Trimethylamine *N*-Oxide, the Microbiome, and Heart and Kidney Disease

Steven H. Zeisel and Manya Warrier

Nutrition Research Institute and Department of Nutrition, University of North Carolina, Kannapolis, North Carolina 28081; email: steven_zeisel@unc.edu

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Abstract

Trimethylamine *N*-oxide (TMAO) is a biologically active molecule and is a putative promoter of chronic diseases including atherosclerosis in humans. Host intestinal bacteria produce its precursor trimethylamine (TMA) from carnitine, choline, or choline-containing compounds. Most of the TMA produced is passively absorbed into portal circulation, and hepatic flavin-dependent monooxygenases (FMOs) efficiently oxidize TMA to TMAO. Both observational and experimental studies suggest a strong positive correlation between increased plasma TMAO concentrations and adverse cardiovascular events, such as myocardial infarction, stroke, and death. However, a clear mechanistic link between TMAO and such diseases is not yet validated. Therefore, it is debated whether increased TMAO concentrations are the cause or result of these diseases. Here, we have tried to review the current understanding of the properties and physiological functions of TMAO, its dietary sources, and its effects on human metabolism. Studies that describe the potential role of TMAO in the etiology of cardiovascular and other diseases are also discussed.

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1. INTRODUCTION

Within the last decade, the gut microbiome has emerged as an important metabolic organ that links nutrient metabolism to molecules that contribute to chronic diseases (97). Among these molecules, trimethylamine *N*-oxide (TMAO) has gained much attention due to its potential role as a promoter of atherosclerosis, causing cardiovascular and kidney diseases (19, 61, 109). Cardiovascular disease (CVD) accounts for roughly 17 million deaths worldwide each year and remains the number one cause of mortality in the United States (17). Ten to 15% of the population has chronic kidney disease (122). These numbers are expected to rise owing to the epidemic of obesity, diabetes, and metabolic syndrome (5). Atherosclerosis, caused by accumulation of cholesterol-laden macrophages in the artery wall, is the underlying cause of most CVD and much kidney disease (84). TMAO-mediated atherosclerosis could potentially be a significant cause of heart and kidney disease, but the underlying mechanisms whereby TMAO is related to heart and kidney disease need to be fully elucidated and validated.

In people, TMAO is formed from trimethylamine (TMA) (**Figure 1**). This TMA is obtained either directly from foods high in TMA, such as fish (98, 126), or obtained indirectly from bacterial

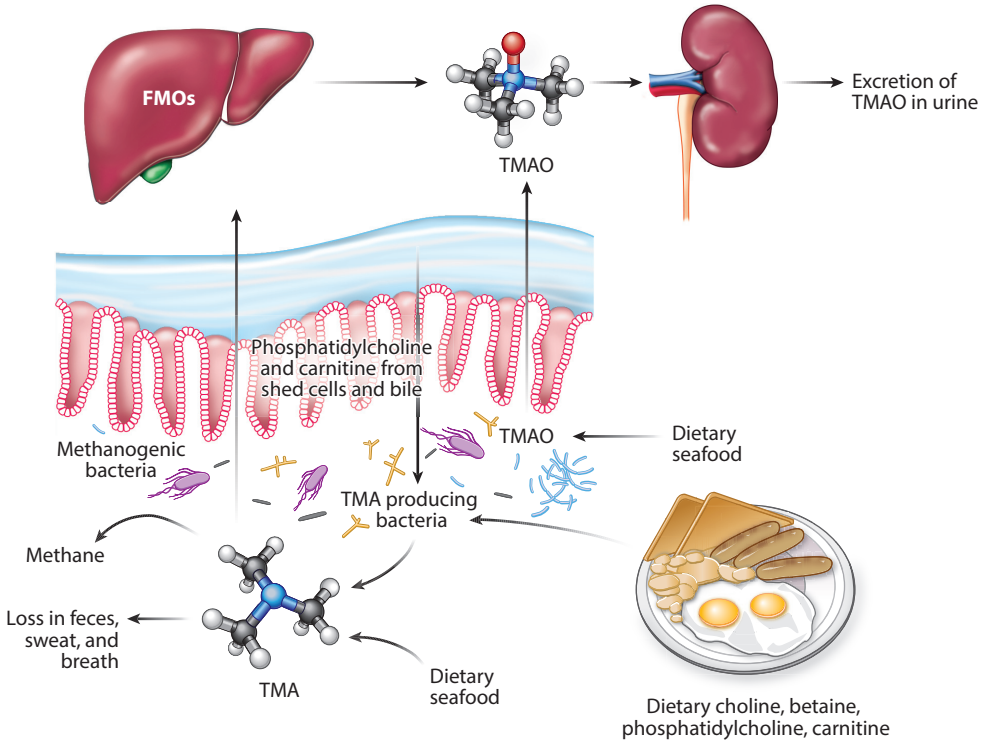

Figure 1

Pathways for trimethylamine *N*-oxide (TMAO) formation and removal. Trimethylamine (TMA) is formed in the intestinal lumen when gut microbiota metabolize carnitine, choline, and choline-containing compounds in the diet. Dietary seafood contains large amounts of both TMA and TMAO. TMA also is formed by gut microbiota from carnitine and choline-containing compounds in the bile and sloughed cells that are delivered to the intestinal lumen. TMA in the intestine can be used as a substrate to make methane by gut methanogenic microbiota, and TMA can be absorbed from the intestine. This absorbed TMA is delivered to the liver where flavin-dependent monooxygenase (FMO) isoforms 1 and 3 convert it to TMAO. TMAO is excreted by the kidney into urine, and is excreted also in breath and in sweat.

metabolism of dietary choline [and choline-containing compounds such as phosphatidylcholine (PtdCho)], of dietary betaine (a metabolite of choline), and of dietary L-carnitine (and its metabolite γ-butyrobetaine) in the intestine (19, 63, 118, 127, 128). TMA also may be derived from the bacterial recycling of biliary PtdCho and enteric tract cell debris (9).

TMA is absorbed from the intestine and then oxidized in the liver by flavin-dependent monooxygenase isoforms 1 and 3 (FMO1 and FMO3) to produce TMAO (13). In people, TMAO is excreted through urine, sweat, and breath (9). Thus, human exposure to TMAO depends on diet, composition of the gut microbiome, liver function, and the capacity to excrete it (kidney function, lung function, etc.).

In people, TMAO was originally only thought of as a waste product of choline metabolism. Now, we recognize that this molecule is biologically active. TMAO modulates both lipid and glucose homeostasis, and as discussed in the first paragraph, it may exacerbate several chronic

diseases including atherosclerosis, diabetes, and chronic kidney diseases (20). Even though the exact mechanism(s) by which TMAO promotes these diseases is currently under investigation, present studies suggest that reducing plasma TMAO concentration could be a potential therapeutic approach for the treatment of such chronic disorders. It is also possible that this biomarker is not causal but rather reflects renal disease caused by atherosclerosis, which reduces excretion of TMAO, thereby elevating concentrations in plasma. We summarize the current state of knowledge about TMAO, the role of nutrients in its production, and discuss potential causal relationships whereby TMAO could contribute to the development of chronic diseases with an emphasis on CVD and renal diseases.

2. METABOLISM OF TRIMETHYLAMINE AND TRIMETHYLAMINE *N*-OXIDE

2.1. Trimethylamine Formation by Bacteria

As discussed in Section 1, TMAO is produced from TMA, and TMA can be formed when intestinal bacteria metabolize choline, choline-containing compounds, betaine, and L-carnitine ingested in the diet or recycled in the gut. We know that gut microbiota are required for these nutrients to be converted to TMA because gnotobiotic mice do not produce such TMA (1, 96), and antibiotic treatment of standard mice decreases TMA formation (1). Colonization of gnotobiotic mice with choline-converting bacteria not only increases cecal TMA production but also lowers serum choline concentration (96). Although most of the production of TMA occurs in the cecum and colon, oral flora (*Streptococcus sanguis*, a facultative anaerobe) also can form TMA from choline (25).

A choline-utilization gene cluster (*Cut*) responsible for anaerobic conversion of choline to TMA was identified in the sulfate-reducing bacterium *Desulfovibrio desulfuricans* (32, 112). Crucial genes in the cluster are *CutC* and *CutD*, encoding for choline TMA-lyase and its activating protein, respectively. Deletion of the *CutC* gene in *Desulfovibrio alaskensis* G20 and heterologous expression of the *CutC* and *CutD* genes from *D. alaskensis* G20 in *Escherichia coli* confirmed that these genes are essential for the conversion of choline to TMA (32). The pathway involves a radical C–N bond cleavage of choline to generate TMA and acetaldehyde (32).

A microbial metabolic pathway generating TMA and malic semialdehyde from L-carnitine hydroxylation are active in human colon isolates belonging to the genera *Acinetobacter* and *Serratia* (41). Two genes, *CntA* and *CntB*, encode a two-subunit oxidoreductase (*A* encodes the carnitine oxidase, and *B* encodes a reductase) for L-carnitine conversion to TMA (confirmed by a series of knockout and heterologous expression experiments) (137). A second pathway converting carnitine to TMA involves production of the intermediate γ-butyrobetaine in the ileum, with subsequent conversion of γ-butyrobetaine to TMA in the cecum and colon (62). A *YeaW/YeaX* gene pair encodes for oxygenase and oxidoreductase activities that have substrate promiscuity for γ-butyrobetaine, L-carnitine, choline, and betaine. Orthologs and homologs of the CntA/CntB and *YeaW/YeaX* gene pairs are found in a variety of gut microbiota: Gammaproteobacteria (*Klebsiella pneumoniae*, *E. coli*, *Citrobacter*, Providencia, and *Shigella*), Betaproteobacteria (*Achromobacter*), Firmicutes (*Sporosarcina*), and *Actinobacteria*; they appear to be absent in *Bacteroidetes* (41). A recent study in men observed that people with higher Firmicutes to Bacteroidetes enrichment generated more TMA after a challenge meal (28). Only *Edwardsiella tarda* does not express the TMA-producing genes discussed in Section 1 above but produces TMA nonetheless (96). The elucidation of microbial TMA-generating pathways is ongoing, and it is likely that part of TMA-producing microbial metabolism remains undiscovered (41).

2.2. Trimethylamine *N*-Oxide Formation from Trimethylamine in Humans

Once TMA is produced, there are several ways it can be removed from the intestine: through further microbial metabolism, through loss in the feces, and by host absorption into systemic circulation. TMA can be used for methanogenesis in the gastrointestinal tract; in human intestine, this pathway is active in bacteria belonging to the genera *Methanomassiliicoccus* and *Methanomethylophilus* (18, 21, 38) as well as in *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* (41). TMA loss via feces has not been carefully quantitated.

Most TMA is absorbed into the hepatic portal circulation by passive diffusion across the enterocyte membranes and then is efficiently converted to TMAO by hepatic FMO1 and FMO3 (13); about 95% of TMA is oxidized as it is subsequently excreted in urine in a 3:95 TMA:TMAO ratio (132). Physiological concentration ranges for TMAO are ≈10- to 20-fold higher than are those for TMA in human plasma (99). FMO3 has tenfold higher specific activity in the liver than does FMO1 (13). FMO3 catalyzes NADPH-dependent oxygenation of TMA. In addition to TMA, other substrates for FMO3 include the pharmaceutical drugs cimetidine, chlorpromazine, ketoconazole, morphine, propranolol, ranitidine, sulindac, tamoxifen and tyramine (71). TMA is a volatile gas and smells fishy, whereas TMAO is a solid that is less volatile. Due to bacterial activity, TMAO in rotting fish is converted to TMA, imparting the characteristic fish odor. The human ability to detect this odor is very sensitive, perhaps preventing humans from eating rotten fish (113). Thus, mutations in *FMO3* in humans that result in excretion of TMA in sweat and breath present as fish-odor syndrome (trimethylaminuria). In Britain, it is estimated that the incidence of carrier status for trimethylaminuria is 1% (82), and most cases of trimethylaminuria are inherited in an autosomal recessive pattern. Carriers of an *FMO3* mutation may have mild symptoms of trimethylaminuria or experience temporary episodes of fish-like body odor (71). More than 300 variants or single-nucleotide polymorphisms have been reported in *FMO3*.

FMO3 in the liver is expressed differently during the stages of liver development. Both mRNA and protein were found in 30% of fetal livers in the first trimester of pregnancy but not in the second and third trimesters; however, they could be detected again by 21 postnatal days. Expression of *FMO3* then gradually increased to 8% of adult levels at about age nine months, 20% by 11 years, and did not reach adult levels until after 18 years (64, 133). The bile salt, cholic acid, induces *FMO3* via the bile acid–activated nuclear receptor FXR (13). Testosterone suppresses and estrogen induces *FMO3* expression. Thus, FMO3 activity is 100 times higher in female than in male mice; more modest gender-related differences are observed in humans (13). In fact, a large study reported that women have less TMAO formation than do men (89). We do not yet understand what factors limit TMAO production in women and men, and more studies on the contributions of various isoforms of FMO and their differential regulation in men versus women are needed. It may be that FMO1, expression of which is higher in male mice than in female mice, may be partly responsible for some TMAO synthesis in males (13). In some normal healthy women, modest trimethylaminuria occurs just at the onset of and during menstruation and then disappears (82). This menstrual trimethylaminuria is more pronounced in women homozygous for variants that result in a mild decrease in FMO3 enzyme activity (103). Dietary *Crucifera* can decrease FMO3 enzyme activity. After 300 g/day of cooked brussels sprouts for three weeks, people eating brussels sprouts showed significant increases in their urinary TMA/TMAO ratio, suggesting a decrease in TMA N oxidation (24).

FMO1 mRNA and protein are expressed in the human liver during the first trimester, declining gradually in the second and third trimesters, and disappearing completely by about the third postnatal day in the liver and kidney (71). Small amounts of FMO1 mRNA are also found in the adult small intestine (71).

Although *FMO3* and *FMO1* genes are important in the formation of TMAO, a genome-wide association study failed to find any significant host genetic influences on circulating TMAO concentrations and proposed that dietary factors and gut microbes may be the major determinants of circulating TMAO concentrations (49).

2.3. Excretion of Trimethylamine *N*-Oxide

TMAO is a small molecule (75.1 Da) and is readily filtered by the kidneys. It is passively excreted in the proximal tubules of the chicken nephron via a probenecid-sensitive transporter at the luminal side, whereas N-oxidation of TMA occurs via a quinidine-sensitive transporter at the basal side. It has not been determined whether TMAO transporters are present in the human nephron (108). After administration of radiolabeled TMA or TMAO to people, 94.5% of the dose label was excreted in the urine within 24 h; only 4% of the dose was excreted in the feces and <1% in the breath (2). The circulating TMAO concentrations of patients with renal dysfunction increased as kidney function decreased, and abnormally high TMAO concentrations resolved after kidney transplantation (106) (discussed in more detail in Section 5.2.1). However, the degree of rise in TMAO concentrations was not completely concordant with the degree of reduction in the estimated glomerular filtration rate (eGFR) (108). It is interesting that, in rats, urinary TMAO excretion was elimination- and not formation-rate limited (88). Whether an increase in TMAO concentrations in patients with kidney disease is elimination- and not formation-rate limited, or a combination of both, is not known.

2.4. Measurement of Trimethylamine and Trimethylamine *N*-Oxide

TMA is volatile and is more soluble in acidified samples, so it is important to collect samples appropriately. TMAO is not as volatile. Methods used for measuring plasma and urine TMA and TMAO include the following: liquid chromatography mass spectrometry (134), proton nuclear magnetic resonance spectrometry (68), headspace gas chromatography (80), electrospray ionization tandem mass spectrometry (58), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (53).

The European Prospective Investigation into Cancer and Nutrition (EPIC)-Heidelberg study evaluated individual variations in fasting plasma TMAO concentrations for one year. TMAO concentration varied within each individual over time more than it did between people (66). We do not know whether this variation is due to variation in diet, circadian rhythm, hormone status, or other causes.

3. DIETARY SOURCES OF TRIMETHYLAMINE AND TRIMETHYLAMINE *N*-OXIDE AND THEIR PRECURSORS

3.1. Fish

TMAO and TMA are present in high concentrations in fish and other seafood. Marine fish (e.g., cod, haddock, halibut, herring, skate) have higher concentrations than do freshwater fish (e.g., trout) (9). For example, 100 g of cod contains 300 mg TMAO compared to 100 g of beef or of egg, which contain <1 mg TMAO (28). In addition, TMA can be present in commercially available fish oil or choline-containing diet supplements that have been subject to bacterial degradation during preparation or storage (131). There are no data available about TMAO in diet supplements.

3.2. L-Carnitine

TMA is obtained from bacterial metabolism of dietary L-carnitine (and its metabolite γ-butyrobetaine). Only the L-stereoisomer of carnitine is found in foods. Animal products like meat, fish, poultry, and milk (whey fraction) are highest in carnitine, whereas plants contain little carnitine (42). For example, 100 g of asparagus contains 0.2 mg carnitine, 100 g of egg contains <1 mg carnitine, 100 g of cod contains 4 mg carnitine while 100 g of beef contains 39 mg carnitine, (28). Carnitine also can be synthesized endogenously from two essential amino acids, lysine and methionine (63). This occurs in kidney, liver, and brain (42). After administration of an L-carnitine challenge test (consumption of a diet containing an 8-ounce sirloin steak and a capsule containing 250 mg of a heavy isotope–labeled L-carnitine), people produced TMAO from L-carnitine in a gut microbe-dependent manner (63). The bioavailability of L-carnitine in vegetarians (who are adapted to low-carnitine diets) was higher than in carnivores (42); therefore, less carnitine reaches the bacteria of the lower intestine in vegetarians. TMAO production, following a challenge with L-carnitine, was significantly greater in omnivores versus vegans, and there were specific bacterial taxa associated with both dietary status and plasma TMAO concentration (63).

3.3. L-Choline and Choline-Containing Compounds

TMA can be produced from bacterial metabolism of dietary choline and choline-containing compounds, such as PtdCho. The foods that contain the most choline are of animal origin, especially eggs and liver; however, most of the foods we eat contain some amounts of choline or choline compounds (129, 130). The US Department of Agriculture maintains a database on the total choline content in common foods (116) that can be used to calculate dietary choline intake. For example, 100 g of cod contains 89 mg total choline (choline + PtdCho + phosphocholine + sphingomyelin + glycerophosphocholine) compared to 100 g of beef, which contains 73 mg total choline, or 100 g of eggs, which contain 266 mg total choline (28). In 1998, the US Institute of Medicine (Food and Nutrition Board) established adequate intake and tolerable upper intake limit values for choline, based on limited human studies (56). The adequate intake is 550 mg/day for men and 425 mg/day for women, with upward adjustment in pregnant and lactating women; the upper intake limit ranges from 1,000 mg/day in children to 3,500 mg/day in adults (56).

Choline is present as free choline and choline esters (such as PtdCho) in foods (130), and these forms of choline are absorbed differently in the intestines. Choline is absorbed in the small intestine via mediated transport, whereas PtdCho is absorbed intact via the lymphatic system or is hydrolyzed by pancreatic lipases and absorbed as glycerophosphocholine (125). On the basis of these differences, dietary choline should be a substrate for TMA formation by bacteria in the intestine, but dietary PtdCho should not be. However, consumption of ≥2 eggs, which contain mostly PtdCho, results in increased formation of TMAO (79, 110). About 15% of the PtdCho in eggs was converted to TMA, and peak plasma TMA concentrations were achieved between 6 and 8 h after ingestion of the eggs (79).

3.4. Betaine

TMA also is obtained from bacterial metabolism of dietary betaine (120). The foods that contain the most betaine are of plant origin, especially spinach, beets and grains grown in high osmotic environments (129, 130). Plants use betaine in their roots as an osmolyte to help them retain intracellular water (33). The US Department of Agriculture maintains a database on the betaine content in common foods (116) that can be used to calculate dietary betaine intake. For example,

100 g of spinach contains 577 mg betaine, 100 g of cod or beef contain 7 mg betaine, and 100 g of eggs contain <1 mg betaine.

3.5. Diet Patterns and Trimethylamine *N*-Oxide

There have been few studies that examined dietary intake patterns and plasma TMAO concentrations. In a small study in healthy Bavarian subjects, dietary intake of fish, meat, or eggs was not associated with plasma TMAO concentrations, but dairy intake was positively associated with plasma TMAO concentrations (94). In a study of Swedish men, fish consumption was associated with increased urinary TMAO concentrations (107). In obese patients undergoing bariatric surgery, TMAO concentrations were moderately increased after surgery (114).

Little is known about the kinetics of TMAO and TMA in human blood. After people consume a meal containing eggs, TMAO concentrations in plasma peak at 6 to 8 h and return to baseline by 24 h after the meal (79). We do not know whether there is a circadian rhythm in TMAO concentrations in plasma.

4. PHYSIOLOGICAL FUNCTIONS OF TRIMETHYLAMINE AND TRIMETHYLAMINE *N*-OXIDE

4.1. Trimethylamine *N*-Oxide Is an Osmolyte and Protein Stabilizer

TMAO is an osmolyte in tissues and acts as a molecular chaperone that stabilizes proteins. It retains the folded state of proteins and counteracts the effect of denaturants, such as pH, urea, and high pressure (6, 13, 29). The exact mechanism for the chaperoning properties of TMAO remains unknown, but many theories have been proposed (6, 14, 29, 91, 105). In tissues, the protein-stabilizing effects of TMAO are important for maintaining enzymatic activity. Increased TMAO concentrations reduce the loss of catalytic activity of rabbit muscle phosphofructokinase under conditions of pH-induced cold lability (48). In experiments designed to investigate pH-induced hysteretic processes of ischemic myocardium, TMAO increased the rate of reactivation of the phosphofructokinase enzyme purified from rat myocardium (47). In accord with its projected role as a chemical chaperone and nanocrowding particle (29), TMAO stimulated the association of the enzyme phosphorylase kinase with its substrate glycogen (26).

4.2. Trimethylamine Is a Ligand for Trace Amine-Associated Receptor 5

Trace amine-associated receptors (TAARs) belong to the family of G protein–coupled receptors and are olfactory receptors (117). Human TAAR5 can be selectively activated in a concentration-dependent manner by TMA but not by TMAO (117). *TAAR5* is the most highly expressed TAAR gene in humans and may constitute an olfactory detector of TMA that elicits innate behaviors (117). TMA is formed in male ejaculate and vaginal secretions, and as noted in Section 2.2, TMA formation may be regulated by sex hormones (13), and cycle-dependent variations in TMA occur in women (82). Thus, TMA may be a pheromone in humans (117). TMAO also may signal via activation of mitogen-activated protein kinase and nuclear factor-κB (NF-κB) pathways (99, 136).

4.3. Trimethylamine *N*-Oxide and Sterol Metabolism

TMAO regulates multiple aspects of cholesterol and sterol metabolism (**Figure 2**). Mice, fed a choline- (or L-carnitine-) or TMAO-supplemented diet, showed significant decreases (~30% and

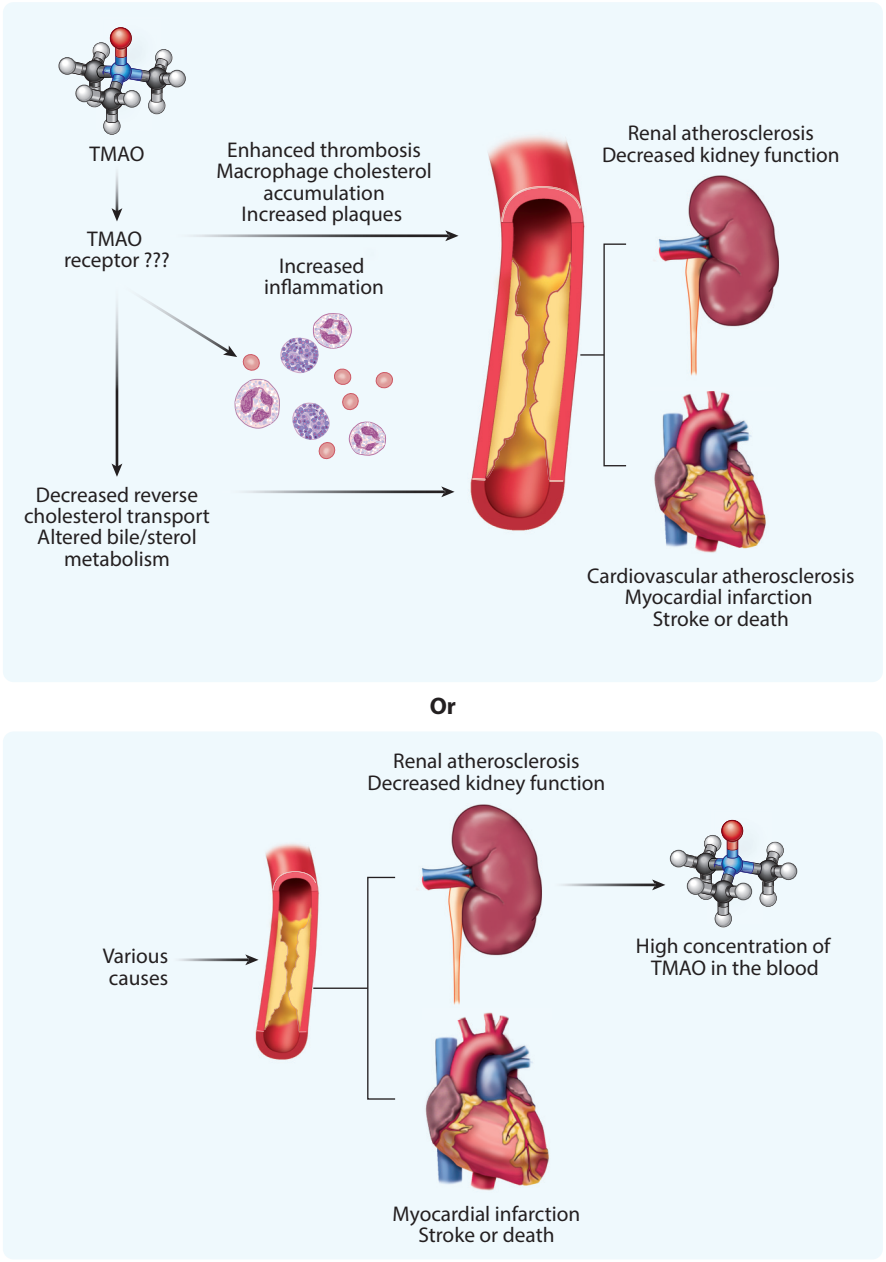

Figure 2

Proposed effects of trimethylamine *N*-oxide (TMAO). TMAO may be causal in enhancing macrophage foam cell formation and development of atherosclerotic plaques. TMAO increases inflammatory macrophage responses, and it decreases reverse cholesterol transport. It is possible that there is an endogenous receptor for TMAO, but it has yet to be identified. There is evidence in a specific genetically modified mouse that TMAO can cause atherosclerosis; however, human data is observational and cannot rule out the possibility that atherosclerosis of the kidney and cardiovascular system are highly related and that as a result of atherosclerotic kidney disease TMAO concentrations are higher in plasma but not causal.

~16%, respectively) in reverse cholesterol transport (RCT; the net movement of cholesterol from peripheral tissues back to the liver via the plasma) (62, 63). Suppression of intestinal microbiota (and therefore plasma TMAO concentrations) completely inhibited diet-dependent reductions in RCT. The molecular mechanism(s) by which TMAO inhibits RCT is not entirely understood but seems to involve multiple steps of both forward and reverse cholesterol transport (19). Even though high concentrations of circulating plasma TMAO increase cholesterol influx to peritoneal macrophages (118), cholesterol efflux or cholesterol biosynthesis appears to be unchanged. In fact, modest but significant increases in many cholesterol transporters, such as Abca1 and Abcg1, were noted (118). These changes do not explain reductions in RCT. TMAO feeding did not change the expression of hepatic cholesterol exporters, but striking reductions in bile acid pool size and species, along with reduced expression of the cytochrome P450 family of enzymes, Cyp7a1 and Cyp27a1, were noted (118). Transcript levels of hepatic bile acid transporters, such as Oatp1, Oatp4, Mrp2, and Ntcp, also showed significant reductions in response to TMAO feeding (118). Dietary supplementation of TMAO also decreased intestinal cholesterol absorption. The effect is consistent with reduced expression of the cholesterol transporter Niemann-Pick C1-like 1 but again does not explain reductions in RCT (118). Taken together these results provide evidence that TMAO alters pathways for cholesterol elimination from the body and causes reductions in RCT.

When C57Bl/6 female mice were fed either a low- or high-cholesterol diet, *FMO3* knockdown greatly reduced hepatic *FMO3* expression with corresponding increases in plasma TMA concentrations and reductions in TMAO concentrations (121). *FMO3* knockdown altered several steps of cholesterol balance, including intestinal cholesterol absorption, hepatic cholesterol ester storage, and macrophage RCT in a dietary cholesterol-dependent manner (121). FMO3 inhibition stimulated fecal neutral sterol loss and basal and liver X receptor-dependent macrophage RCT (121). The administration of antibiotics eliminated the FMO3 substrate TMA and normalized intestinal cholesterol absorption and fecal neutral sterol loss seen in FMO3 antisense oligonucleotide-treated mice (121). However, dietary supplementation of TMAO failed to normalize cholesterol balance. Similar reductions in hepatic lipids, plasma lipids, ketone bodies, glucose, and insulin were also seen in FMO3 antisense oligonucleotide (ASO)-treated LDL receptor knockout mice (102). These results suggest that, although chronic elevation of TMAO can be detrimental in mice, the ability of FMO3 to reorganize cholesterol balance is most likely not mediated through TMAO. Thus, both studies conclude that FMO3 inhibition has definite positive effects on cholesterol and glucose homeostasis and that TMAO does not seem to be a critical player. Rather, these processes appear to be regulated by either TMA or other unidentified enzymatic substrates or products of FMO3.

5. TRIMETHYLAMINE *N*-OXIDE AND DISEASE

5.1. Trimethylamine *N*-Oxide and Cardiovascular Disease

As discussed above in Section 1, there is accumulating evidence suggesting an association between TMAO and risk for developing atherosclerotic CVD. The data supporting the hypothesis that TMAO causes atherosclerosis divides into three categories:

1. People at risk for CVD and stroke have high plasma TMAO concentrations; they also have higher concentrations of the TMAO precursors, i.e., choline, betaine, and carnitine in plasma.

2. Apolipoprotein E (Apo E) knockout mice treated with TMAO precursors or with TMAO develop more atherosclerosis.

3. Mouse peritoneal macrophages incubated with TMA express scavenger receptors, accumulate cholesterol, and form foam cells.

In the following sections, we examine the strengths and weaknesses of this evidence.

5.1.1. High trimethylamine *N*-oxide correlates with cardiovascular disease. In 75 patients undergoing voluntary cardiac assessment who later experienced a heart attack, stroke, or death over a three-year period, nontargeted metabolomics were used to identify small molecules in plasma that might predict increased risk for CVD (118). Choline, betaine, and TMAO were identified as molecules in plasma that were significantly associated with increased atherosclerotic plaque burden in subjects (118). As discussed above in Section 2.1, choline and betaine are precursors for TMA formation by gut bacteria. In both the clinical cohorts, kidney function was not normal [normal glomerular filtration rate (GFR) is ~100 mL/min]; the mean GFRs in both groups were 73 and 74 mL/min, respectively (range 60–87), values compatible with some loss of kidney function. In addition, plasma concentrations of the TMAO precursor L-carnitine in subjects undergoing cardiac evaluation ($n = 2{,}595$) predicted increased risks for CVD and for major adverse cardiac events (myocardial infarction, stroke, and death) (63). This cohort had a mean eGFR of 83 mL/min (63).

During three years of follow-up in patients ($n = 4{,}007$) undergoing elective coronary angiography, increased plasma concentrations of TMAO were associated with increased risks of major adverse cardiovascular events (110). In this study, participants with events had decreased kidney function (median eGFR of 75 mL/min) and median plasma TMAO of 5 μM compared to those without events (median eGFR of 83 mL/min) who had median plasma TMAO of 3.7 μM.

Higher dietary intake of PtdCho, another precursor for TMAO formation, was associated with increased mortality risk in an analysis of 80,978 women from the Nurses' Health Study (1980–2012) and 39,434 men from the Health Professionals Follow-Up Study (1986–2012) (135).

In a cross-sectional study of 227 patients who underwent cardiovascular surgery for coronary artery disease, valvular heart disease, or aortic disease, a significantly increased number of infarcted coronary arteries was identified in patients in the highest quartile of plasma TMAO concentrations compared to the lowest quartile (odds ratio 11.9; 95% confidence interval 3.88–36.7, p value $\leq$ 0.001) (72). Higher TMAO concentrations were observed in people who also had advanced-stage chronic kidney disease (72). In patients in the highest quartile of plasma TMAO concentrations, 38% had greater than stage 3 kidney disease, whereas in lowest quartile for TMAO, none had greater than stage 3 kidney disease (72).

In 292 individuals (99 CVD cases and 193 unmatched control subjects), plasma TMAO concentrations were significantly correlated with prevalent CVD, even after accounting for covariates, such as meat, fish, cholesterol, and energy intake (75). There was no significant association between L-carnitine concentrations and prevalent CVD. There was no association with kidney disease severity either (75). In diabetic patients, elevated plasma betaine concentration is a risk factor for CVD (69); betaine is a metabolite of choline. Plasma TMAO concentrations were also higher in patients with chronic heart failure (115). Plasma TMAO concentrations were studied in HIV-infected patients with myocardial perfusion defects but were not associated with their coronary calcium score or intima media thickness (46).

The prognostic value of plasma TMAO concentrations was assessed in a cohort of patients ($n = 2{,}235$) with stable coronary artery disease, and elevated concentrations were associated with greater long-term mortality risk (101). Elevated concentrations of plasma choline and betaine, assessed in stable cardiac patients, were associated with incident mortality, myocardial infarction, and stroke risk, independent of other traditional risk factors. However, these metabolites were

predictive only when a simultaneous increase in plasma TMAO concentrations was observed (120). A number of other clinical studies observed an association between TMAO and CVD risk. Plasma TMAO concentrations were strongly associated with long-term mortality in heart failure patients, with advanced left-ventricular diastolic dysfunction, and with atherosclerosis burden in patients with atherosclerotic coronary artery disease (100, 101, 109, 111, 120).

Several studies, discussed above in Section 5.1.1, support the hypothesis that there is an association between diets high in TMAO precursors (choline, betaine, L-carnitine) and in high plasma TMAO concentrations and risk for CVD. However, there are inconsistencies that give pause. In a cohort of 339 patients who underwent coronary angiography, plasma concentrations of TMAO or choline were significantly increased with decreasing renal function, but TMAO concentrations were not associated with incidence or symptoms of coronary heart disease. The study concluded that plasma concentrations of TMAO were confounded by impaired kidney function because it was increased with declining GFR (85). A meta-analysis of 13 controlled trials ($n = 3{,}629$ people) reported that low oral dosage of L-carnitine resulted in 27% reduction in all-cause mortality, a 65% reduction in ventricular arrhythmias, and a 40% reduction in angina symptoms in patients who had an acute myocardial infarction (37). Although oral supplementation of L-carnitine was associated with increased plasma TMAO concentrations, it decreased vascular injury markers and oxidative stress markers in hemodialysis patients (43). There was a positive correlation between processed meat and mortality due to CVD observed in a study from the EPIC group ($n > 400{,}000$ people), but this association was specific to processed meat and was not seen with unprocessed meat, which has high L-carnitine and choline content (95). No association was found between dietary choline or betaine intake and incident CVD in the Atherosclerosis Risk in Communities Study ($n = 14{,}430$ middle-aged people) (16). In the Jackson Heart Study, involving 3,924 African-Americans, higher dietary choline was associated with reduced risk of incident ischemic stroke, whereas higher betaine intake was associated with a nonlinear higher risk of incident coronary heart disease (78). Dietary intakes of betaine and choline were not associated with CVD risk in >17,000 postmenopausal Dutch women in the PROSPECT-EPIC cohort (34). Recently, the Coronary Artery Risk Development in Young Adults study evaluated the role of TMAO in early atherosclerosis progression in a cohort of subjects ($n = 817$) of ages 33–45 over a 10-year follow-up. The authors found no association between TMAO concentrations and atherosclerotic progression as measured by carotid artery calcification incidence and carotid intima medial thickness (76). This study was conducted in relatively younger and healthier individuals as compared to other studies (110, 111) and therefore was not confounded by other factors, such as age-related increases in TMAO concentrations. In conclusion, these studies support the need for further research to define the cause-and-effect relationship and mechanisms of action of TMAO in promoting CVD and its related outcomes.

5.1.2. Mice treated with trimethylamine *N*-oxide develop cardiovascular disease. Atherosclerosis-prone mice (C57BL/6J *Apo* $e^{-/-}$) fed a normal diet supplemented with additional choline (118) and L-carnitine (63) or TMAO (118) had increased total aortic root atherosclerotic plaque area compared with mice fed a control diet (118). Feeding the TMAO precursor γ-butyrobetaine (a metabolite of carnitine) also increased aortic plaque size (62). The proatherogenic effects of choline, γ-butyrobetaine, and carnitine were prevented by treatment with antibiotics, suggesting that microbial metabolism of these precursors was required for the effect (63, 118). The proatherogenic effects of feeding choline to *Apoe*$^{-/-}$ mice was also reported by others (27). In the transverse aortic constriction surgical mouse model for pressure overload-induced heart failure, a choline (1.2%)/TMAO (0.12%)-enriched diet significantly increased plasma TMAO concentrations and worsened pulmonary edema, cardiac enlargement, myocardial fibrosis, and

left ventricular ejection fraction (90). Interpretation of such results is difficult, as normal mouse diets contain 0.1% choline, and the authors treated mice with amounts of choline twelve times higher; these levels are not physiologic.

The animal model used in these studies appears to be important. Feeding L-carnitine to *Apo* $e^{-/-}$ transgenic mice overexpressing human cholesterol ester transfer protein (CETP) caused increased plasma TMAO concentrations in these mice, but this elevated TMAO was inversely correlated with aortic lesion size (31).

Chronic choline supplementation of atherosclerosis-prone low-density lipoprotein receptor ($LDLR^{-/-}$) mice increased plasma TMAO concentrations and increased expression of inflammatory genes in vascular cells (52). Aortas from these mice showed enhanced expression of cytokines and adhesion molecules. Acute intraperitoneal administration of TMAO to $LDLR^{-/-}$ mice recapitulated this phenotype, which could be a mechanism for development of atherosclerosis (52). When plasma TMAO concentrations were raised to ~100 μM by intraperitoneal injection of TMAO in an $FeCl_3$-induced carotid artery injury mouse model, thrombus formation was increased compared to controls. In addition, when mice were fed a chemically defined diet supplemented with either 0.12% TMAO or 1% choline, platelet aggregation induced by adenosine diphosphate stimulation was significantly increased (136). Although these studies provide interesting insights to TMAO and CVD risks, the excessively high levels of dietary choline and/or TMAO used in the above studies make it difficult to extrapolate these results directly to humans. High levels of choline itself can be toxic and can induce undesired side effects in humans (56).

5.1.3. Macrophages treated with trimethylamine *N*-oxide develop the atherosclerotic phenotype. Peritoneal macrophages obtained from C57BL/6J mice, fed a TMAO-supplemented diet for more than three weeks, showed increased mRNA levels of two macrophage scavenger receptors (CD36 and SR-A1), which are implicated in atherosclerosis (118). These macrophages had excess cholesterol accumulation and foam cell formation (118). Short-term TMAO treatment of primary mouse peritoneal macrophages did not result in phosphorylation of p38 mitogen-activated protein kinase, ERK1/2, and p65 NF-κB (other inflammatory pathways associated with atherosclerosis) (52).

5.1.4. Trimethylamine *N*-oxide—cause or result of atherosclerosis. Most, but not all, human observational studies support the hypothesis that there is an association between high plasma TMAO concentrations and risk for CVD, especially atherosclerosis. However, such associations do not mean, necessarily, that TMAO causes heart disease. It could be that TMAO is a biomarker for a metabolic perturbation associated with atherosclerosis (like renal dysfunction) and not part of the causal pathway. Or, it could be that the bacteria that can produce TMA from precursors in the gut also produce an atherogenic factor; TMAO would then just be a marker for the presence of these bacteria (**Figure 3**).

As noted above in Section 2.3, TMAO is excreted by the kidneys, and this metabolite is elevated in patients with kidney disease. Many of the studies that identified an association between TMAO and heart disease also found that people with higher TMAO had decreased renal function (61, 109). Uremia accelerates atherosclerosis and arterial calcification in patients with chronic renal failure (22, 74). In renal failure, the underlying mechanisms for accelerated atherosclerosis are not known but could include increased concentrations of calcium, phosphate, and intact parathyroid hormone in blood and/or perturbed cholesterol metabolism (74) and increased homocysteine concentrations (104). Increased TMAO concentrations may or may not be a contributing mechanism. If cardiovascular atherosclerosis is accompanied by renal atherosclerosis and decreased

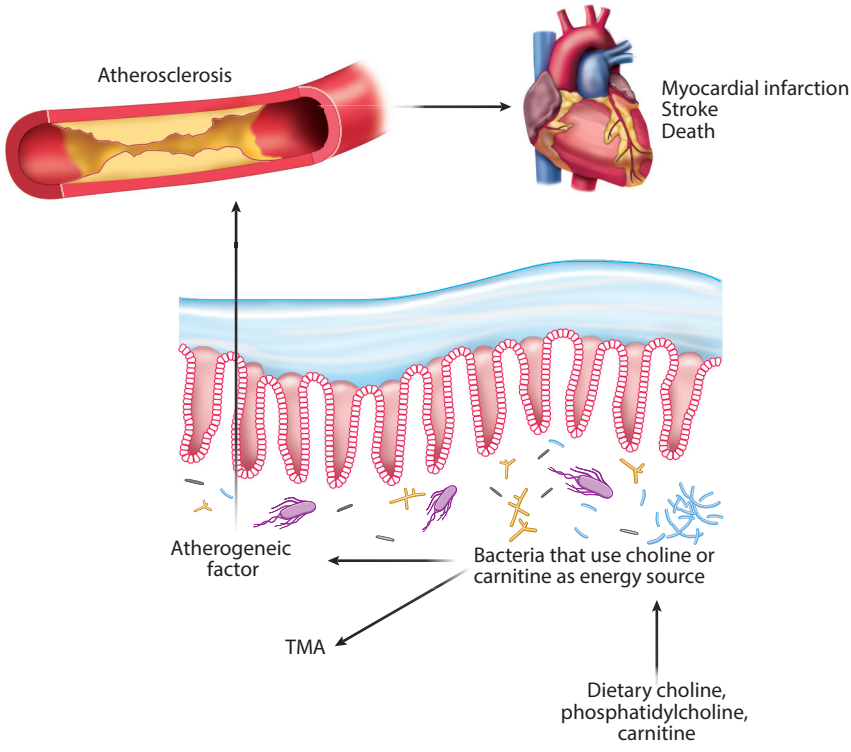

Figure 3

Alternate theory for atherogenesis. Although trimethylamine (TMA) is formed in the intestinal lumen when specific types of gut microbiota metabolize carnitine, choline, and choline-containing compounds in the diet, these bacteria also derive energy and carbon from these substrates that allow them to proliferate. This type of bacteria could make an atherogenic factor other than TMAO derived from TMA. People with these bacteria would thus have high plasma concentrations of TMAO, but the atherogenic factor would be the cause of atherosclerosis rather than TMAO.

kidney function results, then TMAO could be a biomarker for decreased kidney function. Rather than being the cause of atherosclerosis, TMAO would be the result of atherosclerosis.

Fish contain high concentrations of TMA, which are converted to TMAO by bacteria in the gut of people who eat fish. If TMAO causes CVD, then eating more fish should be associated with increased risk for heart disease; it is not. In fact, a large body of evidence links fish consumption to cardio-protective effects. Initial observational studies, involving Greenland Eskimos, Alaskan natives, and Japanese Okinawa islanders, reported lower risk of coronary artery disease death in these populations due to higher dietary fish intake (10, 40, 65). A meta-analysis of 11 eligible studies and 13 cohorts ($n = 222{,}364$) found that fish consumption was inversely associated with CVD and may even reduce mortality due to CVD (50, 51). In the Physicians Health Study, no evidence of association was found between fish consumption and any type of CVD events (3). The Nurses' Health Study compared women who rarely ate fish (<1 per month) with those who ate fish regularly and found that those with a higher intake of fish had a lower risk of CVD (54). In an analysis of 26 prospective cohort studies and 12 randomized controlled trials with aggregate data on 794,000 nonoverlapping people and 34,817 cerebrovascular outcomes, dietary fish intake was protective against stroke (30). This inconsistency, whereby a food that is considered heart healthy

(fish) could be a rich source of TMA and TMAO (the putative causal agents of heart disease), needs further study.

Thus, there are strong data that suggest an association between TMAO and CVD risk, but there are equally strong inconsistencies. The clinical and epidemiological data themselves do not prove that TMAO causes heart disease. Randomized intervention trials with TMAO do not exist, but several studies in mouse models and cell culture, discussed above in Sections 5.1.2 and 5.1.3, provide key evidence that there could be a causal relationship between TMAO and CVD. Are these mouse model data sufficient to conclude that TMAO causes atherosclerosis?

There are important differences in atherogenesis between mice and humans. Humans carry about 75% of their plasma cholesterol in low-density lipoprotein (LDL) particles, whereas mice carry most of their cholesterol in high-density lipoprotein (HDL) particles (57). Mice lack the cholesteryl ester transfer protein that transfers cholesterol ester from HDL to very low-density lipoprotein (VLDL) and LDL (57). Apo E knockout mice are considered to be one of the most relevant models for atherosclerosis because they are hypercholesterolemic and develop spontaneous arterial lesions (87). Apo E is a glycoprotein that is synthesized in the liver, brain, and other tissues in both humans and mice. It is a part of all lipoprotein particles other than LDL and is a high-affinity ligand for ApoB, ApoE, LDL, and chylomicron remnant receptor, thereby allowing the specific uptake of Apo E-containing particles by the liver (57). Apo E-knockout mice have a shift in plasma lipoproteins from HDL, the major lipoprotein in wild-type mice, to cholesterol-enriched remnants of chylomicrons and VLDLs, and these mice have phenotypes similar to those of Apo E-deficient humans (57). Lesion progression and cell types are similar, as is the presence of oxidized lipoproteins. The major difference between this mouse model and humans is that plaque rupture is not observed (57). As discussed above in Section 5.1.2, the effects of TMAO on atherogenesis are completely opposite when *Apo* $e^{-/-}$ transgenic mice overexpressing human CETP are studied. In these mice, increased plasma TMAO concentrations were inversely correlated with aortic lesion size (31). Thus, the existing mouse data may support the hypothesis that TMAO causes atherosclerosis, but given the concerns discussed above, we should be cautious about extrapolating data from a particular mouse model to humans.

5.2. Trimethylamine *N*-Oxide and Kidney Disease

The relationship between TMAO and renal function was discussed above in Sections 2.3 and 5.1.1. TMAO is cleared from plasma by the kidney. Renal atherosclerosis is difficult to separate from CVD, and much of the discussion about the relationships between TMAO and CVD risk therefore applies to the risk for kidney disease. The data supporting the hypothesis that TMAO causes kidney disease divides into two categories:

1. People at risk for renal disease have high plasma TMAO concentrations.
2. Mice treated with TMAO develop renal disease.

In the following sections, we examine the strengths and weaknesses of this evidence.

5.2.1. Trimethylamine *N*-oxide correlates with renal disease. Patients with poor kidney function have elevated plasma TMAO concentrations (93, 109). Metabolomic analysis of urine from 31 patients identified a group of seven metabolites that differed between chronic kidney disease (CKD) and non-CKD urine samples; TMAO concentrations in plasma were higher in CKD (92). Significantly higher concentrations of TMA and TMAO were noted in plasma from 15 patients with end-stage renal disease compared to 15 healthy counterparts (8). Magnetic resonance spectroscopy in 16 patients observed that plasma TMAO concentrations were correlated with

the degree of renal failure (12). In 179 CKD stage 3–5 patients, the GFR and impaired renal function were the main variables affecting plasma TMAO concentrations (81). Increased TMAO concentrations were associated with systemic inflammatory markers and predicted a reduced five-year survival (81). Serum concentration of TMAO was inversely associated with eGFR and was markedly higher in patients receiving dialysis (59, 81); hemodialysis efficiently removed TMAO from blood (8). Renal transplantation corrects the high plasma TMAO concentrations seen in renal failure (81, 106).

Increased TMAO concentrations have been associated with increased mortality from renal disease. In a study of stable patients with kidney disease ($n = 521$), there was an association between fasting plasma TMAO concentrations and all-cause mortality (109). Elevated plasma TMAO concentrations were predictive of five-year mortality risk after adjusting for traditional risk factors, such as high-sensitivity C-reactive protein, glomerular filtration rate, etc. Even among patients who did not have severe kidney disease, elevated plasma TMAO concentrations indicated poor prognosis (109). In 123 individuals (out of 1,434 participants) in the Framingham Heart Study who did not have CKD at baseline but developed it during eight years of follow-up, metabolomic profiling on plasma showed that TMAO was associated with incident CKD even after adjustment for GFR, sex, diabetes, hypertension, and proteinuria at baseline (93). An NMR-based metabolomics study observed an association between urinary TMAO concentrations and tubulointerstitial lesions in patients ($n = 77$) with glomerulonephritis. The study concluded that this increased excretion of TMAO could be due to tubulointerstitial distortions (4).

5.2.2. Mice treated with trimethylamine *N*-oxide develop renal damage. When C57BL/6J mice were fed either a high-choline diet or a diet supplemented with TMAO for six weeks, plasma concentrations of TMAO increased and were associated with corresponding increases in tubulointerstitial fibrosis and collagen deposition, as well as increases in kidney injury marker-1. When mice were fed these diets for 16 weeks, serum cystatin C concentrations rose (a marker of kidney injury) (109). These data support the hypothesis that, in mice, TMAO is a cause of kidney damage.

5.2.3. Trimethylamine *N*-oxide—cause or result of renal dysfunction. As discussed above in Section 5.2.1, TMAO concentrations are high in patients with renal disease. However, such an association is not sufficient evidence that TMAO causes renal disease. Because renal clearance is a determinant of plasma TMAO concentrations, kidney function and TMAO must be correlated even if TMAO does not cause kidney damage. It is also important to note that some studies report no causal association of TMAO with morbidity or mortality from renal diseases. For example, no relationship was observed between serum TMAO concentrations and all-cause mortality or cardiovascular outcomes in the Comprehensive Dialysis Study, a prospective cohort of 235 patients with end-stage renal disease requiring hemodialysis or peritoneal dialysis (59).

It was suggested that the accumulation of TMAO in end-stage renal disease patients who receive chronic hemodialysis is mainly due to the previously unrecognized limitation of chronic dialysis (45). TMAO is cleared more efficiently than urea by the normal kidney function but not by dialysis (45).

The data from the mouse study discussed in Section 5.2.2 (109) are the most compelling evidence that TMAO (and choline a precursor of TMAO) causes renal disease. This study has not been replicated, yet the paper mentions a companion study using *Apo* $e^{-/-}$ mice but fails to present its results. And the investigators did not determine, using antibiotics, whether conversion of choline to TMAO was required for the choline effects. The same cautionary notes as discussed in the CVD sections of this review apply to using mouse data to predict human responses. Thus,

the existing mouse data may support the hypothesis that TMAO causes renal disease, but given the concerns discussed in Section 5.1.4, scientists and clinicians should be cautious about extrapolating data from a particular mouse model to humans.

5.3. Trimethylamine *N*-Oxide and Diabetes

The data supporting the hypothesis that TMAO causes diabetes divides into two categories:

1. People at risk for diabetes have high plasma TMAO concentrations.
2. Animals with altered TMAO metabolism or that are treated with TMAO develop diabetes.

In the following sections, we examine the strengths and weaknesses of this evidence.

5.3.1. People with high trimethylamine *N*-oxide concentrations are at risk for diabetes. Alterations in TMAO metabolism have been linked to diabetes and insulin resistance. In individuals undergoing elective coronary angiography, plasma TMAO concentrations were correlated with increased serum glucose and the patient's diabetic state (110). In diabetic patients, increased plasma TMAO concentrations were associated with death, myocardial infarction, heart failure, unstable angina, and other cardiovascular events (69). In a group of 191 patients undergoing coronary intervention, plasma TMAO concentrations were independently associated with the patients' age, diabetes, and body mass index (35). Plasma TMAO concentrations also were significantly increased after consumption of a low glycemic load diet for 28 days (11). By contrast, patients with diabetes treated with metformin had decreased glucose but increased plasma TMAO concentrations compared with untreated individuals (55).

5.3.2. Animals with altered trimethylamine *N*-oxide metabolism, or treated with trimethylamine *N*-oxide, develop diabetes. Serum and urinary concentrations of methylamines including TMAO were associated with the development of nonalcoholic fatty liver and impairment of glucose metabolism in high-fat-fed 129S6 mice (39). In high-fat-fed C57Bl/6 mice, TMAO feeding increases the glucose intolerance induced by the diet as measured by higher fasting insulin levels and the homeostasis model assessment (HOMA) for insulin resistance scores along with altered insulin signaling and increased inflammatory markers (44). Serum TMAO concentrations were also predictors of insulin resistance in cholesterol and high-fat-fed *Macaca mulatta* (70). Liver insulin receptor knockout (LIRKO) mice are unable to respond to insulin in the liver and display several features of diabetes, such as hyperglycemia, hyperinsulinemia, and dyslipidemia. They are also susceptible to atherosclerosis (15). Transcriptional and metabolomics profiling revealed upregulation of FMO3 and its corresponding metabolite TMAO in LIRKO mice (77). LIRKO mice have increased expression of gluconeogenic genes owing to increased expression of the transcription factor forkhead box O1 (FoxO1). Knockdown of FMO3 in LIRKO mice suppressed FoxO1 expression (and improved glucose tolerance) by upregulating sterol regulatory element binding protein 2 (SREBP2) (77). SREBP2 directly activates microRNA 182, which in turn regulates FoxO1 expression (77). It is interesting that hepatic TMAO concentrations are decreased (not increased) in diabetic *db/db* mice (123).

5.3.3. Trimethylamine *N*-oxide—cause or result of diabetes. As for TMAO and CVD or renal disease, there is some evidence that high plasma TMAO concentrations are correlated with diabetes risk. In addition, there is evidence that feeding TMAO increases insulin resistance in animal models. The same caveats apply as to extrapolation of animal data to humans, but it is clear that additional studies are needed to clarify the potential causal relationship of TMAO to diabetes.

5.4. Trimethylamine *N*-Oxide and Cancer

There is considerably less known about TMAO's relationship to cancer. The Women's Health Initiative and the alpha-tocopherol, beta-carotene cancer prevention observational studies reported that alterations in choline metabolism and plasma TMAO concentrations were predicted risk factors for colorectal cancer (7, 83). Also in a genome-wide analysis study, TMAO was genetically associated with colorectal cancer (124). TMAO was used also as a urinary marker in a mouse model of gastric tumorigenesis (60).

6. TARGETING TRIMETHYLAMINE, HEPATIC FLAVIN MONOOXYGENASE, AND TRIMETHYLAMINE *N*-OXIDE PATHWAYS IN DISEASE THERAPY

The concept that a metabolite of dietary nutrients, formed by gut microbiota, is absorbed and then converted by the liver to a molecule that increases the risk for CVD, CKD, and diabetes is groundbreaking. Although there is reason to be cautious in accepting this as a proven hypothesis, the data to date strongly suggest that the idea deserves serious consideration. If true, this novel pathway suggests new targets for treatment and prevention of these disorders.

Prebiotics and probiotic therapy could be used to alter the composition of gut flora so as to lower those organisms that have the capacity to form TMA. Prebiotic therapy includes ingestion of selected food components, such as nondigestible fiber, or energy substrates that provide a growth advantage to beneficial bacteria. Probiotic therapy includes delivering specific bacterial strains that do not have the genes needed to cleave choline or carnitine to form TMA (20). Even though it needs to be demonstrated in humans, introducing *Lactobacillus paracasei* in mice expressing human baby flora decreased fecal choline and was associated with reduced concentrations of TMA and dimethylamine in the liver and plasma lipoproteins (73). TMA can be used for methanogenesis in the gastrointestinal tract, and bacteria belonging to the genera *Methanomassiliicoccus* and *Methanomethylophilus* have this pathway (18, 21, 38, 41). Probiotic treatment with such bacteria might be an approach to lower exposure to TMA and thus to TMAO (21).

The removal of TMAO or its precursor TMA from the gut by oral nonabsorbent binders could be a promising approach. To this end, oral charcoal adsorbent (AST-120) has been clinically used to remove uremic toxins, such as indoxyl sulfate, in patients with advanced renal failure (86). At this time, specific products that remove TMAO remain undiscovered. Another possible therapeutic intervention relies on the use of broad or class-specific antibiotics to eliminate the production of gut microbes that produce harmful metabolites including TMA. However, long-term usage of antibiotics can promote the emergence of antibiotic-resistant bacterial strains and repopulation of microbes after its withdrawal. Antibiotic treatment also suppresses both good and bad bacteria, making it difficult to produce a desired impact (20).

Inhibition of the bacterial enzymes responsible for the production of TMAO by specific nonlethal microbial inhibitors holds great promise. The bacterial gene cluster that encodes the enzymes for the anaerobic production of TMA from choline have been identified (see Section 2.1). These gene products can be attractive drug targets as their inhibition eliminates the production of TMA. A nontoxic structural analog of choline, 3,3-dimethyl-1-butanol, inhibited the conversion of choline, carnitine, and crotonobetaine (but not γ-butyrobetaine) to TMA (119). It also significantly lowered plasma TMAO concentrations and mitigated choline-enhanced macrophage foam cell formation and atherosclerosis in Apo $e^{-/-}$ mice (119). Enzymes that catabolize TMAO and hepatic FMO enzymes, which convert TMA to TMAO, provide other potential targets of therapeutic intervention (20). Inhibiting FMO enzymes, however, may not be ideal as it causes accumulation

of TMA, leading to a fishy odor and inflammation as observed in antisense oligonucleotide-treated mice (102, 121). One should also consider the side effects associated with inhibiting FMOs as they are major xenobiotic metabolizing enzymes (23). Meldonium, a drug that has cardioprotective action, decreased plasma concentrations of TMAO by increasing urinary excretion in humans. It also decreased intestinal microbial-dependent production of TMAO from L-carnitine (36, 67). Resveratrol, a natural phytoalexin, also reduced plasma TMAO concentrations in Apo $e^{-/-}$ mice by inhibiting TMA production from gut microbiota (27).

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Dr. Warrier is not aware of having any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review. Dr. Zeisel is a founder of Nutrigene Sciences, LLC, a company in which he owns stock equity. He receives research funding from Balchem, Inc., a company that manufactures choline chloride, and therefore could have an interest in TMAO as it affects use of choline as a diet supplement or food ingredient. The funded research is unrelated to TMA or TMAO. He serves on the Scientific Advisory Board of Metabolon. He is a paid consultant for Nestle, Enzymotec, Bayer Consumer Healthcare, DSM, and Pharmavite. None of these entities had input to the manuscript or has a direct interest in the manuscript.

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