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

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***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^{-6}) 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^{-7}) 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

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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.[3] 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 phosphotidylcholine) 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 monooxygenase (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

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Nonstandard Abbreviations and Acronyms	
CAD	coronary artery disease
CARDIoGRAM	Coronary Artery Disease Genome-wide Replication And Meta-Analysis
FMO	flavin monooxygenase
GWAS	genome-wide association study
HMDP	hybrid mouse diversity panel
QTL	quantitative trait loci
SNPs	single nucleotide polymorphisms
Slc30a7	solute carrier family 30 member 7
TMA	trimethylamine
TMAO	trimethylamine *N*-oxide

coronary artery disease (CAD).[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.[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.[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.[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 *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.[13] 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.[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 ≈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 ($P=4.5\times10^{-4}$) that remained significant after correction for multiple testing (0.05/57; Bonferroni-corrected $P=8.8\times10^{-4}$). *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.[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 *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.[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

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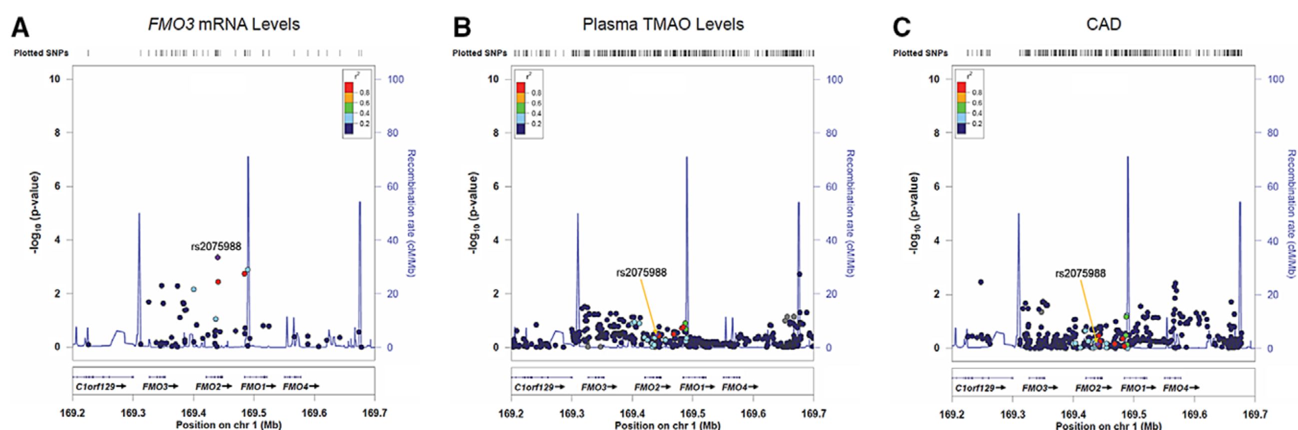

Figure 1. Association of the flavin monooxygenase (*FMO*) locus with FMO3 mRNA levels, plasma trimethylamine *N*-oxide (TMAO) levels, and risk of coronary artery disease (CAD) in humans. Using a publicly available expression quantitative trait loci liver data set, 57 single nucleotide polymorphisms (SNPs) were tested for association with hepatic *FMO3* mRNA levels, one of which (rs2075988) yielded a significant *P* value (4.5×10^{-4}) after Bonferroni correction for multiple testing (**A**). In the GeneBank cohort, none of the 471 SNPs tested in the *FMO* locus yielded significant association with plasma TMAO levels (**B**). Evaluation of the *FMO* locus with risk of CAD using 388 SNPs available from the results of the Coronary Artery Disease Genome-wide Replication And Meta-Analysis (CARDIoGRAM) consortium did not reveal any significant associations (**C**). The same genomic interval spanning ≈451 kb across the *FMO* cluster on chromosome 1q24.3 is shown for all 3 plots, and the variant most strongly associated with *FMO3* mRNA levels is given as the reference SNP (rs2075988). Chr indicates chromosome.

115 Mb that exceeded the genome-wide significance threshold for association in the HMDP (P=2.37×10^{-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=1.07×10^{-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=7.62×10^{-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 *Fmo* gene cluster (162–163 Mb).

Table 1. Clinical Characteristics of the Study Population

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

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 *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=2.8×10^{-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

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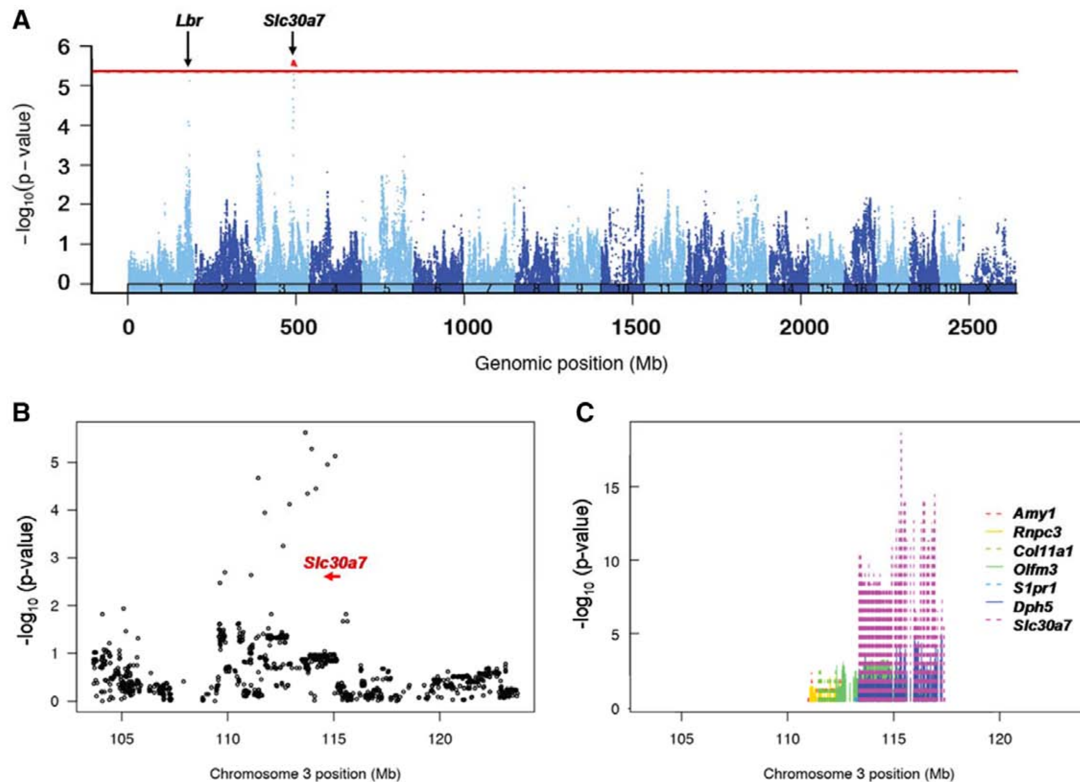

Figure 2. Manhattan plot for genome-wide association study (GWAS) of plasma trimethylamine *N*-oxide (TMAO) levels in mice. A GWAS for plasma TMAO levels in the hybrid mouse diversity panel (HMDP) identifies a significant locus over the solute carrier family 30 member 7 (*Slc30a7*) gene (red dot) at 110 to 115 Mb on chromosome 3 and a suggestive locus on chromosome 1 ≈40 kb away from the *Lbr* gene (**A**). A regional plot for chromosome 3 shows the location and transcriptional orientation of *Slc30a7* (indicated by red arrow) in relation to the peak single nucleotide polymorphisms (SNPs) in this region (**B**). Of the genes in this locus, a highly significant (P=1.07×10^{-20}) *cis*-acting expression quantitative trait loci is observed for *Slc30a7* (**C**). The red line indicates the genome-wide threshold for significance in the HMDP (P=4.1×10^{-6}). Plasma TMAO and hepatic mRNA levels were quantified in male mice from ≈100 HMDP strains (n=3–8 mice per strain) and analyzed for association with ≈107 000 SNPs, after correcting for population structure using the EMMA algorithm.

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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.[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 monoooxygenases has also been shown to use zinc

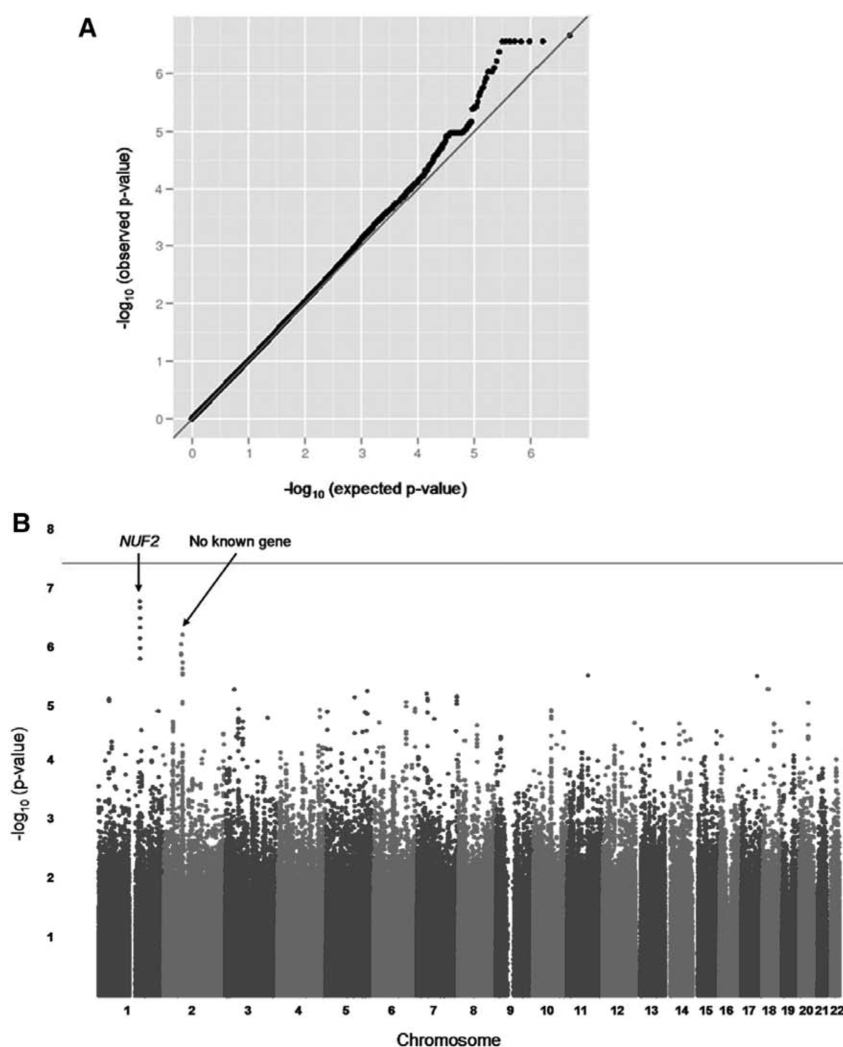

Figure 3. Results of a genome-wide association study (GWAS) for plasma trimethylamine *N*-oxide (TMAO) levels in humans. The quantile-quantile plot of the GWAS results for plasma TMAO levels in humans (n=1973) shows slight deviation of the observed *P* values from the expected distribution under the null hypothesis of no association (**A**). The observed genomic control factor in these analyses was 1.007, indicating that the results are not confounded by underlying population stratification. A GWAS analysis in humans identifies 2 loci on chromosomes 1 and 2 exhibiting suggestive evidence of association with plasma TMAO levels but no locus that exceeds the genome-wide threshold for significance (indicated by the horizontal red line; **B**).

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as a cofactor.[23] 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.[13]

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

	rs17359359				rs12402441			
Stage	GG	AG	AA	*P* Value*	AA	AG	GG	*P* Value*
GWAS	5.3±8.0 (n=1727)	8.2±20.3 (n=238)	9.9±8.1 (n=8)	2.8×10^{-7}	5.8±10.8 (n=1773)	4.5±4.7 (n=186)	4.5±3.7 (n=14)	0.006
Replication	6.6±14.5 (n=1495)	8.3±23.2 (n=186)	4.0±2.9 (n=9)	0.71	6.6±13.3 (n=1598)	8.8±31.2 (n=158)	4.5±1.5 (n=10)	0.68
Combined	5.9±11.5 (n=3222)	8.3±21.6 (n=424)	6.8±6.5 (n=17)	1.1×10^{-4}	6.2±12.1 (n=3371)	6.5±21.5 (n=344)	4.5±2.9 (n=24)	0.14

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 $r^2 \geq 0.8$. 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.

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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 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

1. Zeisel SH, Blusztajn JK. Choline and human nutrition. *Annu Rev Nutr*. 1994;14:269–296.
2. Buchman AL, Ament ME, Sohel M, Dubin M, Jenden DJ, Roch M, Pownall H, Farley W, Awal M, Ahn C. Choline deficiency causes reversible hepatic abnormalities in patients receiving parenteral nutrition: proof of a human choline requirement: a placebo-controlled trial. *JPEN J Parenter Enteral Nutr*. 2001;25:260–268.
3. Hollenbeck CB. The importance of being choline. *J Am Diet Assoc*. 2010;110:1162–1165.
4. Meck WH, Williams CL. Metabolic imprinting of choline by its availability during gestation: implications for memory and attentional processing across the lifespan. *Neurosci Biobehav Rev*. 2003;27:385–399.
5. Cermak JM, Holler T, Jackson DA, Blusztajn JK. Prenatal availability of choline modifies development of the hippocampal cholinergic system. *FASEB J*. 1998;12:349–357.
6. Craciunescu CN, Albright CD, Mar MH, Song J, Zeisel SH. Choline availability during embryonic development alters progenitor cell mitosis in developing mouse hippocampus. *J Nutr.* 2003;133:3614–3618.
7. Yates AA, Schlicker SA, Suitor CW. Dietary Reference Intakes: the new basis for recommendations for calcium and related nutrients, B vitamins, and choline. *J Am Diet Assoc*. 1998;98:699–706.
8. Zeisel SH. Choline: an essential nutrient for humans. *Nutrition*. 2000;16:669–671.
9. Krueger SK, Williams DE. Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. *Pharmacol Ther*. 2005;106:357–387.
10. Phillips IR, Shephard EA. Flavin-containing monooxygenases: mutations, disease and drug response. *Trends Pharmacol Sci*. 2008;29:294–301.
11. Wang Z, Klipfell E, Bennett BJ, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*. 2011;472:57–63.
12. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, Wu Y, Hazen SL. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N Engl J Med*. 2013;368:1575–1584.
13. Bennett BJ, de Aguiar Vallim TQ, Wang Z, Shih DM, Meng Y, Gregory J, Allayee H, Lee R, Graham M, Crooke R, Edwards PA, Hazen SL, Lusis AJ. Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metab*. 2013;17:49–60.
14. Koeth RA, Wang Z, Levison BS, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med.* 2013;19:576–585.
15. Schadt EE, Molony C, Chudin E, et al. Mapping the genetic architecture of gene expression in human liver. *PLoS Biol*. 2008;6:e107.
16. Schunkert H, König IR, Kathiresan S, et al.; Cardiogenics; CARDIoGRAM Consortium. Large-scale association analysis identifies 13 new susceptibility loci for coronary artery disease. *Nat Genet.* 2011;43:333–338.
17. Bennett BJ, Farber CR, Orozco L, et al. A high-resolution association mapping panel for the dissection of complex traits in mice. *Genome Res*. 2010;20:281–290.
18. Farber CR, Bennett BJ, Orozco L, et al. Mouse genome-wide association and systems genetics identify Asxl2 as a regulator of bone mineral density and osteoclastogenesis. *PLoS Genet*. 2011;7:e1002038.
19. Ghazalpour A, Bennett B, Petyuk VA, et al. Comparative analysis of proteome and transcriptome variation in mouse. *PLoS Genet*. 2011;7:e1001393.
20. Park CC, Gale GD, de Jong S, Ghazalpour A, Bennett BJ, Farber CR, Langfelder P, Lin A, Khan AH, Eskin E, Horvath S, Lusis AJ, Ophoff RA, Smith DJ. Gene networks associated with conditional fear in mice identified using a systems genetics approach. *BMC Syst Biol*. 2011;5:43.
21. Huang L, Yu YY, Kirschke CP, Gertz ER, Lloyd KK. Znt7 (Slc30a7)-deficient mice display reduced body zinc status and body fat accumulation. *J Biol Chem*. 2007;282:37053–37063.
22. Luo Z, Hines RN. Regulation of flavin-containing monooxygenase 1 expression by ying yang 1 and hepatic nuclear factors 1 and 4. *Mol Pharmacol.* 2001;60:1421–1430.
23. Ensign SA, Allen JR. Aliphatic epoxide carboxylation. *Annu Rev Biochem*. 2003;72:55–76.
24. Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. *Proc Natl Acad Sci U S A*. 2005;102:11070–11075.
25. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI. Evolution of mammals and their gut microbes. *Science*. 2008;320:1647–1651.

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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