

“The Role of Trimethylamine-N-Oxide as a Mediator of Cardiovascular Complications in Chronic Kidney Disease”

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Abstract

Patients with chronic kidney disease (CKD) have an enhanced risk of cardiovascular (CV) morbidity and mortality when compared to age and gender-matched individuals with normal kidney function. Trimethylamine-N-oxide (TMAO) is a gut-derived amine oxide that has been implicated in the causation of cardiovascular diseases. Plasma TMAO is cleared by the kidney and TMAO levels are elevated in CKD. Experimental studies have identified pathogenic mechanisms by which TMAO may contribute to cardiovascular disease through dysregulation of lipid metabolism, enhanced macrophage foam cell formation and platelet dysfunction. Safe and well tolerated therapeutic interventions such as pre- and probiotics, which modify the gut microbiome, offer the opportunity for interventional studies. This review examines the pathogenicity of TMAO, its value as a biomarker and its potential as a therapeutic target in the context of CKD.

Cardiovascular disease in CKD – Unexplained risk

Patients with chronic kidney disease (CKD) have an enhanced risk of cardiovascular (CV) morbidity and mortality when compared to age and gender-matched individuals with normal kidney function.¹ Advanced kidney disease (stage G5A3; estimated glomerular filtration rate (eGFR) <15 mL/min/1.73m², albumin:creatinine ratio >30 mg/mM) carries an 8-fold increased adjusted CV mortality risk when compared to subjects with no kidney disease.² Traditional CV risk factors such as hypertension and dyslipidaemia are frequently present in

CKD, but they do not provide the same predictive value as for individuals with normal kidney function.^{3,4} This could be because different risk factors contribute to CV disease pathogenesis in CKD, for example hyperphosphataemia, anaemia and other metabolic derangements related to a “uraemic milieu.” Several novel risk markers have been identified in CKD but none have been causally linked to CV disease pathogenesis in interventional studies. For example, promising interventions designed to ameliorate abnormal bone-mineral metabolism in patients receiving hemodialysis such as non-calcium-based oral phosphate binders⁵ and calcimimetics,⁶ failed to reduce mortality or CV events in large randomized trials (although secondary analyses and trends suggested potential benefit in some populations). Indeed, with the exception of combined statin/ezetimibe lipid-lowering therapy,⁷ no interventions have been proven to reduce CV risk in large scale randomized controlled trials specifically recruiting CKD patients.

The gut microbiome in health and disease

The human gastrointestinal tract is colonized by approximately 10-100 trillion (or 10^{14}) microbes, outnumbering the host's own somatic and germ cell population by a factor of ten.⁸ During health, this gut microbiome functions both as a living barrier against ingested noxious substances and liberates otherwise inaccessible nutrients for systemic absorption. Subpopulations of gut microbiota vary widely between individuals and can have a measureable impact upon whole body nutrition, metabolism and immune function.⁸ Potentially toxic metabolites may arise from alterations in gut microbiota in a range of disorders such as obesity,⁹ impaired glucose tolerance¹⁰ and liver disease,¹¹ suggesting that the usual symbiotic relationship between gut microbiota and host may turn *dysbiotic* in these conditions, allowing gut flora to exacerbate disease. In addition, the gut microbiome has been implicated as a source of pathogenic mediators in the context of CKD.¹² In this clinical setting, compounds derived from dysbiotic gut bacteria are absorbed into the circulation and accumulate due to reduced excretion by the kidney. Blood levels of some of these compounds have been shown to correlate with dysregulated immunity, CV disease burden and decline of kidney function.¹² Over the last five years, the role of trimethylamine-N-oxide

(TMAO) as a gut-derived metabolite mediating CV disease pathogenesis has been under particularly intense scrutiny.

Trimethylamine-N-oxide biology

TMAO has two well-described biological functions. Firstly, this oxidized amine promotes bacterial growth by acting as an electron acceptor during anaerobic respiration.¹³ Gaseous trimethylamine (TMA) is produced as a by-product and has a characteristic strong malodor facilitating its use as an indicator of spoilage by the fish industry.¹³ Secondly, TMAO plays a role in preserving protein structure and function *in vivo*. Marine animals experience large fluctuations in urea concentration, salinity and hydrostatic pressure that can denature proteins. To preserve protein conformation and function, TMAO interrupts these destabilizing hydrophobic interactions by serving as a chemical chaperone.^{14,15} Despite evidence of these biological activities, no clear physiological role for TMAO has so far been confirmed in humans. For example, although osmolytes such as TMAO may stabilize protein structure and function in human cells *in vitro*¹⁵ there remains a lack of data to support this function *in vivo*.

TMAO Metabolism in Mammals

Mammalian TMAO production is a two-step process (Figure 1). The first step involves gut bacteria that metabolize TMA-containing substrates such as phosphatidylcholine (lecithin), choline, betaine and L-carnitine (found in dietary red meat, fish and eggs). Experimental studies dating back to the mid 1900s demonstrated increases in urinary trimethylamine-N-oxide (TMAO) after feeding rats dietary choline, but not when the same dose was injected intraperitoneally.¹⁶ Gut flora eradication with antibiotic treatment in both mice and human subjects prevented TMAO production, but when gut bacteria were allowed to repopulate, TMAO-generating capacity was restored.^{17,18} In the second step, bacterially cleaved gaseous TMA is rapidly absorbed into the circulation and oxidized by the liver enzyme flavin monooxygenase isoform 3 (FMO3) to form TMAO.¹⁹ (Figure 1).

Unlike certain species of bacteria and fish, mammals lack the enzymatic capacity to further reduce or demethylate TMAO and >95% of total body TMAO

is excreted unchanged by the kidney²⁰ through glomerular filtration and tubular secretion, the latter accounting for at least half of all TMAO excreted in the urine.²¹ Consistent with this, patients commenced on drugs known to modulate tubular function, such as anti-retroviral protease inhibitors²² and calcineurin inhibitors²³, may exhibit elevations in plasma TMAO or reduced urinary TMAO clearance.^{22,23} Potential cell membrane transporters for TMAO uptake and efflux have been identified. Overexpression of organic cation transporter 2 (Oct2) in HeLa cells results in a 3-fold increase in intracellular TMAO concentration, whilst *in vivo*, global Oct1/2 gene deletion in mice is associated with a 2-fold greater plasma TMAO concentration when compared to wild-type controls.²⁴ Provisional *in vitro* data suggest that multiple efflux transporters of the ATP-binding cassette (ABC) family (variably expressed in liver, kidney and gut epithelia) may also play a role in TMAO efflux and hence excretion.²⁴

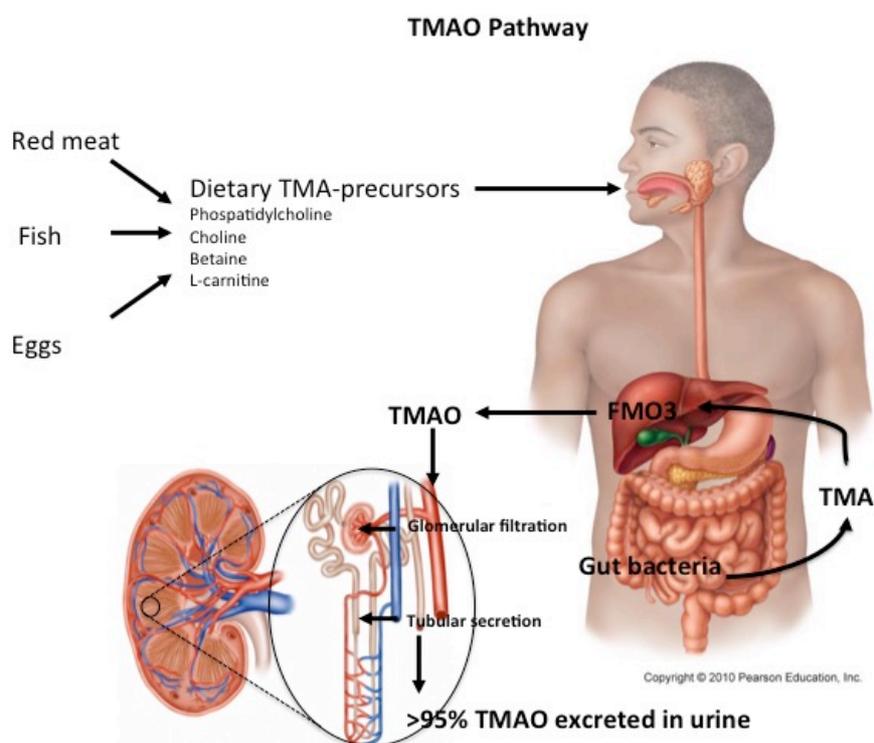


Figure 1. The Mammalian TMAO Pathway.

Gut bacteria metabolize TMA-containing substrates such as phosphatidylcholine (lecithin), choline, betaine and L-carnitine (found in dietary red meat, fish and eggs). Bacterially cleaved gaseous TMA is rapidly absorbed into the circulation and oxidized by the liver enzyme flavin monooxygenase isoform 3 (FMO3) to form TMAO. Mammals cannot metabolize TMAO and >95%

of total body TMAO is excreted unchanged by the kidney through glomerular filtration and tubular secretion.

TMAO as a mediator of CV disease: Clinical Data

The first human study to implicate TMAO as a mediator of CV disease used unbiased plasma metabolomic screening to identify three metabolites of phosphatidylcholine; choline, betaine and TMAO that independently predicted incident CV events (heart attack, stroke and CV death) in 1876 subjects referred for cardiac disease evaluation over a 3-year follow-up period.¹⁷ Subsequently, having extended their cohort to 2595 subjects, these investigators demonstrated that plasma levels of L-carnitine, an alternative TMAO precursor, was also predictive of CV events when raised in association with TMAO, over a 3 year follow-up period.¹⁸ Two other observational studies linked plasma TMAO levels to severity of heart failure. Both a Norwegian study of 155 heart failure patients²⁵ and a North American study of 112 patients²⁶ reported associations between higher plasma TMAO, symptom severity and risk of all-cause mortality or heart transplantation over a 5-year follow-up period.^{25,26} Importantly, increased age, previous myocardial infarction²⁵ and reduced kidney function assessed by estimated glomerular filtration rate (eGFR) strongly predicted blood TMAO levels.

Despite these initial findings, the association between blood TMAO levels and CV disease has not been consistent in all population studies to date. In a North American study of 817 mixed race healthy volunteers aged 33-45 years, plasma TMAO levels were not associated with incident coronary artery calcification assessed by computed tomography or coronary artery intimal-medial wall thickness assessed by ultrasound over a 10-year follow-up period.²⁷

A Chinese study reported that although distinct profiles of gut flora were associated with cerebrovascular disease severity, TMAO concentrations were actually *lower* in patients with prevalent cerebrovascular disease when compared to asymptomatic controls and were not associated with increased intima-media carotid artery thickness or the presence of atherosclerotic plaques.²⁸ Similarly, a European study of 339 patients undergoing coronary angiography

found no association between plasma TMAO and prevalent coronary artery disease or incident events over an 8-year follow-up period.²⁹

Infection with HIV carries a greater risk of developing CV disease. A study comparing coronary calcium scores in HIV-infected patients and non-infected controls found that TMA but not TMAO concentrations correlated with higher plaque burden, but only in the HIV-positive group.³⁰ In another HIV cohort, higher plasma TMAO levels were associated with advanced age, reduced kidney function and use of protease inhibitors, but not with coronary artery calcium scores or myocardial infarction.²²

Study of humans with variants of the FMO3 gene could provide opportunities to assess the impact of reduced TMAO production on CV disease. Trimethylaminuria, or Fish Odor Syndrome, results from a rare FMO3 gene variant with markedly diminished capacity for oxidizing TMA into TMAO. Accumulation of TMA gives a strong fishy odor to the breath, sweat, and urine.³¹ However, despite extremely low TMAO levels, there are no studies suggesting that individuals with this rare condition are protected from CV disease. In terms of more common FMO gene variants, a large consortium genome-wide association study of ~22,000 coronary artery disease patients with ~65,000 control subjects did not reveal an association between 388 FMO gene polymorphisms and coronary artery disease.³² In a separate cohort, no association was identified between 471 FMO gene polymorphisms and plasma TMAO levels in 3865 subjects referred for cardiac evaluation.³²

These apparently conflicting data linking blood TMAO levels with cardiovascular disease risk are likely to have resulted from a combination of issues including differences in study methodology, diet, ethnicity, environment and prevalent patterns of gut microbiome composition in study subjects. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is frequently used to measure plasma TMAO and has high sensitivity and specificity. Despite this, reported inter- and intra-individual ranges of TMAO concentrations are wide. For example, fasting plasma TMAO concentrations in 349 healthy North American subjects ranged between 0.73 - 126uM.³³ A New Zealand study of 243 overweight individuals with type 2 diabetes but no significant kidney disease (eGFR>60 mL/min/1.73m²), revealed high intra-individual variability amongst

four samples taken over 2-years. Log-normal reference change values ranged from 403% to -80% in plasma, whilst by comparison, an alternative TMA-substrate betaine ranged from 54% to -35%.³⁴ Such inter- and intra-individual variability of plasma TMAO could limit its use as a reliable biomarker and provides an explanation as to why reports of TMAO and disease associations can differ.

Additional explanations for discrepant data include the fact that TMAO levels in co-morbid individuals are determined by a variety of intrinsically linked pathogenic factors including an unhealthy diet, a dysbiotic gut microbiome, dysregulated FMO3 activity and perhaps most importantly, reduced clearance via the kidneys.

TMAO as a Mediator of CV Disease: Experimental Data

Experimental data suggest that TMAO may directly enhance atherogenesis and contribute to CV events *via* dysregulation of lipid handling and macrophage function as well as directly causing vascular inflammation and platelet activation leading to thrombosis (Figure 2). Atherosclerosis-prone apolipoprotein E knockout mice (ApoE^{-/-}) fed supplemental choline or TMAO developed elevated plasma TMAO levels, larger aortic atherosclerotic plaques with enriched macrophage content as compared to wild type, although these effects were prevented by pre-treatment with antibiotics.¹⁷ Elevated TMAO in ApoE^{-/-} mice also reduces reverse cholesterol transport¹⁸ and in wild-type mice, causes peritoneal macrophages to express higher scavenger receptor proteins (CD36 and SR-A1) and become cholesterol-laden foam cells.¹⁷ Furthermore, raising plasma TMAO in mice fed another substrate, L-carnitine, is associated with decreased liver mRNA expression of the bile acid synthetic enzymes Cyp7a1 and Cyp27a1, indicating reduced ability to eliminate bile-acid cholesterol.¹⁸

Several pro-inflammatory mediators including Cyclo-oxygenase-2 (COX-2), E-Selectin and Intracellular Adhesion Molecule-1 (ICAM-1) were upregulated in the aortic tissue of low-density lipoprotein receptor knock-out (LDLR^{-/-}) mice receiving dietary choline or intraperitoneal TMAO.³⁵ *In vitro*, TMAO enhanced leucocyte adhesion to endothelial cells in an NF-κB-dependent manner.³⁵ Furthermore, enhanced platelet activation and adhesion has been observed

following intraperitoneal TMAO injections in an *in vivo* carotid artery injury model.³⁶

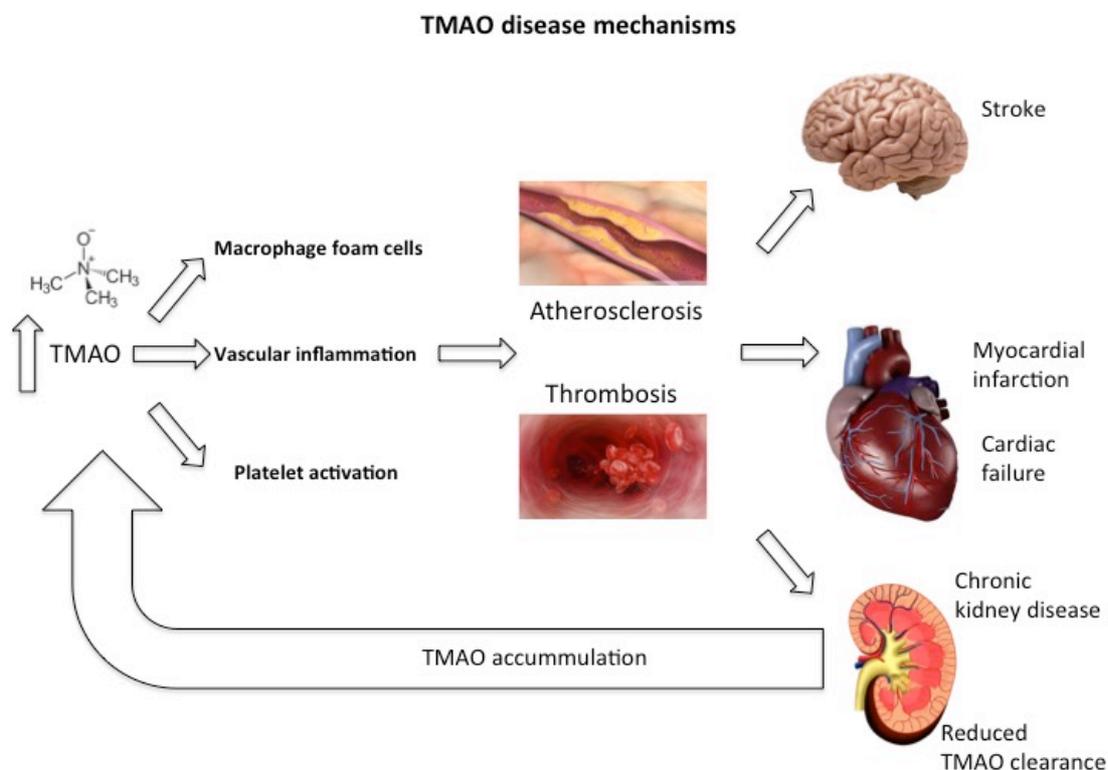


Figure 2. Pathogenic Mechanisms of TMAO contributing to CV and Kidney Disease.

Experimental data suggest that TMAO may directly enhance atherogenesis and contribute to CV events *via* dysregulation of lipid handling and macrophage function as well as directly causing vascular inflammation and platelet activation leading to thrombosis.

What is not clear is how TMAO mediates these cellular responses because no cell surface receptors have been identified. One potential mechanism is the protein-stabilizing effect of TMAO, which provides protection against physiological stress. However, this action may not necessarily be beneficial. Under certain circumstances in mammals, TMAO may reduce protein function or limit degradation of key enzymes or signaling proteins with potentially deleterious downstream effects.¹⁵

TMAO and CV Risk in Chronic Kidney Disease

As kidney function declines from stage 3a (eGFR 45-60 mL/min/1.73m²) and beyond, circulating TMAO concentrations not only rise but also become increasingly variable, one study reporting median concentrations of 3.3 μ M (inter-quartile range 3.1-6.0 μ M) in healthy volunteers versus 94.4 μ M (inter-

quartile range 54.8-133 μM) in people with end-stage kidney disease.³⁷ As in non-CKD populations, elevated TMAO levels have been reported to predict CV events and mortality in cohorts with mild to moderately severe CKD (stages 3-4) even after correction for kidney function and traditional CV risk factors such as age, blood pressure and lipid status.^{37,38} A 1.9-fold increased risk of 5-year all-cause mortality was reported in stage 3 CKD subjects with TMAO levels in the highest quartile as compared to those in the lowest quartile.³⁸ In people with CKD stage 3-4, blood TMAO concentrations independently correlated with both coronary artery disease severity score (assessed by quantitative angiography) and mortality when used as a continuous variable, but this was not significant when tested across TMAO tertiles.³⁷ Following kidney transplantation, TMAO levels fall towards normal, leading to speculation that improved clearance of TMAO may at least in part account for reduced risk of CV observed in kidney allograft recipients.³⁷ Finally, in a large study of over 2500 Canadian patients with CKD, plasma TMAO independently predicted CV events over a 3-year follow-up period in stage 3, but not in stage 4 (in which TMAO was much more variable).³⁹

Some thought is required before interpreting these data as evidence of an independent association between TMAO and CV disease in renal impairment, let alone one of causation. Statistical correction for reduced renal clearance of TMAO using creatinine or cystatin C -based measures of eGFR does not account for tubular function, which has an impact on excretion of TMAO. Tubular function can be highly variable at any given eGFR and often depends on the underlying CKD aetiology.⁴⁰ In addition to the key confounding issue of reduced renal TMAO clearance, gut-related factors specific to CKD are likely to contribute to elevated blood TMAO levels. Gut microbiome subpopulation composition may shift from symbiotic to dysbiotic under the influence of “uraemic” factors such as gut oedema, reduced transit time, anorexia or adherence to a low phosphorus or potassium diet along with oral phosphate binders and iron replacement.¹²

TMAO and CV Risk in Hemodialysis Cohorts

Hemodialysis clears TMAO at a rate similar to creatinine, with re-accumulation to plasma concentrations of approximately 100 μM before the subsequent

dialysis session. ^{21,37} Studies in this population do not indicate a consistent predictive value of plasma TMAO levels for CV disease risk. A recent North American study of 1232 mixed race hemodialysis patients reported a 4-fold higher risk of cardiac mortality in white patients in the highest quintile of plasma TMAO (>135 μ M) compared to their black counterparts over a median follow-up of 2.3-years. ⁴¹ Importantly, the white subgroup contained more males and had increased co-morbidity scores, more prevalent gastrointestinal disease and better residual kidney function than black participants, which potentially confounded results. In a cohort of 235 hemodialysis patients, no association was found between blood TMAO concentrations and cardiovascular mortality, but TMAO did correlate with serum albumin, creatinine and inversely with CRP. ⁴² An attenuated or even reverse association between traditional disease markers such as high blood pressure or blood cholesterol and cardiovascular outcomes has previously been reported in the hemodialysis population, likely due to confounding by comorbidity ^{3,4} and TMAO may be subject to the same phenomenon. For example, much like albumin, lower TMAO levels in some hemodialysis patients could reflect poor nutritional and overall health status. ⁴² Because of such confounding, interventional studies are likely to be the only reliable method to test the hypothesis that TMAO contributes to CV disease in the context of CKD.

Potential treatment strategies to reduce TMAO exposure

Besides renal clearance, current evidence indicates that dietary intake and gut-microbial metabolism are the strongest determinants of plasma TMAO levels and hence hold the most promise for therapeutic intervention. Whilst genetic knock-down of FMO3 protected against atherosclerosis in atherosclerotic-prone mice, ⁴³ human genome-wide association data suggest that single genes contribute little to plasma TMAO concentrations ³² and the wide range of other metabolic functions served by FMO enzymes mean that pharmacological FMO3 inhibition is unlikely to provide overall benefit.

Dietary approaches

Fish and red meat contain high concentrations of TMA-precursors and TMAO, but avoiding dietary fish in order to reduce CV disease risk is a strategy not supported by current evidence. Fish ingestion can elevate TMAO excretion more than 20-times higher when compared to alternative diets ¹⁶ but despite this, higher consumption of fish has long been associated with protection against ischaemic heart disease. ⁴⁴

Dietary L-carnitine from red meat represents a source of TMAO and could thus potentiate CV disease. ¹⁸ This conflicts with a reported CV protective effect of L-carnitine supplementation, which is itself inconsistent between populations. A meta-analysis of 13 controlled trials using L-carnitine supplementation (oral or intravenous) following acute myocardial infarction reported a 27% reduction in mortality compared with placebo or control. ⁴⁵ In a meta-analysis of 49 trials conducted in end-stage kidney disease patients receiving hemodialysis and L-carnitine supplementation (37 using intravenous and 12 oral administration), no such mortality benefit was found although serum low-density-lipoproteins (LDLs) and C-reactive protein (CRP) were both reduced. ⁴⁶ L-carnitine undergoes gut microbial conversion to TMA and intravenous administration bypasses this, thereby providing a means to test the protective effects of L-carnitine, whilst not affecting TMAO concentrations. In the above meta-analysis, intravenous L-carnitine reduced serum cholesterol and hematocrit when compared to oral supplementation⁴⁶. Consistent with this, a small crossover study showed improved lipid profiles (reduced serum fatty acids and increased high-density lipoproteins) after switching from oral to intravenous L-carnitine but it is unclear whether this effect was related to increased bioavailability rather than avoidance of TMAO production, which was not reported. Direct comparison of the long-term effects of oral versus intravenous L-carnitine supplementation would likely be informative but have not yet been tested in large clinical trials.

In addition to being a direct source of TMA-precursors, a diet rich in fish and red meat changes the composition of gut flora favoring TMA-forming bacteria such as Clostridia and Prevotella, ¹⁸ thereby potentiating elevated blood TMAO levels. Whilst outright avoidance of meat and fish is not necessarily beneficial, the potential for the diet to alter gut flora indicates to an opportunity

to recover a symbiotic gut microbiome or at least reduce the component of TMA-producing bacteria.

Therapeutic Manipulation of the Gut Microbiome

Prebiotics (eg. oligosaccharides) are indigestible food additives that promote growth of beneficial bacteria subtypes whilst limiting proliferation of pathogenic bacteria populations. Resveratrol is a naturally occurring polyphenol found in grapes and berries that when fed to ApoE^{-/-} mice, increased gut Lactobacillus, decreased plasma TMAO levels and attenuated aortic atherosclerosis.⁴⁷ Probiotics are live microorganisms (eg. Lactobacilli, Streptococci) that when ingested can alter the pattern of resident gut flora. A small randomized, double-blinded, placebo-controlled trial in 37 stage 4-5 CKD patients demonstrated that a combination of pre- and probiotics altered gut microbiota composition and reduced plasma concentrations of gut-derived uraemic metabolite p-cresyl sulfate⁴⁸ providing support for the use of similar agents in CKD patients to reduce gut bacteria-derived TMAO.

Alternative strategies to reduce absorption of gut-derived TMA include structural choline analogues such as 3,3-dimethyl-1-butanol (DMB), which have been shown to reduce plasma TMAO levels and prevent macrophage foam cell formation in APOE^{-/-} mice, but have not yet been tested in humans.⁴⁹ Oral meldonium, a synthetic structural analogue of L-carnitine precursor γ -butyrobetaine, limited elevations in plasma TMAO after a fish-rich diet and increased urinary TMAO excretion by 35% in human test subjects.⁵⁰ It is not clear if this effect was due to inhibition of reabsorption or enhanced secretion of TMAO by the tubule. If a tubular TMAO transporter is confirmed, targeted therapeutic strategies to enhance urinary TMAO clearance may be feasible.

Conclusion

With the available data it is difficult to make a compelling case for the use of TMAO in clinical practice either as a biomarker or a therapeutic target in any patient population. Although elevated circulating plasma TMAO levels are associated with an increased risk of CV disease in some (including CKD) populations, the available data are inconsistent across observational studies. It is

therefore unclear whether TMAO contributes to CV disease in CKD patients or it is simply yet another marker of reduced renal clearance. To establish a role for TMAO in CKD-associated cardiovascular disease, large-scale interventional trials are required using TMAO reduction strategies with assessment of clinical endpoints relevant to the CV diseases prevalent in this population. If therapies that modify the gut microbiome can be developed with tolerable side effect profiles, such studies may become possible. In our view, more compelling observational and mechanistic data are required before significant amounts of time and money are invested in large clinical trials targeting TMAO reduction in CKD patients.

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