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Review

Trimethylamine-*N*-Oxide: Friend, Foe, or Simply Caught in the Cross-Fire?

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Trimethylamine-*N*-oxide (TMAO), a gut-derived metabolite, has recently emerged as a candidate risk factor for cardiovascular disease and other adverse health outcomes. However, the relation between TMAO and chronic disease can be confounded by several factors, including kidney function, the gut microbiome, and flavin-containing monooxygenase *3* (*FMO3*) genotype. Thus, whether TMAO is a causative agent in human disease development and progression, or simply a marker of an underlying pathology, remains inconclusive. Importantly, dietary sources of TMAO have beneficial health effects and provide nutrients that have critical roles in many biological functions. Pre-emptive dietary strategies to restrict TMAO-generating nutrients as a means to improve human health warrant careful consideration and may not be justified at this time.

Trends

Circulating TMAO is elevated in humans with cardiovascular disease, kidney disease, type 2 diabetes mellitus, and cancer.

Kidney function, the gut microbiome, and a *FMO3* genotype and/or activity influence circulating TMAO and may confound the relation between TMAO and disease risk.

Restriction of animal source foods because of their TMAO-raising properties may be unjustified and could have unintended consequences.

Trimethylamine-*N*-Oxide: A Metabolite Linked to the Gut Microbiome

The relation between diet and health involves complex interactions among nutrients, genes, and many physiological systems, including the gut microbiome. Although long recognized for its role in the processing, biosynthesis, and utilization of nutrients [1], it is now clear that the gut microbiome has additional roles and might be modulating susceptibility to chronic diseases, such as cardiovascular disease, obesity, and cancer [1,2]. One purported mechanism involves the microbial production of trimethylamine (TMA) from dietary substrates and its subsequent conversion in the liver to TMAO, a small organic molecule that has recently emerged as a predictor of cardiovascular disease [3,4]. In addition to the gut microbiome, kidney function and genetics are other factors that can modulate circulating TMAO concentrations and may influence the relation between TMAO and disease outcomes either independently or through interactions with the gut microbiome.

In this review, we provide an overview of TMAO dietary sources, metabolism, and function, followed by a discussion of the role of TMAO in chronic disease risk that considers confounding factors, such as the gut microbiome, kidney function, and genotype. The importance of essential nutrients that act as precursors to TMAO and their role in human health are also highlighted, and future research needs addressed.

Dietary Sources of TMAO

TMAO, an amine oxide with the chemical formula $(CH_3)_3NO$, is found naturally in our diets in the preformed state (e.g., TMAO in fish), or can be generated within the human intestine from choline and carnitine, nutrients that are abundant in eggs and beef (Figure 1). Of these dietary sources, preformed TMAO in fish has the greatest impact on circulating TMAO concentrations. For example, consumption of fish yielded ~50 times higher postprandial circulating TMAO concentrations compared with the consumption of eggs (abundant in choline) or beef (abundant in

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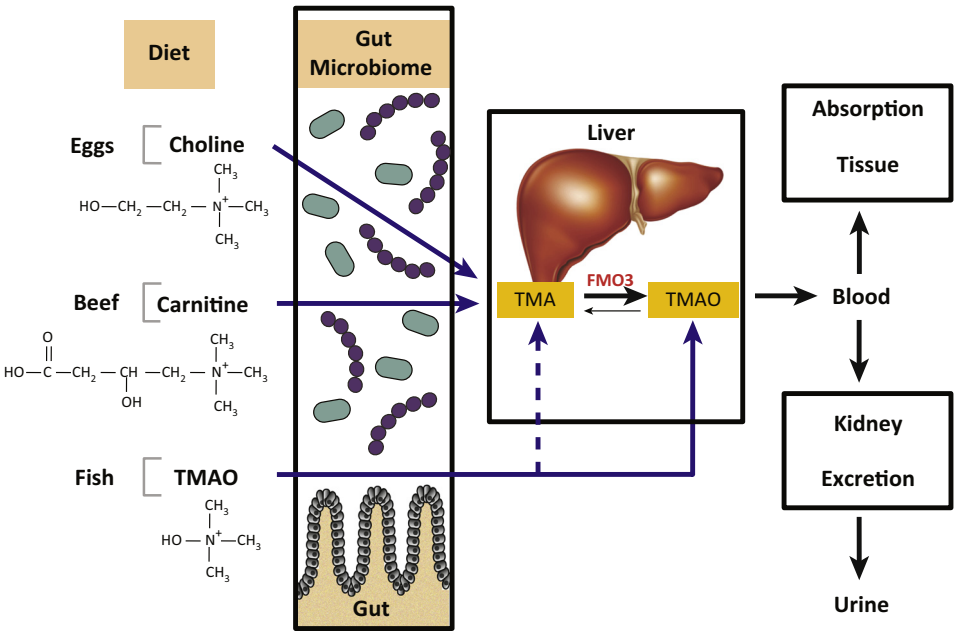

Figure 1. A Simplified Diagram of Trimethylamine-*N*-Oxide (TMAO) Generation and Metabolism. Animal source foods are enriched in TMAO (e.g., fish) or its dietary precursors, choline (e.g., eggs) and carnitine (e.g., meat). While dietary TMAO can bypass processing by the gut microbiome before absorption, both choline and carnitine require conversion to trimethylamine (TMA) by gut microbes. Once formed, TMA can be absorbed and subsequently converted to TMAO by the hepatic enzyme flavin-containing monooxygenase 3 (FMO3). Dietary and endogenously produced TMAO can be released by the liver and taken up by extrahepatic tissues or excreted in urine.

carnitine and choline) among healthy young men [5]. Similarly, others have reported elevated urinary excretion of TMAO and dimethylamine (a derivative of TMA) after consumption of fish but not of meat, dairy, fruits, vegetables, or grain [6,7], as well as higher urinary TMAO excretion in populations with greater fish intake [8,9].

Circulating TMAO concentrations are also influenced by the form and dose of dietary substrates. Human ingestion of large doses of free choline, but not phosphatidylcholine (the main form in food), yielded excessive urinary excretion of TMA and its derivatives [10]. Notably, considerable interindividual variations in circulating and urinary TMAO concentrations have been reported in response to egg consumption [11] and supplemental choline [12]; such findings are indicative of significant diet × host interactions.

A high-fat diet may be another dietary factor that influences circulating TMAO concentrations. An increase in postprandial TMAO concentrations was reported in response to a high-fat meal among healthy, nonobese men [13]. Furthermore, consumption of a hypercaloric (surplus of >1000 kcal/day), high-fat (55% fat) diet for 4 weeks increased fasting concentrations of plasma TMAO compared with baseline concentrations [14]. The mechanisms responsible for these observations are undetermined but could result, in part, from the higher carnitine and choline content of the high-fat meals and/or diet.

TMAO Metabolism

Choline and carnitine, dietary precursors of TMAO, must first undergo bacterial conversion in the mammalian gut to TMA, a fish-smelling odorant that is characteristic of degrading seafood. The obligate role of the gut microbiome in TMAO generation from dietary precursors within the

Box 1. Hepatic Conversion of TMA to TMAO

TMA is a fish-smelling molecule that is absorbed by intestinal cells, enters portal blood, and is subsequently taken up by the liver (Figure 1, main text). Hepatic FMOs, a family of enzymes that oxidize xenobiotics and drugs, thus facilitating their excretion, subsequently catalyze the conversion of TMA to the odorless TMAO [78]. Genetic deficiency of *FMO3*, the isoform with a predominant role in this conversion, results in a metabolic disease known as trimethylaminuria or the 'fish odor syndrome', and is characterized by an excessive excretion of TMA in urine, sweat, and breath [79]. Small amounts of TMA may also be converted to dimethylamine and monomethylamine. Some of these methylamine derivatives may be used as substrates to form a nitrosated compound, nitrosodimethylamine [80]. TMAO retroreduction to TMA can also occur in liver.

intestine has been demonstrated through manipulation of the gut. Studies in humans revealed that circulating TMAO concentrations in response to choline and carnitine are suppressed after antibiotic treatment but return to normal upon withdrawal of antibiotics and recolonization of the gut bacteria [3,15]. Similarly, reduced plasma choline concentrations were observed after colonization of TMA-producing bacteria in germ-free mice [16], secondary to the increased use of this dietary substrate for TMA generation by microbes. Microbial conversion of choline to TMA is catalyzed by choline TMA-lyase, a glycyl radical enzyme encoded by Cut gene clusters present in three gut bacteria phyla: *Firmicutes*, *Proteobacteria*, and *Actinobacteria* [17]. The gut microbiome-generated TMA is subsequently converted to TMAO in a reversible reaction catalyzed by the enzyme FMO3, in the liver (Box 1). Once produced, TMAO is mostly eliminated unchanged in urine within 24 h [6].

Interestingly, long-term dietary habits (e.g., vegan/vegetarian versus omnivore/carnivore) have been shown to alter gut microbiota composition and, consequently, the generation of TMAO from dietary substrates [15]. During a carnitine challenge, greater concentrations of circulating TMAO were observed in those classified as meat-eaters versus those who were vegetarian [15]. Although the quality of the background diet may modulate this outcome, analysis of fecal microbial composition revealed differences between the two eating patterns with an enterotype characterized by enriched proportions of the genus *Prevotella* (rather than *Bacteroides*) among the higher TMAO responders [15]. Thus, long-term dietary habits appear to influence the bacterial taxa, which may in turn affect TMAO production potential.

In contrast to dietary precursors of TMAO, most preformed TMAO (abundant in fish) in humans [5] and rats [18] appears to be absorbed in a manner that is independent of the gut microbes. A recent study by Cho *et al.* [5] demonstrated that circulating TMAO was elevated within 15 min of fish consumption, a time frame that is too short to allow for gut microbial conversion and hepatic processing.

Functions of TMAO

TMAO has a range of biological effects across numerous species and tissue types. As an organic osmolyte, TMAO is used by water-stressed organisms and tissues to maintain cell volume. Mammalian kidneys accumulate TMAO to counteract the destabilizing effects of urea (and inorganic ions) on macromolecular structures (e.g., proteins and nucleic acids), and to offset the inhibitory effects of urea on functions such as ligand binding [19]. TMAO is also suggested to offset the destabilizing effects of hydrostatic and thermal pressures on protein structure and ligand binding in deep-sea animals [19]. The protein-stabilizing effect of TMAO may be achieved (at least in part) by decreasing the hydrogen-bonding ability of water (and, hence, the stability of the unfolded state), and by acting as a molecular crowder that can increase the stability of the folded state via exclusion of the volume effect [20]. TMAO may also enhance protein stability (and favor protein folding) by suppressing the activity of the actomyosin motor in muscular proteins [21].

TMAO also functions as a 'chemical chaperone', where it accumulates in the endoplasmic reticulum (ER) to promote protein folding, thereby inhibiting ER stress and attenuating the

unfolded protein response. Administration of TMAO as a chemical chaperone has been shown to reduce experimental diabetic peripheral neuropathy [22], asthma [23], and cataract formation [24] in rodent models of disease. In addition, treating human cell lines with TMAO reduced markers of oxidative damage in neuroblastoma cells [25], improved protein folding and secretion of a myocilin mutant protein in trabecular meshwork cells of glaucoma [26], and attenuated heat-induced keratin aggregates in keratinocytes from patients with epidermolysis bullosa simplex, a blistering skin disease caused by mutations in genes encoding keratin [27].

TMAO and Chronic Disease Risk

Cardiovascular Disease

A link between TMAO and cardiovascular disease risk first emerged in 2011. Using an untargeted metabolomics approach, investigators found a dose-dependent association between plasma concentrations of TMAO (as well as of choline and betaine) and cardiovascular disease risk among cardiac patients [4]. In a follow-up study with a different cohort of cardiac patients, this group showed that the highest quartile of fasting plasma concentrations of TMAO was predictive of death, myocardial infarction, or stroke [3]. Similar findings have been reported by others. A small cross-sectional study in a multiethnic population in Canada observed that serum TMAO (but not L-carnitine) showed a graded association with prevalent cardiovascular disease [28]. Circulating TMAO concentrations were also associated with infarcted coronary artery number in patients undergoing cardiovascular surgery [29], and urinary dimethylamine levels have been associated with coronary artery disease [30].

The mechanism by which TMAO may contribute to cardiovascular disease could involve enhanced cholesterol accumulation in macrophages. In the ApoE$^{-/-}$ genetic model of atherosclerosis-prone mice, administration of TMAO (and its dietary precursors) enhanced foam cell formation in a microbiota-dependent manner by increasing cell surface expression of two proatherogenic scavenger receptors: cluster of differentiation 36 (CD36) and scavenger receptor A [4]. In addition, TMAO was subsequently reported to reduce reverse cholesterol transport in ApoE$^{-/-}$ mice by downregulating hepatic CYP7A1 activity, the rate-limiting step in the bile acid synthetic pathway and a major route for cholesterol elimination from the body [15]. Notably, however, TMAO was found to slow aortic lesion formation, indicating a protective effect in cardiovascular disease, in ApoE$^{-/-}$ mice that had been transfected with an adeno-associated viral vector containing the human cholesteryl ester transfer protein, a key enzyme in reverse cholesterol transport [31]. Other mechanisms by which TMAO may contribute to cardiovascular disease pathogenesis include prolongation of the hypertensive effect of angiotensin II, as shown in rats [32], and enhanced platelet activation that may contribute to platelet hyper-responsiveness and thrombosis potential [33]. Whether TMAO has the role of a mediator in the cause of cardiovascular diseases or whether its high concentration only coexists with factors hindering cholesterol metabolism and homeostasis of the circulatory system remains to be determined.

Kidney Disease

Circulating concentrations of TMAO are elevated in chronic kidney disease and inversely associated with glomerular filtration rate [34,35]. Studies have also suggested that circulating TMAO is an independent risk factor for chronic kidney disease mortality. In patients with chronic kidney disease ranging from mild-moderate to end-stage renal disease, higher TMAO levels were associated with an increased risk of all-cause mortality, which remained significant after controlling for glomerular filtration rate and other covariates [36]. Circulating TMAO concentrations were also shown to identify those at the highest risk for cardiovascular events among patients with advanced chronic kidney disease [37]. Nonetheless, a recent study conducted in cardiac patients that controlled for an array of cardiometabolic risk factors, including kidney

function, did not find any significant association of TMAO, choline, or betaine plasma concentrations with the presence or the risk of incident major cardiovascular events [38].

Although the mechanism by which TMAO may exacerbate kidney impairment is not well studied, a diet containing an excess of choline or TMAO associated with corresponding increases in tubulointerstitial fibrosis and collagen deposition relative to the control diet [39]. Increased phosphorylation of Smad3, a regulator of the profibrotic transforming growth factor-β signal transduction, was also observed [39]. Whether consumption of physiologically relevant amounts of dietary choline and TMAO would contribute to renal tubulointerstitial fibrosis and dysfunction remains unclear. Moreover, TMAO is synthesized by the renal medulla and released into plasma upon kidney damage [40], suggesting that circulating TMAO is a proxy for the extent of renal medulla injury.

Type 2 Diabetes Mellitus

Elevations in TMAO are also associated with type 2 diabetes mellitus (T2DM). Animal models of diabetes (i.e., db/db mice) exhibited circulating TMAO concentrations up to tenfold higher compared with lean control animals [41]. Similarly, higher circulating TMAO concentrations were independently associated with T2DM in humans [41]. Notably, T2DM appears to augment the association between TMAO and cardiovascular events. Among those with T2DM, high TMAO was associated with excess risk for several cardiovascular events, including death, myocardial infarction, heart failure, and unstable angina; whereas, in subjects without diabetes, TMAO was associated only with death and heart failure [42]. Similarly, increased carotid intima-media thickness was observed with higher serum TMAO concentrations among subjects with a history of T2DM, impaired glucose tolerance, or gestational diabetes, or a body mass index (BMI) >27 kg/m^2, independent of age, sex, and visceral fat mass [43].

The mechanism by which TMAO may influence T2D pathogenesis is relatively unexplored. In mice, the addition of supplemental TMAO to a high-fat diet impaired glucose tolerance to a greater extent than did a high-fat diet alone [44]. Perturbations in the hepatic insulin signaling pathway and increases in adipose tissue inflammation were also observed among the mice receiving the supplemental TMAO [44]. Nonetheless, the more advanced cardiometabolic risk profile among those with T2DM may also arise from alterations in pathways interrelated with TMAO metabolism, including those that involve lipids, phospholipids, and methylation [45].

Cancer

Elevations in circulating TMAO have been associated with greater risk of certain cancers. In the Women's Health Initiative Observational Study, a higher TMAO concentration was associated with a greater risk of colorectal cancer among postmenopausal women with low vitamin B_{12} status [46]. Furthermore, a strong link between colorectal cancer and TMAO was detected in a genome-wide systems analysis that demonstrated TMAO and colorectal cancer share many of the same genetic pathways [47]. Lastly, a positive association between TMAO and aggressive prostate cancer was observed in a metabolomic analysis of prostate cancer risk in a prospective cohort [48].

Important Modulators of TMAO in Relation to Disease Risk

At present, it is unclear whether TMAO contributes to disease pathogenesis or is simply a marker of an underlying pathogenic factor. In addition, fasting plasma concentrations of TMAO exhibit a relatively high degree of intraindividual variation, such that measurements taken from the same individual 1 year apart are weakly correlated [49]. This modest correlation of TMAO levels over time may confound the relation between TMAO and disease endpoints in longitudinal studies [49]. Furthermore, circulating TMAO concentrations are affected by several factors, including

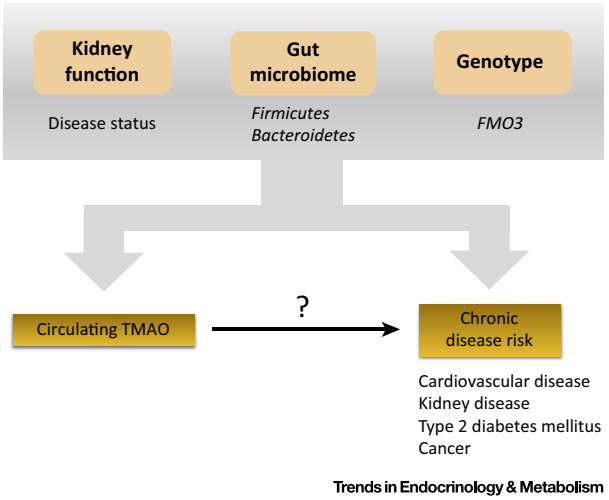

Figure 2. Overview of Factors that Can Influence the Relation between Trimethylamine-*N*-Oxide (TMAO) and Chronic Disease Risk. Kidney function, the gut microbiome, and a flavin-containing monooxygenase 3 *(FMO3)* genotype may independently or interactively modulate circulating TMAO concentrations, thereby confounding the relation between TMAO and chronic disease risk.

kidney function, the gut microbiome, and FMO3 activity and/or genotype, all of which may have a direct role in disease etiology and progression (summarized in Figure 2).

Kidney Function

Given that declining kidney function is a common comorbidity in people with cardiovascular disease [50] and is a well-established risk factor for cardiovascular disease [51], kidney function is an important confounder in studies examining the relation between TMAO and cardiovascular outcomes. While glomerular filtration rate is considered the gold standard for assessing kidney function, estimates of glomerular filtration rate can be imprecise and relatively insensitive for detecting early renal disease and for monitoring its progression [52]. Thus, clinically significant impairments in renal function may go undetected, preventing absolute control of this metabolic confounder. Furthermore, elevated TMAO concentrations may reflect renal medullary damage as a result of the hypertension associated with cardiovascular disease [39,53].

Gut Microbiome

Several disease states are now being linked with pathological variation in the gut microbiome, and data from rodent studies suggest that dysbiosis contributes to disease pathogenesis [54,55]. Given that TMAO is a gut microbiome-derived metabolite, circulating TMAO concentrations may be a biomarker of gut microbiota composition. Indeed, a lower microbial diversity and a greater enrichment of *Firmicutes* relative to *Bacteroidetes* were detected among healthy young men who exhibited a greater postprandial increase in circulating TMAO following egg and beef consumption [5]. Notably, this variation in gut microbiome composition, which can be marked by elevated TMAO, could be a direct contributor to disease pathogenesis and progression. This is supported by the finding that feces transplanted in ApoE$^{-/-}$ mice from a strain of atherosclerosis-prone, high TMAO-producing mice, resulted in higher atherosclerotic plaque formation compared with feces transplanted from a strain of atherosclerosis-resistant, low TMAO-producing mice [56]. A metagenomics analysis also revealed that patients with atherosclerosis had greater abundance of *Collinsella* compared with the age- and gender-matched control group, which showed enrichment in *Bacteroides*, *Eubacterium* and *Roseburia* [57]. These findings raise the distinct possibility that elevations in circulating TMAO may arise from a dysbiotic microbiome, which in turn could be the causative factor underlying disease

pathogenesis and progression. Alternatively, circulating TMAO could reflect differences in gut microbiome composition that transpired during the disease process. In either scenario, TMAO would be a marker of disease rather than a direct contributor.

FMO3 Activity and/or Genotype

Although FMO3 activity is well recognized for its catalysis of TMA to TMAO [58], it also functions to regulate aspects of cholesterol metabolism and insulin sensitivity [59,60]. Knockdown of *FMO3* in cholesterol-fed mice altered biliary lipid secretion, blunted intestinal cholesterol absorption and limited the production of hepatic oxysterols and cholesteryl esters [60]. Furthermore, knockdown of *FMO3* in insulin-resistant mice suppressed FoxO1, a central node for metabolic control, and prevented the development of hyperglycemia, hyperlipidemia, and atherosclerosis [61]. Thus, FMO activity may influence disease outcomes via routes that are independent of TMAO.

Notably, functional differences in FMO3 activity can occur in humans secondary to variations within the *FMO3* gene. In addition to rare genetic mutations in the FMO3 enzyme in trimethylaminuria, single nucleotide polymorphisms (e.g., E158K and E308G) have been reported to reduce the metabolic efficiency of this enzyme (and lower TMA conversion to TMAO under normal physiological conditions) [62]. Moreover, several studies have reported associations between these genetic variants and disease risk. In patients with hypertension, greater risk of ischemic stroke was observed among heterozygote *FMO3* E158K and E308G genotype carriers [63]. The polymorphism E158K was also associated with increased risk of essential hypertension susceptibility in a Russian population [64], and increased risk of mortality among patients with chronic kidney disease [65]. However, among obese individuals, the wildtype genotypes for both *FMO3* E158K and E308G increased the risk of ischemic stroke by five to six times compared with nonobese individuals [63]. Although the nature of the relation between modifiers of FMO3 activity and disease risk is complex and poorly understood, these data collectively suggest that FMO3 activity has a role in disease pathology that is extraneous to its role in TMAO metabolism.

Beneficial Effects of Diets Enriched in TMAO Precursors

While fish consumption (high in TMAO) has long been associated with reduced risk for cardiovascular disease [66], diets enriched in choline or carnitine are also associated with beneficial effects on human health [67]. Furthermore, animal source foods containing TMAO precursors are important sources of other nutrients, such as omega-3 fatty acids, iron and vitamin B_{12} [67].

As the precursor of phosphatidylcholine and acetylcholine, choline has a critical role in membrane biosynthesis and neurotransmission [68]. Choline is also a source of labile methyl groups that are used in cellular methylation reactions, including DNA methylation, an epigenetic modification with downstream effects on gene expression and genome stability [68]. The demand for choline is particularly high during pregnancy [12]. Consumption of extra choline during this reproductive state was shown to lower neonatal response to stress via epigenetic mechanisms [69] and improve placental function [69,70], and was associated with a decreased risk of having a baby with a neural tube defect [71]. Diets enriched in choline were also associated with lower plasma levels of inflammatory markers in healthy Greek adults [72] and improved cognitive function in a dementia-free cohort of US adults [73].

L-carnitine is important in energy metabolism, particularly the oxidation of fatty acids [74], and carnitine supplementation has been used as an ergogenic aid [75]. Among patients undergoing hemodialysis, carnitine supplementation reduced markers of vascular injury and oxidative stress, including soluble forms of intracellular adhesion molecule 1, vascular cell adhesion molecule 1,

and malondialdehyde, despite yielding higher plasma TMA and TMAO concentrations [76]. Furthermore, a meta-analysis showed that L-carnitine supplementation may reduce mortality [77].

Concluding Remarks and Future Perspectives

TMAO is a novel predictive risk factor of adverse cardiovascular outcomes mostly in patients with medical conditions or in animal models of disease. Circulating TMAO is also emerging as a risk factor for a growing number of additional chronic diseases, including kidney disease, T2DM, and cancer. Whether TMAO is a causative agent in disease development and progression, or simply a marker of an underlying pathology, remains inconclusive in humans. Important confounding factors that warrant consideration are kidney function, the gut microbiome, and the *FMO3* genotype. Diets enriched in TMAO precursors provide nutrients that are important to health and, thus, proposing restriction of animal source foods because of their TMAO-raising properties may be unjustified and could have unintended adverse consequences.

Clinical studies investigating the effects of lowering circulating TMAO on cardiovascular disease outcomes are needed to clarify the role of TMAO in the disease process (see Outstanding Questions). Since TMAO may serve as a surrogate marker of a microbe community with adverse effects on human health, intervention trials investigating the effects of dietary and pharmaceutical strategies aimed at restoring the symbiotic relation between the gut microbes and their host may be worthwhile. Finally, laboratory studies that enhance our current understanding of the role of TMAO and FMO3 in human organ systems are warranted.

Outstanding Questions

Are TMAO-lowering strategies (that do not involve manipulation of the microbiome) an effective means to improve human health?

Do TMA-generating microbes contribute to the development and progression of chronic diseases? What is the mechanism? Can the gut microbiome be manipulated to produce long-term benefits to health?

Does FMO3 activity contribute to disease risk in humans? Is this influence independent of TMAO production?

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