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Association of moderately elevated trimethylamine N-oxide with cardiovascular risk: is TMAO serving as a marker for hepatic insulin resistance

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ELEVATED TRIMETHYLAMINE-N-OXIDE IS AN ESTABLISHED CARDIOVASCULAR AND METABOLIC RISK FACTOR

To date, at least five prospective cohort studies have concluded that increased plasma levels of trimethylamine N-oxide (TMAO) predict increased risk for major adverse cardiovascular (CV) events in patients with pre-existing coronary heart disease.[1–5] Moreover, though some epidemiology does not support a connection between plasma TMAO and CV risk,[6 7] a recent meta-analysis of 11 prospective cohort studies concludes that higher plasma TMAO correlates with a 23% increase in risk for CV events (HR 1.23, 95% CI 1.07 to 1.42), as well as a 55% increase in all-cause mortality.[8] The possibility that TMAO may be a mediating factor in this regard is raised by rodent studies in which plasma levels have been raised either by direct oral administration of TMAO, or by administration of very high doses (proportionately very much higher than would be employed in human supplementation) of its precursors phosphatidylcholine and carnitine; in these studies, in which the achieved plasma level of TMAO was at least an order of magnitude higher than commonly observed in humans, a proatherogenic effect was documented.[9–14] In vitro studies, likewise employing supraphysiological concentrations of TMAO, have demonstrated effects suggesting proatherogenic potential.[12 13 15–17]

In case–control epidemiology, elevated TMAO has also been linked to substantially increased risk for type 2 diabetes and metabolic syndrome.[18–20] Indeed, the correlations between TMAO and diabetes risk appear to be stronger than those for CV risk.

NUTRITIONAL INTAKES OF TMAO AND ITS PRECURSORS DO NOT CORRELATE WITH CV RISK

Yet the notion that TMAO acts as a human vascular toxin at the plasma concentrations seen in people with reasonably normal renal function is difficult to square with other recent findings. Preformed TMAO is notably high in fish, in which it serves to maintain osmotic balance; levels tend to be higher in deep-sea fish, which must survive at higher pressures.[21–24] This TMAO can be directly absorbed after fish consumption.[25] However, at least in those who do not ingest a very large amount of fish, a high proportion of their plasma TMAO arises from bacterial metabolism of dietary choline (usually ingested as phosphatidylcholine) and carnitine; trimethyllysine also makes a minor contribution in this regard.[9 10 26] Certain gut bacteria can metabolise these compounds to trimethylamine (TMA) via TMA lyase activity; inhibition of this lyase activity prevents induction of atherosclerosis in mice fed high-dose choline.[11 27 28] This TMA can then be absorbed; its subsequent oxidation by hepatic flavin-containing monooxygenases (FMOs) converts it to TMAO.[29 30] Unless choline has cardioprotective properties, we are currently unaware of, a diet relatively rich in choline would be expected to increase CV risk if physiological levels of plasma TMAO can indeed provoke CV disease or CV events. Yet a recent meta-analysis of prospective epidemiological studies concluded that dietary choline intake has no significant impact on risk for incident CV disease or CV mortality; with respect to CV mortality, only two pertinent studies were available, so the conclusion in this respect might not be definitive.[31] Likewise, a recent meta-analysis failed to associate consumption of eggs–a rich source of phosphatidylcholine–with increased CV risk.[32]

With respect to carnitine and CV risk, a meta-analysis of prospective clinical trials in patients who had recently experienced

a myocardial infarction (MI) concluded that carnitine supplementation is markedly protective with respect to total mortality, ventricular arrhythmias and new-onset angina; trends for lower incidence of reinfarction or heart failure did not achieve statistical significance, possibly owing to the modest size of the studies included.[33] Clinical trials have also reported favourable effects of supplemental carnitine or carnitine esters on angina, intermittent claudication and heart failure.[34–39] Moreover, rodent atherogenesis studies, in which carnitine has been administered in doses reasonably proportional to the supplementation doses used clinically, have found that carnitine is antiatherogenic, despite its propensity to raise TMAO.[22 40–42] With respect to fish, the primary dietary source of preformed TMAO, a meta-analysis found that fish consumption correlates dose dependently with CV protection, likely because of the long-chain omega-3 content of fish.[43] While it might be argued that the benefits of omega-3 ingestion are masking a genuine adverse impact of TMAO on CV risk, the impact of moderate supplemental intakes of fish omega-3 on this risk seems to be rather modest in the context of current drug therapy, primarily influencing risk for sudden death arrhythmias.[44–46] Hence, these benefits would seem unlikely to overwhelm the adverse effects of TMAO if these were of important magnitude. In aggregate, these findings are difficult to square with the notion that TMAO is a mediating CV risk factor, at least in commonly occurring levels, since increased ingestion of choline, carnitine or fish would be expected to increase TMAO levels, but is not associated with increased CV risk.

It is, therefore, reasonable to suspect that moderately elevated TMAO, rather than being a mediator of the associated CV risk, is a marker for factors which both promote CV events and increase plasma TMAO.[47 48] Indeed, after a plethora of multicentre supplementation trials, we have learnt something precisely comparable about moderately elevated homocysteine and coronary risk.[49 50] Whereas the highly elevated homocysteine levels seen in genetic hyperhomocysteinaemia are evidently directly pathogenic to the vascular system, and homocysteine at comparably high levels exerts proinflammatory effects on vascular cells in vitro, we were never presented with evidence that the moderately elevated levels of homocysteine associated with increased CV risk–roughly an order of magnitude lower–exerted important effects in vitro. Currently, TMAO appears to be in an analogous position.[22]

DIMINISHED RENAL FUNCTION CAN MARKEDLY ELEVATE TMAO

Evidently, an increase in plasma TMAO can reflect an increase in TMAO synthesis or a reduction in its renal clearance. A promising lead is offered by the observation that plasma TMAO levels are highly dependent on renal function. A study examining plasma TMAO levels in patients with varying degrees of renal compromise and in healthy controls found that TMAO averaged 5.8 µM/L in the controls (with average measured glomerular filtration rate [mGFR]–83 mL/min), 14.6 µM/L in patients with stages 3–4 kidney disease (mGFR 28 mL/min) and 75.5 µM/L (mGFR 7 mL/min) in stage 5 patients.[51] Hence, as mGFR falls, plasma TMAO tends to rise almost proportionally.

Although it is well known that severe kidney disease is associated with a considerable increase in CV risk, a meta-analysis of general population cohort studies has found that even a mild reduction in estimated GFR (eGFR) is a risk factor for CV mortality.[52] Thus, whereas risk for CV mortality was found to be relatively flat for eGFRs in the range of 75–120 mL/min, a significantly higher risk was seen at eGFR 60 mL/min, and this mortality rose progressively as eGFR fell. Hence, even relatively modest reductions of eGFR sometimes considered to be within the 'normal' range of kidney function (eGFR of 60 mL/min or greater) are associated with increased CV risk. This increased risk could presumably reflect an impact of suboptimal kidney function per se (leading to increased levels of phosphate or other uraemic toxins), as well as of vasculotoxic factors inducing reduction of kidney function. These may not have been adequately corrected for in epidemiological analyses focusing on TMAO.

Nonetheless, there is good reason to believe that, whereas uncorrected correlations of TMAO with CV risk are explained in part by the CV risk associated with diminished renal function, this is not the sole explanation for the utility of TMAO as a risk factor. That is because the five cohort studies cited above-included analyses which adjusted for eGFR as a covariate; while this correction markedly decreased the calculated CV risk associated increased TMAO, it by no means eliminated it.[1–5] We can, therefore, conclude that factors, which boost TMAO synthesis, are mediators of some of the risk associated with elevated TMAO.

ARE BAD BACTERIA THE CULPRIT?

Two steps are involved in the synthesis of TMAO: generation of TMA from certain dietary precursors–most notably, choline and carnitine–by the TMA lyase activity of gastrointestinal (GI) bacteria; and oxidation of circulating TMA to TMAO by hepatic FMOs, by far the most active of which in this regard is FMO3.[29] With respect to GI bacteria, rodent studies have led to increasing awareness of the fact that microbiota can notably modulate metabolic health.[53–56] Is it possible that certain commonly occurring GI bacteria are quite proficient at generating TMA, while simultaneously increasing CV risk by certain mechanisms–for example, by suppressing incretin synthesis or maximising bile acid reabsorption (which might elevate LDL cholesterol)? Or could some dietary factor–soluble fibre, perhaps–suppress the capacity of GI bacteria to generate TMA, while simultaneously protecting CV health?

While this is an intriguing hypothesis that merits further follow-up, research studies to date provide little support for it. Controlled clinical studies of supplementation with probiotic micro-organisms linked to improved intestinal health–Lactobacillus casei Shirota and another preparation providing a *Lactobacillus*, *Bifidobacterium* and *Streptococcus thermophilus*–have so far failed to demonstrate reductions in plasma TMAO.[57 58] Faecal microbiota transplantation from vegan donors to recipients with metabolic syndrome, while it did succeed in altering the latter's GI flora, did not lower their plasma TMAO levels.[59] Administration of the cholesterol-lowering prebiotic glucaro-1,4-lactone to rats fed a high-fat diet, which markedly boosted intestinal levels of *Lactobacillus*, *Bifidobacteria* and *Enterococcus*, while suppressing *Escherichia coli*, was associated with an increase of TMAO in urine.[60] Supplementation of mouse diets with either galacto-oligosaccharides/inulin or polydextrose and insoluble bran fibre increased serum TMAO levels, whereas supplementation with both simultaneously failed to influence TMAO.[61]

In one mouse study, supplementation with soluble fibre from wheat bran did lower colonic TMA lyase activity as well as serum cholesterol.[62] However, it seems unlikely that an increased intake of protective soluble fibre explains the association of TMAO with vascular risk, since very ample intakes of soluble fibre are required to achieve a modest reduction in Low-density lipoprotein (LDL) cholesterol–intakes which very few people ingest; and in any case the associated risk persists after adjustment for lipid risk factors such as LDL cholesterol.[63]

While it is feasible to produce mice whose intestines have been colonised with bacteria with limited capacity to generate TMA, there so far is no evidence that this confers any special vascular protection on these mice when they eat normal diets.[27]

ELEVATED HEPATIC FMO3 ACTIVITY CAN REFLECT HEPATIC INSULIN RESISTANCE

Which brings us to the alternative thesis: that modulation of hepatic FMO3 activity by certain factors that can influence CV health, can rationalise the epidemiology of TMAO. The regulation of hepatic FMO3 requires much further research, but several intriguing findings have emerged. Insulin suppresses FMO3 expression at both the messenger RNA (mRNA) and protein level; conversely, glucagon elevates FMO3 expression.[64] Also, the FXR receptor, for which many bile acids serve as activating ligands, stimulates transcription of the FMO3 gene.[29 65] With respect to the impact of insulin, genetically modified mice in which hepatic expression of the insulin receptor has been selectively ablated (Liver-specific insulin receptor knockout mice) have greatly enhanced hepatic expression of FMO3.[64] These mice develop marked hypercholesterolaemia and are exceptionally prone to atherosclerosis when fed a proatherogenic diet, and also understandably have an elevated hepatic glucose output.[66] The pertinence of these findings to humans has been clarified by a study in which liver biopsies were obtained both from obese subjects and lean controls; mRNA expression of FMO3 was about twice as high in the obese subjects, likely reflecting hepatic insulin resistance in the context of hyperinsulinaemia.[64]

Recent studies suggest that the hepatic insulin resistance associated with obesity and metabolic syndrome is mediated by increased hepatic influx of free fatty acids (FFAs), giving rise to increased levels of diacylglycerol; the latter promotes activation of protein kinase C-epsilon, which in term hampers the tyrosine kinase activity of the insulin receptor by phosphorylating threonine-1160 of the beta-chain.[67–69] Other kinase or phosphatase activities stimulated by lipid overload may also impair insulin signalling at points downstream from the insulin receptor.[70 71] Excess FFA influx also drives increased triglyceride synthesis, giving rise to the hepatic steatosis often associated with hepatic insulin resistance. However, increased hepatic triglyceride levels per se may not promote hepatic insulin resistance; such resistance correlates with hepatocyte levels of diacylglycerol, rather than of triglycerides.[69 72]

Hepatic insulin resistance and its common concomitant hepatic steatosis are associated with increased CV risk, as well as elevated risk for type 2 diabetes—risks likewise associated with elevated TMAO.[66 73–77] It is, therefore, straightforward to postulate that TMAO can serve as a marker for hepatic insulin resistance, and that this explains at least a portion of the risk for CV events and diabetes linked to TMAO. Although studies establishing TMAO as an independent CV risk factor have often corrected for certain correlates of obesity, such as body mass index or diabetes, it is unlikely that such corrections fully capture the impact of hepatic insulin resistance.

CORRECTING HEPATIC INSULIN RESISTANCE

This analysis suggests that healthful measures which tend to correct hepatic insulin resistance may favourably impact the vascular and metabolic health of subjects with high TMAO. Evidently, sustained remediation of the visceral obesity which often underlies hepatic insulin resistance should be helpful in this regard; nonetheless, it is easier to recommend this than to achieve it! By improving the insulin sensitivity of hypertrophied adipocytes, thiazolidinediones such as pioglitazone tend to improve hepatic insulin resistance in people with diabetes by quelling excessive fatty acid efflux from adipocytes, even though they tend to increase body fat mass somewhat.[78–81]

Hormones and medications which boost hepatic AMPK activity tend to improve impaired hepatic insulin sensitivity. AMPK achieves this, at least in part, by downregulating mTORC1 activity, which acts indirectly to promote phosphorylations of insulin receptor substrate-1 that impede transmission of the insulin signal.[82] Also, by promoting oxidative disposal of FFAs while suppressing lipogenesis, AMPK could be expected to lessen hepatic diacylglycerol synthesis, thereby getting to the root of

hepatic insulin resistance.[83 84] The favourable impact of metformin on hepatic insulin resistance in diabetes is thought to be mediated by activation of AMPK.[85–88] The phytochemical nutraceutical berberine, widely used in China for the management of type 2 diabetes, is likewise thought to improve glycaemic control via activation of AMPK, and has been shown to counter hepatic insulin resistance in diabetic hamsters.[89–93]

Both adiponectin and glucagon-like peptide-1 (GLP-1) act on the liver to stimulate AMPK activity; moreover, they have been shown to combat hepatic insulin resistance, and work in various ways to promote vascular and metabolic health.[94–106] Hence, elevated TMAO may often be a marker for suboptimal adiponectin and/or GLP-1 activity. The antidiabetic drug pioglitazone tends to boost the diminished adiponectin secretion of hypertrophied adipocytes.[107 108] It seems likely that plant-based diets of rather low-protein content can increase adiponectin production, as these boost the liver's production of fibroblast growth factor-21, one of whose major functions is to promote adiponectin secretion by adipocytes.[109 110] Such diets are also useful for preventing or correcting the obesity that often underlies hepatic insulin resistance.[111–113]

With respect to GLP-1, acarbose, dietary lente carbohydrate, bile acid sequestrants and certain prebiotics can boost GLP-1 production, drugs inhibiting plasma dipeptidyl peptidase-4 can prolong its half-life, and injectable GLP-1 receptor agonists can mimic its bioactivity.[114–117]

PPARalpha agonists, such as fenofibrate, also promote hepatic fatty acid oxidation, owing to induction of a range of mitochondrial enzymes (including carnitine palmitoyl transferases-1a and -2, fatty acyl-CoA dehydrogenase, UCP-2) which catalyse such oxidation.[118 119] Moreover, PPARalpha agonism also acts indirectly to stimulate AMPK in the liver and other tissues by boosting adiponectin production in adipose tissue; PPARalpha enhances hepatic synthesis and release of fibroblast growth factor-21, which in turn stimulates adiponectin synthesis in adipocytes.[120–123] Not surprisingly, fenofibrate has been shown to decrease hepatic levels of diacylglycerol and alleviate hepatic insulin resistance in rodents fed diets high in fat and/or fructose.[124–128] Moreover, fenofibrate therapy has been shown to reduce risk for CV events in patients with metabolic syndrome.[118]

There is recent evidence that the carotenoid antioxidant astaxanthin can also serve as a PPARalpha agonist, and, both in rodents and humans, alleviate the dyslipidaemia associated with metabolic syndrome.[129–135] In obese mice, astaxanthin has been reported to improve hepatic insulin resistance.[136] Krill oil provides esterified forms of astaxanthin which have superior bioavailability, as well as health-protective omega-3 fatty acids, oxidised forms of which likewise serve as PPARalpha agonists.[137–140] Moreover, krill oil supplementation has been found to beneficially modulate serum lipid profile–including, intriguingly, a reduction in LDL cholesterol–in controlled clinical trials.[141] Krill oil, even when compared with fish oil, suppresses hepatic steatosis in rodents.[142–144] This may be due to its astaxanthin content, which is not found in fish oil. Moreover, krill oil, but not fish oil, reduces diacylglycerol and ceramide content in the liver.[145] The phospholipid fraction of krill oil has also been noted to reduce hepatic glucose production, unlike fish oil.[146] Thus, krill oil, being a source of highly bioavailable form of astaxanthin, appears to have additional advantages for reducing hepatic steatosis and hepatic insulin resistance compared with fish oil.

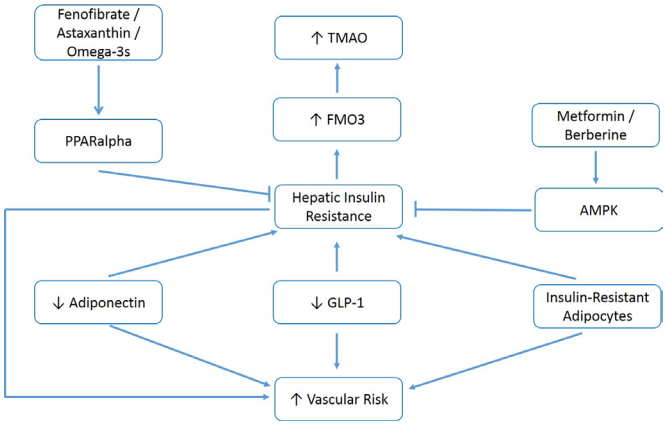

Figure 1 Measures which increase adiponectin, increase GLP-1 activity, control metabolic syndrome and activate hepatic AMPK or PPARalpha may decrease elevated TMAO and associated vascular/metabolic risk. GLP-1, glucagon-like peptide-1; TMAO, trimethylamine-N-oxid.

In brief, if this analysis is accurate, various measures which alleviate hepatic insulin resistance–correction of visceral obesity, activation of 5' adenosine monophosphate-activated protein kinase (AMPK) with metformin or berberine, activation of PPARalpha with fenofibrate or astaxanthin, amplification of adiponectin production with pioglitazone or plant-based diets, and clinical strategies which boost the production or bioactivity of GLP-1, could be expected to decrease elevated TMAO while also decreasing the risk for vascular events and diabetes associated with this risk factor. Figure 1 summarises these relationships.

FMO3 MIGHT ALSO MEDIATE RISK ASSOCIATED WITH ELEVATED TMAO

One intriguing observation to emerge from TMAO research is that elevated hepatic expression of FMO3 boosts hepatic lipogenesis and gluconeogenesis, independent of its impact on TMAO levels; this might reflect FMO3's ability to somehow support expression of FoxO1.[30 64] This raises the interesting prospect that drugs selectively targeting FMO3 might have some utility in diabetes and hyperlipidaemia, particularly when elevated TMAO levels suggest that hepatic FMO3 expression is high. However, since FMO3 plays a systemic role in catecholamine metabolism, suppressing its function might not prove to be innocuous; genetic absence of FMO3 activity has been associated with hypertension.[147]

In any case, when hepatic insulin resistance is present, correcting this should lessen hepatic FMO3 expression.

OVERVIEW

Accumulating evidence points to elevated plasma TMAO as a risk factor for both atherosclerosis, CV events and type 2 diabetes, and rodent studies have found that extremely high dietary intakes of TMAO per se or its dietary precursors choline and carnitine are proatherogenic. Moreover, supraphysiological concentrations of TMAO exert proinflammatory effects in cell culture studies. These findings have led some observers to recommend that dietary or supplementary consumption of choline and carnitine should be minimised–although these analysts have rarely recommended abstinence from fish, the richest dietary source of preformed TMAO. In fact, a meta-analysis of pertinent nutritional epidemiology has failed to observe an impact of dietary choline on CV risk. Supplemental use of carnitine has been found to reduce mortality and diminish risk for arrhythmias and new-onset angina in patients who have suffered a previous MI, has shown clinical utility in angina, intermittent claudication and heart failure, and exerts antiatherogenic effects in rodents when fed at moderate levels comparable to human supplemental intake. And, fish consumption correlates dose dependently with favourable vascular outcomes. These findings point ineluctably to the conclusion that TMAO is not a mediating risk factor, at least in the concentrations seen in people whose renal function is not severely defective.

Hence, moderately elevated TMAO must be viewed as a marker for other factors that both raise TMAO and confer increased risk for vascular disease and diabetes. Plasma levels of TMAO are highly reflective of renal function, and hence a portion of the risk associated with elevated TMAO is mediated either by impaired renal function, or renotoxic factors that are also vasculotoxic or promote diabetes. Nonetheless, TMAO remains predictive of vascular risk after statistical correction for eGFR; factors influencing TMAO synthesis evidently mediate some of this risk. While it is theoretically possible that certain strains of GI bacteria possessing high TMA lyase activity exert adverse effects on vascular and metabolic health, this remains to be demonstrated, and efforts to lower plasma TMAO with probiotics thought to be health protective have so far failed.

Factors which upregulate hepatic expression and activity of FMO3, chiefly responsible for conversion of TMA to TMAO, therefore, fall under suspicion. In this regard, it is notable that subnormal hepatic insulin activity reflecting hepatic insulin resistance has been found to boost hepatic FMO3 expression. Hepatic insulin resistance is typically induced by the excessive FFA influx associated with metabolic syndrome and visceral obesity, well-known risk factors for vascular disease and diabetes. This excessive FFA influx also gives rise to hepatic steatosis; although excessive accumulation of triglycerides in the liver does not appear to mediate hepatic insulin resistance, it serves as a marker for the increased FFA influx that does. Subnormal activities of either adiponectin or GLP-1–both of which exert favourable vascular and metabolic effects–can also promote hepatic insulin resistance. It is, therefore, reasonable to speculate that lifestyle measures which reverse visceral obesity, or nutraceutical/drug/dietary measures which boost the production or bioactivity of adiponectin and/or GLP-1, will alleviate the risk associated with elevated TMAO by ameliorating hepatic insulin resistance. Activation of AMPK with metformin or berberine, or of PPARalpha with fenofibrate or astaxanthin, could also be expected to have a favourable impact in this regard, in part by accelerating the oxidative disposal of excessive hepatic FFAs. Finally, elevated FMO3 activity per se may mediate some of the risk associated with high TMAO via upregulation of hepatic lipogenesis and gluconeogenesis.

Importantly, this analysis does not exclude the possibility that TMAO might be directly pathogenic at the very elevated levels typically seen in severe kidney dysfunction. Indeed, cell culture studies suggest that TMAO can be proinflammatory in the plasma concentrations achieved during kidney failure. It generally is wise to minimise the consumption of nitrogenous compounds in this context.

In conclusion, there is a reason to suspect that the elevated risk for vascular events and type 2 diabetes associated with elevated TMAO, after correction for recognised risk factors, is mediated largely by hepatic insulin resistance and the metabolic factors which induce it. This implies that a range of measures which typically improve hepatic insulin sensitivity, as catalogued above, could be expected to decrease elevated TMAO–a proposition that is readily clinically testable–while ameliorating the vascular and metabolic risk associated with high TMAO.

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