Title:

The Role of Microbiota in Cardiovascular Risk: Focus on Trimethylamine Oxide

Authors:

Hussein Al-Rubaye

Medical school, St George's, University of London, Cranmer terrace, Tooting, London SW17 0SZ, UK

Giulia Perfetti

Università Vita e Salute San Raffaele, Via Olgettina, 58, 20132 Milan, Italy

Juan-Carlos Kaski DSc, MD, DM (Hons), FRCP, FACC, FESC, FAHA

Molecular and Clinical Sciences Research Institute, St. George's, University of London, London SW17 0SZ, UK.

Corresponding author:

Juan-Carlos Kaski DSc, MD, DM (Hons), FRCP, FACC, FESC, FAHA

Molecular and Clinical Sciences Research Institute, St. George's, University of London, London, UK.

jkaski@sgul.ac.uk

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Abstract

The extensive collection of bacteria cohabiting within the host collaborates with human functions and metabolisms in both health and disease. The fine equilibrium of commensals is tightly controlled and an imbalance ("dysbiosis") in the gut microbiota can play different roles in human disease. The development of new genome sequencing techniques has allowed a better understanding of the role of human gut microbiota. This led to the identification of numerous metabolites produced in the gut, which have been suggested to play a role in human disease. Among these, TMAO appears to be of particular importance as a risk factor and potentially as a causative agent of various pathologies, most remarkably cardiovascular and disease and other associated conditions. Mechanistic links are yet to be established, however, increased levels of TMAO have been shown to augment the risk of developing renal failure, metabolic syndrome, diabetes mellitus, heart failure, hypertension, atherosclerosis and dyslipidemia ultimately leading to increased risk of serious cardiovascular events. This article reviews the potential impact of TMAO in human cardiovascular disease.

Introduction

Cardiovascular disease is the first cause of mortality worldwide.[1] Defining risk factors for prevention and control of its prognosis is of utmost importance. The rise of genome sequencing technologies has provided opportunities to characterize potential effect of commensal organisms on pathogenesis of human disease.[2, 3] Of course, this brought great interest for investigation of the putative role of gut microbiota in cardiovascular risk.
This article analyses evidence gathered in recent years regarding the role of microbiota in cardiovascular disease, as reported in original studies and meta-analyses. The aim of this review is to provide a critical view on the topic, with a specific focus on trimethylamine oxide (TMAO), a metabolite derived from choline and L-carnitine, which are nutrients found in ingested red meat and other animal products.

The microbiota in health and disease

Microbiota is a collection of organisms that coexist within the host, mainly colonizing nutrient rich sites of the gastrointestinal tract and utilizing anaerobic metabolism to survive.[4] While benefiting from the colonization, gut microbiota also has a role in the saccharolytic and proteolytic digestion pathways.[4] Gut microbiota performs multiple functions around the body, including stimulation of the immune system, promoting innate immunity against pathogens [4] and regulating mucosal barriers.[5] Aside from their commensal physiological roles, gut microbiota can have unfavorable effects on different organs in the body. With all these factors in place, gut microbiota has different roles in the general health of individuals.[6]

The human gut mainly hosts five phyla i.e. Actinobacteria, Bacteroidetes, Verrucomicrobia, Proteobacteria and Firmicutes.[6] The relative abundance and ratios of each phyla among individuals has been studied in relation to morbidity risk. Studies have shown that an increase of Firmicutes to Bacteroidetes ratio can cause obesity, metabolic syndrome and hypertension.[7] Factors such as diet, antibiotic use and lifestyle can affect ratios differently in different individuals.

Gut microbiota can also have an impact through its biologically active metabolites. Multiple products are released by bacterial fermentation, such as short chain fatty acids (SCFAs), choline metabolites, bile acids (BAs) and uremic toxins.[8-10] Pathogenic mechanisms of different metabolites on cardio-metabolic disorders are highlighted in Figure 1.

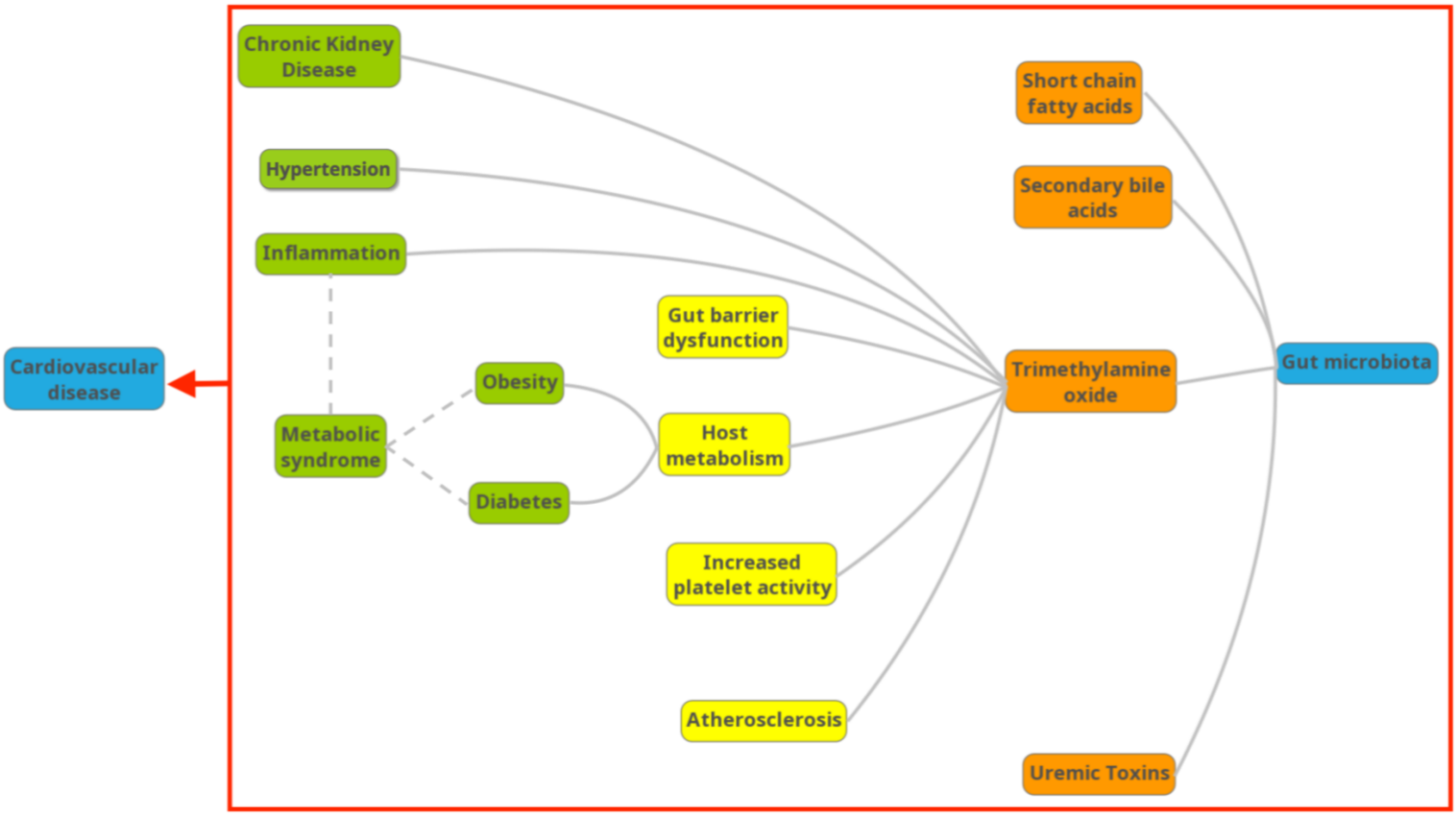

Figure 1: Gut Microbiota and Cardiovascular Disease
Adapted from Ahmadmehrabi et al.[11]

A metabolite that has caught the interest of scientists and clinicians is the choline metabolite trimethylamine (TMA), derived from choline and L-carnitine, which are nutrients found in ingested red meat and other animal products. TMA is oxidized in the liver to trimethylamine oxide (TMAO) by Flavin-containing monooxygenases (FMO), proven to show detrimental effects on the cardiovascular system through multiple pathways, later discussed in this review.[10, 12]

TMAO and all-cause mortality

Recent meta-analyses have shown that elevated levels of plasma TMAO are associated with increased all-cause mortality (HR: 1.91; 95% CI:1.40-2.61),[13] even after correcting for conventional cardiovascular risk factors and chronic kidney disease.[13, 14]

Of interest, controversy exist regarding the impact of geographical location on the results of the studies showing a link between TMAO and impaired clinical outcomes,[14] as race, ethnicity and dietary habits, may have an impact on the findings. In a recent study, Schiattarella et al.[13] stratified study individuals to assess whether the relation between increased all-cause mortality and elevated levels of TMAO persists across countries and

found this to be the case. They also carried out the first dose-response meta-analysis for TMAO and all-cause mortality, revealing an increased relative risk with increased concentrations (RR: 1.07, mortality increase by 7.6% per each 10 μmol/L increment of TMAO), but this was a nonlinear association. With the notable exception of pre-existing CAD, that strengthens the relation, the association between elevated plasma TMAO levels and increased mortality seems not to be altered by population baseline characteristics. Nonetheless, the distribution of TMAO has no standardized values, since studies have not been carried out in the general population; thus, the possibility to establish a systematic association is limited, until randomized multicenter general population studies are performed. Due to its wide impact on the host metabolism, TMAO levels influence morbidity too.[13, 14]

The link between TMAO and obesity, metabolic syndrome and type 2 diabetes mellitus

Studies have shown that predisposition to obesity might be linked to a higher ratio of Firmicutes to Bacteroidetes.[15] Conversely, other trials have shown that obesity itself alters the microbial flora.[16] This modification in gut microbiota composition, leads to an alteration in gut microbiota metabolites i.e. increased secondary bile acids, short chain fatty acids, lipopolysaccharides and TMAO.[17] As previously mentioned, all these metabolites have been described to have a pathogenic role in human health. In fact, tightly linked with obesity, they seem to be concurrently causing metabolic syndrome.[18] Perry et al. showed that increased production of metabolites (acetate) stimulates a parasympathetic response consequently promoting abnormal glucose-stimulated insulin secretion, increased ghrelin secretion, hyperphagia, and eventually, obesity, metabolic syndrome and their sequelae.[18] Based on these assumptions, a study demonstrated that fecal microbial transplantation from lean male donors (allogenic) to patients with metabolic syndrome improved insulin sensitivity after six weeks, compared to autologous fecal transplant.[19]

Gut microbiota is also known to differ in composition and to have various grades of microbial dysbiosis in patients with type 2 diabetes mellitus compared with individuals without diabetes. In particular, patients with T2DM have decreased quantity of butyrate-producing bacteria and augmented Lactobacillus spp.[20, 21] It is now know that a decrease in butyrate producing bacteria, normally protective against different type of diseases and T2DM complications, leads to reduced insulin sensitivity and promotes the development of diabetes.[20, 22]

Chronic kidney disease

A large cohort study by Kim et al. involving 2529 patients found that individuals with CKD have very high levels of TMAO, with increasing levels among higher CKD stage patients.[23] Tang et al assessed 3687 CKD patients and found that TMAO was associated with a 1.7-fold increase (HR 1.70, 95%CI 1.25–2.30, $p<0.001$) in all-cause mortality after adjusting for traditional CVD risk factors and stratifying results according to median TMAO levels. The study further explored the effects of TMAO on animal models and concluded that TMAO promotes renal fibrosis and kidney dysfunction.[24] It is not known however whether TMAO contributes to the development of CKD or pre-existing CKD favors a decreased clearance of TMAO thus resulting in high concentrations of the metabolite. Randomized controlled trials are required to answer this question.

Hypertension

Being the most prevalent modifiable cardiovascular risk factor, hypertension became a matter of interest to microbiota research teams. Studies have suggested a possible impact of microbial gut dysbiosis on blood pressure control. In particular, Yang et al. observed an imbalance in the Firmicutes to Bacteroidetes ratio and a decrease in butyrate and acetate producing bacteria both in spontaneously hypertensive rats and in patients, albeit the latter were small cohorts.[25] Along these lines, overweight and obese women with higher butyrate producing bacteria typically have lower blood pressure readings.[26] Thus, according to these results, the gut microbiota appears to have effects on blood pressure control. These findings indicate that communication channels probably exist between the gut enteric nervous system and the CNS through sympathetic innervation and metabolites signaling, as proposed by Santisteban et al.[27] In fact, it appears that intestinal wall permeability allows metabolites produced by gut microbes to have an effect on distant organs and systems.[28]

Authors have suggested that antibiotics and probiotics could be used for better pressure control, particularly in patients with resistant hypertension [29] and gut microbial dysbiosis.[30]

Dyslipidemia

Gut microbiome is known to have influence on body mass, triglycerides and high-density lipoproteins. It has also been reported that the effect of microbiota on triglycerides and HDL is independent from patient's body mass, suggesting a direct mechanistic effect of its metabolites.[31] This biological mechanism, though, remains unknown.[17] What is known,

however, is that TMAO-generating enzyme FMO3 reduces reverse cholesterol transport, alters tissue cholesterol and sterol metabolism and changes bile acids composition and pool size. This happens through modification of bile acid secretion, reduction of intestinal cholesterol absorption and limitation of hepatic oxysterols and cholesteryl esters' production. Thus, the microbiota could also affect cholesterol levels thus probably contributing to atherogenesis. Warrier et al. [32] also showed that in FMO3 knock out models, liver-X receptor function is stimulated, thus promoting reverse cholesterol transport and ameliorating cholesterol levels and equilibrium. These findings depict the FMO3 /TMAO pathway as a key player in the regulation of cholesterol levels and metabolism potentially, playing an important pro-atherogenic role.

Atherosclerosis and myocardial infarction

One of the strongest links between gut microbiota and human disease has been demonstrated in relation to atherosclerosis. In fact, after developing the hypothesis that bacterial infection could be a cause of atherosclerotic plaque formation, Ott el al. investigated atheromatous plaques from heart disease patients and healthy donors for bacterial DNA identification. They detected a very high variety of bacterial signature in the material, among which Staphylococcus, Proteus vulgaris, Klebsiella pneumoniae, and Streptococcus species featured prominently. Although these observations do not represent proof that the microbiota plays a causative role in atherosclerosis, they suggest that bacterial agents could act as an additional factor accelerating disease progression.[33] The same concept, but involving oral microbiota, has been demonstrated to be plausible by Koren et al. [2] Their trial also proposed to use bacteria from the oral cavity as disease markers for atherosclerosis, since the bacterial DNA present in the analyzed material, was the same of the taxa in patients' oral cavity and gut microbiota.

Other than bacterial signatures, also higher levels of TMAO have exhibited a positive correlation with atherosclerotic plaque size. Wang et al. showed that TMAO and betaine upregulate scavenger receptors on macrophages, consequently promoting atherogenesis; TMAO itself fosters cholesterol accumulation and foam cell formation.[8] Furthermore, Karlsson et al., through meta-genomic sequencing, showed that the composition of gut microbiota seems to be different in patients with symptomatic atherosclerosis, compared to asymptomatic ones. This could be due to the fact that, through their metabolites, bacteria can promote a pro-inflammatory state in the host, fostering clinical instability and plaque rupture,

leading to symptoms and an increased number of cardiovascular events. According to their study, more symptomatic patients have decreased levels of Rosebarium species in their stool samples, species known to promote anti-inflammatory effects in humans.[34]

Taken all of the above information together it seems plausible that microbial dysbiosis could lead to high levels of TMAO, plaque instability and myocardial infarction. Indeed, Lam et al. demonstrated that the composition of intestinal microbiota might have an impact of the severity of myocardial infarction in rats.[35] A mechanistic link was proposed in these and other studies, administering broad spectrum antibiotics in mice with induced myocardial infarction. Compared to untreated mice, those receiving antibiotic treatment showed smaller size of myocardial infarction and improved left ventricular function during recovery time. This finding was linked to a reduction in leptin concentrations, associated with the use of antibiotics.[22] Similarly, Gan et al., again in an experimental animal study, demonstrated that probiotic administration i.e. Lactobacillus rhamnosus GR-1 in coronary artery occlusion mice models lessened post-infarction myocardial hypertrophy.[36] The mechanism found was leptin reduction, as postulated by other investigators previously..

Heart failure

It has been hypothesized that inflammatory mechanisms seen in heart failure patients could be due to decreased cardiac output to the gut leading to disruption of the intestinal barrier, allowing the passage of bacteria and endotoxins.[37, 38] These toxins activate toll-like receptors fueling the inflammatory process and possibly fostering atherogenesis.[39] Another possible mechanistic link between heart failure and high TMAO levels is that TMAO prolongs the effect of angiotensin, inducing adverse cardiac remodelling.[40] From the information summarized above it is apparent that there are bidirectional effects of TMAO on heart failure.

Different studies confirmed that concentrations of TMAO and its precursors (betaine and choline) are higher in patients with chronic heart failure and levels correlate with New York Heart Association (NYHA) classes.[38, 41, 42] A study by Tang et al. showed that there is a correlation between TMAO and brain natriuretic peptide (BNP), a marker of heart failure ($r = 0.23$; $p < 0.001$). Elevated TMAO levels are predictive of worsening 5-year mortality risk after adjusting for traditional risk factors and BNP (HR: 2.2; 95% CI: 1.42 to 3.43; $p < 0.001$).[38] Tang et al. showed that in 112 chronic heart failure patients, only higher TMAO rather than choline or betaine levels resulted in adverse clinical events independent of age and BNP levels (HR 1.46 [1.03 2.14], p=0.03).[42]

Increased Major Adverse Cardiovascular Events and morbidity

Major Adverse Cardiovascular Events (MACE) development in relation to gut metabolites have been investigated in several clinical studies, and further assessed and reviewed in various qualitative and quantitative meta-analyses.

A meta-analysis by Heianza et al. reviewing 19 prospective studies estimated the risk of MACE incidence and all cause death in association with TMAO levels. Results showed a strong increase in relative risk of both outcomes in patients with higher TMAO levels (62% increase RR in MACE, 63% increase in all-cause death).[43] Interestingly, TMAO levels came as an independent predictor of MACE, not being influenced by past cardiovascular history and diabetes in comparable populations. This counter-intuitive finding was further supported by dose response analyses that conveyed a dose dependent relation between TMAO and its precursors and the incidence of MACE.

A meta-analysis by Schiattarella et al. reported similar outcomes underlying that the presence of CKD as a comorbidity does not seem to influence results.[13]

As previously mentioned, the postulated potential mechanisms underlying this increased relative risk are several, i.e. enhanced development of larger, more unstable atherosclerotic plaques; alteration of hepatic cholesterol and lipid metabolism; fostering a pro-inflammatory condition and promotion of endothelial dysfunction with heightened platelet activity due to modified calcium signaling.

It has to be said however that a case of reverse causality cannot be excluded. At present, no study demonstrated whether the composition of the gut microbiota is affected after an ischemic event, thus increasing TMAO production, or high TMAO plasma levels are the primary cause of augmented risk of CAD. It should be underlined that most clinical studies investigating the matter have been carried out in known CVD and high-risk patients. Still, no standardized normal level and life-trend of TMAO has been studied in the general population and no prospective cohort study has been carried out in patients at different levels of cardiovascular risk.

Is gut bacterial dysbiosis a therapeutic target?

Several therapeutic interventions have been investigated to further assert the effect of TMAO on cardiovascular disease. Diet interventions have been utilized in high risk individuals to improve chronic metabolic disorders. As mentioned earlier, the composition of microbiota is

affected by the individual's dietary habits. Studies have shown that a diet resulting in decreased synthesis of TMAO has beneficial effects.[44-47] Despite these findings, other studies have shown that the composition of gut microbiota remains practically unchanged over a person's lifetime.[22] Am emerging therapeutic intervention is the use of Fecal Microbiota Transplantation (FMT) to manage cardio-metabolic disorders. This strategy aims to displace intestinal pathogens and introduces the fecal content of healthy individuals into high risk patients. The effects of autologous fecal transfer –Transfer of one's own feces acting as placebo- and allogenic fecal transfer -transfer from healthy lean individuals- were compared in metabolic syndrome patients, and it resulted in a significant improvement of hepatic and peripheral insulin sensitivity after 6 weeks follow-up.[48] Despite initial encouraging studies, limitations are still to be overcome regarding FMT, particularly the introduction of endotoxins, as well as rejection.[49, 50]

Theories surrounding the potential of TMAO as a causative agent for platelet aggregation could potentially prove to be extremely beneficial. This is due to the fact that targeting TMAO's presence is speculated to have an anti-aggregation effect comparable to that of conventional antiplatelet drugs, with less bleeding risk.[10]

Furthermore, intervening with probiotics and prebiotics has shown to influence the intestinal microbiota content. Probiotics are beneficial bacterial organisms that inhabit the gut while prebiotics are non-bacterial food ingredients that affect the microbial community. Probiotics have shown to positively influence glucose intolerance associated with developing metabolic syndrome and decrease the amount of toxins produced by the gut during metabolism.

Gaps in knowledge and future perspectives

Even though many of the studies mentioned in this review highlight the potential importance of TMAO and its metabolites levels, numerous gaps in knowledge do exist. Firstly, no detailed mechanistic or causal evidence has been provided on its role in morbidity, the development of atherosclerosis and/or increased incidence of MACE. Moreover, work is needed to identify potential molecular targets as well as defining other therapeutic strategies. Secondly, studies are required to define TMAO plasma levels that represent increased risk and to establish correlation between concentrations of the metabolite and increased cardiovascular risk, as well as risk of disease in general. Matters like inhibition of TMA production in order to decrease TMAO levels and -possibly- beneficially affect the heart

failure phenotype. Of great interest, it would be to understand the mechanisms and clinical significance of TMAO elevation in CKD patients, which has been associated with worsening of renal fibrosis and kidney dysfunction.[22] Moreover, investigating the relative risk of new incidence of CVD in previously healthy individuals with high TMAO plasma levels would be of utmost importance to clarify the role of this metabolite in human disease.

All the clinical trials performed thus far have involved hospitalized patients and high-risk individuals, probably resulting in bias regarding result interpretation and making it difficult to extrapolate these findings to the general population or unselected patient groups who are at lower risk.

Future perspective

Possibility of using TMAO as a novel biomarker in primary prevention of cardiovascular disease could be further established in mechanistic studies. This will lead to a shift from traditional CVD risk factors and open doors for new treatments and modulation therapies specifically targeted towards gut microbiota.

Possible interventions such as the use of antibiotics to alter the microbial composition of the gut have been studied in young mice, where adiposity was increased in subjects that were administered with a subtherapeutic dose of an antibiotic. This is further proven by studies that have shown that obesity can be influenced by the use of antibiotics in infancy, showing that there could be a possible mechanistic link between the development of obesity and microbial composition in the gut.

Another future possible intervention in gut microbiota modulation is targeting enzymes within the TMAO pathway, such as TMA lyase, which influence the plasma levels of TMAO as shown through in vivo studies on mice.[51] This grows the potential of controlling metabolite production leading to a further improvement in cardiovascular risk management.

Conclusion

The links between TMAO and the development of CVD as well as related conditions have been investigated in several studies. Further large randomized studies, however, are necessary in unselected population as well as in carefully characterized patient subgroups to establish the true importance of TMAO as a marker of disease, a causative agent and a therapeutic target.

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