Trimethylamine *N*-oxide: heart of the microbiota–CVD nexus?

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Abstract

We critically review potential involvement of trimethylamine *N*-oxide (TMAO) as a link between diet, the gut microbiota and CVD. Generated primarily from dietary choline and carnitine by gut bacteria and hepatic flavin-containing mono-oxygenase (FMO) activity, TMAO could promote cardiometabolic disease when chronically elevated. However, control of circulating TMAO is poorly understood, and diet, age, body mass, sex hormones, renal clearance, FMO3 expression and genetic background may explain as little as 25 % of TMAO variance. The basis of elevations with obesity, diabetes, atherosclerosis or CHD is similarly ill-defined, although gut microbiota profiles/remodelling appear critical. Elevated TMAO could promote CVD via inflammation, oxidative stress, scavenger receptor up-regulation, reverse cholesterol transport (RCT) inhibition, and cardiovascular dysfunction. However, concentrations influencing inflammation, scavenger receptors and RCT ($\geq$100 µM) are only achieved in advanced heart failure or chronic kidney disease (CKD), and greatly exceed pathogenicity of <1–5 µM levels implied in some TMAO–CVD associations. There is also evidence that CVD risk is insensitive to TMAO variance beyond these levels in omnivores and vegetarians, and that major TMAO sources are cardioprotective. Assessing available evidence suggests that modest elevations in TMAO ($\leq$10 µM) are a non-pathogenic consequence of diverse risk factors (ageing, obesity, dyslipidaemia, insulin resistance/diabetes, renal dysfunction), indirectly reflecting CVD risk without participating mechanistically. Nonetheless, TMAO may surpass a pathogenic threshold as a consequence of CVD/CKD, secondarily promoting disease progression. TMAO might thus reflect early CVD risk while providing a prognostic biomarker or secondary target in established disease, although mechanistic contributions to CVD await confirmation.

Key words: **Atherosclerosis: CVD: Carnitine: Choline: Gut microbiota: Vascular dysfunction: Vegetarians**

Introduction

CVD, particularly CHD, remains a dominant cause of morbidity/mortality globally. Nonetheless, CHD is largely preventable, involving modifiable factors such as poor diet and physical inactivity, associated dyslipidaemia and obesity, smoking, psychosocial stressors and socio-economic adversity. Recently there has been considerable focus on the role of the body's microbiota in non-communicable diseases. The gut microbiota may be a key nexus linking major modifiable risk factors (diet, obesity, inactivity, stress) to diverse chronic diseases[1], with trimethylamine (TMA) *N*-oxide (TMAO) potentially participating in these linkages[2] (Fig. 1). Indeed, evidence that dietary lipids are not a major CVD risk factor[3] encourages investigation of such alternative diet-linked drivers of disease.

Bolstered by evidence of detrimental effects of high TMAO concentrations in cell[4-7] and animal models[8-13], recently reported associations between TMAO and CVD risk and outcomes[2,14-17] have focused attention on TMAO as a potential determinant of disease. This may provide both a circulating biomarker of CVD risk, and a potential therapeutic target (Fig. 2).

Nonetheless, whether the effects and disease associations of TMAO reflect a primary causal or secondary reinforcing role in CVD is unclear, and critical unknowns remain unresolved: how are TMAO levels elevated, and is this a consequence or cause of disease; what do association studies indicate in terms of the role of TMAO in CVD; are associations consistent with current knowledge regarding TMAO pathogenicity? More broadly, is a primary role for TMAO reconcilable with known dietary risks for and determinants of CVD?

Pathogenic effects of trimethylamine *N*-oxide

The pathogenic effects and potency of TMAO are fundamental to its potential role in CVD. Evidence to date supports detrimental effects of high TMAO levels on atherosclerotic processes, and vascular and myocardial function, a suite of actions that could well promote CVD. However, there are inconsistencies between the concentration dependence of TMAO effects in experimental systems and pathogenic sensitivities implied in some TMAO–CVD associations: the latter suggest markedly increased CVD

Abbreviations: ABCA1, ATP-binding cassette transporter A1; AMI, acute myocardial infarction; CD36, cluster of differentiation 36; CKD, chronic kidney disease; DMB, 3,3-dimethyl-1-butanol; eNOS, endothelial NO synthase; FMO, flavin-containing mono-oxygenase; FMO3, flavin-containing mono-oxygenase 3; HF, heart failure; ICAM-1, intercellular adhesion molecule 1; MACE, major adverse cardiac events; NLRP3, NOD-like receptor family, pyrin domain containing 3; RCT, reverse cholesterol transport; ROS, reactive oxygen species; SR, scavenger receptor; SR-A, scavenger receptor A; SR-B1, scavenger receptor B1; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide.

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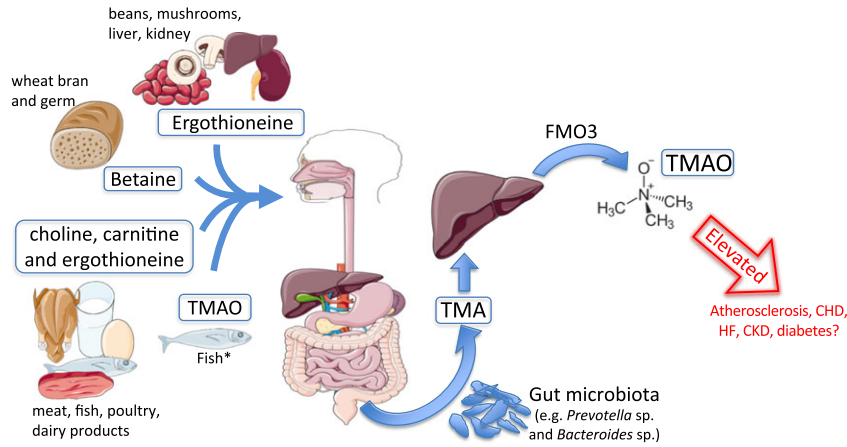
Fig. 1. Trimethylamine-*N*-oxide (TMAO) formation. Process of TMAO intake or generation from dietary sources. Substantial elevations in circulating TMAO are hypothesised to promote cardiometabolic and renal diseases. * Fish represent a significant and direct dietary source of TMAO. CKD, chronic kidney disease; FMO3, flavin-containing mono-oxygenase enzyme 3; HF, heart failure; TMA, trimethylamine.
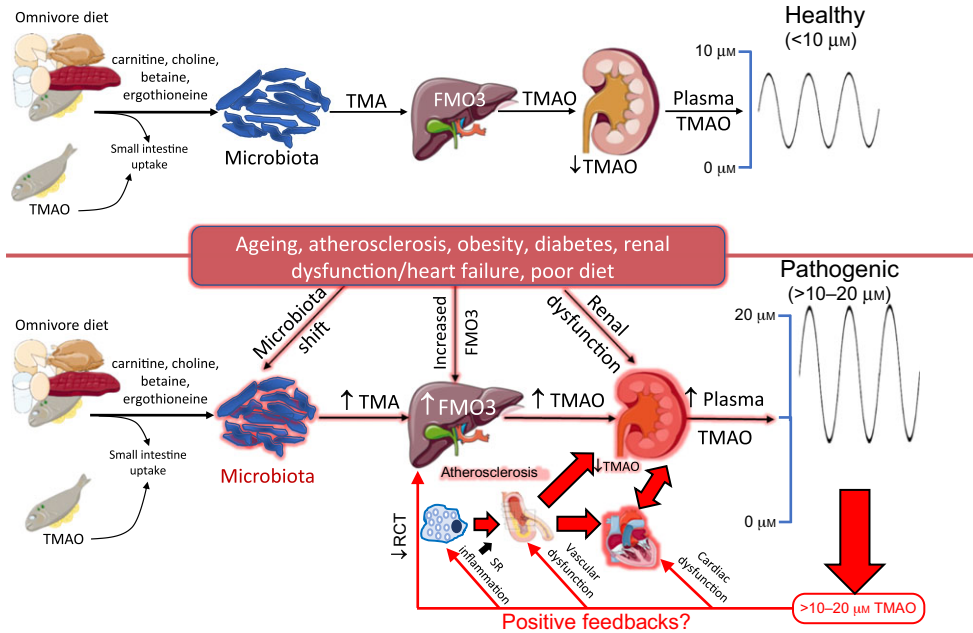

Fig. 2. Trimethylamine-*N*-oxide (TMAO) responses in health and diseased omnivores. Variations in circulating TMAO as a result of dietary substrates in omnivores may be insufficient to promote disease. However, a combination of major risk factors (ageing, obesity, insulin resistance, shift in gut microbiota) and/or disease (diabetes, chronic kidney disease) – as in increasingly common multimorbidity – may cumulatively and chronically elevate circulating TMAO to >10–20 μM. These concentrations may be sufficient to promote vascular and cardiac dysfunction/disease. Speculative positive feedbacks are also presented, based on putative roles of TMAO in inflammation, cardiac and vascular dysfunction, and flavin-containing mono-oxygenase 3 (FMO3) activity: evolving CVD can induce renal dysfunction to elevate TMAO; inflammation promotes TMAO generation and FMO3 expression; and TMAO itself together with renal dysfunction may up-regulate FMO3 activity. These putative feedbacks await experimental confirmation. RCT, reverse cholesterol transport; SR, scavenger receptor; TMA, trimethylamine.

risk and complications with <5 μM TMAO[2,14,17-19], despite little direct evidence for detrimental effects at these concentrations. Indeed, pathological concentrations in experimental systems may be rarely encountered *in vivo*, except in advanced CVD or renal disease.

Pro-atherosclerotic actions

There has been a focus on roles of TMAO in atherosclerosis and vascular dysfunction. At substantially elevated concentrations, TMAO is pro-inflammatory, impairs vascular function and

structure, and may up-regulate scavenger receptors (SR) while inhibiting reverse cholesterol transport (RCT)[6,7,19,20] (Fig. 3). High TMAO concentrations can also induce platelet hyper-reactivity, enhancing thrombotic potential via amplified intracellular Ca^{2+} release[21]. A 10-fold elevation in serum TMAO with choline supplementation in mice (1 % w/w, about 12× normal) also augments platelet reactivity to ADP, though failing to influence other indices including surface phosphatidylserine content in ADP-stimulated platelets, levels of Von Willebrand factor, α granule release, or baseline pro-thrombotic microvesicle release[22]. These mixed actions could contribute to vascular disease when
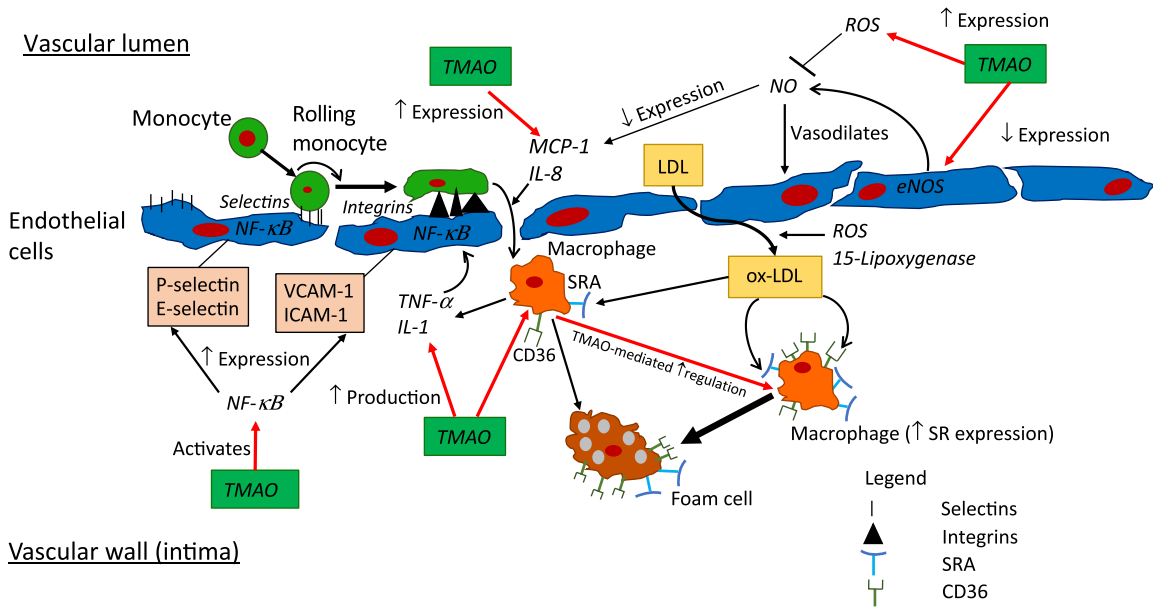
Fig. 3. Putative pro-inflammatory and -atherosclerotic actions of trimethylamine-*N*-oxide (TMAO). Pronounced elevations in TMAO to >10–20 μM, for example in advanced heart failure or chronic kidney disease, may be sufficient to modify multiple determinants of inflammation and atherosclerosis, as detailed here. Whether levels of circulating TMAO in obesity, diabetes or CHD are sufficient to significantly influence these processes is presently unclear. CD36, cluster of differentiation 36; eNOS, endothelial nitric oxide synthase; ICAM-1, intracellular adhesion molecule-1; MCP-1; monocyte chemoattractant protein 1; NF-κB; nuclear factor κ-light-chain-enhancer of activated B cells; ox-LDL, oxidised-LDL; ROS, reactive oxygen species; SR, scavenger receptor; SRA, scavenger receptor A, VCAM-1, vascular adhesion protein-1.

TMAO exceeds 10–20 μM, for example in heart failure (HF) and chronic kidney disease (CKD); however, involvement in the initial pathogenesis of atherosclerosis and CHD is questionable[23].

Studies in genetic models predisposed to disease indicate that major elevations in TMAO with high-level carnitine supplementation (1–1·3 % in drinking water) promote atherogenesis. Carnitine supplementation exaggerates atherosclerosis in ApoE−/− mice, an effect countered by broad-spectrum antibiotics[20] and the choline analogue inhibitor of TMA lyase, 3,3-dimethyl-1-butanol (DMB)[23]. Pro-atherosclerotic effects of intermittent hypoxia/hypercapnia (relevant to sleep apnoea) in ApoE−/− and LDL receptor knockout mice are also partially DMB sensitive[24]. These observations support some role for TMAO in high-risk settings where both propensity to atherosclerosis and TMAO concentrations are artificially augmented; however, their broader relevance is less clear. A word of caution is also warranted regarding the applicability of the ApoE−/− model to human atherosclerosis, given lack of the cholesteryl ester transfer protein important in human RCT. Contrasting these studies, analysis of outcomes in cholesteryl ester transfer protein-transfected ApoE−/− mice supports a protective rather than detrimental role for TMAO[25]. Other work reveals no relationship between TMAO, plasma cholesterol and atherogenesis in mice fed normal diets[26], or in ApoE−/− mice fed high-fat diets[27]. Differences in TMAO within inbred strains of mice are also estimated to explain less than 10 % of the variance in atherosclerosis[28].

Up-regulation of scavenger receptors. Increased expression of atherogenic SR, for example cluster of differentiation 36 (CD36) and scavenger receptor A (SR-A), is a putative mechanism for TMAO-sensitive atherogenesis[2,20,27,29] (Fig. 2 and Fig. 3). The

membrane glycoprotein CD36 is essential in plaque formation, internalising oxidised LDL within the vascular intima[30,31], while SR-A1 is key to macrophage cholesterol accumulation/foam cell formation, and modulates immune function, cell proliferation and death[30,31]. Importantly, effects of TMAO on macrophage SR-A1 expression are only evident at exceedingly high *in vitro* concentrations (≥300 μM)[2,4,5], unlikely to be encountered *in vivo*. Macrophage stress responses are similarly evident at very high TMAO concentrations[5]. Macrophage CD36 and SR-A1 expression in atherosclerotic mice are increased in response to an excessive 350 μM plasma concentration (achieved with TMAO supplementation)[2,4,5]. Geng *et al.*[27] recently showed that TMAO supplementation (1 mM in drinking water) increases plaque CD36 in ApoE−/− mice, and that 100 μM TMAO exaggerates oxidised LDL-dependent changes in CD36 and foam cell formation (via p38 MAPK/JNK1/2 signalling) without influencing baseline CD36 expression[27]. These data suggest a possible role for high concentrations of TMAO in exaggerating SR changes in advanced disease, without effects in otherwise healthy animals. Nonetheless, the causal involvement of SR-A1 or CD36 in TMAO-sensitive atherosclerosis has yet to be established.

Inhibition of reverse cholesterol transport. Additional to promoting sequestration of oxidised LDL, elevated TMAO may inhibit the RCT that underlies hepatic catabolism and biliary excretion of lipids[32] (Fig. 2). Dietary supplementation with carnitine (1·3 %), choline (1·0 %) or TMAO (0·12 %) decreases RCT in mice, with effects of carnitine and choline eliminated by antibiotics[2,20]. The basis of this effect awaits clarification; however, shifts in transporters such as ATP-binding cassette transporter A1 (ABCA1) and scavenger receptor B1 (SR-B1) could contribute.

High concentrations of TMAO (75–150 µM) may reduce macrophage ABCA1 expression *in vitro*[5], although others report increased macrophage ABCA1 with 100 µM TMAO, no change in SR-B1 with 50–100 µM TMAO, and no changes in hepatic SR-B1 protein or ABCA1 mRNA with TMAO supplementation in atherosclerotic mice[20]. Similarly excessive concentrations of TMAO (50–200 µM) moderately increase ABCA1-dependent cholesterol efflux[2,20], while Trenteseaux *et al.*[33] report opposing increases in SR-B1 mRNA in male offspring of hypercholesterolaemic mice *v.* decreases in female offspring. Other effects of TMAO that might influence RCT and atherosclerosis include reductions in bile acid pool size, synthetic enzymes and transport proteins[20], including Cyp7a1, a major rate-limiting synthetic enzyme in cholesterol catabolism[34]. Potential modulation of RCT by TMAO requires further study, and causal roles for ABCA1 or SR-B1 in TMAO-dependent changes in RCT and atherosclerosis remain to be demonstrated.

Pro-inflammatory actions. Inflammation is important in atherogenesis, vascular dysfunction and remodelling[35,36], and participates broadly in cardiometabolic dysfunction and disease. Studies *in vitro* and in animal models indicate that elevated TMAO can be pro-inflammatory[6-8,19,27], although again the concentrations required are up to an order of magnitude higher than observed in different disease states. Sufficiently high TMAO concentrations may influence multiple determinants of inflammation and associated vascular dysfunction, including integrin expression, reactive oxygen species (ROS) generation, NF-κB signalling and cytokine production, the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome, and anti-inflammatory sirtuins (Fig. 3). Consistent with pro-inflammatory effects of age-dependent elevations in TMAO, inhibiting TMA formation limits changes in TNF-α, IL-1β and endothelial NO synthase (eNOS) in ageing rats (in which TMAO was elevated >2-fold), whereas expression was unaltered in young animals[6,8].

Sun *et al.*[19] report concentration- and time-dependent effects of 100–300 µM TMAO on IL-1β, IL-18, NLRP3 and oxidative stress in human endothelial cells, with eNOS expression and NO release repressed. Chen *et al.*[7] also document increased human endothelial expression of IL-1β and NLRP3, together with ICAM-1, matrix metallopeptidase-9 (MMP-9), and caspase-1, on exposure to extremely high (300–900 µM) TMAO. These effects, together with NLRP3 inflammasome activation, appear related to decreased superoxide dismutase 2 (SOD2) and sirtuin-3, and increased mitochondrial ROS generation[7]. Similar sensitivities are reported in rat vascular smooth muscle cells, with IL-1β, NF-κB and NLRP3 increased in response to 100–600 µM TMAO[37]. Expression of TNF-α, IL-6 and intercellular adhesion molecule 1 (ICAM-1) are also increased by TMAO in the ApoE$^{-/-}$ model and in cultured macrophages, facilitating macrophage recruitment; however, the required TMAO concentrations were unreported[27].

Choline supplementation (1·3 % in drinking water) to increase plasma TMAO >10-fold (about 55 µM) significantly increases vascular transcription of inflammation-related factors in the atherosclerosis-prone LDL receptor knockout mouse, including monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 2 (MIP-2), TNF-α, ICAM-1, keratinocyte chemoattractant (mouse analogue of IL-8), cyclo-oxygenase-2 (COX-2), E-selectin, vascular adhesion protein-1 (VCAM-1) and the macrophage marker CD68[6]. Effects in wild-type mice were not assessed, while even higher TMAO concentrations (100–200 µM) were found to increase inflammatory mediators in human vascular endothelial and smooth muscle cells, with 400 µM TMAO increasing endothelial recruitment of leucocytes[6]. These effects were suppressed by inhibitors of NF-κB and the βγ G-protein subunit of G-protein coupled receptors (GPCR)[6], though whether the latter reflects direct TMAO–GPCR interactions or modulation of protein conformation is unclear[20]. Interaction between TMA and GPCR has been reported[38]. Contrasting evidence of pro-inflammatory actions, TMAO appears to have no effect on toll-like receptor 4 signalling[19]. Overall, while some studies support pro-inflammatory effects of high TMAO levels, these concentrations are only relevant to advanced HF and CKD, whereas concentrations observed in obesity, diabetes, atherosclerosis and CHD are orders of magnitude lower. Further work is essential to clarify whether and how TMAO influences inflammation in these disease settings.

Vascular dysfunction and senescence

Recent studies also suggest that very high concentrations of TMAO may impair eNOS- or EDHF-dependent vascular control, and facilitate vessel remodelling and senescence[8,39-41]. Elevated TMAO can increase formation of ROS that degrade NO, and may also inhibit eNOS expression, compounding vascular dysfunction (Fig. 3)[19]. Sun *et al.*[19] found that 100–300 µM TMAO suppresses endothelial eNOS expression and NO release, an effect involving ROS-dependent activation of the NLRP3 inflammasome[19]. Additionally, Matsumoto *et al.*[41] report a modest inhibitory effect of 300 µM TMAO on EDHF-mediated relaxation in the femoral (but not superior mesenteric) artery. Suggestive of some vascular influences, chronic TMAO infusion in rats to achieve a 58 µM plasma concentration did not modify blood pressure, yet prolonged the hypertensive effect of angiotensin II[42]. Whether this reflects a direct vascular influence is unclear. Supporting TMAO-dependent vascular dysfunction in disease, inhibition of TMA production with DMB reportedly preserves aortic relaxation and eNOS phosphorylation, and eliminates vascular inflammation and oxidative stress in a rat model of CKD associated with a doubling of plasma TMAO[39]. This study, remarkable in implicating a modest elevation in TMA/TMAO as the sole driver of inflammation, oxidative stress and eNOS and vascular dysregulation in CKD, requires broader confirmation and reconciliation both with evidence of other mechanistic involvement (including nuclear factor erythroid 2-related factor 2 (Nrf2) suppression, inducible NO synthase (iNOS) induction, altered parathyroid hormone and Ca^{2+} handling) and with the excessive TMAO concentrations required to induce such effects *in vitro*. There is other support for TMAO-dependent vascular damage in CKD, with vessel calcification in CKD patients correlating with TMAO concentrations above 100 µM, and evidence that 100–600 µM TMAO can promote vascular calcification and expression of bone-related and inflammatory molecules *in vitro* and *in vivo*[37].

Ageing is an independent risk factor for CVD and involves vascular remodelling and progressive reductions in endothelial function and NO bioavailability, coupled with increased cyclo-oxygenase (COX)-derived vasoconstrictors. Circulating TMAO increases with age in humans and rodents[8,40,43,44], and recent work indicates that inhibition of TMAO formation reduces[8] whereas TMAO supplementation mimics or accelerates[40] ageing-dependent vascular dysfunction and inflammation in rodents. Pro-senescence effects were associated with exaggerated oxidative stress, suppression of sirtuin-1 and activation of p53/p21/Rb signalling[40]. Antibiotic treatment in ageing mice also improves vascular reactivity, in association with a fall in circulating TMAO; however, the role of TMAO was not directly tested[44]. These studies, together with evidence for TMAO-dependent brain ageing[43], suggest elevated TMAO could promote biological ageing, predisposing to strongly age-dependent diseases such as CHD and CKD. Indeed, Li *et al.*[8] report that a moderate elevation in TMAO from about 6 to 14 μM is solely responsible for age-dependent vascular dysfunction, inflammation and eNOS depletion, with the aged vascular phenotype in senescent rats reversed by DMB treatment. This surprising observation also demands broader confirmation, and reconciliation with evidence for diverse mechanistic elements of vascular ageing. More fundamentally, TMAO concentrations required to modify viability and senescence markers in human endothelial cells[40] are so extreme (100 mM) as to have no biological or pathological relevance in humans or animal models.

Myocardial dysfunction

Although there are relatively few studies of the myocardial effects of TMAO, emerging evidence suggests cardiac sensitivity to concentrations that are approached or surpassed in HF and CKD. These pronounced elevations in TMAO may thus promote cardiac dysfunction, as suggested by select effects of DMB in diseased but not healthy animals[10,45]. In addition to evidence of TMAO-dependent exaggeration of cardiac fibrosis and dysfunction in disease models[46], TMAO may disrupt myocyte structure, contractile function and energy metabolism, and promote oxidative stress[47,48]. Inhibitory effects on contraction, relaxation and Ca^{2+} dynamics are evident with 20 μM TMAO[47], although the latter effects appeared concentration-independent in the limited range tested. However, other work indicates that 0·3–3 μM TMAO disrupts T-tubule organisation and Ca^{2+} handling in isolated murine cardiomyocytes[49], suggesting a curious scenario in which concentrations more than 10-fold lower than circulating levels in healthy mice[10] are significantly damaging. Distinct from these studies, recent work supports modest positive inotropic effects of TMAO when applied at extremely high (0·3–3 mM) levels[50]. Further work is needed to clarify the cardiac functional influences of relevant concentrations of TMAO.

Potential impacts of TMAO on cardiac energy metabolism are supported by TMAO-dependent inhibition of mitochondrial pyruvate and fatty acid oxidation, also apparent *in vitro* with 20 μM TMAO[48]. Another recent study suggests a threshold of about 100 μM TMAO for inhibition of oxidoreductase capacity in cultured cardiomyoblasts, although TMA appeared substantially more potent, reducing metabolic capacity at 1–10 μM[51]. It has

also been reported that exogenously applied TMAO can destabilise atrial electrophysiology to promote fibrillation (potentially via inflammation)[52]; however, concentrations mediating this effect are difficult to ascertain. There is also some indirect evidence that myocardial protection with physical activity may be inhibited by TMAO levels exceeding 20 μM[13]; however, involvement of TMAO was not tested and is inconsistent with a lack of effect of TMAO supplementation.

Apparent sensitivities to about 20 μM TMAO suggest that cardiac dysfunction could arise as a direct result of elevations observed in HF and CKD, or following acute myocardial infarction (AMI). Renal hypo-perfusion and neuroendocrine changes characteristic of HF probably contribute to the associated rise in TMAO[15,53]. Ageing, obesity, insulin resistance and diabetes[54] – associated with and promoting HF – may additionally contribute to increased TMAO, for example via altered flavin-containing mono-oxygenase 3 (FMO3) expression[44,55,56]. These TMAO changes could constitute an important cardio-renal linkage, with TMAO functioning as a uraemic toxin that promotes cardiac and renal dysfunction and remodelling[57] (Fig. 2). Elevated TMAO does appear to worsen pressure overload-induced HF in mice[46], and promotes renal inflammation and fibrosis[8,11]. Causal involvement in cardiac dysfunction and remodelling is supported by the observation that TMAO is elevated to about 20 μM in a rodent model of obesity, with DMB preventing diastolic dysfunction, inflammation and cardiac fibrosis independently of metabolic disruption[10]. The observation that myocardial impacts of dietary obesity appear entirely dependent on a 2-fold rise in TMAO (to about 20 μM) has important implications, though awaits confirmation. Li *et al.*[45] present evidence that post-infarct HF in rats may also involve an associated increase in circulating TMAO (to about 25 μM), with cardiac outcomes improved upon DMB treatment[45]. Nonetheless, involvement of circulating TMAO in the pathogenesis of HF awaits more extensive analysis, as does its potential role as a uraemic toxin in the cardio-renal syndrome. There is contrary evidence, for example, that chronic elevations in TMAO may actually protect against myocardial dysfunction and fibrosis[58].

Associations between trimethylamine *N*-oxide and CVD: cause and/or effect?

Experimental *in vitro* and *in vivo* studies to date support potentially pathogenic effects of as little as about 20 μM TMAO on vascular and cardiac function, and ≥100 μM TMAO on inflammation and other pro-atherosclerotic processes. How does this pattern of pathogenicity mesh with concentrations reported in healthy and diseased cohorts, and apparent associations between TMAO and CVD?

Measures of circulating TMAO in healthy humans (largely via stable isotope dilution and LC/MS-MS) generally range from 2 to 5 μM[2,14,15,17,18,20,40,53,56,59-67], with lower estimates of 0·2–1 μM[18] (Table 1). Although underlying mechanisms remain obscure, circulating concentrations increase moderately (30–50 %) with body weight[56] or diabetes[56,64], rise to about 10 μM with ageing[40,43], and increase more profoundly in HF (>10 μM; linked to disease severity/renal dysfunction)[2,18,60] and CKD

Table 1. Estimates of human serum trimethylamine *N*-oxide (TMAO) concentrations in healthy and diseased cohorts*

Study	Subjects	Serum TMAO	Sample size	Age (years)	BMI (kg/m^2)	Sex (% male)	TMAO analysis
Healthy or broad cohort							
Mente et al. (2015)[17]	TMAO Q1 (19 % CVD, 2 % diabetes)	0·46 (SD 0·14) μM (Q1)	44	51 (SD 9)	25·4 (SD 4·0)	64	LC/MS
	TMAO Q5 (43 % CVD, 19 % diabetes)	4·93 (SD 5·69) μM (Q5)	59	53 (SD 12)	28·7 (SD 4·8)	75	
Wang et al. (2014)[59]	Healthy	Median 3·45 (IQR 2·25–5·79) μM	349	54 (SD 16)	25 (23–29)	33	LC-MS/MS
Krüger et al. (2017)[61]	Healthy (n 297 total)	<2·9 μM (Q1)	74	40 (SD 16)	23·3 (SD 2·7)	54	LC/MS
		>6 μM (Q4)	74	57 (SD 14)	24·6 (SD 3·0)	54	
Cho et al. (2017)[62]	Healthy	3·3 (SD 0·2) μM	40	28 (SD 1)	24·2 (SD 0·4)	100	LC-MS/MS
Meyer et al. (2016)[65]	Healthy (n 817 in CARDIA)	Median 1·3 (IQR 1·1–1·5) μmol/l (Q1)	203	40 (SD 4)	28·8 (SD 6·8)	38	LC/SIDA/MS
		Median 6·6 (IQR 5·1–10·1) μmol/l (Q4)	201	40 (SD 4)	28·7 (SD 5·7)	59	
Gruppen et al. (2017)[227]	PREVEND (CVD n 61 (4·5 %) in Q1, n 96 (7·0 %) in Q4)	<1·7 μM (Q1)	1361	49 (SD 11)	26·1 (SD 4·3)	46	^{1}H-NMR
		≥5·7 μM (Q4)	1368	54 (SD 12)	27·1 (SD 4·6)	44	
CVD							
Wang et al. (2011)[2]	Total cohort (65 % CVD, 27 % MI, 31 % diabetes, Framingham Risk Score 10)	Median 3·9 (IQR 2·6–6·3) μM	1876	64 (SD 10)	NA	49	MS^n, LC-MS/MS, GC-MS/MS
Tang et al. (2013)[14]	Healthy (30 % diabetes, 40 % MI)	Median 3·5 (IQR 2·9–5·9) μM	3494	62 (SD 11)	Median 28·7 (IQR 25·7–32·5)	65	SIDA/LC/MS
	MACE (43 % diabetes, 53 % MI)	Median 5·0 (IQR 3·0–8·8) μM	513	68 (SD 10)	Median 28·1 (IQR 24·8–32·4)	62	
Wang et al. (2014)[170]	EDCA (65 % CVD, 41 % MI)	Median 3·7 (IQR 2·4–6·2) μM	3903	Mean 63 (SD 11)	NA	64	LC-MS/MS
Diabetes							
Dambrova et al. (2016)[64]	PCI non-diabetic	1·73 (SD 1·05) μM	112	63 (total)	Median 28·4 (IQR 25·8–31·8) (total)	75 (total)	UPLC-MS/MS
	PCI diabetes (34 % prior PCI, 30 % MI, 70 % hypertension)	2·25 (SD 1·09) μM	39				
Obeid et al. (2016)[66]	Healthy	Mean 5·4 (SD 5·2) μmol/l	185	67 (SD 9)	26·4 (SD 3·5)	57	LC/MS
	T2DM	Mean 8·6 (SD 12·2) μmol/l	98	67 (SD 10)	29·3 (SD 4·8)		
Shan et al. (2017)[54]	Healthy	Median 1·47 (IQR 0·81–2·20) μmol/l	1348	55 (SD 10)	23·6 (SD 3·4)	56	SIDA-MS
	T2DM	Median 1·77 (IQR 1·09–2·20) μmol/l	1346	53 (SD 10)	25·0 (SD 3·6)	57	
Al-Obaide et al. (2017)[228]	Healthy	Mean 0·18 (SEM 0·05) μg/ml	20	54 (SEM 3)	28·2 (SEM 1·1)	NA	LC/MS
	T2DM-CKD	Mean 1·52 (SEM 0·29) μg/ml	20	64 (SEM 2)	33·2 (SEM 2·9)		
Ageing							
Ke et al. (2018)[40]	Healthy (30 years of age)	Mean 2·93 (SD 2·89) μM	109	30 (SD 7)	21·8 (SD 2·8)	47	LC-MS/MS
	Advanced age (>65 years)	Mean 10·52 (SD 8·09) μM	77	72 (SD 5)	22·4 (SD 3·0)	56	
Li et al. (2018)[43]	Healthy (31 years of age)	Mean 2·85 (SD 3·1) μmol/l	168	31 (SD 7)	21·8 (SD 3·0)	48	LC-MS/MS
	Advanced age (>65 years)	Mean 9·83 (SD 10·6) μmol/l	141	71 (SD 5)	22·5 (SD 3·2)	59	
Kidney disease							
Stubbs et al. (2016)[15]	Healthy	Median 3·3 (IQR 3·1–6·0) μM	17	44 (SD 15)	29·1 (SD 6·6)	29	UHPLC-MS/MS
	ESRD	Median 94·4 (IQR 54·8–133·0) μM	25	64 (SD 10)	29·1 (SD 4·9)	76	

Table 1. (*Continued*)

Study	Subjects	Serum TMAO	Sample size	Age (years)	BMI (kg/m²)	Sex (% male)	TMAO analysis
Missailidis *et al.* (2016)[53]	Healthy (13 % CVD)	Median 5·8 (10th–90th percentile 3·1–13·3) µmol/l	80	62 (SD 12)	26 (SD 4)	71	LC-MS/MS
	Stage 3–5 CKD (28 % CVD)	Median 53·4 (10th–90th percentile 9·3–170·0) µmol/l	179	55 (SD 14)	25 (SD 5)	65	
Xu *et al.* (2017)[68]	Healthy (0 % CVD)	Mean 2·08 (SD 1·89) µmol/l	32	55 (SD 10)	NA	50	LC-MS/MS
	Stage 4–5 CKD (78 % CVD)	Mean 30·33 (SD 27·35) µmol/l	32	53 (SD 15)	NA	50	
Kaysen *et al.* (2015)[165]	Healthy	Mean 1·41 (SD 0·49) µmol/l	NA	NA	NA	NA	UPLC-MS
	ESRD (59 % diabetes, 34 % HF, 36 % atherosclerosis)	Mean 50 (SD 32) µmol/l	235	62 (SD 14)	29·7 (SD 7·3)	55	

CARDIA, Coronary Artery Risk Development in Young Adults Study; CKD, complex kidney disease; EDCA, elective diagnostic coronary angiography; ESRD, end-stage renal disease; ^{1}H-NMR, proton-NMR; IQR, interquartile range; LC, liquid chromatography; MACE, major adverse cardiovascular events; MI, myocardial infarction; MS*, multiple-stage MS; NA, not available; PCI, percutaneous coronary intervention; SIDA, stable-isotope-dilution assay; PREVEND, prevention of renal and vascular end-stage disease; T2DM, type 2 diabetes mellitus; UHPLC, ultra-high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography.
* Summary of studies in human subjects detailing TMAO concentrations in healthy and pathological conditions. Values are variably expressed as: means and standard deviations, medians and IQR, or medians and 10th percentile<->90th percentile, quartile 1 (Q1) and quartile 4 (Q4), or quintile 1 (Q1) and quintile 5 (Q5).

($\geq$90 µM in end stages; also correlating with renal dysfunction)[15,18,53,68] (Table 1). Studies in rodents evidence 2- to 3-fold higher baseline concentrations than in human subjects[8-13], though some murine studies report equivalent 1–5 µM levels[40,44,46,64]. As in human disease, plasma TMAO increases in animal models of diet-induced obesity[10,11,13], atherosclerosis[2], diabetes[64] and CKD[39], and with ageing[39,44].

A number of studies link circulating TMAO with CVD[2,14,15,53,64], suggesting that chronic elevations might mechanistically contribute to disease (Table 1). Several link TMAO elevations to major adverse cardiac events (MACE) in those with high cardiometabolic risk and a history of CVD[2,17,20], congruent with pro-atherosclerotic, vascular and myocardial effects of high TMAO in experimental models[2,7,14,20,27,47,69,70]. Similarly, TMAO concentrations in stable HF independently predict 5-year mortality when adjusted for traditional risk factors[71], and MACE either in the presence or absence of traditional CVD risks[14]. Meta-analysis supports a concentration-dependent association between TMAO and cardiovascular risk and mortality[72]. These analyses collectively evidence links between TMAO concentration and CVD risk, progression and mortality in high-risk subjects.

Other evidence suggests that such associations may be limited to high-risk cohorts with existing disease or multimorbidity[2,14,17,20,71,73]. No association was evident between TMAO, coronary artery Ca^{2+} and carotid intima-media thickness in a cohort of >800 participants aged 33–55 years old without CVD[65]. Association between TMAO and the metabolic syndrome in patients undergoing coronary angiography appears dependent on renal function, with no links between TMAO and history of infarction, or CHD and MACE over 8 years of follow-up[74]. These observations agree with lack of association between TMAO, plasma cholesterol and atherogenesis under normal dietary conditions in animal models[26]. Evidence that TMAO is more predictive of CVD and its outcomes in those with existing disease or co-morbidities such as diabetes[75] and CKD[76] indicates that TMAO may play a secondary or biomarker role in CVD. This is consistent with the experimental evidence that TMAO only influences disease processes under pathological conditions[27], without modifying vascular[8] or cardiac phenotype[10] in young healthy animals. Indeed, recent Mendelian randomisation analysis detected no relationship between genetically predicted high TMAO (or carnitine) and risk of CHD or AMI (or atrial fibrillation, stroke, type 2 diabetes mellitus or CKD), while type 2 diabetes mellitus and CKD were causally associated with elevated TMAO[77]. These diseases may thus mechanistically drive increases in TMAO, meaning that correlations with CVD reflect a reverse causality. Critically, while a number of association studies suggest markedly increased CVD risk at $\leq$5 µM TMAO[2,14,17-19], there is little evidence of pathological effects of TMAO at these concentrations.

Implied cardiovascular pathogenicity of trimethylamine *N*-oxide

The nature of reported TMAO–CVD associations raises important questions regarding causality: the TMAO concentrations

linked to major increases in CVD risk or adverse outcomes are well within ranges reported in healthy cohorts (Table 1), and orders of magnitude lower than those mediating pathological effects in experimental models[6,7,19,20]. For example, it seems implausible that the TMAO–CVD association reported by Wang et al.[2] reflects causal involvement, as it implies that a rise in TMAO from <1 to 5 μM increases CVD risk 4-fold, despite these levels falling within the range observed in healthy subjects, and no evidence of pathological responses to ≤5 μM TMAO. Similarly, the data of Tang et al.[14] implies that a modest 40 % rise in TMAO from 3·5 μM to 5 μM significantly promotes CVD; the association in Mente et al.[17] suggests that a rise from <1 to 5 μM increases risk of CVD about 9-fold; and Mafune et al.[18] link small elevations in TMAO from <3 to 4, 5 and 6 μM to increases in diseased coronaries from 0 to 1, 2 and 3 in surgical patients with CVD[18]. While association between TMAO and thrombosis risk suggests markedly increased thrombotic events with a rise in TMAO from 2 to 6 μM, evidence supports a 10–100 μM threshold for functional effects of TMAO in platelets[21]. Meta-analysis suggests a somewhat lower sensitivity of all-cause mortality, which increases about 8 % per 10 μM increment in plasma TMAO[72], and highlights an unexplained disparity between TMAO levels and outcomes: mortality exhibited an unexpected 10-fold greater dependence on TMAO in the low 1–10 μM v. higher concentration ranges. In short, were these varied associations reflective of causal involvement of TMAO as an early driver of disease, normal variance observed in TMAO concentrations across healthy populations[43,59,62,65] would be predicted to profoundly increase CVD risk and mortality. However, there is little evidence for pathological effects of ≤10 μM TMAO on inflammation, atherosclerosis or thrombosis, and no pathobiological basis for markedly higher dependence of mortality on <10 μM v. >10 μM TMAO. These findings contrast evidence of a higher TMAO threshold for cardiometabolic risk approaching 10 μM[78]. Fundamentally complicating matters, the basis of the variance in TMAO in healthy populations and of elevated concentrations with CVD risks, co-morbidities and disease itself remain unclear, constraining capacity to address the question of TMAO causal involvement.

Determinants of circulating trimethylamine N-oxide in health and disease

The question of how circulating TMAO levels are regulated and increase beyond a rather broad 'normal' range[61,79] to achieve pathological levels is fundamental to delineating the roles of TMAO in disease. Circulating TMAO, predominantly derived from bacterially generated trimethylamine (TMA) and its subsequent oxidation by hepatic FMO3, is influenced by multiple factors including age, body weight, renal clearance, metabolic and endocrine status, and diet (among others) (Fig. 2). However, these determinants only partially explain the variance in circulating TMAO in different cohorts. In subjects with dyslipidaemia, for example, TMAO concentrations correlate with age and to a limited extent BMI, yet these factors together with sex, choline concentration, renal function, and polymorphisms in FMO3 and the ABCG2 transporter may explain < 20–25 % of TMAO variance[80]. The basis of TMAO elevations with CHD risk factors

including obesity[10,11,13], insulin resistance[67,81], diabetes[54,64,75] and the metabolic syndrome[78] is similarly poorly defined, as is the basis of elevations in CHD. This knowledge gap, and the failure of current models to explain TMAO variance in health[61] and cardiometabolic disease[80], point to a critical though incompletely defined role for diet–microbiota interactions and biota remodelling[79]. Existence of sub-populations of biota-dependent high v. low TMAO producers[20,62] may also be relevant to reported links between TMAO and disease.

Dietary substrates

Excessive intake of substrates for TMA generation may contribute to elevations in circulating TMAO, influenced by gut microbiota profiles[62]. That said, evidence that major TMAO substrates protect against cardiometabolic disease[82] and that red meat intake is a weak CVD risk[83-85] appears difficult to reconcile with an important mechanistic role for TMAO in CVD. Generation of TMAO increases with intake of choline and carnitine substrates for TMA generation[86] (Fig. 1). Increased formation occurs when sufficient choline/carnitine reaches regions of bacterial TMA production – the caecum appears to have the highest TMA generation capacity in mouse models[20,87], though a recent report (albeit for a single human subject) suggests that TMA may be generated and absorbed specifically in the small intestine[88]. Such localisation, if confirmed, could increase the diet sensitivity of TMA/TMAO generation in humans v. rodents. Following gut formation and absorption, the amine is oxidised by hepatic FMO3 to TMAO (Fig. 1)[20,89,90]. Additional forms of FMO in humans (FMO1, FMO2, FMO4, FMO5) do not appear to participate significantly in TMA oxidation[91], whereas recent work indicates that FMO1 is responsible for about 10 % of TMA conversion in mice[26].

Choline. Foods rich in lecithin, such as eggs, milk, liver, red meat, poultry and seafoods, are major dietary sources of choline[2], which can be derived from fat-soluble phosphatidylcholine (the dominant source) and sphingomyelin, and water-soluble phosphocholine, glycerophosphocholine and free choline[92]. The abundant choline moiety within bile presents another potential TMA substrate[22]. Choline, which contains a trimethylammonium moiety, is directly converted to TMA by gut bacteria, or may be initially metabolised to γ-butyrobetaine before subsequent conversion[87]. There appears to be a threshold intake beyond which choline reaches the large intestine in sufficient quantities for bacterial metabolism[93,94]. Choline is absorbed from the small intestine via transport proteins that are 50 % saturated at 200–300 μM in mice[95,96]. With transporter saturation, choline can reach distal sites of bacterial conversion to TMA and dimethylamine[97], though preliminary evidence of TMA generation within the small intestine[88] suggests that transit to the large intestine may be unnecessary. While details of choline transport and metabolism to TMA and TMAO in humans remain to be clarified, consumption of two hard-boiled eggs (about 250 mg of choline each) together with a 250 mg radiolabelled phosphatidylcholine supplement transiently doubles plasma TMAO[14], similar to other studies of egg consumption[62,98] (Table 2). Consistent with transience of diet-induced TMAO changes, baseline plasma

Table 2. Effects of diet or supplementary interventions on plasma trimethylamine *N*-oxide (TMAO) concentrations*

Study	Subjects	Sample size	Sex (% male)	Intervention/design	Baseline concentration (μM)	Fed concentration (μM)
Diet intervention						
DiMarco *et al.* (2017)[63]	24·1 years, BMI 24·3 kg/m² Fasted	36	50	3 Eggs/d, 4 weeks	3·7 (SD 4·7)	3·4 (SD 5·2)
West *et al.* (2014)[93]	Lacto-ovo vegetarians, 35·7 years, BMI 23·7 kg/m² Fasted	15	0	6 Eggs/week, 8 weeks	2·3 (SD 0·3)	2·1 (SD 0·3)
Zhu *et al.* (2017)[100]	46 years, ten omnivores, eight vegetarians	8	40	About 450 mg choline/d, 2 months	2·6 (SD 0·6) (vegetarians)	28·9 (SD 5·5) (vegetarians)
		10			2·5 (SD 0·3) (omnivores)	36·8 (SD 9·4) (omnivores)
Boutagy *et al.* (2015)[229]	22·1 years, BMI 22·3 kg/m²	10	100	High-fat feeding (55 % fat), 5 d	1·6 (SD 0·2)	1·8 (SD 0·2) (2·5 (SD 0·3) 4 h postprandial *v.* 1·6 (SD 0·1) for control diet)
Single or 24 h supplement						
Tang *et al.* (2013)[14]	Healthy adults	40		2 Eggs + 250 mg phosphatidylcholine	4·5 (SD 1·1)	Peak 7·6 μM at 1 h, recovered to baseline at 4 h
Cho *et al.* (2017)[62]	27·8 years, BMI 24·2 kg/m²	40	100	Fish, beef meal/6 h	3·3 (SD 0·2) (about 20 μmol x 6 h, AUC)	Fish: about 700 (μmol x 6 h, AUC) Beef: about 21 (μmol x 6 h, AUC)
Miller *et al.* 2014[98]	42·5 years, BMI 30·7 kg/m²	6	33	6 Egg yolks/24 h	30 (SD 6) (μmol x 24 h, AUC)	142 (SD 26) (μmol x 24 h, AUC)
Dietary makeup						
Koeth *et al.* (2013)[20]	Healthy male and female	26		Vegetarian	2·1 (95 % CI 1·1, 4·2)	
		51		Omnivore	2·8 (95 % CI 1·3, 7·2)	
Rohrmann *et al.* (2016)[86]	Male 50 years, female 44 years, BMI 26·1 kg/m² male, 25·2 kg/m² female	104	100	Male	2·55 (95 % CI 2·17, 2·99)	
		167	0	Female	2·52 (95 % CI 2·22, 2·86)	
	Diet makeup based on ≥2 × 24 h dietary recalls				3·35 (95 % CI 1·88, 5·97)	
	Non-fasted	8		Vegetarian	2·51 (95 % CI 2·27, 2·78)	
		NA		Omnivore	2·5 (95 % CI 2·13, 2·93)	
				Red meat	2·37 (95 % CI 1·95, 2·89)	
		111		0 g/d		
		68		≥55 g/d	2·08 (95 % CI 1·69, 2·57)	
				Milk and dairy products	3·13 (95 % CI 2·56, 3·84)	
		68		<68·2 g/d	2·56 (95 % CI 2·25, 2·92)	
		67		>253·3 g/d	2·51 (95 % CI 2·07, 3·04)	
				Eggs	2·53 (95 % CI 2·24, 2·87)	
		161		0 g/d	2·45 (95 % CI 2·07, 2·91)	
		72		>17·1 g/d		
				Fish		
		180		−		
		91		+		

NA, not available.

* Summary of human studies examining dietary influences or effects of dietary interventions on plasma TMAO concentrations. Levels of TMAO are shown as concentration or as cumulative AUC over defined periods post-interventions, and are variably expressed as: means and standard deviations, or as means or medians and 95 % CI.

TMAO in healthy subjects is not altered with 8 weeks' consumption of six eggs per week[93]. In high-fat-fed mice, supplementation with 1·2 % (w/w) of the alternative choline source sphingomyelin also increases serum TMAO, although concentrations are relatively low and insufficient to influence atherosclerosis[99]. Summarising studies of choline feeding: high intakes 10- to 15-fold above normal can elevate TMAO in animals[6,39,76], as can dietary supplementation in humans[14,100] (Table 2); however, dietary elevations appear transient, and continuous high-level intakes may be necessary to chronically elevate TMAO to pathological levels. This may involve longer-term effects of dietary substrates on TMA-generating bacteria. Further analysis of choline's role in influencing TMAO levels and CVD is warranted, since studies indicate dietary choline may mitigate against CVD[82] or exert no effect[101,102], contrary to proposed pathogenicity of TMAO.

Carnitine. L-Carnitine is produced from lysine in eukaryotes, and is catabolised by prokaryotic organisms[103], the latter begin able to yield TMA and malic semialdehyde via cleavage of the backbone carbon–nitrogen bond of carnitine[104]. Carnitine is an essential component of fatty acid metabolism, transporting activated long-chain fatty acyl groups into the mitochondrial matrix[105]. Similar to choline, carnitine uptake from the human small intestine is not well defined and deserves further study. Mucosal carnitine uptake appears saturated with 2 g orally administered L-carnitine[106]. Saturation is also reported with 3 × 1 g doses of carnitine, significantly elevating plasma TMAO[107], although baseline concentrations of about 35 μM in this study are over an order of magnitude higher than widely reported[59]. Consumption of about 225 g of sirloin steak (about 180 mg of carnitine) transiently increases plasma TMAO concentration[20]. Prolonged daily supplementation of L-carnitine (1 g/d over more than 1 year) has been shown to increase median plasma TMAO by about 12-fold in a cohort of nine patients with mitochondrial disorders[108]. The gut microbiota may also be able to produce γ-butyrobetaine from L-carnitine metabolism[103], a metabolite that bacteria can subsequently convert to TMA[91]. Collective evidence indicates that high and chronic dietary loads of carnitine are required to elevate TMAO towards pathological concentrations (>10 μM) in the long term. Again, this may reflect substrate-driven remodelling of the gut microbiota, favouring TMAO generation (see below). Importantly, while carnitine intake increases TMAO concentrations, it reduces the risk of CVD and metabolic disorders[109], protecting against diabetes[110] and the metabolic syndrome[111]. Meta-analysis indicates that carnitine reduces all-cause mortality, ventricular arrhythmias and angina symptoms in infarct patients[112]. As for choline and seafood, the proposed pathogenic role of TMAO awaits reconciliation with these observations.

Betaine. Trimethylglycine, commonly known as betaine, provides an additional source of TMA, thus TMAO. Betaine functions predominantly as a methyl donor via the methionine cycle[113], and as an osmolyte[114]. The former function reduces the dietary methionine and choline required for optimal nutrition[113]. Betaine is mainly acquired through the consumption of betaine-rich foods such as wheat bran, wheat germ and spinach[115],

though irreversible oxidation of choline to betaine within the host[116] and gut[117] represents another source. Certain gut bacteria can also convert L-carnitine into betaine[104]. Betaine itself can be metabolised to TMA via multiple paths: initial conversion to choline and carnitine, as already noted; reduction to form TMA and acetate via a Stickland-type redox reaction[118]; or initial demethylation to dimethylglycine, which undergoes decarboxylation to form TMA[119]. In terms of relationships with disease, betaine supplementation has a moderate effect on total plasma cholesterol, but not LDL, HDL or TAG[120], and high plasma betaine appears to have no effect on CVD risk[101,121] while protecting against type 2 diabetes mellitus[122].

Ergothioneine. Histidine-derived ergothioneine represents another dietary precursor for TMA[123]. It appears ubiquitous across cells and tissues[124], though is primarily concentrated in the liver, kidney, bone marrow and erythrocytes[125]. Ergothioneine is not synthesised in animals, but is readily derived from an array of foods including mushrooms (where it is synthesised), black and red beans, oat bran and liver or kidney[126]. Cellular uptake occurs via organic cation transporter 1[127], and it may have antioxidant roles within cells[124,128]. The ergothionase enzyme in some gut bacteria can catalyse degradation of ergothioneine to form TMA[123]. To date, no studies have investigated the biological relevance of ergothioneine-dependent TMA/TMAO generation, or associations with CVD. Potential antioxidant effects of ergothioneine itself are counter to pro-oxidant and inflammatory effects of its downstream product TMAO. In terms of dietary ergothioneine sources, there is no evidence of a relationship between mushroom intake and CVD risk[129], while there is support for increased CVD mortality *v.* decreased diabetes risk and cancer mortality with increased legume intake[130,131], though the basis of these distinct disease outcomes is unknown.

Seafood. TMAO can be absorbed without gut and hepatic processing following the consumption of seafood, with plasma levels increasing within 15 min[62,107]. Consumption of a 1·67 g dose of TMAO results in about 50 % of the molecule being absorbed unchanged[132], while the remainder is believed to be reduced via bacterial TMAO reductase to form TMA which is subsequently absorbed and re-oxidised to TMAO[132]. Fish are rich in TMAO, owing to physiological roles in buoyancy and osmotic control[133,134], with the free TMAO content of seafood several-fold higher than choline/carnitine contents of red meat and eggs[133]. Populations consuming more fish exhibit high urinary TMAO concentrations[61,135,136], and analysis of diverse foods initially revealed that only seafood substantially elevated TMAO generation and excretion in humans (>50–100-fold higher than red meat or eggs)[132]. Others confirm that eggs and red meat have relatively minor effects on postprandial plasma TMAO compared with seafood (whereas fruits reduce concentrations)[62]. More recently, Schmedes *et al.*[137] showed that a seafood meal significantly increases postprandial TMAO while a non-seafood meal does not. These potent effects of seafood intake on TMAO also await reconciliation with proposed roles of TMAO in CVD and well-established protection with seafood.

Hepatic flavin-containing mono-oxygenase 3

Circulating TMAO concentrations are dependent on TMA oxidation by hepatic FMO3, and correlate with FMO3 expression in mice and human subjects[2].The *N*-oxygenation of TMA via FMO3 proceeds with a K_m of 28 μM and a k_{cat} (substrate to end-product conversions per unit time) of >30/min, based on analysis of heterologously expressed FMO *in vitro*[89]. Thus, rapid conversion to TMAO will occur in a substrate-dependent manner in healthy individuals (TMA about 20–25 μM)[51]. There is evidence that hepatic FMO3 expression or activity is sensitive to sex steroids[28,138,139], dietary factors[140] and energy intake[141,142], NO signalling[143] and the transcriptional regulator CCAAT/enhancer-binding protein β[144]. Hepatic FMO3 expression also increases postnatally in humans, with an apparent plateau after early adulthood[145,146]. Conversely, in female mice hepatic FMO3 transcript may decline following this phase of maturational up-regulation[147]. While there is some debate regarding hepatic FMO expression in male rodents, an ageing-dependent increase in FMO3 protein is reported in male mice[44,141,142]. Sex is a particularly strong determinant of FMO3 expression patterns, a linkage not strictly consistent with involvement of TMAO in CVD risk: hepatic FMO3 is substantially higher in female *v.* male mice and human subjects[28], paralleled by dimorphism in TMAO concentrations in mice[8,28,40] and to a lesser extent humans[148], yet females enjoy protection against CVD over much of the lifespan. Pregnancy further up-regulates FMO3[149], and trimethylaminuria (a disorder in which TMA conversion to TMAO is impaired, leading to odour in sweat, urine and breath from rising TMA) is also exacerbated in women during menstruation[149]. Circadian patterns of FMO3 expression may also differ markedly between sexes, a dimorphism reduced by cardioprotective energy restriction which up-regulates FMO3 in males, 'feminising' the transcriptional profile[141,142]. However, there remains some debate regarding hepatic expression of FMO3 in male mice: there is evidence that FMO3 mRNA is below detection limits[147] or detectable at 0·1–10 % of the levels in females[141,142]; and that FMO3 protein is not detectable in males[9,28], or lowly expressed and augmented with adenoviral delivery, energy restriction or ageing[44,141,142]. These observations raise questions regarding the utility of male murine models in assessing the role of FMO3 and TMAO in human disease.

Expression of FMO3 is modified by chronic disease and CVD risk factors, including renal dysfunction and disease[150], inflammation[151], insulin resistance and diabetes[2,14,54,55], ageing[44,141,142], pollutants and hepatic toxins[152]. Elevations in TMAO itself may up-regulate FMO3 expression, with evidence of up to 5-fold induction in male mice supplemented with TMAO[40]. Though untested (and questions remain regarding hepatic FMO3 in male mice), this suggests that a potentially detrimental positive feedback could emerge in disease settings associated with significantly elevated TMAO. Kidney dysfunction and disease not only reduce TMAO excretion but increase FMO3 expression in mice[150], further promoting elevations in TMAO. Expression of FMO3 is also increased with insulin resistance in both mice and humans[2,14,54,55], providing a basis for TMAO elevations in diabetes and a link between TMAO and CVD[2,14,54,55]. Plasma TMAO in CHD patients undergoing coronary intervention can be independently linked to diabetes, together with age and BMI[64]. Whether FMO3-dependent elevations in TMAO might contribute to diabetes remains to be established, though FMO3 knockdown reduces hyperglycaemia in a murine model of hepatic insulin resistance[55], and circulating TMAO is predictive of insulin resistance with obesogenic feeding in macaques[81]. On the other hand, Liao *et al.*[153] report beneficial effects of FMO3 on glucose homeostasis independent of the insulin-signalling pathway. Furthermore: hepatic TMAO concentrations decline and renal concentrations are unaltered in the *db/db* mouse model of diabetes[154]; a low-glycaemic index diet increases TMAO[155] despite known benefit in obesity, diabetes and on CVD risk[156]; and metformin increases TMAO levels while reducing blood glucose in type 2 diabetic patients[157]. Disruption of glycaemic control and emergence of the metabolic syndrome in mice is also independent of TMAO levels and insensitive to inhibition of TMA generation[22]. Interestingly, anti-diabetic and cardioprotective resveratrol up-regulates FMO3 yet reduces TMAO levels via microbiota remodelling[9,28], an effect associated with reduced atherosclerosis in ApoE$^{-/-}$ mice[9]. Conversely, recent work indicates that resveratrol does not influence plasma TMAO in mice on a choline-supplemented (1 % w/w) diet[22].

Importantly, links between FMO3, TMAO and disease development are clouded by TMAO-independent effects of the enzyme. Knockdown of FMO3 improves cholesterol balance while augmenting hepatic stress and inflammation in cholesterol-fed mice independently of TMAO levels and TMA generation[158]. Metabolic effects of FMO3 manipulation in LDL receptor knockout mice are TMA/TMAO independent[28,159]. Furthermore, while FMO3-dependent elevations in TMAO are suggested to promote tissue ageing[8,40,43], and FMO3 expression may rise with age[44], recent evidence indicates that increased FMO3 expression is protective, exerting anti-ageing effects that mimic those of energy restriction[142]. Thus, not only does FMO3 exert beneficial effects, but linkages between FMO3 and disease do not necessarily reflect a role for TMAO.

Cellular transport and renal excretion

Renal function is a key determinant of circulating TMAO, contributing to elevations in inter-related CKD[15,18,53,68], CVD[2,18,60], diabetes[64] and ageing[8,40,53]. The cellular influx of TMAO may occur predominantly via organic cation transporter 2, which facilitates renal tubular TMAO uptake[80]. Efflux occurs via ATP-binding cassette family transporters, with genetic variation in the ABCG2 transporter potentially contributing to altered TMAO concentrations in dyslipidaemic subjects[80]. The *ABCG2* gene has oestrogen and hypoxia response elements, is down-regulated by parathyroid hormone and up-regulated by aryl hydrocarbon receptors and DNA methylation, and polymorphisms influence renal excretion[160,161].

In healthy volunteers with normal kidney function, fractional renal clearance of TMAO exhibits a broad dynamic range[79], while patients with renal dysfunction exhibit significantly elevated plasma TMAO as a consequence of functional deficit[15,53,162,163]. Elevations in TMAO in CKD and HF involve changes in glomerular filtration rate and renal function[53], with

renal medullary damage with hypoperfusion additionally increasing TMAO[164]. Recent findings suggest that chronic ingestion of red meat may reduce excretion of TMAO[79]. Though speculative, detrimental feedback may also emerge at the level of kidney function: as for uraemic toxins in the cardio-renal syndrome, increases in circulating TMAO due to renal hypoperfusion/injury with atherosclerosis, CHD and HF may surpass a pathological threshold to exacerbate vascular[8,11] and myocardial dysfunction[47,48], renal inflammation, fibrosis and dysfunction[8,11,76] (Fig. 2). Though this remains to be directly tested, elevations in TMAO predict mortality in CKD[53,76], and mimicking TMAO concentrations in advanced CKD and HF (80–100 μM) with either choline (1·0 % total) or TMAO (0·12 %) supplementation promotes renal fibrosis and injury in mice[76]. On the other hand, others report no relationship between TMAO and mortality or cardiovascular outcomes in end-stage renal disease[165].

The gut microbiota and human trimethylamine N-oxide concentrations

Up to about 75 % of the variance in plasma TMAO in healthy humans appears unexplained, with meat and fish consumption estimated to account for <15 % and renal function <5 % of variance[61]. Age is an important factor, with substantial age-dependent elevations in TMAO in animal models and humans[8,40,43]. Curiously, diet has been estimated to be a surprisingly weak determinant of the variance in TMAO in healthy humans[61,80], and analysis of the heritability of high TMAO concentrations indicates that host genetics also plays a minor role[166], consistent with failure of gene association analysis to identify TMAO-linked loci[167]. This poor understanding of the control of TMAO levels reflects in part our incomplete (though evolving) understanding of the roles and control of gut bacterial populations in determining TMAO concentrations. Since circulating TMAO is critically dependent on gut bacteria, elevations may reflect microbiota remodelling with diet and disease. The microbiota of vegans has minimal TMAO-generation capacity[20], and Cho *et al*.[62] present evidence that distinct bacterial profiles govern TMAO generation in healthy male omnivores, identifying 'high' *v*. 'low' producers (42 and 58 % of volunteers, respectively). A substantial proportion of the population may thus, owing to specific microbiota profiles, be specifically susceptible to diet-dependent elevations in TMAO. Apparently paradoxical benefits of TMAO substrate intake (choline, carnitine, seafood) on cardiovascular health may also reflect dominant influences of microbiota makeup and remodelling.

The fundamental importance of the microbiota is evidenced by absence of urinary TMA in germ-free mice, and its reduction with antibiotic treatment in conventional mice[168]. Effects of antibiotics on carnitine-dependent elevations in TMAO confirm microbiota dependence in humans: in female omnivores, increases in plasma TMAO with carnitine intake are abolished by antibiotics and restored after 3 weeks' recovery for microbiota re-population[20]. Subsequent investigations confirm the essential role of the microbiota in humans[2,14], and differences in TMAO generation in vegans *v*. non-vegans further emphasise the microbiota's importance: carnitine challenge increases

plasma and urinary TMAO in omnivores but not vegans, a dimorphism eliminated by antibiotic treatment[20]. This suggests that intake of animal protein favours carnitine/choline-metabolising bacteria[20,169], while a non-meat diet low in these substrates and relatively enriched with betaine (a less favourable TMA substrate[170,171]) promotes non-TMA-generating species. Recent identification of distinct bacterial profiles in high and low TMAO producers (high and low responses to dietary substrate)[62], and effects of red *v*. white meat on bacterial carnitine *v*. choline metabolism[79], further confirm the importance of microbiota changes in driving TMAO elevations in humans.

Fennema *et al*.[91] provide a comprehensive review and summary detailing bacterial species involved in the formation of TMA. Bacteria including *Prevotella*, *Deferribacteres* and *Tenericutes* species can metabolise choline and carnitine to TMA, while *Bacteroides* appears less effective than *Prevotella*[20,69]. A recent cross-sectional analysis of 1653 multi-ethnic participants linked circulating TMAO to the abundance of thirteen genera (six Firmicutes, three Bacteroidetes, three Protobacteria, one Fusobacteria), including *Prevotella*, *Mitsuokella*, *Fusobacterium*, *Desulfovibrio*, *Bilophila* and Ruminococcaceae and Lachnospiraceae family members[67]. Studies indicate that omnivores possess more *Firmicutes* than *Bacteroidetes* species and a less diverse microbiota compared with vegans/vegetarians, who also have relatively lower *Clostridium* species[62,172,173]. In general, meat intake appears to promote *Bacteroides*, *Alistipes*, *Ruminococcus*, *Clostridia* and *Bilophila*, while decreasing *Bifidobacterium* species[174]. Other work links a vegan diet to decreased *Clostridium v*. enrichment of *Anaerostipes caccae* and *Lachnobacterium* species in subjects with the metabolic syndrome[175]. Although a majority of TMA production occurs within the gut, it is also worth noting that formation can also occur in the mouth via *Streptococcus sanguis* metabolism of choline[176]. Recent studies identify three biochemical paths for bacterial TMA production, involving the genes *cutC*[177], *cntA*[178] and *YeaW*[87]. Genomic assessment of TMA production potential in human bacteria, based on these pathways, identifies *Firmicutes*, *Proteobacteria* and *Actinobacteria* species, whereas these paths appear absent in *Bacteroidetes*[179,180]. Interestingly, in terms of observed increases in circulating TMAO with ageing, Brunt *et al*.[44] report select ageing-dependent changes in mouse gut phyla (increased Proteobacteria, Verrucomicrobia, and candidate division TM7) and genera (increased *Bacteroides*, *Akkermansia* and pro-inflammatory, TMA-generating *Desulfovibrio*; and reductions in several *Firmicutes*).

Interactions between diet, microbiota makeup and TMAO generation remain to be better detailed in humans, with findings somewhat contradictory. For example, some studies indicate that vegetarian (but not vegan) diets can increase relative proportions of *Prevotella*, known to produce TMA[181,182]. One study found that plasma TMAO did not differ significantly between vegans and lacto-ovo-vegetarians[183], though the period the groups adhered to these diets was not provided. Recent evidence indicates that red *v*. white meat may selectively increase bacterial metabolism of carnitine (not choline) to TMA, while reducing renal TMAO excretion[79]. Further studies are needed to clarify roles of microbiota constituents in governing TMA

and thus TMAO production, and the influences of animal- and plant-derived nutrients. It is important that broader biochemical influences of TMA-generating bacteria are also detailed to better understand how the gut influences cardiovascular health – additional co-modified factors may contribute to or influence associations between TMAO and CVD. This is critical in addressing the key question of whether elevated TMAO is a biomarker of microbiota changes secondary to different CVD risk factors and co-morbidities, or is a causal factor contributing to CVD development.

Trimethylamine *N*-oxide: early risk biomarker and target in CVD?

Though many aspects of the regulation of TMAO accumulation and its pathogenicity remain to be resolved, assessment of evidence reviewed here leads us to speculate that modest elevations in TMAO (<10–20 μm) are unlikely to be independently pathogenic, though may serve as a biomarker of collective CVD risk with diverse factors (ageing, inactivity, hyperenergetic diet/obesity, dyslipidaemia, atherosclerosis, insulin resistance, diabetes). Each of these factors moderately elevates TMAO; however, below a 10–20 μm threshold TMAO is unlikely to mechanistically participate in CVD development. Apparent dependence of CVD risk or outcomes on these low TMAO concentrations in association studies[132,137] is thus indirect, reflecting collective influences of multiple CVD risk factors and also protectants (for example, exercise, vegan diet), influenced by diet–microbiota interactions (Fig. 4). Targeting TMAO within this range, without attention to the underlying causal factors, may be of limited if any therapeutic value. On the other hand, elevations beyond a 10–20 μm threshold as a consequence of individual or co-morbid diseases (HF, CHD/AMI, CKD) may play an important secondary role, promoting disease progression and sequelae (Fig. 2 and Fig. 4). This is congruent with evidence that TMAO–CVD associations reflect a reverse causality (TMAO changes resulting from disease, for example, CKD or type 2 diabetes mellitus)[77]; the restriction of TMAO–CVD associations to complex cohorts with multiple risk factors[60,71,75,76,184], and evidence that such associations require one or more extant disorders[8,39]; and select cardiovascular effects of inhibition of TMAO production in aged/diseased yet not young and otherwise healthy animals[8,39,45]. We thus suggest, as outlined in Fig. 4, that apparent associations between TMAO and CVD involve two phases: an early indirect phase (TMAO variance a biomarker of the balance of CVD risk factors/protectants), and a later mechanistic phase where disease-dependent elevations in TMAO may secondarily participate in cardiovascular disruption, potentially in a positive feedback manner (as speculated in Fig. 2). Nonetheless, even secondary mechanistic involvement of TMAO in CVD requires further study and confirmation.

Caveats to this interpretation include unknowns regarding the importance of the chronicity and temporal patterns of TMAO change, and whether TMAO may interact positively (additively, synergistically) with other risk factors to facilitate dysfunction and disease. While data are lacking, sustained low-grade elevations in TMAO could exert pathogenic effects
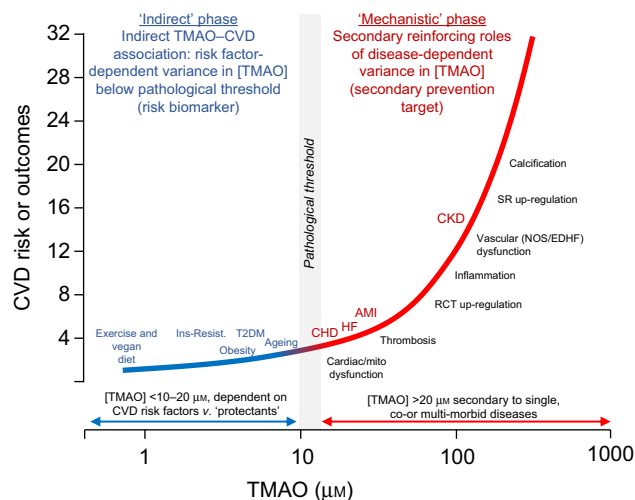
Fig. 4. Speculated linkages between trimethylamine-*N*-oxide (TMAO) and CVD. Whether associations between circulating TMAO and CVD risks/outcomes reflect a causal role in disease remains unclear. Consideration of available evidence suggests indirect associations with CVD: below 10–20 μm ('indirect' phase), variance in TMAO reflects stimulatory influences of well-established CVD risk factors (for example, ageing, inactivity, obesity, insulin resistance, diabetes) and inhibitory influences of cardioprotectants (physical activity, vegan diets). These concentrations are insufficient to influence CVD, though may be of value as a measure of composite CVD risk. Elevations beyond this range (the 'mechanistic' phase) are only achieved with disease (for example, chronic kidney disease, heart failure), acute myocardial infarction in CHD patients, or co-morbid conditions (for example, type 2 diabetes mellitus (T2DM) + CHD, cardiorenal syndrome). These disease-dependent elevations in TMAO may participate in reinforcing disease development (in a potentially positive feedback manner, as speculated in Fig. 2), though this awaits confirmation. Relative effects of risk factors/disease on TMAO concentration ([TMAO]) are shown, together with approximate concentrations for TMAO-dependent pathological effects. Note the illustrative 'CVD risk or outcomes' *y*-axis range is somewhat arbitrary. EDHF, endothelium-derived hyperpolarising factor; Ins-Resist., insulin resistance; mito, mitochondrial; NOS, nitric oxide synthase; RCT, reverse cholesterol transport; SR, scavenger receptor.

at concentrations below thresholds for acute effects, and/or in the presence of other pro-disease factors, such as low-grade inflammation. The latter is consistent with reports that TMAO selectively exaggerates oxidised LDL-dependent but not baseline CD36 expression[27], and that inhibition of TMAO formation selectively inhibits inflammation, oxidative stress or cardiovascular function in aged/diseased but not young/healthy animals[6,8]. In terms of temporal patterns, one might also speculate that transient elevations or spikes in circulating TMAO concentration could be beneficial via hormesis effects, with this transient 'stressor' augmenting resistance to injury/stress. However, chronic low-grade elevations can exhaust such adaptive homeostasis, promoting ageing and chronic disease[185].

Manipulating trimethylamine N-oxide in CVD

As discussed above, modulation of TMAO accumulation might be beneficial in limiting disease progression and impacts in those with existing CVD, or at particularly high risk (including high TMAO producers[62]). Reductions in TMAO in CVD might be achievable via modulation of both diet and the gut microbiota, though unknowns and challenges arise with each approach. Given the importance of the gut microbiota in determining

TMAO production[2,14,20,62], its manipulation is an obvious candidate for lowering TMAO in those with CVD. Unfortunately, this is not presently feasible, as what constitutes a healthy microbiota and the roles of microbiota composition in governing TMAO concentrations are not fully understood, and our ability to selectively remodel the gut microbiota (suppressing/promoting individual species or their functionality) is not currently feasible. Antibiotics suppress TMAO production and TMAO-dependent atherosclerosis[20,87]; however, this 'shotgun' approach is not viable given broad impacts on microbiota and host health, immunity and antibiotic resistance[186]. Ongoing investigations into bacterial control of TMAO generation may reveal strategies for manipulating the biota genetically, pharmacologically or via other means. For example, a recent study shows that halogen-substituted choline analogues alter the caecal microbiota and suppress elevations in TMA, TMAO and thrombosis associated with choline supplementation[22]. Many drugs can modify the gut microbiota[187,188], and a recent study indicates a quarter of 1000 drugs examined possess antibiotic-like side effects[189]. While this broadly supports the potential for pharmacological manipulation of the microbiota, the challenge of specificity (and untoward side effects) remains. This hurdle might be overcome with our evolving understanding of bacterial TMAO production (for example, identification of biochemical paths, and gene determinants such as *cutC*[177], *cntA*[178] and *YeaW*[87]), revealing molecular targets for microbiota-directed genetic or pharmacological therapy.

Probiotics offer a low-cost and -risk approach to microbiota manipulation; however, findings are equivocal, particularly in a CVD context. Supplementation with *Lactobacillus casei* Shirota for >3 months (19·5 × 10^9 colony-forming units/d) failed to influence TMAO production in patients with the metabolic syndrome[190], and 3-month probiotic supplementation in haemodialysis patients (9 × 10^{13} colony-forming units/d, including *Streptococcus thermophilus*, *Lactobacillus acidophilus* and *Bifidobacteria longum*) failed to influence plasma TMAO[191]. Meta-analyses support some cardiovascular benefits of probiotics, though outcomes are modest[192-194]. Unexpectedly, a small double-blind randomised controlled trial of faecal microbiota transplantation from vegans into metabolic syndrome patients successfully modified intestinal microbiota composition yet failed to alter TMAO production[175]. It was unclear what period of time donors adhered to vegan diets, thus whether a vegan microbiota phenotype was established, although shifts between meat and vegetarian diets induce rapid microbiota changes[195]. While FMO3 inhibition might also appear to be a target for manipulating TMAO in patients with CVD, sufficiently effective reductions in FMO3 lead to TMA accumulation and trimethylaminuria[196]. More fundamentally, FMO3 mediates diverse effects on metabolism, tissue stress and ageing independently of TMA/TMAO[142,158].

Dietary modification. Despite evidence that some cardioprotective diets and foods have either no effect[197] or increase circulating TMAO levels[132,137], dietary targeting of TMAO may nonetheless be of value in those with CVD. Limiting the intake of foods containing TMA precursors while increasing those favouring non-TMA-producing bacteria (for example,

vegetables/fruits) or suppressing FMO3 activity (for example, indole-containing vegetables) may offer the simplest approach to reducing TMAO. Although some estimate that dietary factors are relatively weak determinants of fasting TMAO in omnivores[61,80], recent work supports up to 3-fold differences in circulating TMAO with red meat- *v.* white meat-enriched (or non-meat) diets[79]. Circulating TMAO is also reportedly 2-fold higher in omnivores *v.* vegans[20], though such differences are not always observed[86] and warrant further study. Carnitine itself can be specifically reduced or omitted from diets; however, despite favouring TMAO accumulation, carnitine intake reduces risk of CVD and metabolic disorders[109]. Meta-analysis indicates that carnitine also reduces all-cause mortality, ventricular arrhythmias and angina symptoms in infarct patients[112]. Carnitine additionally induces benefits that may counter the effects of TMAO; maintaining the fermentation capacity of colonic microbiota, protecting against microbiota stressors and promoting metabolism of SCFA in association with reduced CVD risk[198].

Since choline is an essential nutrient[199] it may be reduced but not eliminated from the diet. However, dietary choline has been shown to have no effect[101,102] or mitigate against CVD[82], though there is also some evidence that choline may increase (whereas betaine reduces) cardiometabolic risk[73]. Studies also link choline to CVD[121] and MACE[170] in those at high risk or with a history of CVD, and 2-month choline supplementation (about 450 mg total choline/d, equivalent to an additional 80 % of recommended intake) reportedly enhances ADP-dependent platelet aggregation in association with more than a 10-fold rise in TMAO[100]. Dietary sphingomyelin, which contains a choline head group, could also be reduced: it is believed to be pro-atherogenic[200,201], and high circulating concentrations are linked to CVD risk[200,202]. However, recent findings indicate that long-term (16 weeks) sphingomyelin supplementation (1·2 % w/w) does not influence serum TMAO or atherosclerosis in ApoE$^{-/-}$ mice, and reduces lesion development in high-fat-fed animals[99]. A better understanding of choline and carnitine handling, their interactions with the gut biota, and their contributions to elevated TMA/TMAO generation is necessary in informing the manipulation of TMAO in CVD.

Shifting from an omnivorous to vegetarian diet is predicted to moderately lower baseline and postprandial TMAO concentrations[20], via changes in substrate and remodelling of the microbiota. Microbiota changes with animal- *v.* plant-based diets are evident in as little as 5 d[195], and increased fruit and vegetable intake is strongly linked to protection against CVD[203-207]. Trials confirm that plant-based diets reduce disease progression, angina and mortality in CVD patients[208]. Nonetheless, there is limited evidence to suggest that the cardioprotective effects of vegetarian diets[205,206,209-211] stem from reductions in TMAO. Relatively few investigations detail the specific impact of red meat intake on TMAO levels, an effect much less pronounced than that for seafood intake[20,61,62] which reduces CVD risk[212]. Though yet to be established, long-term patterns of high animal protein consumption could exacerbate risk factors or disease-dependent elevations in TMAO, through shifts in gut bacteria, FMO3 activity and renal function[79] (for example, tubulointerstitial injury with increased dietary acid load[213,214]), promoting age-dependent disorders (cardiovascular and renal

dysfunction, diabetes) that in turn favour further TMAO elevations (Fig. 2). Such effects might be compounded by parallel influences of metabolic state, BMI and age itself on TMAO levels. That said, although a red meat-rich diet does increase plasma TMAO[79], it is a rather weak risk for CVD[83-85], contrasting its strong association with other chronic diseases and the high risks attributed to small changes in TMAO[2,17,20,71,170]. Processed meats, on the other hand, strongly promote CVD[215], yet studies demonstrating links to TMAO are lacking. Importantly, the basis of cardioprotection with a vegetarian diet or high vegetable/fruit intake is complex, and may be accounted for by non-protein components[216], reductions in cholesterol and saturated fats, differing amino acid contents, and availability of dietary fibre and bioactive constituents such as isoflavones and polyphenols that counter disease processes[217,218]. Furthermore, reducing animal protein intake (a coarse surrogate for TMAO potential) has very modest effects on CVD risk and outcomes[2,17,20,71,83-85,170] and benefit is difficult to attribute specifically to protein source[218]. Other studies indicate that high protein intake reduces cardiometabolic risk, BMI and blood pressure, effects more pronounced for plant yet still evident with animal protein[219,220]. There is also evidence that the link between high animal protein intake and mortality requires co-existence of one or more additional risk factors[221], consistent with evidence that links between TMAO and disease require the existence of one or more chronic disorders[8,39]. Finally, as seafood is cardioprotective and recommended for CVD risk reduction, omission from diets may be detrimental. Well-established protection with seafood is itself difficult to reconcile with an important role for TMAO as a diet-related risk factor, since seafood increases TMAO[61,135,136] well beyond levels achieved with other foods[132,137]. Although a recent experimental study suggests that fish intake might promote atherosclerosis in rodents[222], this controversial finding contrasts anti-atherosclerotic and cardioprotective effects of seafood in humans and awaits confirmation.

Specific foods do have potential to modulate TMAO: grapefruit juice and indole-containing vegetables can decrease FMO3 activity and alter TMAO metabolism[223]. Recently, grape pomace polyphenol supplementation has been shown to decrease serum TMAO in a cohort of healthy[224] and overweight/obese patients[225]. Whereas Brussels sprouts have the highest choline content among vegetables, increased intake for 3 weeks significantly decreases circulating TMAO, an effect attributed to decreased FMO3 activity without generating trimethylaminuria[140,226]. Other foods associated with a cardioprotective Mediterranean diet appear to be significant sources of endogenous DMB, including some balsamic vinegars, red wines, cold-pressed extra-virgin olive oils and grape seed oils, with levels as high as 25 m$_M$[23].

Conclusions and future directions

CVD remains the leading cause of morbidity and mortality globally, placing an enormous burden on health systems, economies and the individuals directly and indirectly affected. A proposed role for the microbiota-dependent amine TMAO as a new and modifiable determinant of CVD has thus generated much excitement. However, much remains to be clarified regarding the control of TMAO concentrations and its potential involvement in disease. Variations in human TMAO concentrations remain largely unexplained, and whether pathologically relevant elevations arise independently of other disorders is unclear. Although increased concentrations of TMAO can promote inflammation, atherosclerosis, vascular and cardiac dysfunction and remodelling, levels inducing these effects may only be achieved in HF or CKD, or potentially CHD with co-morbid conditions (or AMI). In these select settings TMAO could play a secondary reinforcing role (Fig. 2 and Fig. 4), though even this mechanistic contribution awaits confirmation. A mechanistic role for TMAO in the development of CVD also requires reconciliation with the protective effects of its dietary precursors (particularly seafood and carnitine), and the low CVD risk associated with red meat intake. Future studies should more directly test the mechanistic relevance of TMAO in CVD, clarify the effects of chronic low-grade changes in TMAO, and test whether speculative positive feedbacks (as outlined in Fig. 2) might lead to progressive elevations in TMAO and dysfunction in CVD. This model is untested, though informed by knowledge that putative effects of TMAO (for example, inflammation, renal dysfunction and hypoperfusion) can further enhance TMAO accumulation, and observations that TMAO and renal dysfunction may up-regulate FMO3[40], for example. Importantly, even a secondary reinforcing role supports both the utility of TMAO as a biomarker of CVD risk, and as a therapeutic target in high-risk subjects with multiple co-morbidities or extant CVD. How to specifically reduce TMAO without potentially detrimental effects nonetheless poses a challenge. Enhanced understanding of the specific roles of bacteria in governing TMAO concentrations and how they respond to dietary modulation, together with factors influencing FMO3 activity and other determinants of TMAO concentration, is necessary before potential benefits of TMAO manipulation might be realised in select disease settings.

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