=0.33; rs12402441, P_int=0.11). The sex-specific effects of rs17359359 and rs12402441 when men and women were analyzed separately are shown in Table I in the online-only Data Supplement.

**Discussion**

Using a combined mouse–human GWAS approach, we sought to identify the genetic determinants of plasma TMAO levels in mammals. Several factors served as the motivation for these studies, including recent studies demonstrating that TMAO can be generated from gut microbiota–mediated metabolism of either dietary choline or l-carnitine and that elevated plasma levels are strongly proatherogenic in both mice and humans. Subsequent reports further showed that plasma TMAO levels in mice are regulated by both sex hormones, which could account, in part, for the observed dimorphism between male and female mice and increased Fmo3 gene expression via bile acid–mediated activation of the farnesoid X receptor. Of note, in humans, no differences in plasma TMAO levels were observed between men and women. The collective results of these comprehensive, albeit limited, observational studies indicate that genes play a marginal role in determining TMAO levels and that any genetic effects are either complex or relatively weak. This is particularly true in humans and raises the possibility that variation in dietary composition or the repertoire of gut microbiota may be more important determinants of plasma TMAO levels.

Using the HMDP, we identified 1 locus on chromosome 3, which contains SLC30A7 and was associated with plasma TMAO levels in male mice at the genome-wide significance threshold. This locus also exhibited evidence for cis gene regulation of SLC30A7, which is a subfamily member of the cation diffusion facilitator family of transporters and has essential functions in dietary zinc absorption. Although a biological mechanism for how SLC30A7 would regulate plasma TMAO levels is not directly evident, it has been reported that the zinc finger protein, YY1, regulates the expression of both rabbit and human FMO1. Interestingly, the activity of certain bacterial monooxygenases has also been shown to use zinc.
as a cofactor. However, more detailed functional studies will be required to determine whether Slc30a7 could affect TMAO levels by influencing zinc-mediated activity of ≥1 of the FMOs in mice. We also note that although this locus on mouse chromosome 3 also yielded a highly significant cis-expression QTL for Slc30a7, we cannot exclude the possibility that another gene in this interval harboring functional coding variation is the causal genetic factor for plasma TMAO levels. Because our GWAS with the HMDP was only with male mice, it is also possible that inclusion of females would provide additional support for the association of the Slc30a7 locus, as well as identify other genomic regions controlling plasma TMAO levels that are potentially specific to females. For example, we previously reported that plasma TMAO levels are several-fold higher in female mice compared with males, a portion of which is attributable to differences in sex hormones.

As a comparative analysis to our studies with the HMDP, we also performed a GWAS for plasma TMAO levels in the GeneBank cohort. This analysis identified 2 suggestive loci in the discovery phase, but our attempt to replicate the NUF2 locus on chromosome 1 was unsuccessful. Based on the Slc30a7 locus identified in the mouse GWAS, we also evaluated the syntenic region on human chromosome 1p21.2 for association with plasma TMAO levels. Although 1 SNP in this region yielded nominal association with plasma TMAO levels in humans, this signal also did not replicate in the stage 2 samples. Given the high concordance rate (>98.8%) for genotypes of the same DNA samples used in stages 1 and 2, we do not think technical variability to have been a factor for the lack of replication in stage 2 and conclude that these loci likely represent false-positive signals. However, despite the lack of genetic variation around the human SLC30A7 ortholog being associated with plasma TMAO levels, it is possible that this transporter still plays a biological role in regulating TMAO levels in both species. Furthermore, we did not obtain any evidence for sex-specific effects at these loci or identify any others when the GWAS was performed in men and women separately. Taken together, these results suggest that variation in plasma TMAO levels in humans may be because of weak genetic effects and that larger sample sizes will be required to identify the underlying regulatory factors.

To date, FMO3 is the only genetic factor known to affect plasma TMAO levels in humans. FMO3 is composed of 10 exons spanning 26.9 kb on chromosome 1q24.3 and encodes a 532-residue enzyme. At the amino acid level, FMO3 shares ≥79% homology with the mouse Fmo3 protein and other members of the human FMO family. Interestingly, we previously demonstrated that FMO1, FMO2, and FMO3 were able...
Table 2. Effect of Single Nucleotide Polymorphisms Identified Through GWAS in Humans and Mice on Plasma TMAO Levels in the GeneBank Cohort

<table>
<thead>
<tr>
<th>Stage</th>
<th>rs1739359</th>
<th>rs12402441</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG</td>
<td>AG</td>
</tr>
<tr>
<td>GWAS</td>
<td>(n=1727)</td>
<td>(n=1459)</td>
</tr>
<tr>
<td></td>
<td>5.3±8.0</td>
<td>8.3±20.3</td>
</tr>
<tr>
<td>Replication</td>
<td>(n=186)</td>
<td>(n=9)</td>
</tr>
<tr>
<td></td>
<td>8.3±19.6</td>
<td>6.8±3.5</td>
</tr>
<tr>
<td>Combined</td>
<td>(n=3222)</td>
<td>(n=424)</td>
</tr>
</tbody>
</table>

Mean (±SD) plasma TMAO levels (µmol/L) are shown as a function of genotype. GWAS indicates genome-wide association study; and TMAO, trimethylamine N-oxide.

*P values obtained using linear regression with natural log-transformed values after adjustment for age and sex.

to generate TMAO from TMA but that FMO3 was by far the most active family member.13 Because the Mendelian disease trimethylaminuria is caused by rare mutations that lead to FMO3 deficiency, we leveraged our own data in GeneBank and those from public sources to evaluate whether common variants at the FMO locus were associated with FMO3 gene expression, plasma TMAO levels, and risk of CAD. However, these analyses in humans did not reveal any strong associations with SNPs surrounding FMO3. It is possible that the imputed genotypes from the GWAS data we used did not provide sufficient coverage of the variation around FMO3 (or the entire FMO locus). Based on data for subjects of European ancestry from the 1000 Genomes Project, 59 tagging SNPs with minor allele frequencies ≥1% would cover FMO3 at an r²≥0.8. However, only 15 tagging SNPs across FMO3 were present in our analyses of plasma TMAO levels in GeneBank. In addition, rare variants in FMO3 that could influence gene expression, TMAO production, and risk of CAD would also not necessarily be represented by our imputed GWAS data. By comparison, our previous studies in mice revealed a relatively strong cis-expression QTL for Fmo3 expression over the Fmo locus. However, the present analyses for plasma TMAO levels in the HMDP did not yield association with the Fmo locus at the genome-wide level (data not shown). These observations suggest that the relationship between FMO3 gene expression and plasma TMAO levels in both mice and humans is complex and that other regulatory mechanisms, including post-transcriptional and post-translational modifications, may exist.

The discordance between rare mutations in FMO3 that dramatically reduce plasma TMAO levels and the lack of common genetic determinants associated with this metabolite implies that variation in TMAO levels in humans and mice may be influenced by other factors, such as gut microbial and dietary composition. For example, we previously defined the relative abundances of bacteria at each taxonomic level in relation to the production of TMAO through pyrosequencing of 16S rRNA genes in both mice and humans. One notable difference in these analyses was the source of gut bacteria because the contents of the cecum were used for mice, whereas stool samples were used for the human analyses. This may explain, at least in part, why a direct comparison of bacterial taxa associated with plasma TMAO concentrations did not identify any genre common to both species. This observation is consistent with previous reports indicating that microbes identified from the distal gut of the mouse do not necessarily represent those typically detected in humans.14,25 Thus, although sharing many taxa, the microbial composition observed in mice is architecturally and globally different than in humans. Despite these differences, we were still able to demonstrate associations between dietary patterns (eg, vegan/vegetarian versus omnivore or normal chow versus choline/carnitine supplemented) and both plasma TMAO levels and proportions of specific taxa of fecal microbes in humans and cecal microbes in mice.14 These observations suggest that high dietary intake of l-carnitine or choline would lead to increased plasma TMAO levels, particularly if specific bacterial taxa that metabolize these nutrients to TMA are present in the gut. It is possible that the effects of host genetic factors would also manifest under such dietary conditions. However, compared with mice housed under standardized environmental conditions, the diet in free-ranging humans is far more heterogeneous, which would add further complexity and diversity to any potential interactions with the gut microbiome.

Despite our comprehensive efforts to identify loci associated with plasma TMAO levels, we also note several potential limitations of our study. First, we used GWAS approaches in mice and humans that mostly test association with common genetic variation, which would not necessarily detect the effects of rare variants on plasma TMAO levels. Second, our human GWAS was performed in subjects of European ancestry, and it is possible that genetic variants that are either specific to or present at higher frequency in other ethnicities could influence TMAO levels. Third, although including ≈100 inbred strains, it is still possible that the HMDP does provide sufficient genetic variation to capture all of the effects on plasma TMAO levels in mice compared with the substantially greater genetic diversity present in outbred human populations. In addition, the pathways leading to variation in TMAO levels in mice and humans may not be entirely similar. Finally, as discussed above, variability in dietary composition, particularly in humans, and the gut microbiome clearly factor into plasma TMAO levels and are thus likely to be strong confounding variables that our study did not take into consideration.

Conclusions

Our results indicate that Slc30a7 may represent a novel gene for TMAO levels in mice but that the contribution of genetic factors in humans is more complex. These observations
suggest that the inter-relationships between dietary choline and l-carnitine levels with the composition of gut microbes are perhaps more likely determinants of variation in plasma TMAO levels. Exploring such interactions as part of future studies may help to identify the intrinsic genetic factors that influence plasma TMAO levels and their influence on the development of atherosclerosis.

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**Disclosures**

S.L. Hazen is named as coinventor on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics. He reports having been paid as a consultant or speaker for the following companies: Cleveland Heart Laboratory, Inc, Esperion, Liposciences Inc, Merck & Co, Inc, and Pfizer Inc. He reports he has received research funds from Abbott, Cleveland Heart Laboratory, Esperion, and Liposciences, Inc. He has the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics from Abbott Laboratories, Cleveland Heart Laboratory, Inc, Frantz Biomarkers, LLC, and Siemens. The other authors report no conflicts.

**References**


**Significance**

Elevated plasma levels of trimethylamine *N*-oxide (TMAO), a metabolite generated from dietary choline and carnitine by intestinal bacteria, have recently been identified as a novel risk factor for coronary artery disease. Notably, elimination of bacteria in the gut through administration of antibiotics reduced TMAO levels in mice and humans and decreased atherosclerosis in mice. However, the genes that control plasma TMAO levels are not well defined. The present study uses a comparative genome-wide association study approach in mice and humans to identify the genetic determinants of plasma TMAO levels. In mice, genetic variants near solute carrier family 30 member 7 were significantly associated with plasma TMAO levels, whereas no locus was identified in humans. Our findings suggest that, at least in humans, plasma TMAO levels are under complex genetic regulation, that the effects of any underlying genes are relatively weak, and that variation in gut bacteria may be more important in determining TMAO levels.
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CARDIoGRAM Consortium

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Materials and Methods

**Study Population.** The Cleveland Clinic GeneBank study is a single site sample repository generated from consecutive patients undergoing elective diagnostic coronary angiography or elective cardiac computed tomographic angiography with extensive clinical and laboratory characterization and longitudinal observation. Subject recruitment occurred between 2001 and 2006. Ethnicity was self-reported and information regarding demographics, medical history, and medication use was obtained by patient interviews and confirmed by chart reviews. All clinical outcome data were verified by source documentation. CAD was defined as adjudicated diagnoses of stable or unstable angina, MI (adjudicated definition based on defined electrocardiographic changes or elevated cardiac enzymes), angiographic evidence of ≥ 50% stenosis of one or more major epicardial vessel, and/or a history of known CAD (documented MI, CAD, or history of revascularization). Prospective cardiovascular risk was assessed by the incidence of major adverse cardiac events (MACE) during three years of follow-up from the time of enrollment, which included nonfatal MI, nonfatal stroke, and all-cause mortality. Nonfatal events were defined as MI or stroke in patients who survived at least 48 hours following the onset of symptoms. Adjudicated outcomes ascertained over the ensuing 3 years for all subjects following enrollment were confirmed using source documentation. The GeneBank Study has been used previously for discovery and replication of novel genes and risk factors for atherosclerotic disease 1-6.

**Animal Husbandry.** Male mice were purchased from The Jackson Laboratory and housed in vivaria accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Animals were on a 12hr light–dark cycle and maintained on a chow diet with 6% fat by
weight (Ralston-Purina Co.) until sacrifice at 16 weeks of age. Following a 16 hr fast, mice were bled retro-orbitally under isoflurane anesthesia and plasma TMAO levels were determined as described below. All procedures were approved by the UCLA Institutional Animal Care and Use Committee.

**Measurement of Plasma TMAO Levels.** TMAO levels in human and mouse plasma was quantitated using stable isotope dilution high performance liquid chromatography (HPLC) with on line electro spray ionization tandem mass spectrometry on an API 5000 triple quadrupole mass spectrometer (AB SCIEX, Foster City, CA) interfaced with a Cohesive HPLC (Franklin, MA) equipped with a phenyl column (4.6 × 2505mm, 5μm RexChrom Phenyl; Regis, Morton Grove, IL). Separation was performed using a gradient starting from 10mM ammonium formate over 0.5 min, then to 5mM ammonium formate, 25 % methanol and 0.1 % formic acid over 3 min, held for 8 min, followed by 100% methanol and water washing for 3 min. TMAO was monitored in multiple reaction monitoring (MRM) mode using characteristic parent-daughter ion transitions at m/z 76→58. The internal standards TMAO-trimethyl-d9 and choline-trimethyl-d9, were added to plasma samples prior to protein precipitation and similarly monitored in MRM mode at m/z 85→68 and m/z 113→69. Various concentrations of TMAO standards and a fixed amount of internal standards were spiked into control plasma to prepare the calibration curves for quantification of plasma analytes.

**Genome-wide Association Mapping and Significance Threshold in Mice.** A GWAS for plasma TMAO levels in mice was carried out using the Hybrid Mouse Diversity Panel (HMDP). The HMDP is comprised of ~100 inbred and recombinant inbred (RI) strains that have
previously been assembled for high-resolution association mapping and subjected to extensive metabolic, transcriptional, and proteomic profiling. Genotypes of single nucleotide polymorphisms (SNPs) for the inbred strains in the HMDP were obtained from the Broad Institute (www.broadinstitute.org/mouse/hapmap) and combined with the genotypes from the Wellcome Trust Center for Human Genetics (WTCHG). Genotypes of RI strains at the Broad SNPs were inferred from the WTCHG genotypes by imputing alleles at polymorphic SNPs among parental strains, with ambiguous genotypes labeled as “missing.” Of the 140,000 SNPs available, 107,145 were informative with an allele frequency greater than 5% and used in the present GWAS analysis.

We applied the following linear mixed model to account for the population structure and genetic relatedness among strains: \( y = \mu + x\beta + u + e \) where \( \mu \) represents mean TMAO levels, \( x \) represents the SNP effect, \( u \) represents random effects due to genetic relatedness with \( \text{Var}(u) = \sigma_g^2 K \) and \( \text{Var}(e) = \sigma_e^2 I \), where \( K \) represents an identity-by-descent kinship matrix across all genotypes. A restricted maximum likelihood estimate of \( \sigma_g^2 \) and \( \sigma_e^2 \) were computed using an efficient mixed model association algorithm (EMMA), and the association mapping was performed based on the estimated variance component with a standard F test to test \( \beta \neq 0 \).

Genome-wide significance threshold in the HMDP was determined by the family-wise error rate as the probability of observing one or more false positives across all SNPs per phenotype. We ran 100 different sets of permutation tests and parametric bootstrapping of size 1,000 and observed that the mean and standard error of the genome-wide significance threshold at FWER of 0.05 were \( 3.9 \times 10^{-6} \pm 0.3 \times 10^{-6} \) and \( 4.0 \times 10^{-6} \pm 0.3 \times 10^{-6} \), respectively. This is approximately an order of magnitude larger than the threshold obtained by Bonferroni correction.
(4.6 \times 10^{-7}), which would be an overly conservative estimate of significance since nearby SNPs among inbred mouse strains are highly correlated with each other.

**Genotyping.** Genome-wide genotyping of SNPs in humans was performed on the Affymetrix Genome-Wide Human Array 6.0 chip. Using these data and those from 120 phased chromosomes from the HapMap CEU samples (HapMap r22 release, NCBI build 36), genotypes were imputed for untyped autosomal SNPs across the genome using MACH 1.0 software. All imputations were done on the forward (+) strand using 562,554 genotyped SNPS that had passed quality control (QC) filters. QC filters for the imputed dataset excluded SNPs with HWE p-values < 0.0001, call rate less than 97% or minor allele frequencies < 1%, and individuals with less than 90% call rates. This resulted in 2,421,770 autosomal SNPs that were available for a GWAS analysis in 1973 GeneBank subjects. Genotyping of the two SNPs selected for replication in stage 2 was performed using the TaqMan Allelic Discrimination system from Applied Biosystems, Inc. (Foster City, CA). In control samples from the GWAS dataset that were also genotyped by Taqman, the concordance rate with genotypes obtained from the Affymetrix chip was >98.8% for the two replication SNPs.

**Statistical Analyses.** A GWAS for plasma TMAO levels in humans was carried out with adjustment for age and sex. Linear regression analyses were performed using natural log transformed values under an additive genetic model. A publicly available liver expression dataset was used to evaluate the association of SNPs in the region containing the FMO gene cluster for cis expression quantitative trait loci (eQTL). Seventy nine SNPs were available in this dataset for the FMO region, of which 22 were excluded due to missing genotypes. Linear
regression was carried out with the remaining 57 SNPs under an additive genetic model with adjustment for age and sex to identify eQTLs for hepatic FMO3 mRNA levels. The results of the Coronary Artery Disease Genome-wide Replication And Meta-Analysis (CARDIoGRAM) Consortium were used to determine whether variants at the FMO locus were associated with CAD. CARDIoGRAM represents a GWAS meta-analysis of CAD comprising a discovery set of ~22,000 cases and ~65,000 controls, in which logistic regression was first used in each cohort to test for association with CAD using a log-additive model with adjustment for age and gender and taking into account the uncertainty of possibly imputed genotypes. Subsequently, a meta-analysis was performed separately for every SNP from each study that passed the quality control criteria using a fixed effect model with inverse variance weighting. The results of this default meta-analysis were used to determine whether SNPs spanning the FMO cluster on chromosome 1 were associated with CAD. All genetic analyses were performed using PLINK 1.07 or SAS 9.3 (SAS Institute Inc, Cary, NC).

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The CARDIoGRAM Consortium

Executive Committee: Sekar Kathiresan\textsuperscript{1,2,3}, Muredach P. Reilly\textsuperscript{4}, Nilesh J. Samani\textsuperscript{5,6}, Heribert Schunkert\textsuperscript{7,79}

Executive Secretary: Jeanette Erdmann\textsuperscript{7,79}

Steering Committee: Themistocles L. Assimes\textsuperscript{8}, Eric Boerwinkle\textsuperscript{9}, Jeanette Erdmann\textsuperscript{7,79} Alistair Hall\textsuperscript{10}, Christian Hengstenberg\textsuperscript{11}, Sekar Kathiresan\textsuperscript{1,2,3}, Inke R. König\textsuperscript{12}, Reijo Laaksonen\textsuperscript{13}, Ruth McPherson\textsuperscript{14}, Muredach P. Reilly\textsuperscript{4}, Nilesh J. Samani\textsuperscript{5,6}, Heribert Schunkert\textsuperscript{7,79}, John R. Thompson\textsuperscript{15}, Unnur Thorsteinsdottir\textsuperscript{16,17}, Andreas Ziegler\textsuperscript{12}

Statisticians: Inke R. König\textsuperscript{12} (chair), John R. Thompson\textsuperscript{15} (chair), Devin Absher\textsuperscript{18}, Li Chen\textsuperscript{19}, L. Adrienne Cupples\textsuperscript{20,21}, Eran Halperin\textsuperscript{22}, Mingyao Li\textsuperscript{23}, Kiran Musunuru\textsuperscript{1,2,3}, Michael Preuss\textsuperscript{12,7}, Arne Schillert\textsuperscript{12}, Gudmar Thorleifsson\textsuperscript{16}, Benjamin F. Voight\textsuperscript{2,3,24}, George A. Wells\textsuperscript{25}

Writing group: Themistocles L. Assimes\textsuperscript{8}, Panos Deloukas\textsuperscript{26}, Jeanette Erdmann\textsuperscript{7,79}, Hilma Holm\textsuperscript{16}, Sekar Kathiresan\textsuperscript{1,2,3}, Inke R. König\textsuperscript{12}, Ruth McPherson\textsuperscript{14}, Muredach P. Reilly\textsuperscript{4}, Robert Roberts\textsuperscript{14}, Nilesh J. Samani\textsuperscript{5,6}, Heribert Schunkert\textsuperscript{7,79}, Alexandre F. R. Stewart\textsuperscript{14}

ADVANCE: Devin Absher\textsuperscript{18}, Themistocles L. Assimes\textsuperscript{8}, Stephen Fortmann\textsuperscript{8}, Alan Go\textsuperscript{27}, Mark Hlatky\textsuperscript{8}, Carlos Iribarren\textsuperscript{27}, Joshua Knowles\textsuperscript{8}, Richard Myers\textsuperscript{18}, Thomas Quertermous\textsuperscript{8}, Steven Sidney\textsuperscript{27}, Neil Risch\textsuperscript{28}, Hua Tang\textsuperscript{29}

CADomics: Stefan Blankenberg\textsuperscript{30*}, Tanja Zeller\textsuperscript{30*}, Arne Schillert\textsuperscript{12}, Philipp Wild\textsuperscript{30}, Andreas Ziegler\textsuperscript{12}, Renate Schnabel\textsuperscript{30*}, Christoph Sinning\textsuperscript{30*}, Karl Lackner\textsuperscript{31}, Laurence Tiret\textsuperscript{32}, Viviane Nicaud\textsuperscript{32}, Francois Cambien\textsuperscript{32}, Christoph Bickel\textsuperscript{30}, Hans J. Rupprecht\textsuperscript{30}, Claire Perret\textsuperscript{32}, Carole Proust\textsuperscript{32}, Thomas Münzel\textsuperscript{30}

CHARGE: Maja Barbalic\textsuperscript{33}, Joshua Bis\textsuperscript{34}, Eric Boerwinkle\textsuperscript{9}, Ida Yii-Der Chen\textsuperscript{35}, L. Adrienne Cupples\textsuperscript{20,21}, Abbas Dehghan\textsuperscript{36}, Serkalem Demissie-Banjaw\textsuperscript{37,21}, Aaron Folsom\textsuperscript{38}, Nicole
Glazer\textsuperscript{39}, Vilmundur Gudnason\textsuperscript{40,41}, Tamara Harris\textsuperscript{42}, Susan Heckbert\textsuperscript{43}, Daniel Levy\textsuperscript{21}, Thomas Lumley\textsuperscript{44}, Kristin Marcianti\textsuperscript{45}, Alanna Morrison\textsuperscript{46}, Christopher J. O’Donnell\textsuperscript{47}, Bruce M. Psaty\textsuperscript{48}, Kenneth Rice\textsuperscript{49}, Jerome I. Rotter\textsuperscript{35}, David S. Siscovick\textsuperscript{50}, Nicholas Smith\textsuperscript{13}, Albert Smith\textsuperscript{40,41}, Kent D. Taylor\textsuperscript{35}, Cornelia van Duijn\textsuperscript{36}, Kelly Volcik\textsuperscript{46}, Jaqueline Whitteman\textsuperscript{36}, Vasan Ramachandran\textsuperscript{51}, Albert Hofman\textsuperscript{36}, Andre Uitterlinden\textsuperscript{52,36}
deCODE: Solveig Gretarsdottir\textsuperscript{16}, Jeffrey R. Gulcher\textsuperscript{16}, Hilma Holm\textsuperscript{16}, Augustine Kong\textsuperscript{16}, Kari Stefansson\textsuperscript{16,17}, Gudmundur Thorgeirsson\textsuperscript{53,17}, Karl Andersen\textsuperscript{53,17}, Gudmar Thorleifsson\textsuperscript{16}, Unnur Thorsteinsdottir\textsuperscript{16,17}

GERMIFS I and II: Jeanette Erdmann\textsuperscript{7,79}, Marcus Fischer\textsuperscript{11}, Anika Grosshennig\textsuperscript{12,7}, Christian Hengstenberg\textsuperscript{11}, Inke R. König\textsuperscript{12}, Wolfgang Lieb\textsuperscript{54}, Patrick Linsel-Nitschke\textsuperscript{7}, Michael Preuss\textsuperscript{12,7}, Klaus Stark\textsuperscript{11}, Stefan Schreiber\textsuperscript{55}, H.-Erich Wichmann\textsuperscript{56,58,59}, Andreas Ziegler\textsuperscript{12}, Heribert Schunkert\textsuperscript{7,79}

GERMIFS III (KORA): Zouhair Aherrahrou\textsuperscript{7,79}, Petra Bruse\textsuperscript{7,79}, Angela Doering\textsuperscript{56}, Jeanette Erdmann\textsuperscript{7,79}, Christian Hengstenberg\textsuperscript{11}, Thomas Illig\textsuperscript{56}, Norman Klopp\textsuperscript{56}, Inke R. König\textsuperscript{12}, Patrick Diemert\textsuperscript{7}, Christina Loley\textsuperscript{12,7}, Anja Medack\textsuperscript{7,79}, Christina Meisinger\textsuperscript{56}, Thomas Meiting\textsuperscript{57,60}, Janja Nahrstedt\textsuperscript{12,7}, Annette Peters\textsuperscript{56}, Michael Preuss\textsuperscript{12,7}, Klaus Stark\textsuperscript{11}, Arnika K. Wagner\textsuperscript{7}, H.-Erich Wichmann\textsuperscript{56,58,59}, Christina Willenborg\textsuperscript{7,79}, Andreas Ziegler\textsuperscript{12}, Heribert Schunkert\textsuperscript{7,79}

LURIC/AtheroRemo: Bernhard O. Böhm\textsuperscript{61}, Harald Dobnig\textsuperscript{62}, Tanja B. Grammer\textsuperscript{63}, Eran Halperin\textsuperscript{22}, Michael M. Hoffmann\textsuperscript{64}, Marcus Kleber\textsuperscript{65}, Reijo Laaksonen\textsuperscript{13}, Winfried März\textsuperscript{63,66,67}, Andreas Meinitzer\textsuperscript{66}, Bernhard R. Winkelmann\textsuperscript{68}, Stefan Pilz\textsuperscript{62}, Wilfried Renner\textsuperscript{66}, Hubert Scharnagl\textsuperscript{66}, Tatjana Stojakovic\textsuperscript{66}, Andreas Tomaschitz\textsuperscript{62}, Karl Winkler\textsuperscript{64}
MIGen: Benjamin F. Voight2,3,24, Kiran Musunuru1,2,3, Candace Guiducci3, Noel Burtt3, Stacey B. Gabriel3, David S. Siscovick50, Christopher J. O’Donnell47, Roberto Elosua69, Leena Peltonen49, Veikko Salomaa70, Stephen M. Schwartz50, Olle Melander26, David Altshuler71,3, Sekar Kathiresan1,2,3

OHGS: Alexandre F. R. Stewart14, Li Chen19, Sonny Dandona14, George A. Wells25, Olga Jarinova14, Ruth McPherson14, Robert Roberts14

PennCATH/MedStar: Muredach P. Reilly4, Mingyao Li23, Liming Qu23, Robert Wilensky4, William Matthai4, Hakon H. Hakonarson72, Joe Devaney73, Mary Susan Burnett73, Augusto D. Pichard73, Kenneth M. Kent73, Lowell Satler73, Joseph M. Lindsay73, Ron Waksman73, Christopher W. Knouff74, Dawn M. Waterworth74, Max C. Walker74, Vincent Mooser74, Stephen E. Epstein73, Daniel J. Rader75,4

WTCCC: Nilesh J. Samani5,6, John R. Thompson15, Peter S. Braund5, Christopher P. Nelson5, Benjamin J. Wright76, Anthony J. Balmforth77, Stephen G. Ball78, Alistair S. Hall10, Wellcome Trust Case Control Consortium

CARDioGRAM Affiliations

1 Cardiovascular Research Center and Cardiology Division, Massachusetts General Hospital, Boston, MA, USA; 2 Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA, USA; 3 Program in Medical and Population Genetics, Broad Institute of Harvard and Massachusetts Institute of Technology (MIT), Cambridge, MA, USA; 4 The Cardiovascular Institute, University of Pennsylvania, Philadelphia, PA, USA; 5 Department of Cardiovascular
Sciences, University of Leicester, Glenfield Hospital, Leicester, UK; 6 Leicester National Institute for Health Research Biomedical Research Unit in Cardiovascular Disease, Glenfield Hospital, Leicester, LE3 9QP, UK; 7 Medizinische Klinik II, Universität zu Lübeck, Lübeck, Germany; 8 Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA; 9 University of Texas Health Science Center, Human Genetics Center and Institute of Molecular Medicine, Houston, TX, USA; 10 Division of Cardiovascular and Neuronal Remodelling, Multidisciplinary Cardiovascular Research Centre, Leeds Institute of Genetics, Health and Therapeutics, University of Leeds, UK; 11 Klinik und Poliklinik für Innere Medizin II, Universität Regensburg, Regensburg, Germany; 12 Institut für Medizinische Biometrie und Statistik, Universität zu Lübeck, Lübeck, Germany; 13 Science Center, Tampere University Hospital, Tampere, Finland; 14 The John & Jennifer Ruddy Canadian Cardiovascular Genetics Centre, University of Ottawa Heart Institute, Ottawa, Canada; 15 Department of Health Sciences, University of Leicester, Leicester, UK; 16 deCODE Genetics, 101 Reykjavik, Iceland; 17 University of Iceland, Faculty of Medicine, 101 Reykjavik, Iceland; 18 Hudson Alpha Institute, Huntsville, Alabama, USA; 19 Cardiovascular Research Methods Centre, University of Ottawa Heart Institute, 40 Ruskin Street, Ottawa, Ontario, Canada, K1Y 4W7; 20 Department of Biostatistics, Boston University School of Public Health, Boston, MA USA; 21 National Heart, Lung and Blood Institute's Framingham Heart Study, Framingham, MA, USA; 22 The Blavatnik School of Computer Science and the Department of Molecular Microbiology and Biotechnology, Tel-Aviv University, Tel-Aviv, Israel, and the International Computer Science Institute, Berkeley, CA, USA; 23 Biostatistics and Epidemiology, University of Pennsylvania, Philadelphia, PA, USA; 24 Department of Medicine, Harvard Medical School, Boston, MA, USA; 25 Research Methods, Univ Ottawa Heart Inst; 26 Department of Clinical Sciences,
Hypertension and Cardiovascular Diseases, Scania University Hospital, Lund University, Malmö, Sweden; 27 Division of Research, Kaiser Permanente, Oakland, CA, USA; 28 Institute for Human Genetics, University of California, San Francisco, San Francisco, CA, USA; 29 Dept Cardiovascular Medicine, Cleveland Clinic; 30 Medizinische Klinik und Poliklinik, Johannes-Gutenberg Universität Mainz, Universitätsmedizin, Mainz, Germany; 31 Institut für Klinische Chemie und Laboratoriumsmediizin, Johannes-Gutenberg Universität Mainz, Universitätsmedizin, Mainz, Germany; 32 INSERM UMRS 937, Pierre and Marie Curie University (UPMC, Paris 6) and Medical School, Paris, France; 33 University of Texas Health Science Center, Human Genetics Center, Houston, TX, USA; 34 Cardiovascular Health Research Unit and Department of Medicine, University of Washington, Seattle, WA USA; 35 Cedars-Sinai Medical Center, Medical Genetics Institute, Los Angeles, CA, USA; 36 Erasmus Medical Center, Department of Epidemiology, Rotterdam, The Netherlands; 37 Boston University, School of Public Health, Boston, MA, USA; 38 University of Minnesota School of Public Health, Division of Epidemiology and Community Health, School of Public Health (A.R.F.), Minneapolis, MN, USA; 39 University of Washington, Cardiovascular Health Research Unit and Department of Medicine, Seattle, WA, USA; 40 Icelandic Heart Association, Kopavogur Iceland; 41 University of Iceland, Reykjavik, Iceland; 42 Laboratory of Epidemiology, Demography, and Biometry, Intramural Research Program, National Institute on Aging, National Institutes of Health, Bethesda MD, USA; 43 University of Washington, Department of Epidemiology, Seattle, WA, USA; 44 University of Washington, Department of Biostatistics, Seattle, WA, USA; 45 University of Washington, Department of Internal Medicine, Seattle, WA, USA; 46 University of Texas, School of Public Health, Houston, TX, USA; 47 National Heart, Lung and Blood Institute, Framingham Heart Study, Framingham, MA and Cardiology Division,
Massachusetts General Hospital, Boston, MA, USA; 48 Center for Health Studies, Group Health, Departments of Medicine, Epidemiology, and Health Services, Seattle, WA, USA; 49 The Wellcome Trust Sanger Institute, The Wellcome Trust Genome Campus, Hinxton, Cambridge, UK; 50 Cardiovascular Health Research Unit, Departments of Medicine and Epidemiology, University of Washington, Seattle; 51 Boston University Medical Center, Boston, MA, USA; 52 Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands; 53 Department of Medicine, Landspitali University Hospital, 101 Reykjavik, Iceland; 54 Boston University School of Medicine, Framingham Heart Study, Framingham, MA, USA; 55 Institut für Klinische Molekularbiologie, Christian-Albrechts Universität, Kiel, Germany; 56 Institute of Epidemiology, Helmholtz Zentrum München – German Research Center for Environmental Health, Neuherberg, Germany; 57 Institut für Humangenetik, Helmholtz Zentrum München, Deutsches Forschungszentrum für Umwelt und Gesundheit, Neuherberg, Germany; 58 Institute of Medical Information Science, Biometry and Epidemiology, Ludwig-Maximilians-Universität München, Germany; 59 Klinikum Grosshadern, Munich, Germany; 60 Institut für Humangenetik, Technische Universität München, Germany; 61 Division of Endocrinology and Diabetes, Graduate School of Molecular Endocrinology and Diabetes, University of Ulm, Ulm, Germany; 62 Division of Endocrinology, Department of Medicine, Medical University of Graz, Austria; 63 Synlab Center of Laboratory Diagnostics Heidelberg, Heidelberg, Germany; 64 Division of Clinical Chemistry, Department of Medicine, Albert Ludwigs University, Freiburg, Germany; 65 LURIC non profit LLC, Freiburg, Germany; 66 Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University Graz, Austria; 67 Institute of Public Health, Social and Preventive Medicine, Medical Faculty Manneim, University of Heidelberg, Germany; 68 Cardiology Group Frankfurt-Sachsenhausen, Frankfurt, Germany; 69
Cardiovascular Epidemiology and Genetics Group, Institut Municipal d’Investigació Mèdica, Barcelona; Ciber Epidemiología y Salud Pública (CIBERSP), Spain; 70 Chronic Disease Epidemiology and Prevention Unit, Department of Chronic Disease Prevention, National Institute for Health and Welfare, Helsinki, Finland; 71 Department of Molecular Biology and Center for Human Genetic Research, Massachusetts General Hospital, Harvard Medical School, Boston, USA; 72 The Center for Applied Genomics, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania, USA; 73 Cardiovascular Research Institute, Medstar Health Research Institute, Washington Hospital Center, Washington, DC 20010, USA; 74 Genetics Division and Drug Discovery, GlaxoSmithKline, King of Prussia, Pennsylvania 19406, USA; 75 The Institute for Translational Medicine and Therapeutics, School of Medicine, University of Pennsylvania, Philadelphia, PA, USA; 76 Department of Cardiovascular Surgery, University of Leicester, Leicester, UK; 77 Division of Cardiovascular and Diabetes Research, Multidisciplinary Cardiovascular Research Centre, Leeds Institute of Genetics, Health and Therapeutics, University of Leeds, Leeds, LS2 9JT, UK; 78 LIGHT Research Institute, Faculty of Medicine and Health, University of Leeds, Leeds, UK; 79 Deutsches Zentrum für Herz-Kreislauft-Forschung (DZHK), Universität zu Lübeck, Lübeck, Germany

*Present Address: Department of General and Interventional Cardiology, University Heart Center Hamburg, Hamburg, Germany.

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Supplemental Table I. Sex-stratified Association of SNPs Identified in Human and Mouse GWAS for Plasma TMAO Levels.

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<td></td>
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<tr>
<td>Males</td>
<td>GWAS</td>
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<tr>
<td></td>
<td>5.0 ± 6.5</td>
<td>8.9 ± 24.9</td>
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<tr>
<td></td>
<td>(n=1231)</td>
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<td></td>
<td>Replication</td>
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<td></td>
<td>6.3 ± 11.8</td>
<td>9.9 ± 30.0</td>
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<td></td>
<td>(n=836)</td>
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<td></td>
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<td></td>
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<tr>
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<tr>
<td></td>
<td>GWAS</td>
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<td></td>
<td>6.1 ± 10.8</td>
<td>6.8 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>(n=496)</td>
<td>(n=84)</td>
</tr>
<tr>
<td></td>
<td>Replication</td>
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</tr>
<tr>
<td></td>
<td>6.9 ± 17.7</td>
<td>6.3 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>(n=612)</td>
<td>(n=71)</td>
</tr>
<tr>
<td></td>
<td>Combined</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.6 ± 15.0</td>
<td>6.6 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>(n=1108)</td>
<td>(n=155)</td>
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</table>

Mean (± SD) plasma TMAO levels (µM) are shown as a function of genotype.

aP-values obtained using linear regression with natural log transformed values after adjustment for age.
Supplemental Figure I

A

Males

B

Females
Supplemental Figure I. Sex-stratified GWAS results for plasma TMAO levels. Manhattan plots derived from GWAS analyses performed in males (A) and females (B) separately did not reveal sex-specific effects on chromosomes 1q23.3 and 2p12 or identify other loci that exceed the genome-wide threshold for significance (indicated by the horizontal red line).