Review Article

Intestinal Microbiota Metabolism and Atherosclerosis

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Abstract

Objective: This review aimed to summarize the relationship between intestinal microbiota metabolism and cardiovascular disease (CVD) and to propose a novel CVD therapeutic target.

Data Sources: This study was based on data obtained from PubMed and EMBASE up to June 30, 2015. Articles were selected using the following search terms: “Intestinal microbiota”, “trimethylamine N-oxide (TMAO)”, “trimethylamine (TMA)”, “cardiovascular”, and “atherosclerosis”.

Study Selection: Studies were eligible if they present information on intestinal microbiota metabolism and atherosclerosis. Studies on TMA-containing nutrients were also included.

Results: A new CVD risk factor, TMAO, was recently identified. It has been observed that several TMA-containing compounds may be catabolized by specific intestinal microbiota, resulting in TMA release. TMA is subsequently converted to TMAO in the liver. Several preliminary studies have linked TMAO to CVD, particularly atherosclerosis; however, the details of this relationship remain unclear.

Conclusions: Intestinal microbiota metabolism is associated with atherosclerosis and may represent a promising therapeutic target with respect to CVD management.

Key words: Atherosclerosis; Cardiovascular; Intestinal Microbiota Metabolism; Trimethylamine; Trimethylamine N-oxide

INTRODUCTION

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality worldwide. Atherosclerotic CVD is a chronic inflammatory disease that primarily involves the large arteries and the medium-sized arteries. Multiple conventional risk factors for atherosclerosis have been identified, including hypertension, dyslipidemia, advanced age, and smoking.[1] Red meat (beef, mutton, and pork) consumption has also been linked to CVD.[2,3] It is believed that saturated and trans-fatty acids, which are abundant in red meat, are to blame.[4-6]

Recent researchers have identified components in meat other than fatty acids that may play important roles in the development of CVD. Phosphatidylcholine (also known as lecithin), choline, betaine, and L-carnitine are abundant in red meat and dairy products, participate in intestinal microorganism metabolism and are converted to trimethylamine (TMA). TMA is subsequently metabolized by flavin monooxygenase (FMO), a liver enzyme family, to trimethylamine N-oxide (TMAO).[7,8] It is observed that saturated and trans-fatty acids, which are abundant in red meat, are to blame.[4-6]

Gut bacteria metabolism and TMAO were recently linked to CVD.[8,16-22] In this review, we will discuss how gut bacteria metabolism is related to CVD and propose novel therapeutic targets that may be useful in the management of CVD.

TRIMETHYLAMINE N-OXIDE METABOLISM AND CARDIOVASCULAR DISEASE

Trimethylamine-containing nutrients are essential for survival and may be cardioprotective

Lecithin, choline, betaine, and L-carnitine each feature the same TMA moiety and play a central role in human...
metabolism. They are particularly abundant in both red meat and milk.

Lecithin is a vital component of the cell membrane and also plays an important role in immune modulation, anti-platelet aggregation, and lipid metabolism. Choline is a moiety found in lecithin, which plays a role in both membrane structure and lipid metabolism. It may be oxidized to betaine. Both choline and betaine have been linked to lower plasma homocysteine levels and reduced inflammation. As dyslipidemia, inflammation, hyperhomocysteinemia, and platelet aggregation have been linked to cardiovascular events, both lecithin and choline supplementation is recommended for cardiovascular protection. Indeed, it has been proven that dietary intake of choline and betaine does not render individuals susceptible to developing CVD and may actually prevent CVD by attenuating inflammation.

L-carnitine transports fatty acids into the mitochondria, particularly in cardiomyocytes, thereby facilitating fatty acid breakdown and energy derivation. A meta-analysis including 13 studies (n = 3629) demonstrated that L-carnitine reduced the incidence of angina by 40% and the all-cause mortality associated with acute myocardial infarction (AMI) by 27%. A randomized controlled trial involving 129 patients suffering from AMI, who were followed for 28 days, determined that carnitine reduces infarction size, cardiac enzyme levels, angina pectoris symptoms, and cardiac death. Another study involving patients receiving hemodialysis determined that although oral carnitine supplementation increases plasma TMA and TMAO levels, the markers of vascular injury were decreased after 6 months.

Therefore, it appears that each of these TMA-containing nutrients may represent a promising drug with respect to the management of angina and CVD. However, there have been no large-scale observation studies assessing the safety and the effectiveness of the supplementation of these TMA-containing compounds.

**Trimethylamine-containing compounds are a source of trimethylamine N-oxide**

TMAO metabolism has been well studied. Gut microbes take in TMA-containing compounds (lecithin, choline, betaine, and L-carnitine) and release TMA, which is subsequently metabolized by the FMO enzyme family in the liver to form TMAO (Figure 1). L-carnitine may also be transformed to γ-butyrobetaine by gut bacteria before being converted to TMA and TMAO. Hence, it is reasonable to predict that TMA-containing compound intake is linked with increased TMAO levels. Studies in both rodents and humans have proven this hypothesis. When either humans or mice were fed isotope-labeled lecithin, choline or L-carnitine, tracer-labeled TMAO was subsequently noted in both the plasma and the urine.

**Increased trimethylamine N-oxide level, not increased level of trimethylamine-containing compounds, is an independent risk factor for atherosclerosis and cardiovascular disease in the general population**

It has been observed that aortic root atheroma sizes in mice and clinical plaque burdens increase in parallel with TMAO levels; however, lipid, lipoprotein, and glucose levels are unchanged. Mice fed extra choline, carnitine or TMAO also exhibited a greater atherosclerotic burden. In both retrospective and prospective studies, researchers have observed that plasma levels of TMAO, choline, lecithin, betaine, and L-carnitine are associated with coronary artery disease (CAD), cerebrovascular events and peripheral artery disease. In a large scale (n = 4007)
cohort study, researchers observed that higher fasting plasma TMAO levels were associated with major adverse cardiovascular events (MACE), a relationship that remained robust even following adjustments for traditional risk factors.[19]

Two cohort studies involving patients followed for 3 years concluded that elevated choline, betaine, and carnitine levels predict MACE only when TMAO levels are elevated. When TMAO levels were added to the adjustment model, the correlations involving choline, carnitine, and MACE disappeared. Following the administration of antibiotics to carnitine-fed mice, plasma carnitine levels increased independently of atherosclerosis, indicating that TMAO promotes CVD rather than its TMA-containing precursors.[18,22] Indeed, as stated previously, some studies have determined that these TMA-containing compounds exert cardioprotective effects.

Higher TMAO concentrations have also been linked to higher SYNTAX scores.[14] Higher carnitine concentrations are predictive of multiple-vessel lesions in the setting of CAD.[18] However, the relationship between TMAO and mortality was not observed among patients receiving dialysis.[35]

**Potential mechanisms underlying the involvement of trimethylamine N-oxide in cardiovascular disease**

TMAO and atherosclerosis are related; however, it is unclear whether TMAO causes atherosclerosis or is merely a biomarker of the disease. If the former is true, what is the underlying mechanism of this phenomenon? Currently, limited amount evidence suggested that TMAO is a pro-atherosclerotic molecule.

The fundamental pathological change associated with atherosclerosis is the formation of lipid-rich macrophages (also known as “foam cells”) within the arterial wall. The beginning of atherosclerosis entails the uptake of oxidized low-density lipoprotein by macrophages within the arterial wall, a process mediated by macrophage “scavenger” receptors. High-density lipoprotein particles are able to transport lipids out of the arterial wall, thereby eliminating atherosclerotic plaques, a process termed “reverse cholesterol transport (RCT).” Theoretically, TMAO may cause lipid accumulation by facilitating lipid influx, enhancing *in situ* lipid synthesis, and suppressing lipid clean-up.

TMAO facilitates foam cell formation. When Apoe<sup>−/−</sup> mice were supplied with choline, betaine or TMAO, the levels of two atherosclerosis-related macrophage scavenger receptors (SAs), cluster determinant 36 (CD36) and SR-A1, were increased. Consequently, lipid-rich macrophages (foam cells) formed. However, oral antibiotics inhibited TMAO-dependent foam cell formation.[8]

TMAO also inhibits cholesterol efflux by suppressing RCT, as well as by blocking bile acid secretion. RCT was suppressed in mice receiving either choline or carnitine supplementation. However, following antibiotic administration, the suppression of RCT was reversed. Mice provided TMAO also exhibited decreased RCT; therefore, TMAO is produced by gut microbes and inhibits RCT. However, the levels of cholesterol transporters in macrophages did not change significantly following TMAO treatment.[13] It remains unclear how TMAO alters RCT. Moreover, the levels of bile acid synthetic enzymes (Cyp7a1 and Cyp27a1) and bile acid transporters (Oatp1, Oatp4, Mrp2, and Ntcp) in the liver were reduced when the mice were provided TMAO.[13]

As the bile acid pathway plays a major role in cholesterol elimination, blocking this pathway may have accelerated atherosclerosis.

However, TMAO does not affect cholesterol synthesis. Researchers have cultured macrophages with cholesterol and observed that regardless of whether TMAO was added, the messenger RNA (mRNA) levels of the low density lipoprotein receptor, cholesterol synthesis genes, and inflammatory genes did not change.[18]

Taken together, these findings indicate that TMAO accelerates atherosclerosis by facilitating cholesterol influx and inhibiting cholesterol efflux. However, it does not alter cholesterol synthesis [Figure 2].

Additional mechanisms underlying the atherogenic ability of TMAO may exist and are awaiting discovery. Several targets are worth studying, including the effects of TMAO on endothelial function, lipid and glucose profiles and the immune system. Further studies are warranted.

**Figure 2:** Mechanisms of the pro-atherosclerotic ability of trimethylamine N-oxide. Trimethylamine N-oxide facilitates cholesterol influx into macrophages and inhibit cholesterol efflux by hindering reverse cholesterol transport (RCT) and bile acid excretion.
Flavin monooxygenase 3 expression correlates positively with trimethylamine N-oxide levels and atherosclerotic burdens

FMO3 is the member of the FMO enzyme family with the highest activity level and is capable of oxidizing TMA to TMAO. The expression of Fmo3 is controlled by androgens and bile acids.[36] A significant positive correlation was observed between Fmo3 expression and TMAO levels, as well as atherosclerosis lesion sizes, in mice.[9] In patients undergoing liver biopsies, a positive correlation was also observed between hepatic Fmo3 expression and TMAO levels.[8] Taken together, FMO3 is essential for the formation of TMAO. It has also been found that loss of function (LOF) mutations in Fmo3 are associated with reduced atherosclerotic burdens in humans.[37]

However, another group observed that although the blockade of FMO3 resulted in decreased TMAO concentrations, increased atherosclerosis was also observed.[38] The mechanism underlying this phenomenon remains unclear.

The Relationship between Microbiota and Trimethylamine N-oxide

Specific intestinal microbiota is associated with trimethylamine N-oxide generation

The human body contains a vast and diverse microbial ecosystem of $10^{11}–10^{13}$ microorganisms, which begin colonizing the human intestine shortly following the birth and remain there throughout life. Both the species and the quantities of the gut microbiota are closely related to human health.[39] Each of us possesses hundreds of microbial species. The majority of these bacteria fall within the following four phyla: *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*. It is believed that gut microbiota influence the risk of specific diseases. The composition of gut flora fluctuates in accordance with the food we eat.[42] Gut flora participates in a variety of physiological processes, including digesting nutrients, regulating epithelial function, and guiding immune responses.[41] Intestinal flora may cause atherosclerosis by secreting lipopolysaccharides, commonly known as endotoxins.[44]

To verify the hypothesis that gut microbes are essential for TMAO generation, researchers administered antibiotics and observed that gut flora were completely suppressed and that TMA and TMAO were decreased in both chow-fed mice and mice receiving TMAO-containing compounds. Meanwhile, plaque burdens in the aorta were decreased, and the numbers of macrophages in the plaques also decreased following antibiotic administration. In another gut flora-free model, germ-free mice were utilized. These mice did not produce TMAO following a carnitine challenge. Following feeding with normal food, intestinal bacteria colonized, and plasma TMAO level rose accordingly.[8,18] Human trials have yielded similar results. Following the administration of broad-spectrum antibiotics, TMAO levels were decreased in volunteers’ plasma and urine L-carnitine samples. The reformation of TMAO was subsequently observed when the antibiotics were discontinued.[18,19] Collectively, these observations demonstrated that gut microbes are essential for TMAO generation.

These TMA-producing bacteria could be transplanted in conjunction with atherosclerotic susceptibility. Microbes from atherosclerosis-prone and high TMAO-producing strains enhanced the development of atherosclerosis in mouse recipients.[45]

To identify the microbes responsible for TMA generation, the microbial species localized to the human intestinal tract were cultured with choline *in vitro*: *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *Clostridium hathewayi*, *Clostridium sporogenes*, *Escherichia fergusonii*, *Proteus penneri*, *Providencia rettgeri*, and *Edwardsiella tarda* consumed choline and generated TMA. However, none was able to consume carnitine. Surprisingly, when these bacteria were colonized in germ-free mice, only a small proportion (0.15%) was enough to consume choline in the cecum and increase TMA and TMAO levels in the serum, respectively. When a large proportion of TMA-producing bacteria were colonized in the germ-free mice, the choline, TMA, and TMAO levels remained unchanged.[46]

Gut flora taxa are influenced by dietary habits

Compared with vegans and vegetarians, omnivores exhibited higher TMAO levels in both the plasma and urine at baseline. Even following an L-carnitine challenge, the vegans exhibited no significant elevations with respect to TMAO levels.[18] The observed differences may be attributed to gut flora taxa. By sequencing bacterial 16S rRNA in fecal samples, several bacterial taxa have been linked with both dietary habits (vegetarians vs. omnivores) and TMAO levels. Among them, *Clostridiaceae* and *Peptostreptococcaceae* are more abundant in omnivores, whereas *Lachnospira* and *Sporobacter* are less abundant in omnivores. Additionally, individuals with enterotype two (enriched of *Prevotella*) exhibited increased TMAO concentrations.[18] We concluded that some components in meat modulated gut flora taxa, thereby influencing the ability of microbes to generate TMAO. TMA-containing nutrients are likely to induce TMA production.

In order to verify this hypothesis, the mice were fed with high L-carnitine chow and several bacterial taxa, including *Anaeroplasma* and *Porphyromonadaceae*, were linked with L-carnitine intake and changes in TMAO concentrations.[18] However, another group observed that choline does not affect the amount of transplanted TMA-producing bacteria in germ-free mice,[46] which may be explained by the fact that the transplanted model is too simple to simulate human gut flora.

Microorganisms produce trimethylamine via several enzymes

The identification of the bacterial enzymes responsible for TMA production may provide more precise therapeutic
targets, as the genes encoding these enzymes may be transferred between bacteria. A two-component enzyme, composed of both an oxygenase (CntA) and a reductase (CntB), is associated with TMA formation from carnitine. A “bridging” glutamate within CntA facilitates electron transfer and is essential for TMA generation.\textsuperscript{[47]} Another group of choline specific TMA-producing enzymes, choline utilization C (CutC) and choline utilization D (CutD), has also been identified. Only the coexpression of CutC and CutD is associated with TMA production, as opposed to the expression of either enzyme alone.\textsuperscript{[48]} The genes encoding these enzymes may be transferred to non-TMA-producing bacteria.\textsuperscript{[47,48]} Additional TMA-producing enzymes must be identified, however.

**The genetic influence on trimethylamine N-oxide levels**

In addition to environmental factors, the genetic impact on TMAO levels was also analyzed. However, previous researchers failed to identify the locus responsible for the elevated TMAO levels observed in human.\textsuperscript{[49]} It appears that food and gut microbes are the primary determinants of TMAO levels.

**Future research and potential therapeutic targets**

TMAO, a novel independent cardiovascular risk factor, has drawn the interest of scientists. TMA-containing nutrients, gut flora, and hepatic FMO enzymes are the three indispensable components of this equation. However, many questions regarding this pathway have yet to be elucidated. It is our hope that clarifying these problems may provide us with potential therapeutic targets with respect to the management of CVD.

It is necessary to determine whether the supplementation of TMA-containing compounds is cardioprotective or proatherogenic. According to the gut microbes-TMAO pathway, compounds containing TMA moieties are likely to be metabolized to TMAO, which subsequently accelerates the development and progression of atherosclerosis. However, whether limiting the intake of these compounds are necessary remains unclear. As stated previously, these TMA-containing compounds do not exert direct atherogenic effects. Additionally, they also exert cardioprotective effects. Additional clinical trials utilizing different doses, durations and routes should be considered to determine whether the cardiovascular benefits of TMA-containing compounds outweigh the risks posed by TMAO formation. It is essential to include intake recommendations regarding these TMA-containing compounds in dietary guidelines, as limiting the intake of TMAO precursors fundamentally alters the production of TMAO.

Additionally, TMAO levels may be modulated by manipulating intestinal microbiota. It is necessary to determine which types of bacteria are associated with elevated TMAO levels and whether specific types of bacteria are atherogenic. We may eventually utilize narrow spectrum antibiotics to manage CVD. Some antibiotics may have potential as anti-atherosclerotic medications, as they reduce TMAO levels and plaque sizes. However, many clinical trials using antibiotics for the secondary prevention of CVD ended in failure.\textsuperscript{[50,51]} It appears that only long-term antibiotic use effectively decreases TMAO levels. However, the prolonged administration of antibiotics may be complicated by other problems such as drug tolerance and organ injury. In addition to antibiotics, several other promising methods may be useful with respect to the manipulation of gut microbe taxa. Most recently, an archaea strain known as *Methanomassiliicoccus luminyensis* B10 was found to be able to catabolize TMA.\textsuperscript{[52]} Prebiotics are chemicals that facilitate the growth of certain bacteria and are beneficial for individual’s health. However, TMA and TMAO levels increased when mice were fed with prebiotics.\textsuperscript{[53]} The effects of archaea, probiotics, and prebiotics on the cardiovascular system have not yet been elucidated. The direct modulation of gut microbes through the use of antibiotics, probiotics, prebiotics, and other interventions represents a novel treatment method with respect to the management of CVD, as gut microbes are essential for the formation of TMAO.

The administration of medications that decrease TMAO levels is promising, as TMAO causes atherosclerosis. Meldonium decreases the plasma levels of TMAO by increasing its urinary excretion and inhibiting the conversion of TMA to TMAO by bacteria. However, meldonium does not affect either the intake or the conversion of carnitine,\textsuperscript{[54,55]} findings somewhat reminiscent of homocysteine, which is associated with both CVD and stroke. However, homocysteine lowering agents such as vitamin B6, vitamin B12, and folic acid do not reduce the risk of cardiovascular events.\textsuperscript{[56]} It is unclear whether meldonium reduces TMAO levels but does not reduce the risk of CVD. Additional observations regarding the effects of meldonium in the setting of CVD are necessary. In addition to meldonium, other agents that reduce TMAO levels await discovery. The mechanisms underlying TMAO’s effects may include either increasing TMAO secretion or suppressing TMAO generation.

The relationship between *Fmo3* mutations and CVD remains unclear and warrants additional clinical research. LOF mutations in *Fmo3* may be cardioprotective, as TMAO levels decrease. Indeed, it was found that LOF mutations involving the *Fmo3* gene in humans were associated with less atherosclerosis.\textsuperscript{[57]} Furthermore, the knockdown of *Fmo3* using antisense oligonucleotide in mice also attenuates atherosclerosis.\textsuperscript{[57]} The knockdown of *Fmo3* not only resulted in decreased TMAO levels but also regulated both lipid metabolism and inflammation, thereby attenuating atherosclerosis.\textsuperscript{[58]} However, individuals without functional FMO3 often exhibit a fishy odor, which is caused by trimethylaminuria.\textsuperscript{[59]} Taken together, these findings indicated that the suppression of FMO3 may represent a potential therapeutic target, a hypothesis that warrants additional basic and clinical research to determine the effects of silencing FMO3 and to weigh the pros and cons of this approach. In addition to using antisense oligonucleotides, another group observed that 17β-estradiol also reduces
**Fmo3** mRNA concentrations. However, the effects on atherosclerosis were not evaluated.\(^{[90]}\)

TMAO is associated with both heart failure and atherosclerosis. The concentrations of TMAO among patients with heart failure are higher compared with healthy individuals, and TMAO is an independent risk factor for all-cause mortality among patients suffering from heart failure.\(^{[20,21,61]}\)

Additional studies are necessary to elucidate the correlation among TMAO, CVD and risk factors such as hypertension, dyslipidemia, arrhythmia, and glucose intolerance.

**Conclusions**

CVD poses a serious threat to human health. Although significant progress has been made regarding both the underlying mechanisms and the treatment of CVD, we remain far from a cure. In addition to the traditional risk factors for CVD, the influence exerted by intestinal microbe metabolism on the pathogenesis of CVD has only recently been recognized. Recent observations indicated that intestinal metabolism may represent a promising therapeutic target with respect to the management of CVD. Measuring TMAO levels in asymptomatic populations to identify individuals with an elevated cardiovascular risk, as well as monitoring changes in TMAO levels to determine the severity of CAD and manipulating gut flora to improve patients’ cardiovascular risk, is anticipated in the future.

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**Conflicts of interest**

There are no conflicts of interest.

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