

Trimethylamine-N-Oxide: Heart of the microbiota-cardiovascular disease nexus?

Saba Naghipour, Amanda J. Cox, Jason N. Peart, Eugene F. Du Toit, John P. Headrick*

School of Medical Science, Griffith University, Southport QLD, Australia

Short Title: *TMAO and Cardiovascular Disease*

Email Addresses:

Saba Naghipour, saba.naghipour@griffithuni.edu.au

Amanda J. Cox, a.cox@griffith.edu.au

Jason N. Peart, j.peart@griffith.edu.au

Eugene F. Du Toit, j.dutoit@griffith.edu.au

John P. Headrick, j.headrick@griffith.edu.au

***Corresponding Author:**

Prof. John Headrick
School of Medical Science,
Griffith University, Southport 4217 QLD
AUSTRALIA

Phone: +61 7 5552 8292

Fax: +61 7 5552 8802

Email: j.headrick@griffith.edu.au

This peer-reviewed article has been accepted for publication but not yet copyedited or typeset, and so may be subject to change during the production process. The article is considered published and may be cited using its DOI.

10.1017/S0954422420000177

Nutrition Research Reviews is published by Cambridge University Press on behalf of The Nutrition Society

ABSTRACT

We critically review potential involvement of trimethylamine-N-oxide (TMAO) as a link between diet, the gut microbiota and cardiovascular disease (CVD). Generated primarily from dietary choline and carnitine by gut bacteria and hepatic flavin monooxygenase (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 coronary heart disease (CHD) is similarly ill-defined, although gut microbiota profiles/remodelling appear critical. Elevated TMAO could promote CVD via inflammation, oxidative stress, scavenger receptor (SR) up-regulation, reverse cholesterol transport (RCT) inhibition, and cardiovascular dysfunction. However, concentrations influencing inflammation, SRs and RCT ($\geq 100 \mu\text{M}$) are only achieved in advanced heart failure (HF) or chronic kidney disease (CKD), and greatly exceed pathogenicity of $<1\text{--}5 \mu\text{M}$ levels implied in some TMAO-CVD associations. There is also evidence 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 modest elevations in TMAO ($\leq 10 \mu\text{M}$) are a non-pathogenic consequence of diverse risk factors (aging, 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; Cardiovascular disease; Carnitine; Choline; Coronary heart disease; Gut microbiota; Heart failure; Omnivore; Vascular dysfunction; Vegan; Vegetarian

INTRODUCTION

Cardiovascular disease, 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 socioeconomic 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 TMAO potentially participating in these linkages ^[2] (**Figure 1**). Indeed, evidence dietary lipids are not a major CVD risk factor ^[3] encourages investigation of such alternate diet-linked drivers of disease.

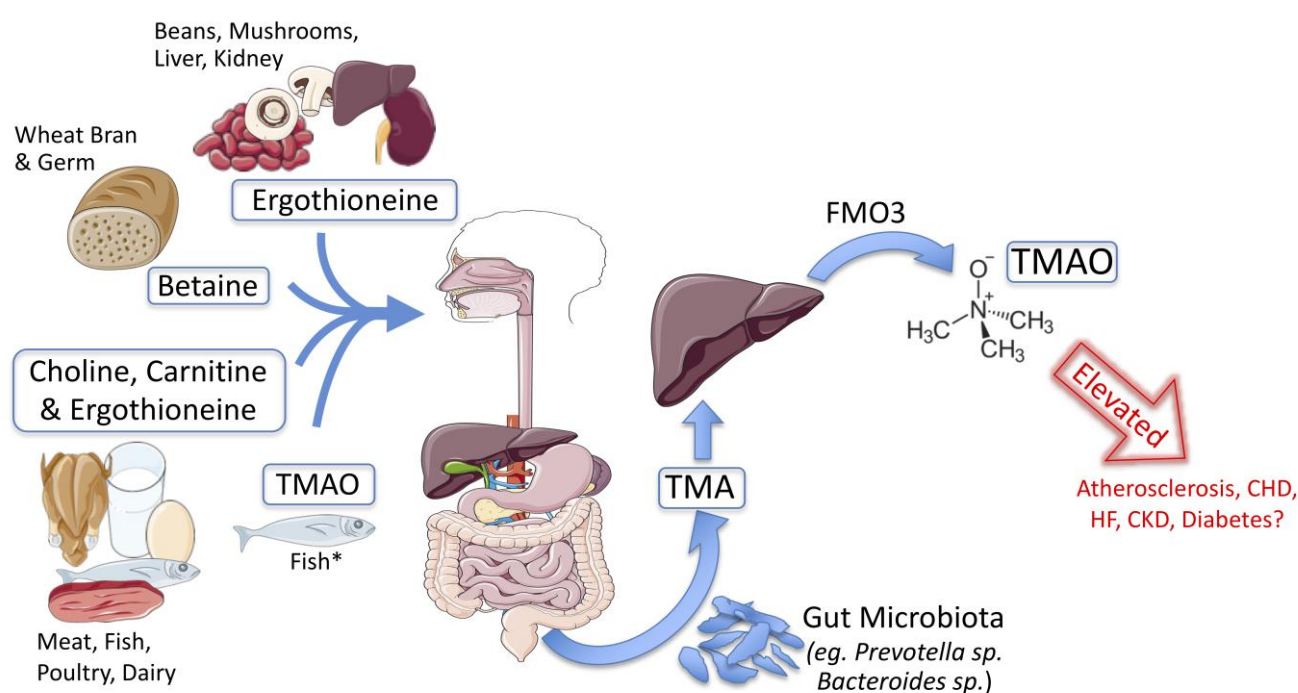


Figure 1. TMAO formation. Process of TMAO intake or generation from dietary sources.

Substantial elevations in circulating TMAO are hypothesised to promote cardiometabolic and renal diseases. *Fish represent a significant and direct dietary source of TMAO. *Abbreviations:* CHD, coronary heart disease; CKD, chronic kidney disease; FMO3, flavin-containing monooxygenase enzyme 3; HF, heart failure; TMA, trimethylamine; TMAO, trimethylamine-N-oxide.

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 focussed attention on TMAO as a potential determinant of disease. This may provide both a circulating biomarker of CVD risk, and a potential therapeutic target (**Figure 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 TMAOs role 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?

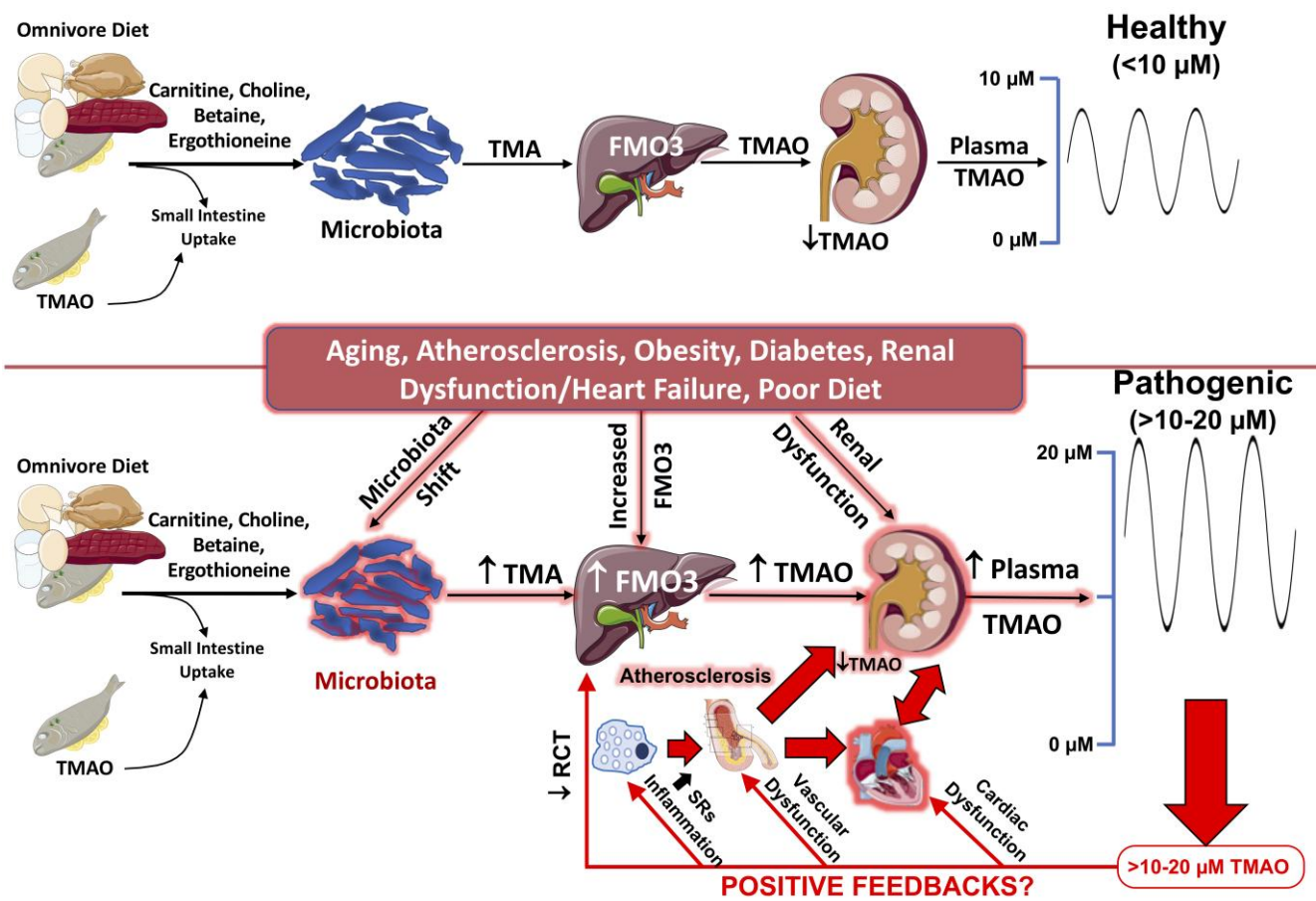


Figure 2. TMAO responses in health and diseased omnivores. Variations in circulating TMAO as a result of dietary substrates in omnivores may be insufficient to promote disease. However, a combination of major risk factors (aging, obesity, insulin-resistance, low testosterone, shift in gut

microbiota) and/or disease (diabetes, CKD) – 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 FMO3 activity: evolving cardiovascular disease 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. *Abbreviations:* CKD, *chronic kidney disease*; FMO3, *flavin-containing monooxygenase 3*; omni, *omnivore*; SR, *scavenger receptor*; TMA, *trimethylamine*; TMAO, *trimethylamine-N-oxide*.

PATHOGENIC EFFECTS OF TMAO

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 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 cardiovascular 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 SRs while inhibiting RCT ^[6, 7, 19, 20] (**Figure 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% wt/wt, ~12x normal) also augments platelet reactivity to ADP, though failed to influence other indices including surface phosphatidylserine content in ADP-stimulated platelets, levels of Von Willebrand factor, alpha granule release, or baseline pro-thrombotic microvesicle release ^[22]. These mixed actions could contribute to vascular disease when TMAO exceeds 10-20 μ M, for example in HF and CKD, however involvement in the initial pathogenesis of atherosclerosis and CHD is questionable ^[23].

Figure 3. Putative pro-inflammatory and -atherosclerotic actions of TMAO. Pronounced elevations in TMAO to >10-20 μ M, for example in advanced HF or CKD, 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. *Abbreviations: CD36, cluster of differentiation 36; CHD, coronary heart disease; CKD, chronic kidney disease; eNOS, endothelial nitric oxide synthase; HF, heart failure; ICAM-1, intracellular adhesion molecule-1; IL, interleukin; LDL, low-density lipoprotein; MCP-1; monocyte chemoattractant protein 1; NF- κ B; nuclear factor kappa-light-chain-enhancer of activated B cells; ox-LDL, oxidised-low-density lipoprotein; ROS, reactive oxygen species; SRA, scavenger receptor A,*

TMAO, *trimethylamine-N-oxide*; TNF- α , *tumour necrosis factor-alpha*; VCAM-1, *vascular adhesion protein-1*.

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 support a protective rather than detrimental role for TMAO^[25]. Other work reveals no relation 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 SRs. Increased expression of atherogenic SRs, 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] (**Figures 2 and 3**). The membrane glycoprotein CD36 is essential in plaque formation, internalizing 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 ($\geq 300 \mu\text{M}$)^[2, 4, 5], unlikely to be encountered *in vivo*. Macrophage stress responses are similarly evident at very high TMAO concentrations^[51]. Macrophage CD36 and SR-A1 expression in atherosclerotic mice are increased in response to an

excessive 350 μ M plasma concentration (achieved with TMAO supplementation) ^[2, 4, 5]. Geng *et al.* recently showed that TMAO supplementation (1 mM in drinking water) increases plaque CD36 in ApoE^{-/-} mice, and that 100 μ M 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, causal involvement of SR-A1 or CD36 in TMAO-sensitive atherosclerosis has yet to be established.

Inhibition of RCT. Additional to promoting sequestration of oxidised LDL, elevated TMAO may inhibit the RCT that underlies hepatic catabolism and biliary excretion of lipids ^[32] (**Figure 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 ABCA1 and SR-B1 could contribute. High concentrations of TMAO (75-150 μ M) may reduce macrophage ABCA1 expression *in vitro* ^[5], although others report *increased* macrophage ABCA1 with 100 μ M TMAO, no change in SR-B1 with 50-100 μ M 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 μ M) moderately increase ABCA1-dependent cholesterol efflux ^[2, 20], while Trenteseaux *et al.* report opposing increases in SR-B1 mRNA in male offspring of hypercholesterolemic mice vs. decreases in female offspring ^[33]. 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 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, ROS generation, NF- κ B signalling and cytokine production, the NLR family pyrin domain containing 3 (NLRP3) inflammasome, and anti-inflammatory sirtuins (**Figure 3**). Consistent with pro-inflammatory effects of age-dependent elevations in TMAO, inhibiting TMA formation limits changes in TNF α , IL-1 β and eNOS in aging 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 μ M TMAO on IL-1 β , 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-1 β and NLRP3, together with ICAM-1, MMP-8, and caspase-1, on exposure to extremely high (300-900 μ M) TMAO. These effects, together with NLRP3 inflammasome activation, appear related to decreased SOD2 and sirtuin-3, and increased mitochondrial ROS generation ^[7]. Similar sensitivities are reported in rat vascular smooth muscle cells, with IL-1 β , NF- κ B and NLRP3 increased in response to 100-600 μ M 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 (~55 μ M) significantly increases vascular transcription of inflammation related factors in the atherosclerosis-prone LDL receptor knockout mouse, including MCP-1, MIP-2, TNF α , ICAM-1, keratinocyte chemoattractant (mouse analogue of IL-8), COX-2, E-selectin, VCAM-1 and the macrophage marker CD68 ^[6]. Effects in wild-type mice were not assessed, while even higher TMAO concentrations (100-200 μ M) were found to increase inflammatory mediators in human vascular endothelial and smooth muscle cells, with 400 μ M TMAO increasing endothelial recruitment of leukocytes ^[6]. These effects were suppressed by inhibitors of NF- κ B and the $\beta\gamma$ G-protein subunit of G-protein coupled receptors (GPCRs) ^[6], though whether the latter reflects direct TMAO-GPCR

interactions or modulation of protein conformation is unclear ^[20]. Interaction between TMA and GPCRs 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 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 (**Figure 3**) ^[19]. Sun *et al.* found that 100-300 μ M TMAO suppresses endothelial eNOS expression and NO release, an effect involving ROS-dependent activation of the NLRP3 inflammasome ^[19]. Additionally, Matsumoto *et al.* report a modest inhibitory effect of 300 μ M TMAO on EDHF mediated relaxation in the femoral (but not superior mesenteric) artery ^[41]. Suggestive of some vascular influences, chronic TMAO infusion in rats to achieve a 58 μ M 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 work, 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 Nrf2 suppression, 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 μ M, and evidence 100-600 μ M TMAO can promote vascular calcification and expression of bone-related and inflammatory molecules *in vitro* and *in vivo* ^[37].

Aging is an independent risk factor for CVD and involves vascular remodelling and progressive reductions in endothelial function and NO bioavailability, coupled with increased COX-derived vasoconstrictors. Circulating TMAO increases with age in humans and rodents ^[8, 40, 43, 44], and recent work indicates inhibition of TMAO formation reduces ^[8] whereas TMAO supplementation mimics or accelerates ^[40] aging-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 aging 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 aging ^[43], suggest elevated TMAO could promote biological aging, predisposing to strongly age-dependent diseases such as CHD and CKD. Indeed, Li *et al.* ^[8] report that a moderate elevation in TMAO from ~6 to 14 μ M 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 aging. 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 0.3-3 μM TMAO disrupts of 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 ~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 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 ~20 μM TMAO suggests 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 likely contribute to the associated rise in TMAO ^[15, 53]. Aging, obesity, insulin-resistance and diabetes ^[54] - associated with and promoting HF - may additionally contribute to increased TMAO, for example via altered FMO3

expression^[44, 55, 56]. These TMAO changes could constitute an important cardio-renal linkage, with TMAO functioning as a uremic toxin that promotes cardiac and renal dysfunction and remodelling^[57] (**Figure 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 ~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 ~20 µM) has important implications, though awaits confirmation. Li *et al.* present evidence post-infarct HF in rats may also involve an associated increase in circulating TMAO (to ~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 uremic toxin in 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 TMAO AND CVD - CAUSE AND/OR EFFECT?

Experimental *in vitro* and *in vivo* studies to date support potentially pathogenic effects of as little as ~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 disease 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 2-5 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 ~10 µM with aging^[40, 43], and increase more profoundly in HF (>10 µM; linked to disease severity/renal dysfunction)^[2, 18, 60] and CKD (≥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 humans ^[8-13], though some murine studies report equivalent 1-5 μ M 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 aging ^[39, 44].

Table 1 Estimates of human serum TMAO concentrations in healthy and disease cohorts

	Serum TMAO (μ M)	Sample Size	Age (Yrs)	BMI (kg/m ²)	Sex (% Male)	TMAO Analysis
HEALTHY OR BROAD COHORT						
Mente <i>et al.</i> 2015 ^[17]						
TMAO Q1 (19% CVD, 2% Diabetes)	0.46 \pm 0.14 (Q1)	44	51 \pm 9	25.4 \pm 4.0	64	LC/MS
TMAO Q5 (43% CVD, 19% Diabetes)	4.93 \pm 5.69 (Q5)	59	53 \pm 12	28.7 \pm 4.8	75	
Wang <i>et al.</i> 2014 ^[59]						
Healthy	3.45 (2.25–5.79)	349	54 \pm 16.00	25 (23–29)	33	LC-MS/MS
Krüger <i>et al.</i> 2017 ^[61]						
Healthy (<i>n</i> =297 total)	<2.9 (Q1) >6 (Q4)		40 (Q1) 57 (Q4)	23.3 (Q1) 24.6 (Q4)	54 (Q1) 54 (Q4)	LC/MS
Cho <i>et al.</i> 2017 ^[62]						
Healthy	3.3 \pm 0.2	40	28 \pm 1	24.2 \pm 0.4	100	LC-MS/MS
Meyer <i>et al.</i> 2016 ^[65]						
Healthy (817 in CARDIA)	1.3 (Q1) 6.6 (Q4)	203 201	40 (Q1) 40 (Q4)	28.8 (Q1) 28.7 (Q4)	38 (Q1) 59 (Q4)	LC/SIDA/MS
Gruppen <i>et al.</i> 2017 ^[69]						
PREVEND (CVD 61% in Q1, 96% in Q4)	<1.7 (Q1) \geq 5.7 (Q4)	1361 1368	49 (Q1) 54 (Q4)	26.1 \pm 4.3 27.1 \pm 4.6	46 44	¹ H-NMR
CVD						
Wang <i>et al.</i> 2011 ^[2]						
Total Cohort (65% CVD, 27% MI, 31% Diabetes, Framingham Risk Score 10)	3.9 (2.6-6.3)	1876	64 \pm 10	NA	49	MS ⁿ , LC-MS/MS, GC-MS/MS

Tang et al. 2013 ^[14]						
<i>Healthy</i> (30% diabetes, 40% MI)	3.5	3494	62 ± 11	28.7 (25.7 – 32.5)	65	SIDA/LC/MS
<i>MACE</i> (40% diabetes, 53% MI)	5	513	68 ± 10	28.1 (24.8 – 32.4)	62	
DIABETES						
Dambrova et al. 2016 ^[64]			63 (total)	28.4 (25.8–31.8) (total)	75 (total)	
<i>PCI Non-Diabetic</i>	1.73 ± 1.05	112				UPLC-MS/MS
<i>PCI Diabetes</i>	2.25 ± 1.09	39				
(34% prior PCI, 30% MI, 70% hypertension)						
Obeid et al. 2016 ^[66]					57	
<i>Healthy</i>	5.4±5.2	185	67±9	26.4±3.5		LC/MS
<i>T2DM</i>	8.6±12.2	98	67±10	29.3±4.8		
Shan et al. 2017 ^[54]						
<i>Healthy</i>	1.47 (0.81–2.20)	1348	55±10	23.6±3.4	56	SIDA-MS
<i>T2DM</i>	1.77 (1.09–2.20)	1346	53±10	25.0±3.6	57	
Al-Obaide et al. 2017 ^[70]						
<i>Healthy</i>	0.18±0.05	20	54±3	28.2±1.1	NA	LC/MS
<i>T2DM-CKD</i>	1.52±0.29	20	64±2	33.2±2.9		
AGING						
Ke et al. 2018 ^[40]						
<i>Healthy (30 yrs of age)</i>	2.93±2.89	109	30±7	21.8±2.8	47	LC-MS/MS
<i>Advanced Age (>65 yrs)</i>	10.52±8.09	77	72±5	22.4±3.0	56	
Li et al. 2018 ^[43]						
<i>Healthy (31 yrs of age)</i>	2.85±3.1	168	31±7	21.8±3.0	48	LC-MS/MS
<i>Advanced Age (>65 yrs)</i>	9.83±10.6	141	71±5	22.5±3.2	59	

KIDNEY DISEASE

Stubbs <i>et al.</i> 2016 ^[15]						
<i>Healthy</i>	3.3 (3.1–6.0)	17	44±15	29.1±6.6	29	UHPLC-MS/MS
<i>ESRD</i>	94.4 (54.8–133.0)	25	64±10	29.1±4.9	76	
Missailidis <i>et al.</i> 2016 ^[53]						
<i>Healthy</i> (13% CVD)	5.8 (3.1 <-> 13.3)	80	62±12	26±4	71	LC-MS/MS
<i>Stage 3-5 CKD</i> (28% CVD)	53.4 (9.3 <-> 170.0)	179	55±14	25±5	65	
Xu <i>et al.</i> 2017 ^[68]						
<i>Healthy</i> (0% CVD)	2.08±1.89	32	55±10	NA	50	LC-MS/MS
<i>Stage 4-5 CKD</i> (78% CVD)	30.33±27.35	32	53±15	NA	50	
Wang <i>et al.</i> 2014 ^[71]						
<i>EDCA</i> (65% CVD, 41% MI)	3.7 (2.4-6.2)	3903	63±11	NA	64	LC-MS/MS
Kaysen <i>et al.</i> 2015 ^[72]						
<i>Healthy</i>	1.41±0.49	NA	NA	NA	NA	UPLC-MS
<i>ESRD</i> (59% Diabetes, 34% HF, 36% atherosclerosis)	50±32	235	62±14	29.7±7.3	55	

Summary of studies in human subjects detailing TMAO concentrations in healthy and pathological conditions. Values are variably expressed as: mean±standard deviation, median (inter-quartile range), or median (10th percentile < - >90th percentile, or quartile 1 (Q1) and quartile 4 (Q4). *Abbreviations:* CKD, complex kidney disease; CVD, cardiovascular disease; EDCA, elective diagnostic coronary angiography; ESRD, end stage renal disease; GC, gas chromatography; ¹H-NMR, proton-nuclear magnetic resonance LC, liquid chromatography; MACE, major adverse cardiovascular events; MI, myocardial infarction; MS, mass spectrometry; MSⁿ, multiple-stage mass spectrometry; 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; UPLC, ultra-performance liquid chromatography.

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, 73, 74]. Similarly, TMAO concentrations in stable HF independently predict 5-year mortality when adjusted for traditional risk factors [75], 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 [76]. These analyses collectively evidence links between TMAO concentration and CVD risk, progression and mortality in high-risk subjects.

Other evidence suggests such associations may be limited to high-risk cohorts with existing disease or multimorbidity [2, 14, 17, 20, 75, 77]. No association was evident between TMAO, coronary artery Ca^{2+} and carotid intima-media thickness in a cohort of >800 33-55 year olds without CVD [65]. Association between TMAO and 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 [78]. 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 comorbidities such as diabetes [79] and CKD [80] indicates TMAO may play a secondary or biomarker role in CVD. This is consistent with the experimental evidence TMAO only influences disease processes under pathological conditions [27], without modifying vascular [8] or cardiac phenotype [10] in young healthy animals. Indeed, recent Mendelian randomization analysis detected no relation between genetically predicted high TMAO (or carnitine) and risk of CHD or AMI (or atrial fibrillation, stroke, T2DM or CKD), while T2DM and CKD were causally associated with elevated TMAO [81]. These diseases may thus mechanistically drive increases in TMAO, meaning correlations with CVD reflect a

reverse causality. Critically, while a number of association studies suggest markedly increased CVD risk at $\leq 5 \mu\text{M}$ TMAO ^[2, 14, 17-19], there is little evidence of pathological effects of TMAO at these concentrations.

Implied cardiovascular pathogenicity of TMAO. 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 a rise in TMAO from <1 to $5 \mu\text{M}$ increases CVD risk 4-fold, despite these levels falling within the range observed in healthy subjects, and no evidence of pathological responses to $\leq 5 \mu\text{M}$ TMAO. Similarly, the data of Tang *et al.* implies a modest 40% rise in TMAO from $3.5 \mu\text{M}$ to $5 \mu\text{M}$ significantly promotes CVD ^[14]; the association in Mente *et al.* ^[17] suggests a rise from <1 to $5 \mu\text{M}$ increases risk of CVD ~9-fold; and Mafune *et al.* link small elevations in TMAO from <3 to $4, 5$ and $6 \mu\text{M}$ 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 $6 \mu\text{M}$, evidence supports a $10\text{--}100 \mu\text{M}$ threshold for functional effects of TMAO in platelets ^[21]. Meta-analysis suggests a somewhat lower sensitivity of all-cause mortality, which increases ~8% per $10 \mu\text{M}$ increment in plasma TMAO ^[76], and highlights an unexplained disparity between TMAO levels and outcomes: mortality exhibited an unexpected 10-fold greater dependence on TMAO in the low $1\text{--}10 \mu\text{M}$ vs. 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 $\leq 10 \mu\text{M}$ TMAO on inflammation, atherosclerosis or thrombosis, and no pathobiological basis for markedly higher dependence of mortality on $<10 \mu\text{M}$ vs. $>10 \mu\text{M}$ TMAO. These findings contrast evidence of a higher TMAO threshold for cardiometabolic risk

approaching 10 μM ^[82]. Fundamentally complicating matters, the basis of the variance in TMAO in healthy populations and of elevated concentrations with CVD risks, comorbidities and disease itself remain unclear, constraining capacity to address the question of TMAOs causal involvement.

DETERMINANTS OF CIRCULATING TMAO IN HEALTH AND DISEASE

The question of how circulating TMAO levels are regulated and increase beyond a rather broad 'normal' range ^[61, 83] 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) (**Figure 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 ^[84]. The basis of TMAO elevations with CHD risk factors including obesity ^[10, 11, 13], insulin-resistance ^[67, 85], diabetes ^[54, 64, 79] and metabolic syndrome ^[82] 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 ^[84], point to a critical though incompletely defined role for diet-microbiota interactions and biota remodelling ^[83]. Existence of sub-populations of biota-dependent high vs. 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 ^[86] and that red meat intake is a weak CVD risk ^[87-89], appear

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 ^[90] (**Figure 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, 91], though a recent report (albeit for a single human subject) suggests TMA may be generated and absorbed specifically in the small intestine ^[92]. Such localisation, if confirmed, could increase the diet sensitivity of TMA/TMAO generation in humans vs. rodents. Following gut formation and absorption, the amine is oxidised by hepatic FMO3 to TMAO (**Figure 1**) ^[20, 93, 94]. Additional forms of FMO in humans (FMO1, FMO2, FMO4, FMO5) do not appear to participate significantly in TMA oxidation ^[95], whereas recent work indicates FMO1 is responsible for ~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 ^[96]. 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 ^[91]. There appears a threshold intake beyond which choline reaches the large intestine in sufficient quantities for bacterial metabolism ^[97, 98]. Choline is absorbed from the small intestine via transport proteins that are 50% saturated at 200-300 μ M in mice ^[99, 100]. With transporter saturation, choline can reach distal sites of bacterial conversion to TMA and dimethylamine ^[101], though preliminary evidence of TMA generation within the small intestine ^[92] suggests 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 (~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, 102] (**Table 2**). Consistent with transience of diet-induced TMAO changes, baseline plasma TMAO in healthy subjects is not altered with 8 weeks consumption of 6

eggs per week ^[97]. In high-fat fed mice, supplementation with 1.2% (wt/wt) of the alternate choline source sphingomyelin also increases serum TMAO, although concentrations are relatively low and insufficient to influence atherosclerosis ^[103]. Summarising studies of choline feeding: high intakes 10- to 15-fold above normal can elevate TMAO in animals ^[6, 39, 80] as can dietary supplementation in humans ^[14, 104] (**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 ^[86] or exert no effect ^[105, 106], contrary to proposed pathogenicity of TMAO.

Table 2 Effects of diet or supplementary interventions on plasma TMAO concentrations

	<i>Sample Size</i>	<i>Sex (% male)</i>	<i>Intervention / Design</i>	<i>Baseline Concentration (μM)</i>	<i>Fed Concentration (μM)</i>
DIET INTERVENTION					
DiMarco et al. 2017 ^[63] (24.1 yrs, BMI 24.3) Fasted	36	50	3 Eggs/day, 4 weeks	3.7±4.7	3.4±5.2
West et al. 2014 ^[97] (Lacto-ovo vegetarians, 35.7 yrs, BMI 23.7) Fasted	15	0	6 Eggs/week, 8 weeks	2.3±0.3	2.1±0.3
Zhu et al. 2017 ^[102] (46 yrs, 10 Omnivore, 8 Vegetarian)	8 10	40	~450 mg choline/ day, 2 months	2.6±0.6 (Vege) 2.5±0.3 (Omni)	28.9±5.5 (Vege) 36.8±9.4 (Omni)
Boutagy et al. 2015 ^[107] (22.1 yrs, BMI 22.3)	10	100	High-fat feeding (55% fat), 5 days	1.6±0.2	1.8±0.2 (2.5±0.3 4 hr post-prandial vs. 1.6±0.1 for control diet)
SINGLE OR 24 HR SUPPLEMENT					
Tang et al. 2013 ^[14]	40		2 eggs + 250 mg phosphatidylcholine	4.5±1.1	Peak 7.6 μM at 1 hr, recovered to baseline at 4 hr
Cho et al. 2017 ^[62] (27.8 yrs, BMI 24.2)	40	100	Fish, beef meal/6 hrs	3.3±2 (~20 μmol.6 hr AUC)	<i>Fish</i> ~700 (μmol.6 hr AUC) <i>Beef</i> ~21 (μmol.6 hr AUC)
Miller et al. 2014 ^[102] (42.5 yrs, BMI 30.7)	6	33	6 egg yolks/24 hr	30±6 (μmol.24 hr AUC)	142±26 (μmol.24 hr AUC)
DIETARY MAKEUP					
Koeth et al. 2013 ^[20]	26		Vegetarian	2.1 (1.1, 4.2)	

Healthy male and female	51		Omnivore	2.8 (1.3, 7.2)
Rohrmann et al. 2016^[90]	104	100	Male	2.55 (2.17, 2.99)
(Male 50 yrs, Female 44, BMI 26.1 Male, 25.2 Female)	167	0	Female	2.52 (2.22, 2.86)
Diet makeup based on ≥ 2 x 24 hr dietary recalls.	8		Vegetarian	3.35 (1.88, 5.97)
Non-Fasted	?		Omnivore	2.51 (2.27, 2.78)
	111		<i>Red Meat</i>	
	68		0 g/day	2.5 (2.13, 2.93)
			≥ 55 g/day	2.37 (1.95, 2.89)
	68		<i>Milk & Dairy</i>	
	67		<68.2g/day	2.08 (1.69, 2.57)
			>253.3 g/day	3.13 (2.56, 3.84)
	161		<i>Eggs</i>	
	72		0 g/day	2.56 (2.25, 2.92)
			>17.1 g/day	2.51 (2.07, 3.04)
	180		<i>Fish</i>	2.53 (2.24, 2.87) 2.45(2.07,
	91		-	2.91)
			+	

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 area under the curve over defined periods post interventions, and are variably expressed as:

mean \pm standard deviation; mean or median (95% confidence intervals). *Abbreviations:* AUC, area under curve; BMI, body mass index.

Carnitine. L-Carnitine is produced from lysine in eukaryotes, and is catabolised by prokaryotic organisms^[108], the latter begin able to yield TMA and malic semialdehyde via cleavage of the backbone carbon-nitrogen bond of carnitine^[109]. Carnitine is an essential component of fatty acid metabolism, transporting activated long chain fatty acyl groups into the mitochondrial matrix^[110]. 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^[111]. Saturation is also reported with 3 x 1 g doses of carnitine, significantly elevating plasma TMAO^[112], although baseline concentrations of ~35 μ M in this study are over an order of magnitude higher than widely reported^[59]. Consumption of ~225 g of sirloin steak (~180 mg of carnitine) transiently increases plasma TMAO concentration^[20]. Prolonged daily supplementation of L-carnitine (1 g/day over more than 1 year) has been shown to increase median plasma TMAO by ~12-fold in a cohort of 9 patients with mitochondrial disorders^[113]. The gut microbiota may also be able to produce γ -butyrobetaine from l-carnitine metabolism^[108], a metabolite that bacteria can subsequently convert to TMA^[95]. Collective evidence indicates that high and chronic dietary loads of carnitine are required to elevate TMAO towards pathological concentrations (>10 μ M) 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 risk of CVD and metabolic disorders^[114], protecting against diabetes^[115] and metabolic syndrome^[116]. Meta-analysis indicates carnitine reduces all-cause mortality, ventricular arrhythmias and angina symptoms in infarct patients^[117]. 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^[118], and as an osmolyte^[119]. The former function reduces the dietary methionine and choline required for optimal nutrition^[118]. Betaine is mainly acquired through consumption of betaine rich foods such as

wheat bran, wheat germ, and spinach ^[120], though irreversible oxidation of choline to betaine within the host ^[121] and gut ^[122] represents another source. Certain gut bacteria can also convert l-carnitine into betaine ^[109]. 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 ^[123]; or initial demethylation to dimethylglycine, which undergoes decarboxylation to form TMA ^[124]. In terms of relationships with disease, betaine supplementation has a moderate effect on total plasma cholesterol, but not LDL, HDL or triglycerides ^[125], and high plasma betaine appears to have no effect on CVD risk ^[105, 126] while protecting against T2DM ^[127].

Ergothioneine. Histidine derived ergothioneine represents another dietary precursor for TMA ^[128]. It appears ubiquitous across cells and tissues ^[129], though is primarily concentrated in the liver, kidney, bone marrow and erythrocytes ^[130]. Ergothioneine is not synthesised in animals, but readily derived from an array of foods including mushrooms (where it is synthesised), black and red beans, oat bran and liver or kidney ^[131]. Cellular uptake occurs via organic cation transporter 1 ^[132], and it may have antioxidant roles within cells ^[129, 133]. The ergothionase enzyme in some gut bacteria can catalyse degradation of ergothioneine to form TMA ^[128]. 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 ^[134], while there is support for increased CVD mortality vs. decreased diabetes risk and cancer mortality with increased legume intake ^[135, 136], though the basis of these distinct disease outcomes is unknown.

Seafood. TMAO can be absorbed without gut and hepatic processing following consumption of seafood, with plasma levels increasing within 15 min ^[62, 112]. Consumption of a 1.67g dose of TMAO results in ~50% of the molecule being absorbed unchanged ^[137], while the remainder is believed to be reduced via bacterial TMAO reductase to form TMA which is subsequently absorbed and re-oxidised to TMAO ^[137]. Fish are rich in TMAO owing to physiological roles in buoyancy and osmotic control

[138, 139], with the free TMAO content of seafood several-fold higher than choline/carnitine contents of red meat and eggs [138]. Populations consuming more fish exhibit high urinary TMAO concentrations [61, 140, 141], 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) [137]. Others confirm eggs and red meat have relatively minor effects on post-prandial plasma TMAO compared with seafood (whereas fruits reduce concentrations) [62]. More recently, Schmedes *et al.* showed that a seafood meal significantly increases postprandial TMAO while a non-seafood meal does not [142]. 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 FMO3

Circulating TMAO concentrations are dependent on TMA oxidation by hepatic FMO3, and correlate with FMO3 expression in mice and humans [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 $>30 \text{ minute}^{-1}$, based on analysis of heterologously expressed FMOs *in vitro* [93]. Thus, rapid conversion to TMAO will occur in a substrate-dependent manner in healthy individuals (TMA $\sim 20\text{--}25 \mu\text{M}$) [51]. There is evidence hepatic FMO3 expression or activity is sensitive to sex steroids [28, 143, 144], dietary factors [145] and calorie intake [146, 147], NO signalling [148] and the transcriptional regulator CCAAT/enhancer-binding protein beta [149]. Hepatic FMO3 expression also increases postnatally in humans, with an apparent plateau after early adulthood [150, 151]. Conversely, in female mice hepatic FMO3 transcript may decline following this phase of maturational up-regulation [152]. While there is some debate regarding hepatic FMO expression in male rodents, an aging dependent increase in FMO3 protein is reported in male mice [44, 146, 147]. 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 vs. male mice and humans [28], paralleled by dimorphism in TMAO concentrations in mice [8, 28, 40] and to a lesser extent humans [153], yet females enjoy protection

against CVD over much of the lifespan. Pregnancy further up-regulates FMO3^[154], 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^[154]. Circadian patterns of FMO3 expression may also differ markedly between sexes, a dimorphism reduced by cardioprotective calorie restriction which up-regulates FMO3 in males, 'feminising' the transcriptional profile^[146, 147]. However, there remains some debate regarding hepatic expression of FMO3 in male mice: there is evidence FMO3 mRNA is below detection limits^[152] or detectable at 0.1-10% of the levels in females^[146, 147]; and that FMO3 protein is not detectable in males^[9, 28], or lowly expressed and augmented with adenoviral delivery, calorie restriction or aging^[44, 146, 147]. 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^[155], inflammation^[156], insulin-resistance and diabetes^[2, 14, 54, 55], aging^[44, 146, 147], pollutants and hepatic toxins^[157]. 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 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^[155], 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^[85]. On the other hand, Liao *et al.* report beneficial effects of FMO3 on glucose homeostasis independent of the

insulin-signalling pathway^[158]. Furthermore: hepatic TMAO concentrations decline and renal concentrations are unaltered in the *db/db* mouse model of diabetes^[159]; a low glycaemic index diet increases TMAO^[160] despite known benefit in obesity, diabetes and on CVD risk^[161]; and metformin increases TMAO levels while reducing blood glucose in type 2 diabetic patients^[162]. Disruption of glycemic control and emergence of 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 resveratrol does not influence plasma TMAO in mice on a choline supplemented (1% wt/wt) 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^[163]. Metabolic effects of FMO3 manipulation in LDL receptor knockout mice are TMA/TMAO independent^[28, 164]. Furthermore, while FMO3 dependent elevations in TMAO are suggested to promote tissue aging^[8, 40, 43], and FMO3 expression may rise with age^[44], recent evidence indicates increased FMO3 expression is protective, exerting anti-aging effects that mimic those of calorie restriction^[147]. 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 aging^[8, 40, 53]. The cellular influx of TMAO may occur predominantly via organic cation transporter 2, which facilitates renal tubular TMAO uptake^[84]. Efflux occurs via ATP-binding cassette family transporters, with genetic variation in the ABCG2 transporter potentially contributing to altered TMAO concentrations in dyslipidaemic subjects^[84]. 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 ^[165, 166].

In healthy volunteers with normal kidney function, fractional renal clearance of TMAO exhibits a broad dynamic range ^[83], while patients with renal dysfunction exhibit significantly elevated plasma TMAO as a consequence of functional deficit ^[15, 53, 167, 168]. 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 ^[169]. Recent findings suggest chronic ingestion of red meat may reduce excretion of TMAO ^[83]. Though speculative, detrimental feedback may also emerge at the level of kidney function: as for uremic toxins in 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, 80] (**Figure 2**). Though this remains to be directly tested, elevations in TMAO predict mortality in CKD ^[53, 80], and mimicking TMAO concentrations in advanced CKD and HF (80-100 μ M) with either choline (1.0% total) or TMAO (0.12%) supplementation promotes renal fibrosis and injury in mice ^[80]. On the other hand, others report no relation between TMAO and mortality or cardiovascular outcomes in end-stage renal disease ^[72].

The gut microbiota and human TMAO concentrations

Up to ~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, 84], and analysis of the heritability of high TMAO concentrations indicates host genetics also plays a minor role ^[170], consistent with failure of gene association analysis to identify TMAO linked loci ^[171]. 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 distinct bacterial profiles govern TMAO generation in healthy male omnivores, identifying 'high' vs. 'low' producers (42% and 58% of volunteers, respectively). A substantial portion 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 ^[172]. 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 vs. non-vegans further emphasise the microbiotas importance: carnitine challenge increases plasma and urinary TMAO in omnivores but not vegans, a dimorphism eliminated by antibiotic treatment ^[20]. This suggests intake of animal protein favours carnitine/choline metabolising bacteria ^[20, 173], while a non-meat diet low in these substrates and relatively enriched with betaine (a less favourable TMA substrate ^[71, 174]) 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 vs. white meat on bacterial carnitine vs. choline metabolism ^[83], further confirm the importance of microbiota changes in driving TMAO elevations in humans.

Fennema *et al* provide a comprehensive review and summary detailing bacterial species involved in formation of TMA ^[95]. Bacteria including *Prevotella*, *Deferribacteres* and *Teneriticutes* species can metabolise choline and carnitine to TMA, while *Bacteroides* appear less effective than

Prevotella ^[20, 73]. Recent cross-sectional analysis of 1653 multi-ethnic participants linked circulating TMAO to the abundance of 13 genera (6 Firmicutes, 3 Bacteroidetes, 3 Proteobacteria, 1 Fusobacteria), including *Prevotella*, *Mitsuokella*, *Fusobacterium*, *Desulfovibrio*, *Bilophila* and Ruminococcaceae and Lachnospiraceae family members ^[67]. Studies indicate omnivores possess more *Firmicutes* than *Bacteroidetes* species and a less diverse microbiota compared to vegans/vegetarians, who also have relatively lower *Clostridium* species ^[62, 175, 176]. In general, meat intake appears to promote *Bacteroides*, *Alistipes*, *Ruminococcus*, *Clostridia* and *Bilophila*, while decreasing *Bifidobacterium* species ^[177]. Other work links a vegan diet to decreased *Clostridium* vs. enrichment of *Anaerostipes caccae* and *Lachnobacterium* species in subjects with metabolic syndrome ^[178]. 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 ^[179]. Recent studies identify three biochemical paths for bacterial TMA production, involving the genes *cutC* ^[180], *cntA* ^[181] and *YeaW* ^[91]. 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* ^[182, 183]. Interestingly, in terms of observed increases in circulating TMAO with aging, Brunt *et al.* ^[44] report select aging-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 vegetarian (but not vegan) diets can increase relative proportions of *Prevotella*, known to produce TMA ^[184, 185]. One study found plasma TMAO did not differ significantly between vegans and lacto-ovo-vegetarians ^[186], though the period the groups adhered to these diets was not provided. Recent evidence indicates red vs. white meat may selectively increase bacterial metabolism of carnitine (not choline) to TMA, while reducing renal TMAO excretion ^[183]. 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 comorbidities, or is a causal factor contributing to CVD development.

TMAO - 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 (aging, inactivity, hypercaloric 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 ^[137, 142] is thus indirect, reflecting collective influences of multiple CVD risk factors and also protectants (e.g. exercise, vegan diet), influenced by diet-microbiota interactions (**Figure 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 comorbid diseases (HF, CHD/AMI, CKD), may play an important secondary role, promoting disease progression and sequelae (**Figures 2 and 4**). This is congruent with evidence TMAO-CVD associations reflect a reverse causality (TMAO changes resulting from disease, for example CKD or T2DM) ^[81]; the restriction of TMAO-CVD associations to complex cohorts with multiple risk factors ^[60, 75, 79, 80, 187], and evidence 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 **Figure 4**, that

apparent associations between TMAO and CVD involve 2 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 **Figure 2**). Nonetheless, even secondary mechanistic involvement of TMAO in CVD requires further study and confirmation.

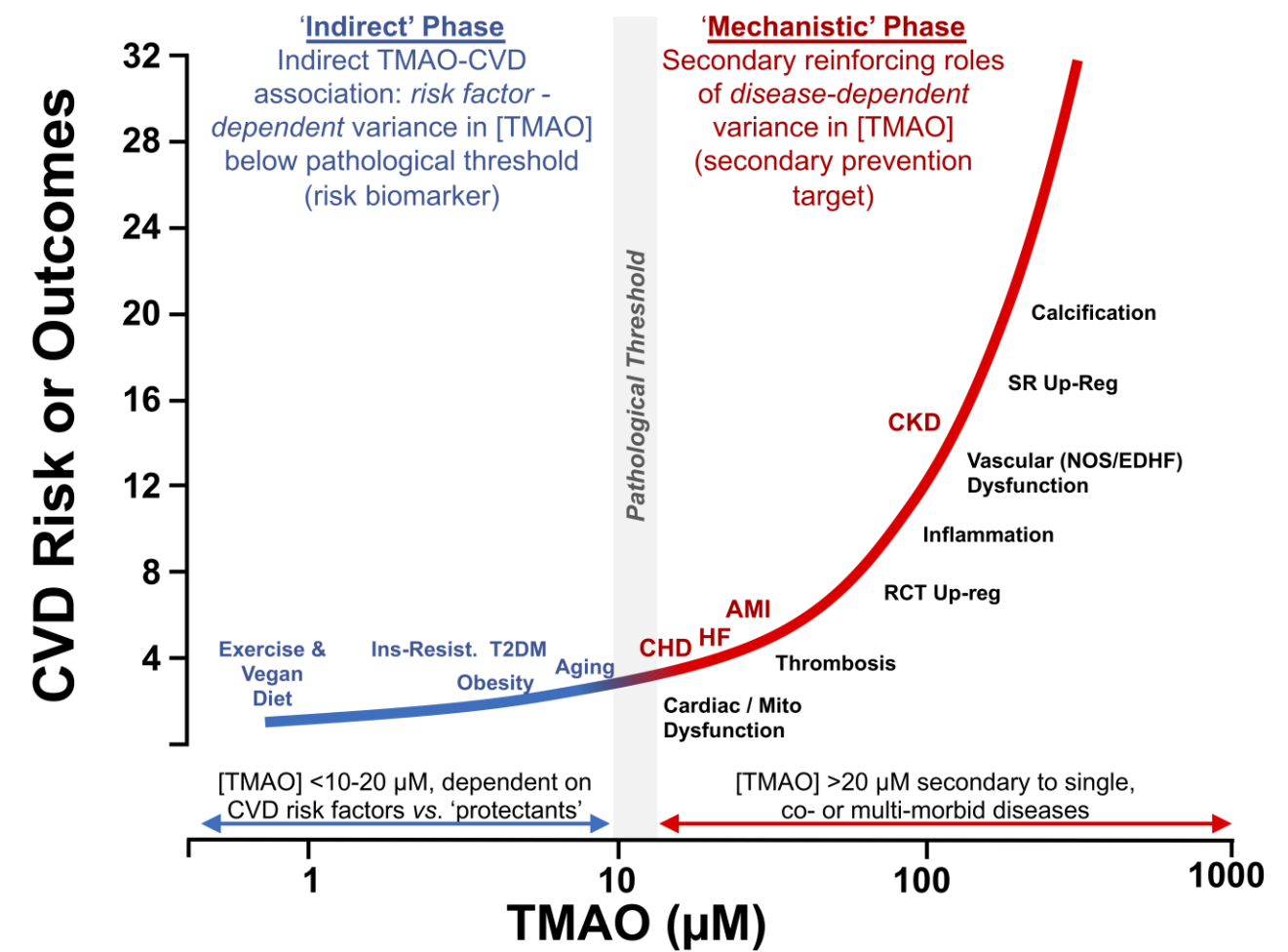


Figure 4. Speculated linkages between TMAO and CVD. Whether associations between circulating TMAO and CVD risks/outcomes reflect a causal role in disease remains unclear. Consideration of available evidence suggests indirect associations with CVD: below 10-20 μM ('Indirect' phase), variance in TMAO reflects stimulatory influences of well-established CVD risk factors (e.g. aging, 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 (e.g. CKD, HF), acute myocardial infarction (AMI) in CHD patients, or co-morbid conditions (e.g. 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 **Figure 2**), though this awaits confirmation. Relative effects of risk factors/disease on TMAO concentration are shown, together with approximate concentrations for TMAO-dependent pathological effects. Note the illustrative 'CVD Risk or Outcomes' y-axis range is somewhat arbitrary.

Abbreviations: AMI, *acute myocardial infarction*; CHD, *coronary heart disease*; CKD, *chronic kidney disease*; CVD, *cardiovascular disease*; EDHF, *endothelium-derived hyperpolarising factor*; HF, *heart failure*; Ins-Resist, *insulin-resistance*; mito, *mitochondrial*; NOS, *nitric oxide synthase*; RCT, *reverse cholesterol transport*; SR, *scavenger receptor*; T2DM, *type II diabetes mellites*; TMAO, *trimethylamine-N-oxide*.

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 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 aging and chronic disease ^[188].

Manipulating TMAO 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, 91], however this 'shotgun' approach is not viable given broad impacts on microbiota and host health, immunity and antibiotic-resistance ^[189]. 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 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 ^[190, 191], and a recent study indicates a quarter of 1,000 drugs examined possess antibiotic-like side effects ^[192]. While this broadly supports the potential for pharmacologic 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 (e.g. identification of biochemical paths, and gene determinants such as *cutC* ^[180], *cntA* ^[181] and *YeaW* ^[91]), 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/day) failed to influence TMAO production in patients with metabolic syndrome ^[193], and 3 month probiotic supplementation in haemodialysis patients (9×10^{13}

colony-forming units/day, including *Streptococcus thermophilus*, *Lactobacillus acidophilus* and *Bifidobacteria longum*) failed to influence plasma TMAO ^[194]. Meta-analyses support some cardiovascular benefits of probiotics, though outcomes are modest ^[195-197]. Unexpectedly, a small double-blind randomized controlled trial of faecal microbiota transplantation from vegans into metabolic syndrome patients successfully modified intestinal microbiota composition yet failed to alter TMAO production ^[178]. 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 ^[198]. While FMO3 inhibition might also appear a target for manipulating TMAO in patients with CVD, sufficiently effective reductions in FMO3 lead to TMA accumulation and trimethylaminuria, ^[199]. More fundamentally, FMO3 mediates diverse effects on metabolism, tissue stress and aging independently of TMA/TMAO ^[147, 163].

Dietary modification. Despite evidence some cardioprotective diets and foods have either no effect ^[200] or increase circulating TMAO levels ^[137, 142], 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 (e.g. vegetables/fruits) or suppressing FMO3 activity (e.g. 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, 84], recent work supports up to 3-fold differences in circulating TMAO with red vs. white meat enriched (or non-meat) diets ^[83]. Circulating TMAO is also reportedly 2-fold higher in omnivores vs. vegans ^[20], though such differences are not always observed ^[90] 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 ^[114]. Meta-analysis indicates carnitine also reduces all-cause mortality, ventricular arrhythmias and angina symptoms in infarct patients ^[117]. 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 short chain fatty acids in association with reduced CVD risk

Since choline is an essential nutrient ^[202] it may be reduced but not eliminated from the diet. However, dietary choline has been shown to have no effect ^[105, 106] or mitigate against CVD ^[86], though there is also some evidence choline may increase (whereas betaine reduces) cardiometabolic risk ^[77]. Studies also link choline to CVD ^[126] and MACE ^[71] in those at high risk or with a history of CVD, and 2-month choline supplementation (~450 mg total choline/day, 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 ^[104]. Dietary sphingomyelin, which contains a choline head group, could also be reduced: it is believed to be pro-atherogenic ^[203, 204], and high circulating concentrations are linked to CVD risk ^[203, 205]. However, recent findings indicate that long-term (16 weeks) sphingomyelin supplementation (1.2% wt/wt) does not influence serum TMAO or atherosclerosis in ApoE^{-/-} mice, and *reduces* lesion development in high-fat fed animals ^[103]. 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 vs. plant-based diets are evident in as little as 5 days ^[198], and increased fruit and vegetable intake is strongly linked to protection against CVD ^[206-210]. Trials confirm plant-based diets reduce disease progression, angina and mortality in CVD patients ^[211]. Nonetheless, there is limited evidence to suggest the cardioprotective effects of vegetarian diets ^[208, 209, 212-214] 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 ^[215]. 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 ^[83] (e.g. tubulointerstitial injury with increased dietary

acid load ^[216, 217]), promoting age-dependent disorders (cardiovascular and renal dysfunction, diabetes) that in turn favour further TMAO elevations (**Figure 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 ^[83], it is a rather weak risk for CVD ^[87-89], contrasting its strong association with other chronic diseases and the high risks attributed to small changes in TMAO ^[2, 17, 20, 71, 75]. Processed meats, on the other hand, strongly promote CVD ^[218], 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 ^[219], 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 ^[220, 221]. Furthermore, reducing animal protein intake (a coarse surrogate for TMAO potential) has very modest effects on CVD risk and outcomes ^[2, 17, 20, 71, 75, 87-89] and benefit is difficult to attribute specifically to protein source ^[221]. Other studies indicate high protein intake reduces cardiometabolic risk, BMI and blood pressure, effects more pronounced for plant yet still evident with animal protein ^[222, 223]. There is also evidence the link between high animal protein intake and mortality requires co-existence of one or more additional risk factors ^[224], consistent with evidence 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, 140, 141] well beyond levels achieved with other foods ^[137, 142]. Although a recent experimental study suggests fish intake might promote atherosclerosis in rodents ^[225], 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 ^[226]. Recently, grape pomace polyphenol supplementation has been shown to decrease serum TMAO in a cohort of healthy ^[227] and

overweight/obese patients ^[228]. 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 ^[145, 229]. 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 mM ^[23].

CONCLUSIONS AND FUTURE DIRECTIONS

Cardiovascular disease 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 comorbid conditions (or AMI). In these select settings TMAO could play a secondary reinforcing role (**Figures 2 and 4**), though even this mechanistic contribution awaits confirmation. A mechanistic role for TMAO in 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 **Figure 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 (e.g. 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 comorbidities 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.

Acknowledgments

Figures 1 & 2 were created using illustrations sourced from Smart Servier Medical Art.

Author contributions. S. Naghipour and J.P. Headrick wrote the first draft. A.J. Cox, E.F. Du Toit and J.N. Peart revised the manuscript and approved the final draft.

Funding/support. The authors were supported by research funds from Griffith University. S. Naghipour is supported by the Abedian Foundation Top Up Scholarship (AbedianTOP).

Declaration of interest. The authors have no relevant interests to declare.

References

1. Blum HE. The human microbiome. *Adv Med Sci*. 2017;62(2):414-20.
2. Wang Z, Klipfell E, Bennett BJ, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*. 2011;472(7341):57-63.
3. Siri-Tarino PW, Sun Q, Hu FB, et al. Meta-analysis of prospective cohort studies evaluating the association of saturated fat with cardiovascular disease. *Am J Clin Nutr*. 2010;91(3):535-46.
4. Mohammadi A, Vahabzadeh Z, Jamalzadeh S, et al. Trimethylamine-N-oxide, as a risk factor for atherosclerosis, induces stress in J774A.1 murine macrophages. *Adv Med Sci*. 2018;63(1):57-63.
5. Mohammadi A, Najar AG, Yaghoobi MM, et al. Trimethylamine-N-oxide treatment induces changes in the ATP-binding cassette transporter A1 and scavenger receptor A1 in murine macrophage J774A.1 cells. *Inflammation*. 2016;39(1):393-404.
6. Seldin MM, Meng Y, Qi H, et al. Trimethylamine N-oxide promotes vascular inflammation through signaling of mitogen-activated protein kinase and nuclear factor-kappaB. *J Am Heart Assoc*. 2016;5(2).
7. Chen ML, Zhu XH, Ran L, et al. Trimethylamine-N-oxide induces vascular inflammation by activating the NLRP3 inflammasome through the SIRT3-SOD2-mtROS signaling pathway. *J Am Heart Assoc*. 2017;6(9).
8. Li T, Chen Y, Gua C, et al. Elevated circulating trimethylamine N-oxide levels contribute to endothelial dysfunction in aged rats through vascular inflammation and oxidative stress. *Front Physiol*. 2017;8:350.
9. Chen ML, Yi L, Zhang Y, et al. Resveratrol attenuates trimethylamine-N-oxide (TMAO)-induced atherosclerosis by regulating TMAO synthesis and bile acid metabolism via remodeling of the gut microbiota. *MBio*. 2016;7(2):e02210-15.
10. Chen K, Zheng X, Feng M, et al. Gut microbiota-dependent metabolite trimethylamine N-oxide contributes to cardiac dysfunction in western diet-induced obese mice. *Front Physiol*. 2017;8:139.

11. Sun G, Yin Z, Liu N, et al. Gut microbial metabolite TMAO contributes to renal dysfunction in a mouse model of diet-induced obesity. *Biochem Biophys Res Commun*. 2017;493(2):964-70.
12. Gao X, Liu X, Xu J, et al. Dietary trimethylamine N-oxide exacerbates impaired glucose tolerance in mice fed a high fat diet. *J Biosci Bioeng*. 2014;118(4):476-81.
13. Zhang H, Meng J, Yu H. Trimethylamine N-oxide supplementation abolishes the cardioprotective effects of voluntary exercise in mice fed a western diet. *Front Physiol*. 2017;8:944.
14. Tang WH, Wang Z, Levison BS, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N Engl J Med*. 2013;368(17):1575-84.
15. Stubbs JR, House JA, Ocque AJ, et al. Serum trimethylamine-N-oxide is elevated in CKD and correlates with coronary atherosclerosis burden. *J Am Soc Nephrol*. 2016;27(1):305-13.
16. Heianza Y, Ma W, Manson JE, et al. Gut microbiota metabolites and risk of major adverse cardiovascular disease events and death: A systematic review and meta-analysis of prospective studies. *J Am Heart Assoc*. 2017;6(7).
17. Mente A, Chalcraft K, Ak H, et al. The relationship between trimethylamine-N-oxide and prevalent cardiovascular disease in a multiethnic population living in Canada. *Can J Cardiol*. 2015;31(9):1189-94.
18. Mafune A, Iwamoto T, Tsutsumi Y, et al. Associations among serum trimethylamine-N-oxide (TMAO) levels, kidney function and infarcted coronary artery number in patients undergoing cardiovascular surgery: a cross-sectional study. *Clin Exp Nephrol*. 2016;20(5):731-9.
19. Sun X, Jiao X, Ma Y, et al. Trimethylamine N-oxide induces inflammation and endothelial dysfunction in human umbilical vein endothelial cells via activating ROS-TXNIP-NLRP3 inflammasome. *Biochem Biophys Res Commun*. 2016;481(1-2):63-70.
20. Koeth RA, Wang Z, Levison BS, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med*. 2013;19(5):576-85.
21. Zhu W, Gregory JC, Org E, et al. Gut microbial metabolite TMAO enhances platelet hyperreactivity and thrombosis risk. *Cell*. 2016;165(1):111-24.

22. Roberts AB, Gu X, Buffa JA, et al. Development of a gut microbe-targeted nonlethal therapeutic to inhibit thrombosis potential. *Nat Med*. 2018;24(9):1407-17.
23. Wang Z, Roberts AB, Buffa JA, et al. Non-lethal inhibition of gut microbial trimethylamine production for the treatment of atherosclerosis. *Cell*. 2015;163(7):1585-95.
24. Xue J, Zhou D, Poulsen O, et al. Intermittent hypoxia and hypercapnia accelerate atherosclerosis, partially via trimethylamine-oxide. *Am J Respir Cell Mol Biol*. 2017;57(5):581-8.
25. Collins HL, Drazul-Schrader D, Sulpizio AC, et al. L-Carnitine intake and high trimethylamine N-oxide plasma levels correlate with low aortic lesions in ApoE(-/-) transgenic mice expressing CETP. *Atherosclerosis*. 2016;244:29-37.
26. Veeravalli S, Karu K, Scott F, et al. Effect of flavin-containing monooxygenase genotype, mouse strain, and gender on trimethylamine N-oxide production, plasma cholesterol concentration, and an index of atherosclerosis. *Drug Metab Dispos*. 2018;46(1):20-5.
27. Geng J, Yang C, Wang B, et al. Trimethylamine N-oxide promotes atherosclerosis via CD36-dependent MAPK/JNK pathway. *Biomed Pharmacother*. 2018;97:941-7.
28. Bennett BJ, de Aguiar Vallim TQ, Wang Z, et al. Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metab*. 2013;17(1):49-60.
29. Velasquez MT, Ramezani A, Manal A, et al. Trimethylamine N-Oxide: The Good, the bad and the unknown. *Toxins (Basel)*. 2016;8(11).
30. Kunjathoor VV, Febbraio M, Podrez EA, et al. Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *J Biol Chem*. 2002;277(51):49982-8.
31. Ben J, Zhu X, Zhang H, et al. Class A1 scavenger receptors in cardiovascular diseases. *Br J Pharmacol*. 2015;172(23):5523-30.
32. Cucuianu M, Coca M, Hancu N. Reverse cholesterol transport and atherosclerosis. A mini review. *Rom J Intern Med*. 2007;45(1):17-27.

33. Trenteseaux C, Gaston AT, Aguesse A, et al. Perinatal hypercholesterolemia exacerbates atherosclerosis lesions in offspring by altering metabolism of trimethylamine-N-oxide and bile acids. *Arterioscler Thromb Vasc Biol.* 2017;37(11):2053-63.
34. Pullinger CR, Eng C, Salen G, et al. Human cholesterol 7 α -hydroxylase (CYP7A1) deficiency has a hypercholesterolemic phenotype. *J Clin Invest.* 2002;110(1):109-17.
35. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med.* 1999;340(2):115-26.
36. Wong BW, Meredith A, Lin D, et al. The biological role of inflammation in atherosclerosis. *Can J Cardiol.* 2012;28(6):631-41.
37. Zhang X, Li Y, Yang P, et al. Trimethylamine-N-oxide promotes vascular calcification through activation of NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome and NF- κ B (nuclear factor κ b) signals. *Arterioscler Thromb Vasc Biol.* 2020;40(3):751-65.
38. Suska A, Ibanez AB, Lundstrom I, et al. G protein-coupled receptor mediated trimethylamine sensing. *Biosens Bioelectron.* 2009;25(4):715-20.
39. Li T, Gua C, Wu B, et al. Increased circulating trimethylamine N-oxide contributes to endothelial dysfunction in a rat model of chronic kidney disease. *Biochem Biophys Res Commun.* 2018;495(2):2071-7.
40. Ke Y, Li D, Zhao M, et al. Gut flora-dependent metabolite trimethylamine-N-oxide accelerates endothelial cell senescence and vascular aging through oxidative stress. *Free Radic Biol Med.* 2018;116:88-100.
41. Matsumoto T, Kojima M, Takayanagi K, et al. Trimethylamine-N-oxide specifically impairs endothelium-derived hyperpolarizing factor-type relaxation in rat femoral artery. *Biol Pharm Bull.* 2020;43(3):569-73.
42. Ufnal M, Jazwiec R, Dadlez M, et al. Trimethylamine-N-oxide: a carnitine-derived metabolite that prolongs the hypertensive effect of angiotensin II in rats. *Can J Cardiol.* 2014;30(12):1700-5.

43. Li D, Ke Y, Zhan R, et al. Trimethylamine-N-oxide promotes brain aging and cognitive impairment in mice. *Aging Cell*. 2018:e12768.
44. Brunt VE, Gioscia-Ryan RA, Richey JJ, et al. Suppression of the gut microbiome ameliorates age-related arterial dysfunction and oxidative stress in mice. *J Physiol*. 2019;597(9):2361-78.
45. Li X, Sun Y, Zhang X, et al. Reductions in gut microbiota-derived metabolite trimethylamine N-oxide in the circulation may ameliorate myocardial infarction-induced heart failure in rats, possibly by inhibiting interleukin-8 secretion. *Mol Med Rep*. 2019;20(1):779-86.
46. Organ CL, Otsuka H, Bhushan S, et al. Choline diet and its gut microbe derived metabolite, trimethylamine N-oxide (TMAO), exacerbate pressure overload-induced heart failure. *Circ Heart Fail*. 2016;9(1):e002314.
47. Savi M, Bocchi L, Bresciani L, et al. Trimethylamine-N-oxide (TMAO)-induced impairment of cardiomyocyte function and the protective role of urolithin B-glucuronide. *Molecules*. 2018;23(3).
48. Makrecka-Kuka M, Volska K, Antone U, et al. Trimethylamine N-oxide impairs pyruvate and fatty acid oxidation in cardiac mitochondria. *Toxicol Lett*. 2017;267:32-8.
49. Jin B, Ji F, Zuo A, et al. Destructive role of TMAO in T-tubule and excitation-contraction coupling in the adult cardiomyocytes. *Int Heart J*. 2020;61(2):355-63.
50. Oakley CI, Vallejo JA, Wang D, et al. Trimethylamine-N-oxide acutely increases cardiac muscle contractility. *Am J Physiol Heart Circ Physiol*. 2020;318(5):H1272-H82.
51. Jaworska K, Hering D, Mosieniak G, et al. TMA, a forgotten uremic toxin, but not TMAO, is involved in cardiovascular pathology. *Toxins (Basel)*. 2019;11(9).
52. Yu L, Meng G, Huang B, et al. A potential relationship between gut microbes and atrial fibrillation: Trimethylamine N-oxide, a gut microbe-derived metabolite, facilitates the progression of atrial fibrillation. *Int J Cardiol*. 2018;255:92-8.
53. Missailidis C, Hällqvist J, Qureshi AR, et al. Serum trimethylamine-N-oxide is strongly related to renal function and predicts outcome in chronic kidney disease. *PLoS One*. 2016;11(1):e0141738.

54. Shan Z, Sun T, Huang H, et al. Association between microbiota-dependent metabolite trimethylamine-N-oxide and type 2 diabetes. *Am J Clin Nutr.* 2017;106(3):888-94.
55. Miao J, Ling AV, Manthena PV, et al. Flavin-containing monooxygenase 3 as a potential player in diabetes-associated atherosclerosis. *Nat Commun.* 2015;6:6498.
56. Schugar RC, Shih DM, Warriar M, et al. The TMAO-producing enzyme flavin-containing monooxygenase 3 regulates obesity and the beiging of white adipose tissue. *Cell Rep.* 2017;19(12):2451-61.
57. Ronco C, Haapio M, House AA, et al. Cardiorenal syndrome. *J Am Coll Cardiol.* 2008;52(19):1527-39.
58. Huc T, Drapala A, Gawrys M, et al. Chronic, low-dose TMAO treatment reduces diastolic dysfunction and heart fibrosis in hypertensive rats. *Am J Physiol Heart Circ Physiol.* 2018;315(6):H1805-H20.
59. Wang Z, Levison BS, Hazen JE, et al. Measurement of trimethylamine-N-oxide by stable isotope dilution liquid chromatography tandem mass spectrometry. *Anal Biochem.* 2014;455:35-40.
60. Tang WH, Wang Z, Shrestha K, et al. Intestinal microbiota-dependent phosphatidylcholine metabolites, diastolic dysfunction, and adverse clinical outcomes in chronic systolic heart failure. *J Card Fail.* 2015;21(2):91-6.
61. Kruger R, Merz B, Rist MJ, et al. Associations of current diet with plasma and urine TMAO in the KarMeN study: direct and indirect contributions. *Mol Nutr Food Res.* 2017;61(11).
62. Cho CE, Taesuwan S, Malysheva OV, et al. Trimethylamine-N-oxide (TMAO) response to animal source foods varies among healthy young men and is influenced by their gut microbiota composition: A randomized controlled trial. *Mol Nutr Food Res.* 2017;61(1).
63. DiMarco DM, Missimer A, Murillo AG, et al. Intake of up to 3 eggs/day increases HDL cholesterol and plasma choline while plasma trimethylamine-N-oxide is unchanged in a healthy population. *Lipids.* 2017;52(3):255-63.

64. Dambrova M, Latkovskis G, Kuka J, et al. Diabetes is associated with higher trimethylamine N-oxide plasma levels. *Exp Clin Endocrinol Diabetes*. 2016;124(4):251-6.
65. Meyer KA, Benton TZ, Bennett BJ, et al. Microbiota-dependent metabolite trimethylamine N-oxide and coronary artery calcium in the coronary artery risk development in young adults study (CARDIA). *J Am Heart Assoc*. 2016;5(10).
66. Obeid R, Awwad HM, Rabagny Y, et al. Plasma trimethylamine N-oxide concentration is associated with choline, phospholipids, and methyl metabolism. *Am J Clin Nutr*. 2016;103(3):703-11.
67. Fu BC, Hullar MAJ, Randolph TW, et al. Associations of plasma trimethylamine N-oxide, choline, carnitine, and betaine with inflammatory and cardiometabolic risk biomarkers and the fecal microbiome in the Multiethnic Cohort Adiposity Phenotype Study. *Am J Clin Nutr*. 2020;111(6):1226-34.
68. Xu K-Y, Xia G-H, Lu J-Q, et al. Impaired renal function and dysbiosis of gut microbiota contribute to increased trimethylamine-N-oxide in chronic kidney disease patients. *Scientific Reports*. 2017;7:1445.
69. Gruppen EG, Garcia E, Connelly MA, et al. TMAO is associated with mortality: Impact of modestly impaired renal function. *Scientific Reports*. 2017;7(1):13781.
70. Al-Obaide MAI, Singh R, Datta P, et al. Gut microbiota-dependent trimethylamine-N-oxide and serum biomarkers in patients with T2DM and advanced CKD. *J Clin Med*. 2017;6(9).
71. Wang Z, Tang WH, Buffa JA, et al. Prognostic value of choline and betaine depends on intestinal microbiota-generated metabolite trimethylamine-N-oxide. *Eur Heart J*. 2014;35(14):904-10.
72. Kaysen GA, Johansen KL, Chertow GM, et al. Associations of trimethylamine N-oxide with nutritional and inflammatory biomarkers and cardiovascular outcomes in patients new to dialysis. *J Ren Nutr*. 2015;25(4):351-6.
73. Chistiakov DA, Bobryshev YV, Kozarov E, et al. Role of gut microbiota in the modulation of atherosclerosis-associated immune response. *Front Microbiol*. 2015;6:671.

74. Brown JM, Hazen SL. Metaorganismal nutrient metabolism as a basis of cardiovascular disease. *Curr Opin Lipidol*. 2014;25(1):48-53.
75. Tang WH, Wang Z, Fan Y, et al. Prognostic value of elevated levels of intestinal microbe-generated metabolite trimethylamine-N-oxide in patients with heart failure: refining the gut hypothesis. *J Am Coll Cardiol*. 2014;64(18):1908-14.
76. Schiattarella GG, Sannino A, Toscano E, et al. Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker: a systematic review and dose-response meta-analysis. *Eur Heart J*. 2017;38(39):2948-56.
77. Roe AJ, Zhang S, Bhadelia RA, et al. Choline and its metabolites are differently associated with cardiometabolic risk factors, history of cardiovascular disease, and MRI-documented cerebrovascular disease in older adults. *Am J Clin Nutr*. 2017;105(6):1283-90.
78. Mueller DM, Allenspach M, Othman A, et al. Plasma levels of trimethylamine-N-oxide are confounded by impaired kidney function and poor metabolic control. *Atherosclerosis*. 2015;243(2):638-44.
79. Lever M, George PM, Slow S, et al. Betaine and trimethylamine-N-oxide as predictors of cardiovascular outcomes show different patterns in diabetes mellitus: An observational study. *PLoS One*. 2014;9(12):e114969.
80. Tang WH, Wang Z, Kennedy DJ, et al. Gut microbiota-dependent trimethylamine N-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease. *Circ Res*. 2015;116(3):448-55.
81. Jia J, Dou P, Gao M, et al. Assessment of causal direction between gut microbiota-dependent metabolites and cardiometabolic health: A bidirectional mendelian randomization analysis. *Diabetes*. 2019;68(9):1747-55.
82. Barrea L, Annunziata G, Muscogiuri G, et al. Trimethylamine-N-oxide (TMAO) as novel potential biomarker of early predictors of metabolic syndrome. *Nutrients*. 2018;10(12).

83. Wang Z, Bergeron N, Levison BS, et al. Impact of chronic dietary red meat, white meat, or non-meat protein on trimethylamine N-oxide metabolism and renal excretion in healthy men and women. *Eur Heart J*. 2019;40(7):583-94.
84. Teft WA, Morse BL, Leake BF, et al. Identification and characterization of trimethylamine-N-oxide uptake and efflux transporters. *Mol Pharm*. 2017;14(1):310-8.
85. Li X, Chen Y, Liu J, et al. Serum metabolic variables associated with impaired glucose tolerance induced by high-fat-high-cholesterol diet in *Macaca mulatta*. *Exp Biol Med (Maywood)*. 2012;237(11):1310-21.
86. Millard HR, Musani SK, Dibaba DT, et al. Dietary choline and betaine; associations with subclinical markers of cardiovascular disease risk and incidence of CVD, coronary heart disease and stroke: the Jackson Heart Study. *Eur J Nutr*. 2018;57(1):51-60.
87. Haring B, Gronroos N, Nettleton JA, et al. Dietary protein intake and coronary heart disease in a large community based cohort: results from the Atherosclerosis Risk in Communities (ARIC) study [corrected]. *PLoS One*. 2014;9(10):e109552.
88. Abete I, Romaguera D, Vieira AR, et al. Association between total, processed, red and white meat consumption and all-cause, CVD and IHD mortality: a meta-analysis of cohort studies. *Br J Nutr*. 2014;112(5):762-75.
89. Wang X, Lin X, Ouyang YY, et al. Red and processed meat consumption and mortality: dose-response meta-analysis of prospective cohort studies. *Public Health Nutr*. 2016;19(5):893-905.
90. Rohrmann S, Linseisen J, Allenspach M, et al. Plasma concentrations of trimethylamine-N-oxide are directly associated with dairy food consumption and low-grade inflammation in a German adult population. *J Nutr*. 2016;146(2):283-9.
91. Koeth RA, Levison BS, Culley MK, et al. gamma-Butyrobetaine is a proatherogenic intermediate in gut microbial metabolism of L-carnitine to TMAO. *Cell Metab*. 2014;20(5):799-812.

92. Stremmel W, Schmidt KV, Schuhmann V, et al. Blood trimethylamine-N-oxide originates from microbiota mediated breakdown of phosphatidylcholine and absorption from small intestine. *PLoS One*. 2017;12(1):e0170742.
93. Lang DH, Yeung CK, Peter RM, et al. Isoform specificity of trimethylamine N-oxygenation by human flavin-containing monooxygenase (FMO) and P450 enzymes: selective catalysis by FMO3. *Biochem Pharmacol*. 1998;56(8):1005-12.
94. Dolphin CT, Janmohamed A, Smith RL, et al. Missense mutation in flavin-containing monooxygenase 3 gene, FMO3, underlies fish-odour syndrome. *Nat Genet*. 1997;17(4):491-4.
95. Fennema D, Phillips IR, Shephard EA. Trimethylamine and trimethylamine N-oxide, a flavin-containing monooxygenase 3 (FMO3)-mediated host-microbiome metabolic axis implicated in health and disease. *Drug Metab Dispos*. 2016;44(11):1839-50.
96. Wiedeman AM, Barr SI, Green TJ, et al. Dietary choline intake: Current state of knowledge across the life cycle. *Nutrients*. 2018;10(10).
97. West AA, Shih Y, Wang W, et al. Egg n-3 fatty acid composition modulates biomarkers of choline metabolism in free-living lacto-ovo-vegetarian women of reproductive age. *J Acad Nutr Diet*. 2014;114(10):1594-600.
98. Ufnal M, Zadlo A, Ostaszewski R. TMAO: A small molecule of great expectations. *Nutrition*. 2015;31(11-12):1317-23.
99. Sheard NF, Zeisel SH. An in vitro study of choline uptake by intestine from neonatal and adult rats. *Pediatr Res*. 1986;20(8):768-72.
100. Sanford PA, Smyth DH. Intestinal transfer of choline in rat and hamster. *J Physiol*. 1971;215(3):769-88.
101. Zeisel SH, DaCosta KA, Fox JG. Endogenous formation of dimethylamine. *Biochem J*. 1985;232(2):403-8.

102. Miller CA, Corbin KD, da Costa KA, et al. Effect of egg ingestion on trimethylamine-N-oxide production in humans: a randomized, controlled, dose-response study. *Am J Clin Nutr*. 2014;100(3):778-86.
103. Chung RWS, Wang Z, Bursill CA, et al. Effect of long-term dietary sphingomyelin supplementation on atherosclerosis in mice. *PLoS One*. 2017;12(12):e0189523.
104. Zhu W, Wang Z, Tang WHW, et al. Gut microbe-generated trimethylamine N-oxide from dietary choline is prothrombotic in subjects. *Circulation*. 2017;135(17):1671-3.
105. Meyer KA, Shea JW. Dietary choline and betaine and risk of CVD: A systematic review and meta-analysis of prospective studies. *Nutrients*. 2017;9(7).
106. Nagata C, Wada K, Tamura T, et al. Choline and betaine intakes are not associated with cardiovascular disease mortality risk in Japanese men and women. *J Nutr*. 2015;145(8):1787-92.
107. Boutagy NE, Neilson AP, Osterberg KL, et al. Short-term high-fat diet increases postprandial trimethylamine-N-oxide in humans. *Nutr Res*. 2015;35(10):858-64.
108. Rebouche CJ, Seim H. Carnitine metabolism and its regulation in microorganisms and mammals. *Annu Rev Nutr*. 1998;18:39-61.
109. Meadows JA, Wargo MJ. Carnitine in bacterial physiology and metabolism. *Microbiology*. 2015;161(6):1161-74.
110. Hoppel C. The role of carnitine in normal and altered fatty acid metabolism. *Am J Kidney Dis*. 2003;41(4 Suppl 4):S4-12.
111. Harper P, Elwin CE, Cederblad G. Pharmacokinetics of intravenous and oral bolus doses of L-carnitine in healthy subjects. *Eur J Clin Pharmacol*. 1988;35(5):555-62.
112. Bain MA, Milne RW, Evans AM. Disposition and metabolite kinetics of oral L-carnitine in humans. *J Clin Pharmacol*. 2006;46(10):1163-70.
113. Vallance HD, Koochin A, Branov J, et al. Marked elevation in plasma trimethylamine-N-oxide (TMAO) in patients with mitochondrial disorders treated with oral l-carnitine. *Mol Genet Metab Rep*. 2018;15:130-3.

114. Ussher JR, Lopaschuk GD, Arduini A. Gut microbiota metabolism of L-carnitine and cardiovascular risk. *Atherosclerosis*. 2013;231(2):456-61.
115. Bene J, Hadzsiev K, Melegh B. Role of carnitine and its derivatives in the development and management of type 2 diabetes. *Nutr Diabetes*. 2018;8(1):8.
116. Noland RC, Koves TR, Seiler SE, et al. Carnitine insufficiency caused by aging and overnutrition compromises mitochondrial performance and metabolic control. *J Biol Chem*. 2009;284(34):22840-52.
117. DiNicolantonio JJ, Lavie CJ, Fares H, et al. L-carnitine in the secondary prevention of cardiovascular disease: systematic review and meta-analysis. *Mayo Clin Proc*. 2013;88(6):544-51.
118. Craig SA. Betaine in human nutrition. *Am J Clin Nutr*. 2004;80(3):539-49.
119. Hoffmann L, Brauers G, Gehrman T, et al. Osmotic regulation of hepatic betaine metabolism. *Am J Physiol Gastrointest Liver Physiol*. 2013;304(9):G835-46.
120. Zeisel SH, Mar MH, Howe JC, et al. Concentrations of choline-containing compounds and betaine in common foods. *J Nutr*. 2003;133(5):1302-7.
121. Weinhold PA, Sanders R. The oxidation of choline by liver slices and mitochondria during liver development in the rat. *Life Sciences*. 1973;13(5):621-9.
122. Andresen PA, Kaasen I, Styrvold OB, et al. Molecular cloning, physical mapping and expression of the bet genes governing the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. *J Gen Microbiol*. 1988;134(6):1737-46.
123. Naumann E, Hippe H, Gottschalk G. Betaine: New oxidant in the Stickland reaction and methanogenesis from betaine and l-alanine by a *Clostridium sporogenes*-*Methanosarcina barkeri* coculture. *Appl Environ Microbiol*. 1983;45(2):474-83.
124. Wood AP, Warren FJ, Kelly DP. Methylotrophic bacteria in trimethylaminuria In: K.N. T, editor. *Handbook of Hydrocarbon and Lipid Microbiology*. Berlin, Heidelberg: Springer; 2010. p. 3227-40.

125. Zawieja EE, Zawieja B, Chmurzynska A. Betaine supplementation moderately increases total cholesterol levels: A systematic review and meta-analysis. *J Diet Suppl.* 2019;1-13.
126. Guasch-Ferre M, Hu FB, Ruiz-Canela M, et al. Plasma Metabolites From Choline Pathway and Risk of Cardiovascular Disease in the PREDIMED (Prevention With Mediterranean Diet) Study. *J Am Heart Assoc.* 2017;6(11).
127. Garcia E, Osté MCJ, Bennett DW, et al. High betaine, a trimethylamine N-oxide related metabolite, is prospectively associated with low future risk of type 2 diabetes mellitus in the PREVEND study. *J Clin Med.* 2019;8(11).
128. Muramatsu H, Matsuo H, Okada N, et al. Characterization of ergothionase from *Burkholderia* sp. HME13 and its application to enzymatic quantification of ergothioneine. *Appl Microbiol Biotechnol.* 2013;97(12):5389-400.
129. Cheah IK, Halliwell B. Ergothioneine; antioxidant potential, physiological function and role in disease. *Biochim Biophys Acta.* 2012;1822(5):784-93.
130. Melville DB, Horner WH, Lubschez R. Tissue ergothioneine. *J Biol Chem.* 1954;206(1):221-8.
131. Ey J, Schömig E, Taubert D. Dietary sources and antioxidant effects of ergothioneine. *J Agric Food Chem.* 2007;55(16):6466-74.
132. Nakamura T, Sugiura S, Kobayashi D, et al. Decreased proliferation and erythroid differentiation of K562 cells by siRNA-induced depression of OCTN1 (SLC22A4) transporter gene. *Pharm Res.* 2007;24(9):1628-35.
133. Halliwell B, Cheah IK, Tang RMY. Ergothioneine - a diet-derived antioxidant with therapeutic potential. *FEBS Lett.* 2018;592(20):3357-66.
134. Lee DH, Yang M, Giovannucci EL, et al. Mushroom consumption, biomarkers, and risk of cardiovascular disease and type 2 diabetes: A prospective cohort study of US women and men. *Am J Clin Nutr.* 2019;110(3):666-74.
135. Papandreou C, Becerra-Tomás N, Bulló M, et al. Legume consumption and risk of all-cause, cardiovascular, and cancer mortality in the PREDIMED study. *Clin Nutr.* 2019;38(1):348-56.

136. Becerra-Tomás N, Díaz-López A, Rosique-Esteban N, et al. Legume consumption is inversely associated with type 2 diabetes incidence in adults: A prospective assessment from the PREDIMED study. *Clin Nutr*. 2018;37(3):906-13.
137. Zhang AQ, Mitchell SC, Smith RL. Dietary precursors of trimethylamine in man: a pilot study. *Food Chem Toxicol*. 1999;37(5):515-20.
138. Landfald B, Valeur J, Berstad A, et al. Microbial trimethylamine-N-oxide as a disease marker: something fishy? *Microb Ecol Health Dis*. 2017;28(1):1327309.
139. Withers PC MG, Guppy M Buoyancy role of urea and TMAO in an elasmobranch fish, the port Jackson Shark, *Heterodontus portusjacksoni*. *Physiol Zool*. 1994;67(3):693-705.
140. Lenz EM, Bright J, Wilson ID, et al. Metabonomics, dietary influences and cultural differences: a ¹H NMR-based study of urine samples obtained from healthy British and Swedish subjects. *J Pharm Biomed Anal*. 2004;36(4):841-9.
141. Dumas ME, Maibaum EC, Teague C, et al. Assessment of analytical reproducibility of ¹H NMR spectroscopy based metabonomics for large-scale epidemiological research: the INTERMAP Study. *Anal Chem*. 2006;78(7):2199-208.
142. Schmedes M, Brejnrod AD, Aadland EK, et al. The effect of lean-seafood and non-seafood diets on fecal metabolites and gut microbiome: Results from a randomized crossover intervention study. *Mol Nutr Food Res*. 2018.
143. Falls JG, Ryu DY, Cao Y, et al. Regulation of mouse liver flavin-containing monooxygenases 1 and 3 by sex steroids. *Arch Biochem Biophys*. 1997;342(2):212-23.
144. Esposito T, Varriale B, D'Angelo R, et al. Regulation of flavin-containing mono-oxygenase (Fmo3) gene expression by steroids in mice and humans. *Horm Mol Biol Clin Investig*. 2014;20(3):99-109.
145. Cashman JR, Xiong Y, Lin J, et al. In vitro and in vivo inhibition of human flavin-containing monooxygenase form 3 (FMO3) in the presence of dietary indoles. *Biochem Pharmacol*. 1999;58(6):1047-55.

146. Astafev AA, Patel SA, Kondratov RV. Calorie restriction effects on circadian rhythms in gene expression are sex dependent. *Scientific Reports*. 2017;7(1):9716.
147. Guo D, Shen Y, Li W, et al. Upregulation of flavin-containing monooxygenase 3 mimics calorie restriction to retard liver aging by inducing autophagy. *Aging (Albany NY)*. 2020;12(1):931-44.
148. Tanino T, Bando T, Komada A, et al. Hepatic flavin-containing monooxygenase 3 enzyme suppressed by type 1 allergy-produced nitric oxide. *Drug Metab Dispos*. 2017;45(11):1189-96.
149. Klick DE, Shadley JD, Hines RN. Differential regulation of human hepatic flavin containing monooxygenase 3 (FMO3) by CCAAT/enhancer-binding protein beta (C/EBPbeta) liver inhibitory and liver activating proteins. *Biochem Pharmacol*. 2008;76(2):268-78.
150. Koukouritaki SB, Simpson P, Yeung CK, et al. Human hepatic flavin-containing monooxygenases 1 (FMO1) and 3 (FMO3) developmental expression. *Pediatr Res*. 2002;51(2):236-43.
151. Xu M, Bhatt DK, Yeung CK, et al. Genetic and nongenetic factors associated with protein abundance of flavin-containing monooxygenase 3 in human liver. *J Pharmacol Exp Ther*. 2017;363(2):265-74.
152. Fu ZD, Csanaky IL, Klaassen CD. Effects of aging on mRNA profiles for drug-metabolizing enzymes and transporters in livers of male and female mice. *Drug Metab Dispos*. 2012;40(6):1216-25.
153. Shimizu M, Cashman JR, Yamazaki H. Transient trimethylaminuria related to menstruation. *BMC Med Genet*. 2007;8:2.
154. Hukkanen J, Dempsey D, Jacob P, 3rd, et al. Effect of pregnancy on a measure of FMO3 activity. *Br J Clin Pharmacol*. 2005;60(2):224-6.
155. Johnson C, Prokopienko AJ, West RE, 3rd, et al. Decreased kidney function is associated with enhanced hepatic flavin monooxygenase activity and increased circulating trimethylamine N-oxide concentrations in mice. *Drug Metab Dispos*. 2018;46(9):1304-9.

156. Zhang J, Chaluvadi MR, Reddy R, et al. Hepatic flavin-containing monooxygenase gene regulation in different mouse inflammation models. *Drug Metab Dispos.* 2009;37(3):462-8.
157. Petriello MC, Hoffman JB, Sunkara M, et al. Dioxin-like pollutants increase hepatic flavin containing monooxygenase (FMO3) expression to promote synthesis of the pro-atherogenic nutrient biomarker trimethylamine N-oxide from dietary precursors. *J Nutr Biochem.* 2016;33:145-53.
158. Liao BM, McManus SA, Hughes WE, et al. Flavin-containing monooxygenase 3 reduces endoplasmic reticulum stress in lipid-treated hepatocytes. *Mol Endocrinol.* 2016;30(4):417-28.
159. Xu J, Zhang J, Cai S, et al. Metabonomics studies of intact hepatic and renal cortical tissues from diabetic db/db mice using high-resolution magic-angle spinning ¹H NMR spectroscopy. *Anal Bioanal Chem.* 2009;393(6-7):1657-68.
160. Barton S, Navarro SL, Buas MF, et al. Targeted plasma metabolome response to variations in dietary glycemic load in a randomized, controlled, crossover feeding trial in healthy adults. *Food Funct.* 2015;6(9):2949-56.
161. Augustin LS, Franceschi S, Jenkins DJ, et al. Glycemic index in chronic disease: a review. *Eur J Clin Nutr.* 2002;56(11):1049-71.
162. Huo T, Cai S, Lu X, et al. Metabonomic study of biochemical changes in the serum of type 2 diabetes mellitus patients after the treatment of metformin hydrochloride. *J Pharm Biomed Anal.* 2009;49(4):976-82.
163. Warriar M, Shih DM, Burrows AC, et al. The TMAO-generating enzyme flavin monooxygenase 3 is a central regulator of cholesterol balance. *Cell Rep.* 2015.
164. Shih DM, Wang Z, Lee R, et al. Flavin containing monooxygenase 3 exerts broad effects on glucose and lipid metabolism and atherosclerosis. *J Lipid Res.* 2015;56(1):22-37.
165. Kusuhara H, Sugiyama Y. ATP-binding cassette, subfamily G (ABCG family). *Pflugers Arch.* 2007;453(5):735-44.
166. Horsey A, Cox M, Sarwat S, et al. The multidrug transporter ABCG2: still more questions than answers. *Biochem Soc Trans.* 2016;44(3):824-30.

167. Al-Waiz M, Mitchell SC, Idle JR, et al. The metabolism of ¹⁴C-labelled trimethylamine and its N-oxide in man. *Xenobiotica*. 1987;17(5):551-8.
168. Bell JD, Lee JA, Lee HA, et al. Nuclear magnetic resonance studies of blood plasma and urine from subjects with chronic renal failure: identification of trimethylamine-N-oxide. *Biochim Biophys Acta*. 1991;1096(2):101-7.
169. Doucet C, Dutheil D, Petit I, et al. Influence of colloid, preservation medium and trimetazidine on renal medulla injury. *Biochim Biophys Acta*. 2004;1673(3):105-14.
170. Xu R, Wang Q, Li L. A genome-wide systems analysis reveals strong link between colorectal cancer and trimethylamine N-oxide (TMAO), a gut microbial metabolite of dietary meat and fat. *BMC Genomics*. 2015;16(Suppl 7):S4.
171. Hartiala J, Bennett BJ, Tang WH, et al. Comparative genome-wide association studies in mice and humans for trimethylamine N-oxide, a proatherogenic metabolite of choline and L-carnitine. *Arterioscler Thromb Vasc Biol*. 2014;34(6):1307-13.
172. al-Waiz M, Mikov M, Mitchell SC, et al. The exogenous origin of trimethylamine in the mouse. *Metabolism*. 1992;41(2):135-6.
173. Ferguson JF. Meat-loving microbes: do steak-eating bacteria promote atherosclerosis? *Circ Cardiovasc Genet*. 2013;6(3):308-9.
174. Ross AB, Zangger A, Guiraud SP. Cereal foods are the major source of betaine in the Western diet--analysis of betaine and free choline in cereal foods and updated assessments of betaine intake. *Food Chem*. 2014;145:859-65.
175. Matijasic BB, Obermajer T, Lipoglavsek L, et al. Association of dietary type with fecal microbiota in vegetarians and omnivores in Slovenia. *Eur J Nutr*. 2014;53(4):1051-64.
176. Kabeerdoss J, Devi RS, Mary RR, et al. Faecal microbiota composition in vegetarians: comparison with omnivores in a cohort of young women in southern India. *Br J Nutr*. 2012;108(6):953-7.

177. Tomova A, Bukovsky I, Rembert E, et al. The effects of vegetarian and vegan diets on gut microbiota. *Front Nutr*. 2019;6:47.
178. Smits LP, Kootte RS, Levin E, et al. Effect of vegan fecal microbiota transplantation on carnitine- and choline-derived trimethylamine-N-oxide production and vascular inflammation in patients With metabolic syndrome. *J Am Heart Assoc*. 2018;7(7).
179. Chao CK, Zeisel SH. Formation of trimethylamine from dietary choline by *Streptococcus sanguis* I, which colonizes the mouth. *J Nutr Biochem*. 1990;1(2):89-97.
180. Craciun S, Balskus EP. Microbial conversion of choline to trimethylamine requires a glycy radical enzyme. *Proc Natl Acad Sci U S A*. 2012;109(52):21307-12.
181. Zhu Y, Jameson E, Crosatti M, et al. Carnitine metabolism to trimethylamine by an unusual Rieske-type oxygenase from human microbiota. *Proc Natl Acad Sci U S A*. 2014;111(11):4268-73.
182. Martinez-del Campo A, Bodea S, Hamer HA, et al. Characterization and detection of a widely distributed gene cluster that predicts anaerobic choline utilization by human gut bacteria. *MBio*. 2015;6(2).
183. Falony G, Vieira-Silva S, Raes J. Microbiology meets big data: The case of gut microbiota-derived trimethylamine. *Annu Rev Microbiol*. 2015;69:305-21.
184. Wu GD, Chen J, Hoffmann C, et al. Linking long-term dietary patterns with gut microbial enterotypes. *Science*. 2011;334(6052):105-8.
185. Ruengsomwong S, Korenori Y, Sakamoto N, et al. Senior Thai fecal microbiota comparison between vegetarians and non-vegetarians using PCR-DGGE and real-time PCR. *J Microbiol Biotechnol*. 2014;24(8):1026-33.
186. Obeid R, Awwad HM, Keller M, et al. Trimethylamine-N-oxide and its biological variations in vegetarians. *Eur J Nutr*. 2017;56(8):2599-609.
187. Troseid M, Ueland T, Hov JR, et al. Microbiota-dependent metabolite trimethylamine-N-oxide is associated with disease severity and survival of patients with chronic heart failure. *J Intern Med*. 2015;277(6):717-26.

188. Lomeli N, Bota DA, Davies KJA. Diminished stress resistance and defective adaptive homeostasis in age-related diseases. *Clin Sci (Lond)*. 2017;131(21):2573-99.
189. Brown JM, Hazen SL. The gut microbial endocrine organ: Bacterially-derived signals driving cardiometabolic diseases. *Annu Rev Med*. 2015;66:343-59.
190. Imhann F, Bonder MJ, Vich Vila A, et al. Proton pump inhibitors affect the gut microbiome. *Gut*. 2016;65(5):740-8.
191. Rogers MAM, Aronoff DM. The influence of non-steroidal anti-inflammatory drugs on the gut microbiome. *Clin Microbiol Infect*. 2016;22(2):178 e1- e9.
192. Maier L, Pruteanu M, Kuhn M, et al. Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature*. 2018;555(7698):623-8.
193. Tripolt NJ, Leber B, Triebl A, et al. Effect of *Lactobacillus casei* Shirota supplementation on trimethylamine-N-oxide levels in patients with metabolic syndrome: An open-label, randomized study. *Atherosclerosis*. 2015;242(1):141-4.
194. Borges NA, Stenvinkel P, Bergman P, et al. Effects of probiotic supplementation on trimethylamine-N-oxide plasma levels in hemodialysis patients: A pilot study. *Probiotics Antimicrob Proteins*. 2018.
195. Khalesi S, Sun J, Buys N, et al. Effect of probiotics on blood pressure: a systematic review and meta-analysis of randomized, controlled trials. *Hypertension*. 2014;64(4):897-903.
196. Shimizu M, Hashiguchi M, Shiga T, et al. Meta-analysis: Effects of probiotic supplementation on lipid profiles in normal to mildly hypercholesterolemic individuals. *PLoS One*. 2015;10(10):e0139795.
197. Khalesi S, Bellissimo N, Vandelanotte C, et al. A review of probiotic supplementation in healthy adults: helpful or hype? *Eur J Clin Nutr*. 2018.
198. David LA, Maurice CF, Carmody RN, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature*. 2014;505(7484):559-63.

199. Mitchell SC, Smith RL. Trimethylaminuria: the fish malodor syndrome. *Drug Metab Dispos.* 2001;29(4 Pt 2):517-21.
200. Griffin LE, Djuric Z, Angiletta CJ, et al. A Mediterranean diet does not alter plasma trimethylamine N-oxide concentrations in healthy adults at risk for colon cancer. *Food Funct.* 2019;10(4):2138-47.
201. Ghonimy A, Zhang DM, Farouk MH, et al. The impact of carnitine on dietary fiber and gut bacteria metabolism and their mutual interaction in monogastrics. *Int J Mol Sci.* 2018;19(4).
202. Zabell A, Tang WHW. Targeting the microbiome in heart failure. *Curr Treat Options Cardiovasc Med.* 2017;19(4):27.
203. Jiang XC, Paultre F, Pearson TA, et al. Plasma sphingomyelin level as a risk factor for coronary artery disease. *Arterioscler Thromb Vasc Biol.* 2000;20(12):2614-8.
204. Li Z, Fan Y, Liu J, et al. Impact of sphingomyelin synthase 1 deficiency on sphingolipid metabolism and atherosclerosis in mice. *Arterioscler Thromb Vasc Biol.* 2012;32(7):1577-84.
205. Nelson JC, Jiang XC, Tabas I, et al. Plasma sphingomyelin and subclinical atherosclerosis: findings from the multi-ethnic study of atherosclerosis. *Am J Epidemiol.* 2006;163(10):903-12.
206. Wang F, Zheng J, Yang B, et al. Effects of vegetarian diets on blood lipids: A systematic review and meta-analysis of randomized controlled trials. *J Am Heart Assoc.* 2015;4(10):e002408.
207. Massera D, Graf L, Barba S, et al. Angina rapidly improved with a plant-based diet and returned after resuming a Western diet. *J Geriatr Cardiol.* 2016;13(4):364-6.
208. Wang X, Ouyang Y, Liu J, et al. Fruit and vegetable consumption and mortality from all causes, cardiovascular disease, and cancer: systematic review and dose-response meta-analysis of prospective cohort studies. *BMJ.* 2014;349:g4490.
209. Bazzano LA, He J, Ogden LG, et al. Fruit and vegetable intake and risk of cardiovascular disease in US adults: the first National Health and Nutrition Examination Survey Epidemiologic Follow-up Study. *Am J Clin Nutr.* 2002;76(1):93-9.

210. Glick-Bauer M, Yeh MC. The health advantage of a vegan diet: exploring the gut microbiota connection. *Nutrients*. 2014;6(11):4822-38.
211. Esselstyn CB, Jr. Resolving the coronary artery disease epidemic through plant-based nutrition. *Prev Cardiol*. 2001;4(4):171-7.
212. Nagura J, Iso H, Watanabe Y, et al. Fruit, vegetable and bean intake and mortality from cardiovascular disease among Japanese men and women: the JACC Study. *Br J Nutr*. 2009;102(2):285-92.
213. Strandhagen E, Hansson PO, Bosaeus I, et al. High fruit intake may reduce mortality among middle-aged and elderly men. The Study of men born in 1913. *Eur J Clin Nutr*. 2000;54(4):337-41.
214. He FJ, Nowson CA, MacGregor GA. Fruit and vegetable consumption and stroke: meta-analysis of cohort studies. *Lancet*. 2006;367(9507):320-6.
215. Streppel MT, Ocke MC, Boshuizen HC, et al. Long-term fish consumption and n-3 fatty acid intake in relation to (sudden) coronary heart disease death: the Zutphen study. *Eur Heart J*. 2008;29(16):2024-30.
216. Odermatt A. The Western-style diet: a major risk factor for impaired kidney function and chronic kidney disease. *Am J Physiol Renal Physiol*. 2011;301(5):F919-31.
217. van den Berg E, Hospers FA, Navis G, et al. Dietary acid load and rapid progression to end-stage renal disease of diabetic nephropathy in Westernized South Asian people. *J Nephrol*. 2011;24(1):11-7.
218. Rohrmann S, Linseisen J. Processed meat: the real villain? *Proc Nutr Soc*. 2016;75(3):233-41.
219. Chen X, Wei G, Jalili T, et al. The associations of plant protein intake with all-cause mortality in CKD. *Am J Kidney Dis*. 2016;67(3):423-30.
220. Li SS, Blanco Mejia S, Lytvyn L, et al. Effect of plant protein on blood lipids: A systematic review and meta-analysis of randomized controlled trials. *J Am Heart Assoc*. 2017;6(12).
221. Richter CK, Skulas-Ray AC, Champagne CM, et al. Plant protein and animal proteins: do they differentially affect cardiovascular disease risk? *Adv Nutr*. 2015;6(6):712-28.

222. Berryman CE, Agarwal S, Lieberman HR, et al. Diets higher in animal and plant protein are associated with lower adiposity and do not impair kidney function in US adults. *Am J Clin Nutr*. 2016;104(3):743-9.
223. Mehrabani S, Asemi M, Najafian J, et al. Association of animal and plant proteins intake with hypertension in Iranian adult population: Isfahan Healthy Heart Program. *Adv Biomed Res*. 2017;6:112.
224. Song M, Fung TT, Hu FB, et al. Association of animal and plant protein intake with all-cause and cause-specific mortality. *JAMA Intern Med*. 2016;176(10):1453-63.
225. Yazdekhosti N, Brandsch C, Schmidt N, et al. Fish protein increases circulating levels of trimethylamine-N-oxide and accelerates aortic lesion formation in apoE null mice. *Mol Nutr Food Res*. 2016;60(2):358-68.
226. Bain MA, Fornasini G, Evans AM. Trimethylamine: metabolic, pharmacokinetic and safety aspects. *Curr Drug Metab*. 2005;6(3):227-40.
227. Annunziata G, Maisto M, Schisano C, et al. Effects of Grape Pomace Polyphenolic Extract (Taurisolo((R))) in Reducing TMAO Serum Levels in Humans: Preliminary Results from a Randomized, Placebo-Controlled, Cross-Over Study. *Nutrients*. 2019;11(1).
228. Annunziata G, Maisto M, Schisano C, et al. Effect of Grape Pomace Polyphenols With or Without Pectin on TMAO Serum Levels Assessed by LC/MS-Based Assay: A Preliminary Clinical Study on Overweight/Obese Subjects. *Front Pharmacol*. 2019;10:575.
229. Busby MG, Fischer L, da Costa KA, et al. Choline- and betaine-defined diets for use in clinical research and for the management of trimethylaminuria. *J Am Diet Assoc*. 2004;104(12):1836-45.