Review Article

Impact of trimethylamine N-oxide (TMAO) metaorganismal pathway on cardiovascular disease

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Abstract: Host-microbes interaction plays a crucial role in cardiovascular disease (CVD) pathogenesis, mechanistically via metaorganismal pathways. The trimethylamine N-oxide (TMAO) metaorganismal pathway is the most deeply investigated one, which comprises trimethylamine precursors, such as choline, trimethylamine lyase, trimethylamine, host liver FMO3, TMAO, and downstream effectors involving unfolded protein response (UPR), NF-κB and NLRP3 inflammasome. Accumulating data from clinical investigations of CVD patient cohorts and rodent models have supported the critical role of this metaorganismal pathway in the pathogenesis of CVD. We summarize an array of significant animal studies especially for arthrosclerosis with an emphasis on downstream molecular effectors of this metaorganismal pathway. We highlight clinical investigations of the prognostic value of plasma TMAO levels in predicting prospective risk for future major adverse cardiac events (MACE) indicated by composite end points of myocardial infarction (MI), stroke, heart failure (HF), other ischemic cardiovascular events, or death. Further, we discuss the latest advances of preclinical models targeting the gut microbiota trimethylamine lyase of the TMAO metaorganismal pathway for CVD intervention, as well as the catalog of gut microbiota TMA lyase genes and microbes in the human gut as the prerequisite for potential clinical intervention. In-depth characterization of TMAO metaorganismal pathway holds great promise for CVD clinical metagenomics, diagnostics and therapeutics.

Keywords: Trimethylamine N-oxide (TMAO); cardiovascular disease (CVD); major adverse cardiac events (MACE)

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Introduction

Trillions of microbes inhabit the human gut, which affect human health and disease. The 16S rRNA gene sequencing and next generation sequencing (NGS) allow culture free detection of bacterium community in human gut, which speed up the advancement of gut microbiome research (1-4). The association between gut microbiome and cardiovascular disease (CVD) received attention during the past several years. Patients with CVD shows different gut microbiota community structure from healthy controls, which are related to CVD phenotypes and cohorts (5-8).

Although there is no consistence in gut microbiota community with CVD among different groups in the world, a consensus has been reached that gut microbiota impacts cardiovascular health and disease via various metabolites, such as trimethylamine-N-oxide (TMAO), short-chain fatty acids and secondary bile acids (9-12). Host-microbes interaction plays crucial role in CVD pathophysiology, mechanistically via metaorganismal pathways. The TMAO metaorganismal pathway is the most deeply investigated one, which comprises trimethylamine precursors, such as choline, TMA lyase, TMA, host liver FMO3, TMAO, and

downstream effectors involving unfolded protein response (UPR), NF-κB and NLRP3 inflammasome (13-20) (*Figure 1*).

TMAO has been widely validated as a pro-atherogenic and pro-thrombotic microbe-host co-metabolite, causally linked to CVD (13-15,20-23), which affects one third of adults as the leading cause of death worldwide (24,25). TMAO is an oxidative product of TMA, catalyzed by hepatic flavin monooxygenases (FMOs) (26,27). Multiple nutrients with structural formula containing TMA group can be cleaved to produce free TMA by enzymes in microbes (28-30). Chronic red meat consumption results in an average of 3 times higher circulating TMAO than does non-meat or white meat (31). The two steps of TMAO production from nutrients constitute a metaorganismal pathway, linking to a number of complex diseases (19). Clinical investigations have highlighted the prognostic value of plasma TMAO levels in predicting prospective risk of complex diseases, including CVD, kidney diseases, diabetes and liver steatosis as well (32-35). Targeting this pathway holds great promise for CVD intervention as supported in preclinical models.

Gut microbiome and CVD

Initially gut microbiome was reported to contribute to metabolic diseases through the modulation of energy balance (increased energy harvest) and immunity (inflammation and autoimmunity) (36,37). Several years later our group reported that gut microbiota metabolism of dietary phosphatidylcholine (PC) promotes CVD (21) and the association between gut microbiome and CVD started to receive attention. The gut microbiome discrimination between patients with CVD and healthy controls were reported in several groups. Karlsson *et al.* used shotgun sequencing to analyze fecal microbiome from 12 patients with symptomatic atherosclerosis and 13 age and gender matched controls and found that the genus *Collinsella* was enriched in patients with symptomatic atherosclerosis, whereas *Roseburia* and *Eubacterium* were enriched in healthy controls (6). Yin *et al.* compared fecal microbiota community using 16S rRNA gene sequencing between 141 patients with stroke and transient ischemic attack and 94 asymptomatic controls and found more opportunistic pathogens, such as *Enterobacter*, *Megasphaera*, *Oscillibacter*, and *Desulfovibrio*, and fewer commensal or beneficial genera such as *Bacteroides*, *Prevotella*, and *Faecalibacterium* in patients (7). Emoto *et al.* compared fecal microbiota community by terminal restriction fragment length polymorphism (T-RFLP) of 16S rDNA amplicons in 39 patients with coronary artery disease (CAD), 30 age- and sex-matched no-CAD controls, and found that increased *Frimicutes/Bacteroidetes* ratio and *Lactobacillus* and decreased *Bacteroides* plus *Preveotella* in CAD (8). Jie *et al.* compared fecal microbiome in 218 patients with atherosclerotic CVD (ACVD) and 187 healthy controls and found that ACVD is rich in *Enterobacteriaceae* including *Escherichia coli*, *Klebsiella* spp., and *Enterobacter aerogenes* and *Streptococcus spp.*, and *Eubacterium eligens*, *Faecalibacterium prausnitzii*, and *Clostridiales sp* and has diminished *Bacteroides spp.*, *Prevotella copri* and *Alistipes shahii* (5). It seems that we cannot find a unique gut microbiome community structure that contributes to CVD, but a consensus was reached that gut microbiome contributes to CVD through gut microbiota derived metabolites including TMAO, aromatic acid metabolites, p-cresyl sulfate and indoxyl sulfate (19,38). Indoxyl sulfate contributes to CVD progression through stimulation of oxidative stress and inhibition of AMPK/UCP2 signaling (39) and activation of multiple signaling pathways including mitogen activated protein kinase (MAPK) and nuclear factor-κB (NFκB) (40). P-cresyl sulfate mediates MCP-1 production in vascular smooth muscle cells through NF-kB pathway, contributing to the inflammatory response initiation involved in atherosclerosis lesion formation (41).

TMAO, a metaorganismal product

The role of TMAO in CVD was initially discovered by Wang *et al.* through untargeted mass spectrometry (MS) based metabolomics approach (21) that was applied to identify metabolites in human plasma to discriminate healthy controls from CVD patients in two cohorts, randomly selected from GeneBank, a large data/sample bank containing more than 10,000 human plasma samples from patients undergone left selective coronary angiography with outcome data monitored after enrollment (42). The learning cohort was comprised of 50 cases subjected to risk for non-fatal myocardial infarction (MI), stroke or death within 3 years versus 50 controls without risk for non-fatal MI, stroke or death, and the validation cohort was comprised of 25 cases versus 25 controls (21). A metabolite with m/z =76 was found to have the smallest molecular weight while highly correlated to the other two analytes in the same metabolism pathway (21). The analyte with m/z =76 was eventually identified as TMAO via multiple MS and different liquid chromatography (LC) conditions by comparison with authentic compound (21). Because

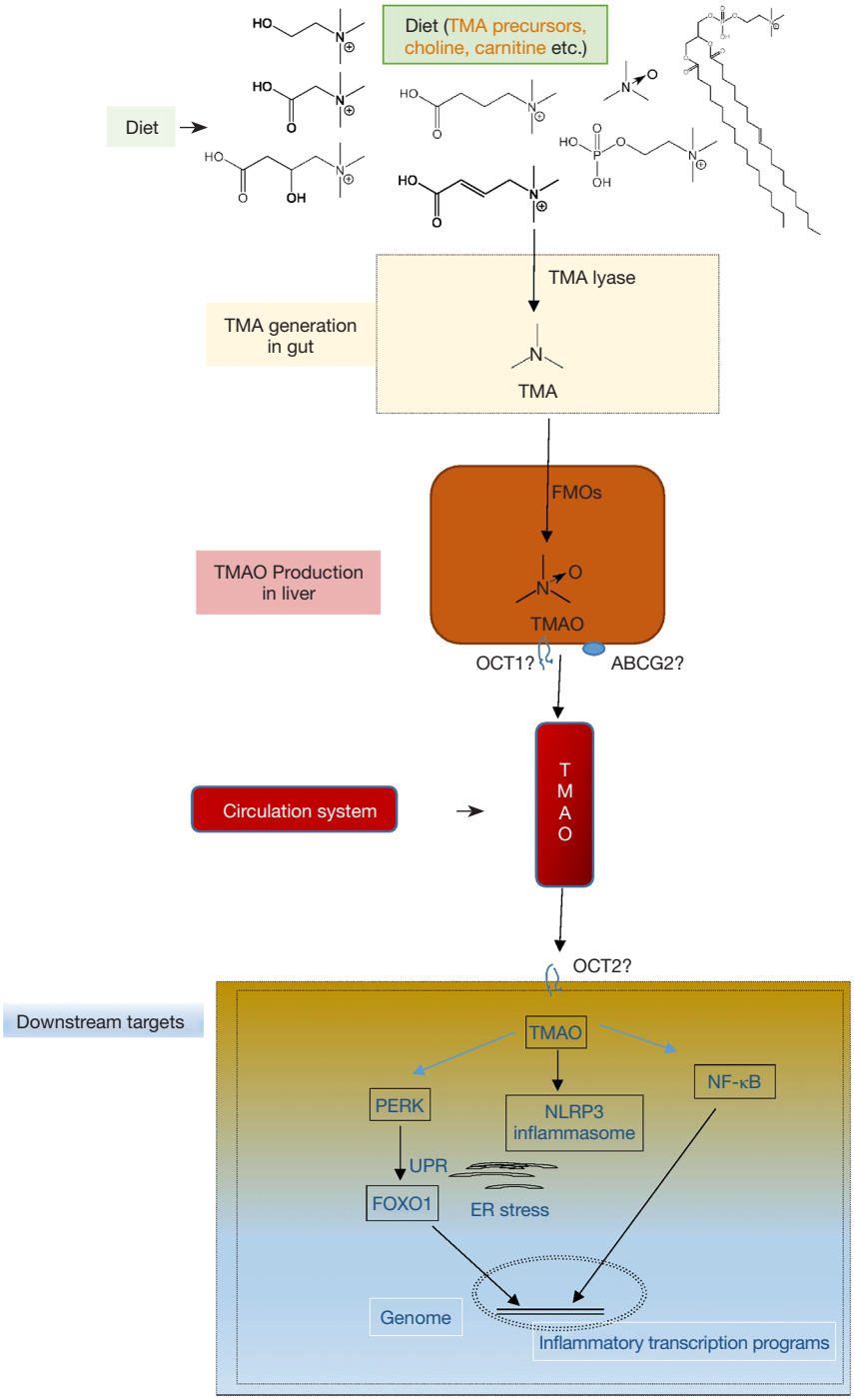

Figure 1 The trimethylamine N-oxide (TMAO) metaorganismal pathway. Key components of this metaorganismal pathway are illustrated, including trimethylamine precursors, such as choline, trimethylamine lyase, trimethylamine, host liver FMOs, TMAO, and downstream effectors involving UPR, NF-κB and NLRP3 inflammasome. TMA, trimethylamine; TMAO, trimethylamine N-oxide; FMOs, flavin monooxygenase; ER, endoplasmic reticulum; PERK, protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK); OCT1, organic cation transporter 1; OCT2, organic cation transporter 2; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; NF-κB, Nuclear factor kappa B; ABCG2, ATP Binding Cassette Subfamily G Member 2.

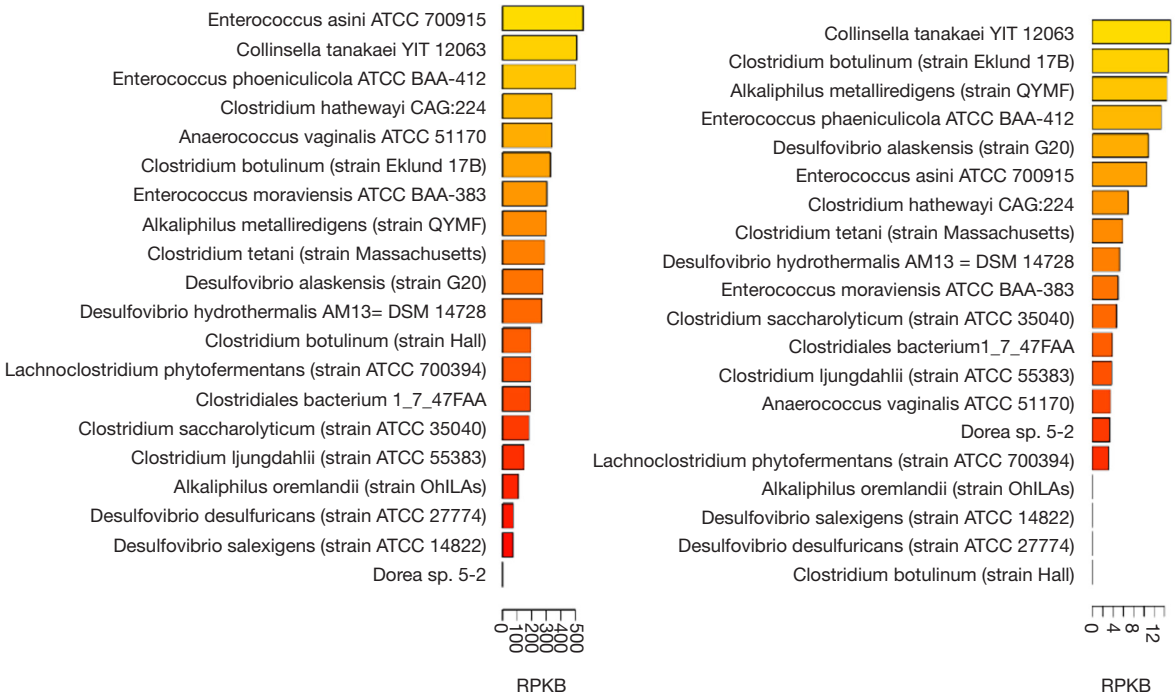

Figure 2 The distribution and abundance of cutC encoding bacteria in the samples of the human microbiome project 1(HMP1) (https://www.hmpdacc.org/resources/). The cutC abundance in oral cavity (A) is much more than that of the stool cutC (B) as shown in bar plot of the maximum abundance (RPKB, strain numbers per billion reads) among all samples in the cohort. The cutC gene abundances were computed following the method described in Thomas *et al.* (50).

the structural formula of TMAO contains TMA group, it was initially hypothesized that TMAO is the metabolite of dietary PC. This hypothesis was examined by feeding mice and humans with isotope labeled PC where ^{1}H atoms were replaced with deuterium (^{2}H) at the TMA functional group. The corresponding isotope labeled TMAO product, alongside two metabolism related metabolites, choline and betaine, was detected for *de novo* biosynthesis (21,43). These data also showed that the broad spectrum antibiotics treatment to deprive gut microbiota suppresses the production of TMAO while the TMAO production recovers after the removal of antibiotics (21,43). In parallel, if d9-PC was intraperitoneally injected, no d9-TMAO was detectable in the conventional mice (21). Thus, gut microbiota plays an obligatory role in the biosynthesis of TMAO (21,43).

While in the above challenge experiments PC was replaced with free choline, it was further confirmed that choline contributes to the production of TMAO, depending on gut microbiota (43). PC is the main choline source in diet (44,45). In the small intestine, PC can be digested by pancreatic phospholipase D, to release free choline (46). Meanwhile, commensal gut bacterium contains phospholipase D with PC as substrate to release free choline (47). In human gut, the endogenously synthesized PC can be released from bile (48). Choline can be then cleaved to produce TMA, which is catalyzed by choline TMA lyase, a glycyl radical enzyme in microbes (28). Choline TMA lyase encoding gene was found via sequence alignment with the operon encoding enzymes of the ethanolamine metabolism (28). The choline TMA lyase gene clusters contain at least two genes, *cutC* and *cutD*, encoding the two subunits of TMA lyase, respectively. The cutD protein activates cutC protein by forming a glycyl radical, which abstracts hydrogen atom from the cysteine residue to form cysteine radical followed by the cysteine radical abstracting hydrogen from choline to release free TMA (28,49).

Choline TMA lyase encoding genes have been found in many bacteria. For instance, in the human microbiome project (HMP1), there are 19 strains with high abundance found in the oral cavity samples while 16 strains were found in the stool samples. Thus, it appears that the abundance of choline TMA lyase encoding bacterium is higher in oral cavity than stool (*Figure 2*). Choline TMA lyases are

associated with other diseases besides CVD, e.g., choline TMA-lyase gene was overabundant in colorectal cancer (50).

Besides choline TMA lyase *CutC/D*, other TMA lyases using different preferential substrates to produce TMA have been reported, including *cntA/B* (the same to *yeaW/X*), betaine reductase and TMAO reductase (29,30,51,52). Multiple FMOs, including FMO1, FMO2 and FMO3, have the capacity to catalyze the oxidation of TMA to TMAO as shown in the HEK293Ad cell line transfected with human FMO1-5 constructs and incubated with d9-TMA with the readout of d9-TMAO monitored by LC/MS/MS (27). After normalization to the corresponding expressing FMO protein level, FMO3 has the highest specific activity (27). In humans, *FMO3* genetic deficiency due to germline mutations cause fish odor syndrome characterized by the accumulation in TMA and excretion in the breath, urine, sweat, saliva and vaginal secretions (53-55).

Dissecting the TMAO metaorganismal pathway in rodent models

The potential causal relationship between plasm TMAO and CVD was examined extensively in mice models (32,33). We summarized functional studies of the TMAO metaorganismal pathway in rodent models in *Table 1*, with an emphasis on the association of TMAO metaorganismal pathway with CVD. Moreover, we described key experimental results as follows, highlighting the complexity of TMAO effectors.

TMA precursors, such as choline, carnitine and -butyrobetaine, promote atherosclerosis via TMAO metaorganismal pathway. *Apoe*$^{-/-}$ mice are similar to humans in atherosclerosis progression (56). When *Apoe*$^{-/-}$ mice were fed choline or TMAO supplemented chow diet for 16 weeks to quantify aortic lesion with oil red O-hematoxylin staining, results showed that both choline and TMAO promote atherosclerosis. In the female mice, the aortic lesion is highly correlated to plasma TMAO (21). In order to test whether choline promoting atherosclerosis is mediated by TMAO, *Apoe*$^{-/-}$ mice were fed choline supplemented chow diet alongside drinking water containing broad spectrum antibiotics (vancomycin, neomycin, ampicillin, metronidazole), to suppress the gut microbiota production of TMAO. The quantification of aortic lesion suggests that gut microbiota plays an important role in choline promoting atherosclerosis and the effect of choline might be mediated through TMAO (21). Further, TMAO inhibits expressions of the key bile acid synthetic enzymes Cyp7a1 and Cyp27a1 in liver, resulting in decreased bile acid pool and cholesterol reverse transport (57). Besides choline promoting atherosclerosis, other TMA containing nutrients such as carnitine and -butyrobetaine, can also promote atherosclerosis via the TMAO metaorganismal pathway (30,57).

TMAO promotes platelet aggregation and thrombosis by inducing Ca^{2+} release. The *Ldlr*$^{-/-}$ mice show decreased plasma level of TMAO after *Fmo3*-knock down by intraperitoneal injection of *Fmo3 antisense* oligonucleotide. Further, atherosclerotic lesion area was decreased with the decreased plasma TMAO (58), suggesting the TMAO metaorganismal pathway is linked to atherosclerosis. TMAO increases platelet hyper-reactivity thereby promoting thrombosis (22). In platelet, TMAO induces Ca^{2+} release from intracellular calcium stores, leading to platelet aggregation and thrombosis (22).

Given that TMAO is linked to inflammatory diseases, it is pivotal to understand the underlying molecular mechanisms of TMAO activating inflammasome, a group of protein complexes built around several proteins, including NLRP3, NLRC4, AIM2 and NLRP6, *et al.* (59-62). TMAO activates the NLRP3 inflammasomes, leading to endothelial dysfunction activation, putatively via pathways of mitogen-activated protein kinase (MAPK) and nuclear factor-B (14,20). Accordingly, TMAO pre-incubated aortic endothelial cells show increased leukocyte adhesion (20). Monocyte adhesion to the vascular endothelial cell constitutes an important step of atherosclerosis progression (63). Importantly, TMAO is linked to UPR during endoplasmic reticulum stress; TMAO binds to PERK at physiologically relevant concentrations; selectively activates the PERK branch of the UPR; and induces the transcription factor FoxO1, a key driver of metabolic disease (18).

The molecular functions and associations with complex disease traits of TMAO metaorganismal pathway have been strengthened by using gnotobiotic models. Gut microbiota transplantation to germ free mice showed that choline diet-induced TMAO production capability and atherosclerosis and thrombosis susceptibility are transmissible. Distinct plasma TMAO levels have been observed in the strains of the hybrid mouse diversity panel (HMDP) (64-66), in which the aortic lesion is positively correlated to plasma TMAO (67). The NZW/LacJ *Apoe*$^{-/-}$ mouse has the lowest plasma TMAO and the lowest aortic lesion, while the C57BL/6J *Apoe*$^{-/-}$ mouse has the highest plasma TMAO and the largest aortic lesion (67).

In a gnotobiotic model, feces from NZW/LacJ *Apoe*$^{-/-}$ mice and from C57BL/6J *Apoe*$^{-/-}$ mice were used as donors

Table 1 Dissecting TMAO metaorganismal pathway in mouse models.

Study	mouse	Genetic model	Diet	intervention	Disease model (trait)	Metaorganismal pathway components	Reference
Wang_2011	C57BL/6J	Apoe$^{-/-}$	Choline supplemented chow diet	Mice were supplied with drinking water containing broad spectrum antibiotics (vancomycin, neomycin, ampicillin, metronidazole)	Atherosclerosis	Choline, Fmo3, TMAO	Wang *et al.*, 2011
Koeth_2013	C57BL/6J	Apoe-$^{-/-}$	Choline or carnitine supplemented chow diet	Mice were supplied with drinking water containing broad spectrum antibiotics (vancomycin, neomycin, ampicillin, metronidazole)	Atherosclerosis	Choline, carnitine, TMA and TMAO, CD36, key bile acid synthetic enzymes Cyp7a1 and Cyp27a1	Koeth *et al.*, 2013
Zhu_2016	C57BL/6J, NZW/LacJ	C57BL/6J versus NZW/LacJ	Chemically defined chow (0.08% total choline) versus chow diet supplemented with 1% choline for 3 weeks before the evaluation of plasma TMAO and platelet function.	FeCl3 carotid artery injury thrombosis model, rose Bengal photochemical injury thrombosis model , cecal microbial transplant study	Platelet hyperresponsiveness and atherothrombotic heart disease	Choline, TMA lyase, FMO3, TMAO, Ca2+	Zhu *et al.*, 2016
Chen_2019	C57BL/6	C57BL/6 insulin receptor liver KO, C57BL/6 FoxO1 liver KO, C57BL/6J Fmo3$^{-/-}$, ob/ob, PERK Flox, C57BL/6 XBP1 Flox	Standard chow diet I (Control for 0.12% TMAO diet,1% Choline diet, 0.25% DIM diet), 0.12% TMAO diet, 1% Choline diet, 0.25% DIM diet, Standard chow diet II (Control for 0.25% I3C diet), 0.25% I3C diet	Germ free female C57BL/6J mice colonized with CutC^{+} or CutC^{-} E. coli strains	Metabolic syndrome	Choline, TMA, cutC, Fmo3, TMAO, PERK, FOXO1	Chen *et al.*, 2019

to transplant to germ free mice, acquired by subjecting conventional mice on drinking water with broad spectrum antibiotics for 3 weeks, initially orally gavaging every other day for 2 weeks, followed by weekly for 11 more weeks and the mice were fed choline supplemented chow diet. At an age of 20 weeks, mice were sacrificed and the aortic plaque was quantified. The germ free mice which received feces from NZW/LacJ *Apoe*$^{-/-}$ mice have a relatively lower plasma TMAO and lower aortic lesion than C57BL/6J *Apoe*$^{-/-}$ mice (67). Thus, this gnotobiotic model of gut microbial transplantation demonstrated that atherosclerosis susceptibility is transferrable via transmission of the choline diet-induced TMAO production.

Wild type *Clostridium sporogenes* in the mice gut contributes to the production of TMAO (68). Intriguingly, disruption of the *cutC* gene in *Clostridium sporogene*s caused the loss of commensal bacterium transmitting thrombotic property (69). Thus, human gut commensals containing *cutC* are sufficient to transmit enhanced platelet reactivity and thrombosis potential.

The TMAO metaorganismal pathway has a molecular mechanism link to inflammation, thereby associated with a variety of inflammatory diseases, including atherosclerosis.

Targeting choline TMA lyase to attenuate atherosclerosis and thrombosis

Multiple choline TMA lyase inhibitors have the capacity of decreasing plasma TMAO levels, thereby ameliorating atherosclerosis and thrombosis. The natural compound, 3,3-dimethyl-1-butanol (DMB), which can be detected in some balsamic vinegars, red wines, and in some cold-pressed extra virgin olive oils and grape seed oils, has been shown to decrease plasma TMAO in mice fed choline or carnitine supplemented chow diet (70). Alongside decreasing plasma TMAO level, DMB can significantly ameliorate aortic lesions in *Apoe*$^{-/-}$ mice fed choline-supplemented chow diet via inhibition of enzymatic activities of multiple TMA lyases (70). In addition, DMB, a microbial choline TMA lyase inhibitor, attenuates choline diet-enhanced platelet responsiveness and *in vivo* rate of thrombus formation (71). In the $FeCl_3$-induced carotid artery injury mice model, the time to cessation of flow appears to be significantly longer in the mice with DMB added to the drinking water when compared with regular water, which is in agreement with the decrease in plasma TMAO (71).

Mice fed western diet developed impaired cardiac systolic and diastolic function by echocardiography accompanied by increased circulating TMAO, the addition of DMB to mice drinking water can decrease plasma TMAO, therefore prevent cardiac systolic and diastolic dysfunction (72). Series choline analogues, including fluromethylcholine (FMC), chloromethylcholine, bromomethylcholine, iodomethylcholine (IMC), were tested as more potent inhibitors to choline TMA lyase. These lead compounds can effectively decrease plasma TMAO to a nearly non-detectable level either fed chow diet or choline supplemented chow diet. FMC and IMC were further functionally tested and both decreased platelet responsiveness from mice fed choline-supplemented chow diet compared with chow diet and increased the time of cessation of flow (71). Inhibitory effects of bacterium growth or changed liver and renal functions have not been observed in the mice administrated with DMB, FMC, or IMC, indicating little potential side effects for these lead compounds (70,71). In addition, some nutraceutical, such as resveratrol, appeared to decrease circulatory TMAO, putatively due to gut microbiota remodeling (73).

TMAO metaorganismal pathway and CVD

Following Wang *et al.* in 2011, measurement of plasma TMAO from subsequent human plasma samples in the GeneBank cohort (4,007 patients) showed that increased plasma TMAO was correlated with increased prospective risk for major adverse cardiac events (MACE, MI, stroke and death) within 3 years (43). In addition, it was reported that high levels of plasma TMAO are independently correlated with plaque rupture in patients with ST-segment—elevation MI (74).

In human gut, there are trillions of microbes, which play important roles in human health, including production of vitamins, such as vitamin K and biotin, serotonin, and fermentation of dietary fiber to produce short chain fatty acids and modulation of immunity as well (75-78). The LifeLines-DEEP population cohort data showed the variance of microbiota can explain 4.5% of the variance in body mass index, 6% in triglycerides, and 4% in high-density lipoproteins, after adjusting for age, sex and genetic risk factors (79). These findings support the concept that gut microbiota contributes to CVD.

The prognostic values of multiple TMA nutrients are dependent on gut microbial metabolite, TMAO

Circulating choline and betaine are significantly correlated

to the prevalence of CVD, and also predict future risk for MACE (80). However, if adjusted with TMAO, the significant differences were not observed (80). The plasma TMAO levels can be stratified into two ranges by the median value, i.e., below median value (LOW) and above median value (HIGH), and the same method was applied to choline or betaine. In the GeneBank cohort, the LOW TMAO, HIGH choline or betaine plasma levels did not show significantly higher prognostic value in predicting risk for future MACE when compared with LOW choline or betaine, while the HIGH TMAO, HIGH choline or betaine levels showed significantly higher prognostic value in predicting risk for future MACE compared with LOW choline or betaine (80). Similarly, for carnitine, another TMAO precursor, the LOW TMAO, HIGH carnitine levels did not show significantly higher prognostic value than LOW carnitine, while the HIGH TMAO, HIGH carnitine levels showed significantly higher prognostic value than LOW carnitine (57). Thus, the prognostic values of multiple TMA nutrients are dependent on gut microbial metabolite, TMAO. Intriguingly, trimethyllysine (TML), a TMA containing compound in structural formula, can be cleaved to produce TMA and further contribute to the production of TMAO. However, the prognostic incident CVD risks of TML is independent of TMAO (81).

Future perspective

Since TMAO is mechanistically linked to CVD and can predict prospective risk for future MACE, the early monitoring of plasma TMAO is predictive to cardiac events. Furthermore, the administration of inibitors to block TMAO production as well as gut microbial transplantation has the potential to mitigate the progression of atherosclerosis and thrombosis. Taken together, TMAO takes the center stage in modulating the processes leading to the development of CVD. Several promising interventions targeting the TMAO metaorganismal pathway could be further investigated and ultimately be applied to patients with CVD.

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Footnote

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Conflicts of Interest: The series "Cardiovascular Precision Medicine" was commissioned by the editorial office without any funding or sponsorship. Z Wang is named as co-inventor on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics, and have the right to receive royalty payment for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland Heart Lab or Proctor & Gamble.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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