The Contribution of Gut-Dependent Microbiota Derived Marker Trimethylamine N-oxide (TMAO) in Coronary Artery Disease

VASANTH KONDA MOHAN and MELVIN GEORGE*

Department of Clinical Pharmacology, SRM Medical College Hospital & Research Centre, Kattankulathur, Chennai.

Abstract

Coronary artery disease (CAD) has a high prevalence and one of the principal drivers of mortality worldwide. Therefore, there is a requirement to develop sensitive diagnostic biomarkers, disease progression control and therapeutic stratification in order to keep a check on the disease rate. Atherosclerosis is a systemic disease, the main cause of heart disease, is associated with hyperlipidemia and lipid oxidation and has always been a common single leading cause of death in well-developed countries. In the attempts to study CAD and the causative agents for the disease, a metabolite circulating in the plasma termed trimethylamine-N-oxide (TMAO) has been found out to be an independent risk factor that increases CAD risk. The use of a metabolomic approachhas proven useful in the recent past, as it can aid in the identification and quantification of several metabolites that play a crucial role for diagnosis and exploring therapeutic targets. TMAO is majorly synthesized by a process which involves the bioconversion of gut microbiota and hepatic flavin monooxygenases (FMOs) from nutrient-containing dietary trimethylamine (TMA). TMA is synthesizedby gut bacterial fermentation from the components present in meat such as phosphatidylcholine (PC), betaine, choline, and L-carnitine. It can accentuate the process of atherosclerosis through the novel meta-organismal metabolic pathway. TMAO leads to atherogenesis by increasing vascular inflammation, reducing vascular functions and disrupting cholesterol homeostasis at various levels. This review article attempts to summarize the pool of evidence collectedon the microbiota-dependent TMAO and its association with atherosclerosis. We performed literature search with Medline, PubMed, and Google Scholar, on "TMAO in CAD", "metabolites in CAD" and "TMAO in other diseases" from the year 1990 to 2020. Although the circulatory TMAO has been identified as an independent marker for CAD, there is still no conclusive evidence to justify its role as a routine marker for CAD diagnosis. Future research must clarify the mechanisms which underpin these complex associations to determine if there is a causal link exists between TMAO and CAD.

CONTACT Melvin George ✉ melvingeorge2003@gmail.com ● Department of Clinical Pharmacology, SRM Medical College Hospital & Research Centre, Kattankulathur, Chennai.

Introduction

Coronary artery disease (CAD), is one of the primary causes of death that involves the formation of fatty deposits or plaque on the interior of the arteries of the heart (coronary arteries). The formation of plaque (mostly cholesterol deposits) within the arteries is called atherosclerosis. Its clinical sequelae including myocardial infarction and heart failure isone of the major causes of morbidity and mortality.[1] In the lastsix decades, CAD has unfortunately become the largest driver of mortality in India, and has risen from 1% in 1960 to 14% in 2011.[2] By the year 2030, the mortality in the Indian subcontinent among individuals below the age of 60, will increase to approximately 17.9 million; data recorded to be higher than that of China, Russia, and even USA.[3]

Owing to the complexity of the disease, the underlying mechanisms regarding the more probable development of coronary macro vascular complications in CAD is yet to beentirely comprehended.[4] It is well-known that CAD is linked with complex metabolic disorders, such as insulin resistance, diabetes, and obesity.[5] Circulating metabolites, such as homocysteine, cholesterol, and triglycerides, have been well established in CAD.[6-8] However, it is still puzzlingas to how mechanistically these risk factors induce atherosclerosis, and whether independent pathways exist that circumvent these conventional factors altogether.[9]

Growing evidence has continued to emerge concerning the development of atherosclerosis with respect to gut microbiota alterations. Trimethylamine-N-oxide (TMAO), a major intestinal microbial metabolite, is derived from dietary choline, betaine, and L-carnitine. It gets metabolized to trimethylamine (TMA) by the microbiota of the gut following which, it is further rapidly oxidized into TMAO by hepatic flavin monooxygenases (FMOs) (Equation 1).[10] TMAO is a small organic compound, that is presentin certain food items such as fish, poultry, red meat, and dairy products.[11] The association between atherosclerosis and TMAO has been explored by recent metabolomics and animal studies.[12,13]

This article compiles the recent developments of gut-dependent microbiota-derived TMAO as an important participant in atherosclerosis and the role it plays in increasing the predilection leading to adverse cardiac events. Additionally, the article also addresses the approach in targeting gut-dependent microbiota and the recently recognized metabolic pathwaysinvolved in the formation of trimethylamine – N-oxide (TMAO) for potential CAD prevention and treatment.

Methodology
Search Strategy

We performed literature searches of research papers indexed in PubMed (including MEDLINE through NCBI) and Google scholar systematically. Broadly, the search strategy combined the following key search terms: "TMAO AND CAD", "metabolites AND CAD" and "TMAO in other diseases" from the year 1990 to 2020.References of retrieved articles were reviewed for additional citations.

Inclusion Criteria

In this analysis, the included studies were to contain the following details i) data analysis on a defined CAD population as entire cohort or stratified subset of mixed cohort; ii) TMAO as an indicator of risk for CAD, either as a single baseline or longitudinal measurements; iii) a measure of association (or ability to calculate) between TMAO and CAD.

Exclusion Criteria

Non-english articles, animal model studies, in-vitro studies, review articles, editorials, commentaries and duplicate articles were excluded.

Origin and Metabolism of TMAO

The hepatic Flavin-containing monooxygenases (FMO) oxidizes the TMA and forms TMAO, a small odorless molecule (mw 75.22g/mol).[14,15] Five members of FMO family are found in humans (FMO1-FMO5), and among them, the oxidation of TMA to TMAO can be catalyzed only by FMO1 and FMO3.[16] FMO3 is the major hepatic isoform and it has been noted that certain uncommon deleterious mutations in the FMO3 gene can lead to reduced or no TMAO formation which in turn results in TMA accumulation.[17-19] This is known as trimethylaminuria, an autosomal recessive condition, which is also referred to as "fish malodor syndrome" (OMIM 602079).[20]

Individuals with this syndrome tend to emit foul odors in their urine, sweat, and breath that resemble the smell of rotten fish.[21] TMA is generated in the colon by the gut microbiome originating from

dietary quaternary ammonium compounds (such as phosphatidylcholine/choline,[22] carnitine,[12] betaine[23]), in casethey fail to becompletely absorbed by the intestinal wall during digestion. TMAO in the blood is mainly eliminated by urinary excretion as well as sweat and breath.[24-27] Individuals excrete aliphatic amine TMA in rare metabolic disorder such as primary trimethylaminuria (TMAU), instead of TMAO.[28]

Equation 1: Oxidation of TMA to TMAO catalyzed by the enzyme hepatic FMO3.

$$TMA + NADPH + O_2 + H^+ \xrightarrow{\text{Hepatic FMO3}} TMAO + NADP^+ + H_2O$$
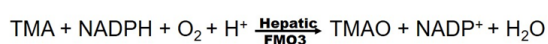
Correlation between CAD and TMAO

Atherosclerosis is a condition in which, the inner walls of the arteries tend to become thicker than their normal width and undergo fatty degeneration which causes a reduction inthe blood flow.[29] The high plasma level of TMAO potentiates the risk of cardiovascular events in established CAD.[11] Patients with CAD usually show higher concentrations of the TMAO. Several investigations have revealedthat TMAO seems to serve as a biomarker in patients for predicting the prevalence of CAD and increased incidence of major adverse cardiovascular events (MACE), such as recurrent heart failure, stroke, and mortality.[30-32]

The association between circulatory TMAO and CAD risk was first revealed in a study by Wang *et al*.[22] In this analysis, targeted metabolomics approach was used to measure the plasma metabolites such as, choline, betaine, and TMAO. Measured levels of these metabolites predict the risk of CVD among subjects. An epic pathway has been explainedwhich links dietary intake of lipids with intestinal micro-flora and atherosclerosis. The identified pathway for TMAO formed by dietary PC/choline through the metabolism of gut-flora is a significant additional nutritional contribution to the pathogenesis of CAD, involving PC and choline metabolism, which plays an essential role for the intestinal microbial population as well as in regulating the surface expression rates of macrophage scavenger receptors known to participate in the atherosclerotic process (Fig 1).

Several studies have opined that increased blood TMAO level is associated with increased CVD risk, consistent with the findings from Wang *et al*. In 2013, the plasma and urinary levels of TMAO, betaine and choline were estimated by using liquid chromatography procedure and online tandem mass spectrometry. In this study, authors found that the elevated plasma concentration of TMAO had a moderate association with increased incident risksfor major cardiovascular events, independent of identified risk factors, and suggested that the intestinal microbes are active in the metabolism of phosphatidylcholine to form circulating plasma and urinary TMAO.[33]

In another study, TMAO was estimated in Chinese subjects with CAD including those with and without concomitant diabetes. Measurement was carried out by LC-MS/MS and it wasobserved that the TMAO plasma levels were significantly higher in patients with CAD as compared to subjects in the control group. It was also found out that the plasma levels of TMAO in patients with T2DM associated with CAD were significantly higher than in CAD patients without T2DM.[34]

In 2019, a study measured the concentration of TMAO by mass spectrometry which was then evaluated for its association with the severity and prognosis of peripheral artery disease. The said study included 262 symptomatic peripheral artery disease (PAD) patients (mean age 70 years, 87% men) categorized as intermittent claudication (IC, n = 147) and critical limb ischemia (CLI, n = 115), all of whom were followed up for a mean average of 4 years (min 1-max 102 months). In the study's result, an increased level of TMAO according to PAD severity as well as an independent association between TMAO and CV-mortality were identified. The design of novel therapeutic approaches for gut-derived metabolite regulation in vascular patients need to be considered not only for intestinal bacterial function, but also for the role of key hepatic enzymes for TMA oxidation (FMO3), as well as renal function.[35] It has been indicated that increased plasma TMAO concentration in patients who are affected by coronary heart disease was strongly correlated with heavy atherosclerotic stress and can also raise the MACE risk in patients living with CHD by 58%. Additionally, it has beenproposed that the TMAO concentration of 5.1 µmol/L may be the ideal cut-off value for prognostication.[36]

The concentration of TMAO can be associated with many other diseases as well. The elevated serum TMAO level have been associated with advanced chronic kidney disease (CKD) stages as well as an increased number of infarcted coronary arteries in patients undergoing cardiovascular surgery.[25]

It has been observed that patients with CKD having elevated plasma TMAO level show higher risk of all-cause mortality.[37] In 2014, Bae *et al* have stated that plasma TMAO has a positive association with colorectal cancer risk in postmenopausal women.[38]
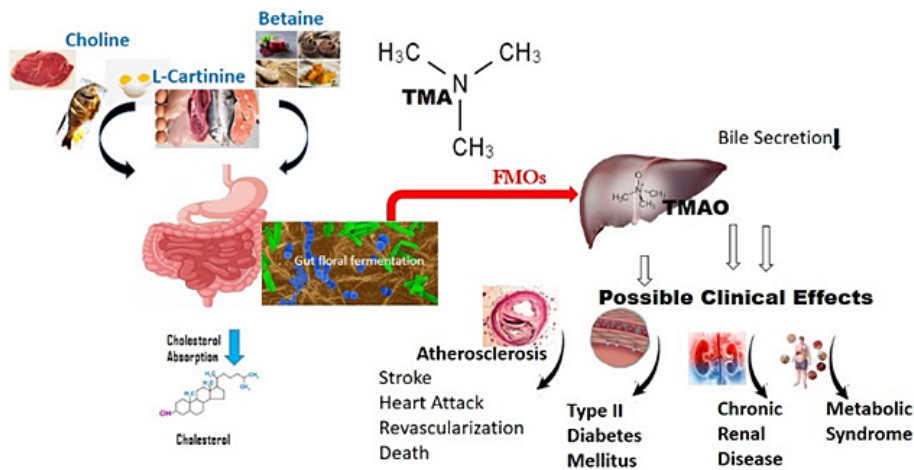

Fig. 1. Synthesisof TMAO and the relationship between diet and floral microbiota

Table 1: Summary of studies that explored TMAO in cardiovascular and related diseases

Populace	Sample Size	Study Design	Main findings/outcomes	Study
T2DM	1463 participants	single-center SURDIAGEN E cohort	TMAO concentrations weresignificantly asso ciated- with diabetes duration, renal function, high-density lipoprotein cholesterol, soluble tumor necrosis factor receptor 1 (sTNFR1) concentrations (R^2 = 0.27)	Croyal *et al.*[45]
Untreated and combination anti-retroviral therapy treated human immu nodeficiency virus (HIV)-infected individuals.	50 untreated and 50 combination antiretroviral therapytreated HIV-infected individuals.	cross-sectional cohort	Similar TMAO levels [3.8 (2.3–6.1), vs. 2.9 µM (1.9–4.8) P = 0.15] were observed in cART treated compared to untreated individuals infected with HIV.	Haissman *et al.*[46]
CHD and T2DM patients	132 control parti -cipants, 243 CHD patients, and 175 CHD patients with T2DM	Three classes of subject: controls, CHD patients, and CHD patients with T2DM	In CHD patients the plasma levels of TMAO were signifi -cantly higher than in control subjects (3.08 ± 0.13 µM versus 1.49 ± 0.05 µM; P < 0 01)	Dong *et al.*[34]

Peripheral Artery Disease	262 patients with symptomatic PAD (intermittent claudi -cation n = 147) and critical limb ischemia n = 115)	Case-control study	Patients with TMAO > 2.26 µmol/L exhibited higher risk of cardiovascular death (subhazard ratios ≥2, P < 0.05)	Roncal *et al.*[35]
Healthy volunteers	N= 113 (all omni -vores, 44 males and 69 females)	Crossover design study of randomi- zed 2-arm (high- or low-saturated fat)	Chronic red meat, but not consumption of white meat or non-meat, increased plasma and urine TMAO (each > two-fold; P < 0.0001)	Wang *et al.*[47]
Peripheral artery disease (PAD) patients	935 patients with PAD	Single-center prospective cohort study	Elevated TMAO levels were associated with 2.7-times higher mortality risk (fourth versus first quartiles, hazard ratio 2.86, 95% CI 1.82–3.97, P<0.001).	Senthong *et al.*[48]
Coronary Heart Disease patients	275 CHD-incident participants and 275 individually matched controls	Nested case- control study	Urinary TMAO was associated with CHD risk but not with its precursors. The odds ratio for the highest versus lowest quartiles of TMAO was 1.91 (95% CI, 1.08–3.35; P_{trend}=0.008)	Yu *et al.*[42]
People living with HIV (PLWH)	175 participants	Cross-sectional analysis and a longitudinal analysis study	The median concentration of serum TMAO were 165 (103-273) ng/mL.An association was observed with age, number of antiretrovirals, serum creatinine, multimorbidity and polypharmacy	Montrucchio *et al.*[49]

Circulating Tmao as Biomarkers of Coronary Artery Disease

A series of cohort studies have demonstrated that raised TMAO levels in patients with either stable CAD or acute coronary syndrome is associated with an increased risk of cardiovascular disease and poor cardiac outcomes.[36,39,40] In a recent study, it was revealed that elevated plasma TMAO levels has an association with plaque rupture in patients with CAD. In patients with ST-segment – elevation myocardial infarction (STEMI) with plaque rupture, there was an association between plasma TMAO levels and culprit lesion morphology than in those with plaque erosion as measured by optical coherence tomography.[41] In the diet-derived, gut microbial–host co-metabolite, it was observed that urinary concentration of TMAO is associated with a higher risk for the general population developing CHD. This was also observed even after adjustment for major CVD risk factors among patients without metabolic conditions such as diabetes mellitus, hypertension, and dyslipidemia.[42] Previous research found that choline or L-carnitine plasma levels were associated with TMAO levels, as well as an increased risk of major adverse cardiovascular events in patients with CAD.[43] A recent study has demonstrated that TMAO has a stronger association with hemorrhagic stroke as compared to ischemic stroke. TMAO promotes vascular inflammation and endothelial cell dysfunction via various signaling pathways, thereby creating the likelihood that TMAO may play a role in hemorrhagic stroke. Nevertheless, due to the presence of a relatively smaller sample size of the hemorrhagic stroke population, their findings need to be explicitly affirmed (Table 1).[44]

Measurement of TMAO and its metabolites in CAD There are several techniques for measuring TMAO in biological samples. In 1990, dimethylamine (DMA), TMA, and TMAO were determined through the use of gas chromatography procedure coupled with mass spectrometric detection method. These were extracted from samples of blood, urine, liver, and kidney of rats and humans, as well as from fish muscles.In this study, DMA, TMA, and TMAO have been extracted from biological samples into acid after deuterated internal standards were added (DMA-d6, TMA-d6, TMAO-d6). DMA derivative tosylamide was formed using p-Toluene sulfonyl chloride. The TMA derivative carbamate was synthesised using 2,2,2,2-Trichloroethyl chloroformate. TMAO combined with titanium (III) chloride was reduced to form TMA, which was then analyzed.[50] However, there are several limitations associated with this particular strategy. TMA is highly liable to quick changes, which precludes its evaluation. Another limitation is that this technique fails todistinguish between TMA, DMA, and TMAO with ease, since all produce a similar by-product. The liquid chromatography or mass spectrometry [LC/MS, electrospray ionization (ESI)] was developed to quantify nitrogen osmolytes (N-osmolytes) in the sample fractions of the natural water samples. This was validated using sea water samples of glycine, betaine, choline and TMAO.[51] A study in 2018 found serum levels of TMAO were measured in samples stored at −80°C.[52] First, serum proteins were precipitated using methanol (serum:methanol, 1:2, v/v); samples were vortex-mixed for 2 min, centrifuged at 14,000 g for 10 min (4 °C)[53] and High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) system was used to separateand analyze the supernatants. According to Beale and Airs, both HPLC-MS conditions and process optimizationwere performed.[51] The HPLC system was coupled with a single quadrupole mass spectrometer equipped with an ESI source, that functioned in a positive ion mode. In combination with a guard column (HILIC), the chromatographic separation was carried out using a Luna HILIC column (150/3 mm, 5 µm particles). A recent study hasauthenticated a procedure for measuring urine TMA and TMAO concentration by using liquid chromatography–mass spectrometry (LC/MS) simultaneously. The TMA and TMAO in the urine can be extracted in alkaline pH using hexane/butanol and transferred for LC/MS quantification to the aqueous cycle after acidification. It is to be noted that none of the nutrients having a chemical structure with a TMA moiety were cleaved spontaneously to yield TMA during sample processing. This also showed that urine acidification prevents TMA from rising after extended storage, as was found in non-acidified urine. Finally, it was observed that TMAO can degrade spontaneously to TMA at a very slow rate..[54]

Tmao and the Risk of Other Diseases

Patients with type 2 diabetes mellitus (T2DM) having an increased concentration of the circulatory gut based metabolite N-oxide (TMAO) was identified by Tang *et al*.[55] Al-Obaide *et al*. have demonstrated that gut microbiota-dependent TMAO increases in patients with diabetic CKD with increased gut permeability and inflammatory as well as endothelial dysfunction.[56] Serum L-carnitine level was found to be associated with an adverse metabolic syndrome profile. Moreover, in 2019, Gao *et al*, identified the relationships between serum TMAO and L-carnitine levels with metabolic syndrome profiles, including obesity, blood pressure, serum glucose, serum lipidsand insulin resistance (IR)-related index in humans.[57] In this analysis, serum L-carnitine levels in males with normal fasting glucose (p<0.05 for all) had a positive correlation with serum insulin, serum triglyceride (TG), IR but only serum TG (p<0.05) in male patients with hyperglycemia. In females, significant positive correlations were seen between serum L-carnitine levels with obesity, total serum cholesterol, glucose, insulin, and IR. Serum TMAO level was only identified to be positively correlated with serum insulin level and IR in hyperglycemic males (p < 0.05 for all).[57]TMAO levels were significantly associated with the percentage of pro-inflammatory intermediate CD14++CD16+ monocytes in patients with ischemic stroke.[58]

Research on humans and mice have shown that plasma TMAO levels increases with ageing[59] and TMAO levels have been significantly associated with body mass index (BMI) in healthy adults with different risk factors.[60] In particular, in untreated HIV-inflicted subjects, sCD14 was independently associated with TMAO.[46]Gut microbial-related choline metabolite TMAO as well as two monocyte activation biomarkers and inflammation biomarkers (SCD14 and sCD163) were also positively associated with carotid artery atherosclerosis in HIV patients.[61]

Conclusion and Future Prospects of TMAO

TMAO is an important gut derived metabolite that contributes vital prognostic information in patients with CAD. It was shown to be helpful in predicting adverse cardiovascular events in the short term as well as long term. This was even after adjustment for traditional risk factors such as diabetes, hypertension and obesity. Even in patients with peripheral artery disease, TMAO showed robust prognostic ability in measuring risk of cardiovascular death. In patients with diabetes and CAD, there is a higher concentration of TMAO than in non-diabetic CAD patients. Future studies should explore it is usefulness as a pharmacological target in CAD.

Conflict of Interest

The authors do not have any conflict of interest.

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