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


Objective—Elevated levels of plasma trimethylamine N-oxide (TMAO), the product of gut microbiome and hepatic-mediated metabolism of dietary choline and l-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=2.37×10⁻⁹) that colocalized with a highly significant (P=1.07×10⁻²⁰) cis-expression quantitative trait locus for solute carrier family 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=3.0×10⁻⁸) 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.1-3 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.4 A variety of animal studies have shown that choline deficiency adversely affects brain and cognitive development during fetal and neonatal life,1,4-6 which has led to specific nutritional guidelines recommending adequate intake of choline for infants and pregnant or lactating women.7,8

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.9 Interestingly, mutations of FMO3 that result in deficiency of this enzyme are the cause of trimethylaminuria, otherwise known as fish malodor syndrome.10 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). 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. More recently, we also demonstrated that t-carnitine, a trimethylamine abundant in red meat, is also metabolized by intestinal microbiota to produce TMAO in mice and humans and that t-carnitine supplementation accelerated atherosclerosis in mice. 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 t-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. 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 of TMAO from dietary choline and t-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 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 FMO Cluster With FMO3 Gene Expression, Plasma TMAO Levels, and CAD in Humans
In previous studies, we reported that 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 Fmo3, suggesting cis-acting regulation in mice. Furthermore, 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 FMO locus on chromosome 1q24.3. To evaluate the association of the FMO cluster with hepatic FMO3 mRNA levels, we used a previously published liver gene expression data set. 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 451-kb region containing FMO3, FMO6P, FMO2, FMO1, and 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 FMO3 mRNA levels (P=4.5×10^-4) that remained significant after correction for multiple testing (0.05/57; Bonferroni-corrected P=8.8×10^-4). Cis-expression quantitative trait loci (QTL) were not observed for any other members of the FMO gene family at this locus (data not shown).

We next determined whether variation in the 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 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 22,000 CAD cases and 65,000 controls. 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=1.3×10^-4; 0.05/388). Furthermore, the SNP that exhibited the strongest association with 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 ~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. 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
115 Mb that exceeded the genome-wide significance threshold for association in the HMIDP ($P=2.37\times10^{-6}$; Figure 2A and 2B). The 10-Mb region centered around the lead SNP on chromosome 3 contained several genes and exhibited a highly significant cis-expression QTL ($P=1.07\times10^{-20}$) for the gene encoding solute carrier family 30 member 7 ($Slc30a7$; 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 ($P=7.62\times10^{-6}$) 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 $Fmo$ gene cluster (162–163 Mb).

### 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 ($\lambda$) 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; $P=2.8\times10^{-7}$) is located ≈47 kb telomeric of $NUF2$, 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; $P=2.8\times10^{-7}$) 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 ($P=1.8\times10^{-4}$; Table 2). Based on the chromosome 3 locus identified in the

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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.
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_interact=0.33; rs12402441, P_interact=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.11,12,14 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.13 Of note, in humans, no differences in plasma TMAO levels were observed between men and women.12 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.21 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 FMO1.22 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...
to generate TMAO from TMA but that FMO3 was by far the most active family member.13 Because the Mendelian disease trimethylammoniauria 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 imputed genotypes from the GWAS data we used 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.24,25 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 t-carnitine or choline would lead to increased plasma TMAO levels, particularly if specific bacterial taxa that metabolize these nutrients to TMA 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

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>rs17359359</th>
<th></th>
<th></th>
<th>rs12402441</th>
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<tr>
<td>Stage</td>
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<td></td>
<td>rs12402441</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>G3</td>
<td>A3</td>
<td>AA</td>
<td>G3</td>
<td>A3</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GWAS</td>
<td>5.3±8.0</td>
<td>8.2±20.3</td>
<td>9.9±8.1</td>
<td>2.8±10⁻²</td>
<td>5.8±10.8</td>
</tr>
<tr>
<td>(n=1727)</td>
<td>(n=238)</td>
<td>(n=8)</td>
<td></td>
<td></td>
<td>(n=1773)</td>
</tr>
<tr>
<td>Replication</td>
<td>6.6±14.5</td>
<td>8.3±23.2</td>
<td>4.0±2.9</td>
<td>0.71</td>
<td>6.6±13.3</td>
</tr>
<tr>
<td>(n=1495)</td>
<td>(n=186)</td>
<td>(n=9)</td>
<td></td>
<td></td>
<td>(n=1598)</td>
</tr>
<tr>
<td>Combined</td>
<td>5.9±11.5</td>
<td>8.3±21.6</td>
<td>6.8±6.5</td>
<td>1.1×10⁻⁴</td>
<td>6.2±12.1</td>
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<tr>
<td>(n=3222)</td>
<td>(n=424)</td>
<td>(n=17)</td>
<td></td>
<td></td>
<td>(n=3371)</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.
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.

Sources of Funding
This study was supported, in part, by National Institutes of Health (NIH) grants K99HL102223, P01HL30568, R01HL08481, R01HL103866, P20HL113452, R01ES021801, a pilot project award from the Southern California Clinical and Translational Science Institute through NIH grant UL1TR000130, and American Heart Association Scientist Development grant 12SDG12050473. GenBank was supported in part by NIH grants P01HL098055, P01HL076491, and R01HL103931. R. Roberts has received research funding from Canadian Institutes of Health Research FOT28210 and Canada Foundation for Innovation 11966. S.L. Hazen is also partially supported by a gift from the Leonard Krieger Fund. Mass spectrometry instrumentation used was housed within the Cleveland Clinic Mass Spectrometry Facility with partial support through a Center of Innovation by AB SCIEX.

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, 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
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Jaana Hartiala, Brian J. Bennett, W.H. Wilson Tang, Zeneng Wang, Alexandre F.R. Stewart,
Robert Roberts, Ruth McPherson, Aldons J. Lusis, Stanley L. Hazen and Hooman Allayee
CARDIoGRAM Consortium

Arterioscler Thromb Vasc Biol. 2014;34:1307-1313; originally published online March 27, 2014;
doi: 10.1161/ATVBAHA.114.303252
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/34/6/1307

Data Supplement (unedited) at:
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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 16hr 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 electrospray 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 3min, held for 8min, followed by 100% methanol and water washing for 3min. 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\textsuperscript{7}. 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 $\text{Var}(u) = \sigma_g^2K$ and $\text{Var}(e) = \sigma_e^2I$, 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)\textsuperscript{8}, and the association mapping was performed based on the estimated variance component with a standard F test to test $\beta\neq0$.

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 × 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 ⁹ 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 $^{10}$. 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 $^{11}$ or SAS 9.3 (SAS Institute Inc, Cary, NC).

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Supplemental Material

Comparative Genome-Wide Association Studies in Mice and Humans for Trimethylamine N-oxide, a Pro-Atherogenic Metabolite of Choline and L-Carnitine

Hartiala et al.
The CARDIoGRAM Consortium

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**CARDIoGRAM Sources of Funding**

The ADVANCE study was supported by a grant from the Reyold's Foundation and NHLBI grant HL087647.
Genetic analyses of CADomics were supported by a research grant from Boehringer Ingelheim. Recruitment and analysis of the CADomics cohort was supported by grants from Boehringer Ingelheim and PHILIPS medical Systems, by the Government of Rheinland-Pfalz in the context of the “Stiftung Rheinland-Pfalz für Innovation”, the research program “Wissen schafft Zukunft” and by the Johannes-Gutenberg University of Mainz in the context of the “Schwerpunkt Vaskuläre Prävention” and the “MAIFOR grant”, by grants from the Fondation de France, the French Ministry of Research, and the Institut National de la Santé et de la Recherche Médicale.

The deCODE CAD/MI Study was sponsored by NIH grant, National Heart, Lung and Blood Institute R01HL089650-02.

The German MI Family Studies (GerMIFS I-III (KORA)) were supported by the Deutsche Forschungsgemeinschaft and the German Federal Ministry of Education and Research (BMBF) in the context of the German National Genome Research Network (NGFN-2 and NGFN-plus), the EU funded integrated project Cardiogenics (LSHM-CT-2006-037593) and ENGAGE, and the bi-national BMBF/ANR funded project CARDomics (01KU0908A).

LURIC has received funding from the EU framework 6 funded Integrated Project “Bloodomics” (LSHM-CT-2004-503485), the EU framework 7 funded Integrated Project AtheroRemo (HEALTH-F2-2008-201668) and from Sanofi/Aventis, Roche, Dade Behring/Siemens, and AstraZeneca. The MIGen study was funded by the US National Institutes of Health (NIH) and National Heart, Lung, and Blood Institute’s STAMPEED genomics research program through R01 HL087676. Ron Do from the MIGen study is supported by a Canada Graduate Doctoral Scholarship from the Canadian Institutes of Health Research.

Recruitment of PennCATH was supported by the Cardiovascular Institute of the University of Pennsylvania. Recruitment of the MedStar sample was supported in part by the MedStar Research Institute and the Washington Hospital Center and a research grant from GlaxoSmithKline. Genotyping of PennCATH and Medstar was performed at the Center for Applied Genomics at the Children’s Hospital of Philadelphia and supported by GlaxoSmithKline through an Alternate Drug Discovery Initiative research alliance award (M. P. R. and D. J. R.) with the University of Pennsylvania School of Medicine.

The Ottawa Heart Genomic Study was supported by CIHR #MOP--82810 (R. R.), CFI #11966 (R. R.), HSFO #NA6001 (R. McP.), CIHR #MOP172605 (R. McP.), CIHR #MOP77682 (A. F. R. S.).

The WTCCC Study was funded by the Wellcome Trust. Recruitment of cases for the WTCCC Study was carried out by the British Heart Foundation (BHF) Family Heart Study Research Group and supported by the BHF and the UK Medical Research Council. N. J. S. and S. G. B. hold chairs funded by the British Heart Foundation. N. J. S. and
A.H.G are also supported by the Leicester NIHR Biomedical Research Unit in Cardiovascular Disease and the work described in this paper is part of the research portfolio of the Leicester NIHR Biomedical Research Unit.

The **Age, Gene/Environment Susceptibility Reykjavik Study** has been funded by NIH contract N01-AG-12100, the NIA Intramural Research Program, Hjartavernd (the Icelandic Heart Association), and the Althingi (the Icelandic Parliament).

The **Cleveland Clinic GeneBank** study was supported by NHLBI grants P01HL098055, P01HL076491, R01HL103866, P20HL113452, and R01HL103931.

The collection of clinical and sociodemographic data in the **Dortmund Health Study** was supported by the German Migraine- & Headache Society (DMKG) and by unrestricted grants of equal share from Astra Zeneca, Berlin Chemie, Boots Healthcare, Glaxo-Smith-Kline, McNeil Pharma (former Woelm Pharma), MSD Sharp & Dohme and Pfizer to the University of Muenster. Blood collection was done through funds from the Institute of Epidemiology and Social Medicine, University of Muenster.

The **EPIC-Norfolk study** is supported by the Medical Research Council UK and Cancer Research UK.

The **EpiDREAM study** is supported by the Canadian Institutes of Health Research, Heart and Stroke Foundation of Ontario, Sanofi-Aventis, GlaxoSmithKline and King Pharmaceuticals. Funding for Andrew Lotery from the **LEEDS** study was provided by the T.F.C. Frost charity and the Macular Disease Society.

The **Rotterdam Study** is supported by the Erasmus Medical Center and Erasmus University Rotterdam; the Netherlands Organization for Scientific Research; the Netherlands Organization for Health Research and Development (ZonMw); the Research Institute for Diseases in the Elderly; The Netherlands Heart Foundation; the Ministry of Education, Culture and Science; the Ministry of Health Welfare and Sports; the European Commission (DG XII); and the Municipality of Rotterdam. Support for genotyping was provided by the Netherlands Organization for Scientific Research (NWO) (175.010.2005.011, 911.03.012), the Netherlands Genomics Initiative (NGI)/NWO project nr. 050-060-810 and Research Institute for Diseases in the Elderly (RIDE). Abbas Dehghan is supported by a grant from NWO (Vici, 918-76-619).

The **SAS** study was funded by the British Heart Foundation.

The Swedish Research Council, the Swedish Heart & Lung Foundation and the Stockholm County Council (ALF) supported the **SHEEP** study.
SMILE was funded by the Netherlands Heart foundation (NHS 92345). Dr Rosendaal is a recipient of the Spinoza Award of the Netherlands Organisation for Scientific Research (NWO) which was used for part of this work.

The Verona Heart Study was funded by grants from the Italian Ministry of University and Research, the Veneto Region, and the Cariverona Foundation, Verona.

The Atherosclerosis Risk in Communities Study is carried out as a collaborative study supported by National Heart, Lung, and Blood Institute contracts N01-HC-55015, N01-HC-55016, N01-HC-55018, N01-HC-55019, N01-HC-55020, N01-HC-55021, and N01-HC-55022. The authors thank the staff and participants of the ARIC study for their important contributions.

The KORA (Kooperative Gesundheitsforschung in der Region Augsburg) research platform was initiated and financed by the Helmholtz Zentrum München - National Research Center for Environmental Health, which is funded by the German Federal Ministry of Education, Science, Research and Technology and by the State of Bavaria. Part of this work was financed by the German National Genome Research Network (NGFN-2 and NGFNPplus) and within the Munich Center of Health Sciences (MC Health) as part of LMUinnovativ.

Work described in this paper is part of the research portfolio supported by the Leicester NIHR Biomedical Research Unit in Cardiovascular Disease.

This work forms part of the research themes contributing to the translational research portfolio of Barts and the London Cardiovascular Biomedical Research Unit which is supported and funded by the National Institute of Health Research.
### Supplemental Table I. Sex-stratified Association of SNPs Identified in Human and Mouse GWAS for Plasma TMAO Levels.

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<td>9.9 ± 30.0</td>
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<td>7.0 ± 15.8</td>
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Mean (± SD) plasma TMAO levels (µM) are shown as a function of genotype.

\(^a\)P-values obtained using linear regression with natural log transformed values after adjustment for age.
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).