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Dietary bioactive ingredients to modulate the gut microbiota-derived metabolite TMAO. New opportunities for functional food development

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There is a growing body of clinical evidence that supports a strong association between elevated circulating trimethylamine *N*-oxide (TMAO) levels with increased risk of developing adverse cardiovascular outcomes such as atherosclerosis and thrombosis. TMAO is synthesized through a *meta*-organismal stepwise process that involves (i) the microbial production of TMA in the gut from dietary precursors and (ii) its subsequent oxidation to TMAO by flavin-containing monooxygenases in the liver. Choline, ʟ-carnitine, betaine, and other TMA-containing compounds are the major dietary precursors of TMA. TMAO can also be absorbed directly from the gastrointestinal tract after the intake of TMAO-rich foods such as fish and shellfish. Thus, diet is an important factor as it provides the nutritional precursors to eventually produce TMAO. A number of studies have attempted to associate circulating TMAO levels with the consumption of diets rich in these foods. On the other hand, there is growing interest for the development of novel food ingredients that reduce either the TMAO-induced damage or the endogenous TMAO levels through the interference with microbiota and host metabolic processes involved in TMAO pathway. Such novel functional food ingredients would offer great opportunities to control circulating TMAO levels or its effects, and potentially contribute to decrease cardiovascular risk. In this review we summarize and discuss current data regarding the effects of TMA precursors-enriched foods or diets on circulating TMAO levels, and recent findings regarding the circulating TMAO-lowering effects of specific foods, food constituents and phytochemicals found in herbs, individually or in extracts, and their potential beneficial effect for cardiovascular health.

1. Introduction

There is mounting evidence that the metabolism of resident microbiota in the gut may have a crucial role in health and disease.[1,2] In recent years, there has been a growing interest in elucidating the interactions between gut microbiota metabolism and cardiovascular disease (CVD). Thus, it has become increasingly apparent that certain gut microbial-derived metabolites, such as some bile acids, short-chain fatty acids, and trimethylamine-*N*-oxide, may contribute to CVD development and progression.[3–11] The topic has attracted much attention as it can be deduced by the extraordinary number of review articles that can be found in a wide variety of scientific journals. Furthermore, new findings and discoveries in this area, such as the recent identification of the gut microbial metabolite phenylacetylglutamine and

its implication in thrombosis potential and CVD, broaden our view of axis diet–microbiota-CVD.[12] At the beginning of the 2010s, a series of groundbreaking studies associated elevated levels of circulating trimethylamine *N*-oxide (TMAO) with the development of atherosclerosis and increased risk of other adverse CVD events.[13–15] Specifically, in a seminal work, Wang *et al.* (2011) uncovered a *meta*-organismal metabolic pathway that involves (i) the transformation of certain dietary trimethylamines (phosphatidylcholine, choline, betaine, ʟ-carnitine, *etc.*) to trimethylamine (TMA) through gut microbiota metabolism, and (ii) its subsequent absorption in the intestine and delivery to the liver through the portal circulation, where it is oxidized to TMAO by flavin-containing monooxygenase family of enzymes (FMOs).[13] Diet plays a key role in the production of TMAO since it provides with the nutritional precursors to produce TMA and TMAO. In addition, as it will be discussed in this review, other dietary constituents have also been investigated for their potential influence on endogenous TMAO levels or/and their effects against TMAO-induced damage, which would confer a dual role to diet for the prevention of CVD.

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2. TMAO: from prognostic factor to therapeutic target

There is a growing body of clinical evidence that supports a strong association between elevated plasma TMAO levels and cardiovascular risk as well as increased risk of adverse cardiovascular and mortality outcomes. The available clinical studies which investigate the role of TMAO in predicting prognostic outcomes, including mortality, in patients with several cardiovascular conditions have been reviewed.[16,17] In addition to its prognostic value, accumulating data suggests that high plasma TMAO is a risk factor for CVD. A *meta*-analysis of prospective studies has shown that higher circulating levels of gut microbiota metabolites, including TMAO and its precursors, are associated with an increased risk of major adverse cardiovascular events, regardless of conventional risk factors.[18] Various excellent reviews covering available studies on the association between circulating TMAO levels and CVD risk have been published in recent years and will not be addressed in the present work.[17,19–22]

In last years, the underlying molecular mechanisms by which TMAO promotes pathogenic effects on the cardiovascular system have attracted much attention. There are convincing evidences observed *in vivo* and *in vitro* studies suggesting that TMAO exerts a broad range of effects in diverse processes such as endothelial dysfunction, foam cell formation, thrombosis, and cholesterol metabolism, causing a variety of responses and outcomes, indicating that the effect of this metabolite is dependent on the cell type and the species. Human umbilical vein endothelial cells (HUVECs) has been one of the most studied *in vitro* models to explore the role of TMAO on the endothelial dysfunction, a hallmark of vascular

damage that leads to atherosclerosis development. An early article by Sun *et al.* (2016) showed that TMAO stimulated the expression of nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome *via* reactive oxygen species and thioredoxin-interactive protein.[23] The immediate effect of such induction was the release of inflammatory cytokines interleukin (IL)-1B and IL-18. Further research using the same *in vitro* cell model supported these findings and concluded that inflammasome activation in part was mediated through inhibition of the SIRT3-SOD2-mitochondial ROS signaling pathway.[24] In a separate report, prolonged exposure of HUVECs to TMAO induced the expression of senescence markers and a reduction of cell proliferation among other effects that were suggestive of senescence and vascular aging.[25] Additionally, Ma *et al.* (2017) observed that TMAO impaired endothelial self-repair and increased monocyte adhesion, processes that accelerate endothelial dysfunction, and subsequently, promote early pathological process of atherosclerosis.[26] Interestingly, TMAO suppressed proliferation and migration of HUVECs compared to control cells, and at the molecular level, TMAO activated protein kinase C and NF-kB, and stimulated vascular cell adhesion molecule-1. The role of TMAO in the activation of inflammatory pathways in cells of the vasculature has also been investigated *in vivo*. A study on LDLR$^{-/-}$ mice explored the impact of TMAO on smooth muscle and endothelial cell function.[27] The elevation of inflammatory gene expression was a common observation in mice that were fed a choline diet or injected with an acute TMAO dose compared to control group. TMAO triggered mitogen-activated protein kinase, extracellular signal-related kinase, and NF-kB signaling cascade. Interestingly, the response observed at the molecular level was also reproduced in primary cultures of human endothelial and

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smooth muscle cells, but it was not observed in mouse peritoneal macrophages subjected to similar treatments. Vascular calcification is a prevalent process in patients with chronic kidney disease (CKD). A recent study aimed at investigating the role of TMAO in vascular calcification revealed that TMAO induced gene expression of molecules involved in the osteogenic differentiation of human vascular smooth muscle cells.[28] In line with other studies, TMAO activated NLRP3 inflammasome and NF-kB signals during vascular calcification in CKD-induced rats, and inhibition of those signals diminished TMAO-induced calcification on vascular smooth muscle cells. Another recent article suggested that high-mobility group B1 (HMGB1) is one of the important mediators of TMAO-induced endothelial dysfunction and it is linked to Toll-like receptor 4 (TLR4) activation.[29] It was found that EOMA endothelial cell monolayers exposed to TMAO exhibited reduced levels of various cell–cell junction proteins compared to untreated control cells, effects that were at least in part dependent of HMGB1 activation. Also related with HMGB1 activation, Cheng *et al.* (2019) have recently reported that exposures of HCAECs to a low TMAO concentration (10 µM) promoted TNF-α or HMGB1-induced Tissue Factor (TF) expression.[30] TF is a molecule implicated in the thrombogenicity of atherosclerotic plaques and TMAO up-regulated its expression and activity in both concentration- and time-fashion, changes that were also dependent on the activation of NF-kB.

Cholesterol accumulation in macrophages is a critical step in atherosclerosis. Wang *et al.* (2011) demonstrated that TMAO promoted uptake of cholesterol in macrophage by increasing the cell surface expression of two proatherogenic scavenger receptors, cluster of differentiation 36 (CD36), scavenger receptor A in macrophages.[13] Further research showed that TMAO also induces the expression of ATP-binding cassette transporter A1 in macrophages which also favors the cholesterol uptake.[14] Another relevant conclusion of the study in mice was that TMAO affects a major pathway for cholesterol elimination from the body. In fact, TMAO inhibited reverse cholesterol transport by affecting bile acid synthetic pathway in the liver at various levels, *i.e.* decreasing mRNA levels of bile acid synthetic enzymes Cyp7a1 and Cyp27a1, and multiple bile acid transporters (OATP1, OATP4, MRP2 and NTCP), observations that were not reproduced in the gut. Further, Geng *et al.* (2018) observed *in vivo* and *in vitro* that TMAO promoted CD36-mediated transformation of ox-LDL induced macrophages into foam cells, a process that seemed to be mediated by MAPK/ JNK pathway in mice.[31]

With regard to the thrombogenic activity of TMAO various studies have been published. In a work by Zhu *et al.* (2016) plasma TMAO levels were independently correlated with incident thrombosis after a follow-up period of 3 years in a cohort with more than 4000 subjects.[32] *In vitro* assays directly exposing platelets to TMAO elevated platelet hyperreactivity, with enhanced agonist-induced platelet activation through calcium release from intracellular stores. In addition, *in vivo* experiments in C57BL/6J mice confirmed the involvement of gut

microbiota and TMAO in modulating platelet hyperresponsiveness. In a further report, Subramaniam *et al.* (2019) observed that cultured HAECs exposed to TMAO induced TF upregulation and procoagulant activity through NF-kB activation.[33] However, contrasting results to those reported by Zhu *et al.* (2016) were found regarding the ability of TMAO to promote thrombosis *in vivo*.[32]

The aforementioned studies have provided valuable information about the molecular events that might explain biological effects on TMAO; however, the lack of understanding about the nature of the events that might trigger the molecular changes observed in the cells in response to TMAO has led to speculate on either two possibilities. On the one side, TMAO could be acting *via* its known effect on protein conformation and stability (*i.e.*, as an allosteric modifier). On the other hand, TMAO could specifically interact with a receptor (*i.e.*, as a ligand). This latter possibility seems to be more plausible considering the kinetics of TMAO-induced signaling responses in mammalian cells. In support of this notion, the recent report by Chen *et al.* (2019) has provided important insights and demonstrated that TMAO can directly and selectively interact with protein kinase R-like endoplasmic reticulum kinase (PERK), one of the three key molecular sensors in endoplasmic reticulum involved in the unfolded protein response signaling.[34] Their results demonstrated *in vivo* and *in vitro* that TMAO-activated PERK induced FoxO1, a relevant transcription factor in metabolic disease; and the inhibition of TMAO formation in liver by FMO3 inhibitors or by manipulating mice gut microbiota could reduce PERK activation and FoxO1 levels and improve glucose tolerance. Considering the already published data supporting that PERK might induce activation of NLRP3 inflammasome,[35] NF-kB,[36] and MAPK signaling,[37] it can be anticipated that further research aimed at disentangling the potential links between these signaling pathways will help on uncovering the intriguing molecular mechanism underlying the pathogenic effects of TMAO in CVD.

3. Key factors in TMA metabolism

3.1. Microbial metabolic pathways leading TMA production in gut

The obligatory role for gut microbiota in TMAO formation from dietary trimethylamines has been demonstrated. Different gut microorganisms have different abilities to form TMA from dietary precursors. This is in part because there are various microbial pathways for its production in the gut (Fig. 1). Choline utilization (*cut*) gene cluster, responsible for anaerobic choline degradation, was shown to contain genes coding for the glycyl radical enzyme choline TMA-lyase (CutC) and its corresponding radical *S*-adenosyl-ʟ-methionine (SAM) activating protein (CutD).[38] Another microbial metabolic pathway generating TMA from ʟ-carnitine is constituted by a two-component CntA/CntB oxygenase/reductase system capable of cleaving ʟ-carnitine into TMA and malic semialdehyde.[39] A closely related bacterial lyase consists of the *yeaW/X*
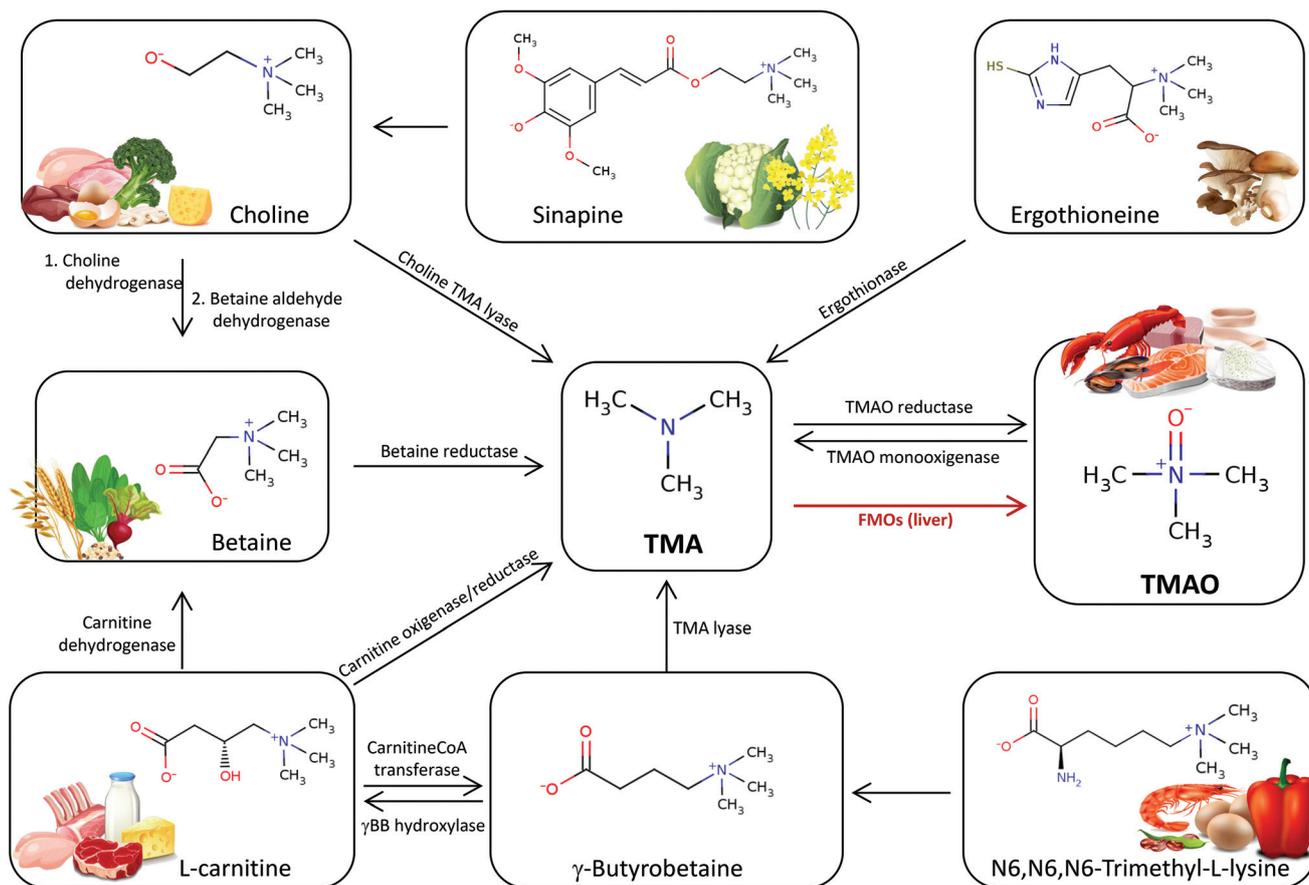
Fig. 1 Chemical formulas of main dietary precursors of TMA and TMAO. Schematic representation of the main metabolic pathways for the production of TMA by the gut microbiota and endogenous enzymes, and metabolism to TMAO by the hepatic FMOs.

gene products (YeaW/X TMA lyase). This promiscuous lyase can convert choline, betaine, L-carnitine, and γ-butyrobetaine to TMA.[40,41] There are other enzymes that could be responsible for production of TMA from glycine betaine through the activity of glycine betaine reductase gene product (GrdH), an enzyme that requires selenium.[42] Besides these TMA-generating pathways from dietary trimethylamines, an additional pathway that reduces TMAO to TMA by the activity of a *torA*-like gene product has been also described in some gut microorganisms.[43] As will be discussed later, in addition to the mentioned phosphatidylcholine, choline and L-carnitine, other food nutrients possess a TMA moiety, suggesting that part of microbial TMA-generating pathways remain to be elucidated. Despite the relevance of TMAO to disease, the specific microorganisms responsible for the anaerobic dietary trimethylamines metabolism in the human gut remain unclear because the genetic and biochemical basis for utilization of these compounds is not fully characterized. Most of the reported attempts to correlate microbiota composition to plasma TMAO levels have been based on 16S ribosomal RNA gene (rDNA) amplicon sequencing technology. However, it has been demonstrated that such associations do not necessarily imply a causal link between the bacteria identified and the poten-

tially enhanced production of TMA in the gut ecosystem. The relevant report by Romano *et al.* (2015) described eight strains, from two different phyla (Firmicutes and Proteobacteria) and obtained from human intestinal isolates, that were capable of producing TMA from choline *in vitro*.[44] On the other hand, the application of advanced bioinformatics to screen reference genome libraries for species carrying the genetic potential to perform anaerobic dietary trimethylamines metabolism has proven to be a straightforward way of identifying potential gut TMA producers. The information derived from such screenings has been used for data mining in public genomic and metagenomic databases to provide a general overview of potential TMA-producing microorganisms in sequenced human gastrointestinal isolates.[40,45] Using this strategy, TMA production potential has been detected in 102 reference genomes, covering 36 bacterial species. Although the TMA production function is widespread in Firmicutes, Proteobacteria, and Actinobacteria, it appears to be absent in Bacteroidetes. Remarkably, Clostridium spp., which were previously associated with an L-carnitine-rich diet using 16S rDNA amplicon sequencing, unexpectedly only pose CutC/D genes in their genome, which is associated with choline utilization, stressing the limitations of 16S rDNA sequencing approach to identify

novel organisms involved in TMA production. A bioinformatic study also suggested that TMAO reduction pathway has the highest detection rate in the gut metagenomes investigated, which indicates an intensive cycling between TMA and TMAO within the gut environment.[45] Besides the interesting data provided by these novel bioinformatic approaches, it would be also important that findings about the metabolic potential of certain TMA producers could be validated biochemically.

3.2. Dietary precursors of TMA and TMAO

TMAO is found in a wide variety of fish and marine invertebrates since it plays an important role in their osmoregulation; it is involved in protein folded state stability and can counteract the effects of destabilizers such as urea and temperature. TMAO is also well known as indicator of freshness or postharvest spoilage degree of seafood products since, under refrigerated conditions, TMAO is reduced into TMA by specific spoilage organisms.[46] TMA is the primary component that imparts the undesirable malodor that finally causes consumer rejection of poor-quality fish products. Marine fish and shellfish are the richest dietary source of TMAO. Concentrations in fresh seafood can vary extensively between species, habitat depth, season, etc.[47] Significant differences in the TMAO concentrations between fish of various genera and species have been found,[48] with values ranging between 8 and 789 mg per 100 g for ray-finned fishes and 262 and 789 mg per 100 g for cartilaginous fishes.

L-Carnitine is considered a conditionally essential nutrient because, under certain conditions, it may require exogenous supplementation. L-Carnitine is essential for energy metabolism and its main function is the transport of long-chain fatty acids from the cytoplasm into mitochondria for β-oxidation. L-Carnitine is endogenously synthesized from methionine and lysine in the liver, kidney, testis, heart, and brain. Besides its endogenous origin, L-carnitine can also be provided through the food intake; although, no dietary reference values have been set. L-Carnitine is generally found at high concentration in foods from animal origin (e.g. meat and dairy products), and in lesser amounts can be found in grains and vegetables.[49] Red meat is the richest dietary source of L-carnitine; it has been reported that the content of L-carnitine in beef range from 45 to 66 mg per 100 g, although higher amounts have been found in the muscle from kangaroo and horse.[50] L-Carnitine concentrations in dairy products vary widely with the different products, consistently with fat content and animal origin, ranged from 3 to 42 mg per 100 g in a wide range of dairy products such as milk, yogurt, cheese and cream.[50]

Choline is also considered a conditionally essential nutrient for humans since the amount produced endogenously in the liver is not sufficient to meet human requirements, and thus, it needs to be obtained from dietary and supplement sources. The Panel on Dietetics Products, Nutrition, and Allergies from the European Food Safety Authority (EFSA) set the adequate intake (AI) at 400 mg day⁻¹ for men and women,[51] while the American Institute of Medicine (IOM) set a different AI for

men (550 mg day⁻¹) and women (425 mg day⁻¹).[52] The main forms of choline present in foods are both water-soluble: free choline, phosphocholine, (PChol), glycerophosphocholine (GPC), and lipid-soluble: phosphatidylcholine (PC) and sphingomyelin (SPM). Other minor amounts are the water-soluble forms cytidine-5-diphosphate-choline (CDP-choline) and acetylcholine. Animal-derived foods contain more choline than plant-based ingredients, but certain plant-based foods are also rich in choline and choline-containing molecules. Eggs and liver represent the major sources of choline in diets, followed by meats and fish, whole grain, cereal, vegetables, fruits, milk, fat and oils.[53] Choline also exists in considerable amounts in the form of phenolic choline esters in the seeds of the *Brassicaceae* family.[54] Sinapine (the choline ester of sinapic acid) is the best-known compound of this class due to its occurrence in foods such as cauliflower, and it has been studied as a dietary precursor of TMAO in rapeseed-derived feed for pigs.[55] Besides sinapine a number of choline related compounds derived from substituted benzoic and cinnamic acids have been identified, however, their role as dietary precursors of TMA has yet to be thoroughly researched.

N6,N6,N6-Trimethyl-L-lysine (TML) is a non-protein amino acid which has an important role as a precursor of L-carnitine. It has been determined that TML is presented as free amino acid in noticeable amounts in vegetables, which represent an important part of daily alimentation for most mammals. Servillo et al. (2014) have reported the occurrence of TML in a number of vegetables sources such as legumes, grains, leafy vegetables, solanaceae vegetables and fruits.[56] The highest concentration in vegetables was found in sweet pepper fruit (0.8–1.8 mg per 100 g). Li et al. (2018) reported the presence of TML in both plant- and animal-derived foods alike, and they found a greater level in animal- than plant-derived products.[57] The TML content in meats from different animal origins ranged from 1 to 8.6 mg per 100 g, and interestingly, amount TML was the highest in eggs (13.7 mg per 100 g) and shrimps (12.2 mg per 100 g).

Betaine, a choline derivative also known as glycine betaine and trimethylglycine, is produced endogenously from choline and the amino acid glycine. Betaine is present in plant- and animal-based foods. Cereal-based foods are one of the major sources of betaine (>1 g per 100 g).[58] Spinach, beets, crustaceans and finfish are also good sources of betaine (650–200 mg per 100 g).[59]

Ergothioneine is an unusual sulfur-containing derivative of histidine that is synthesized only by some fungi and bacteria. In humans, ergothioneine is acquired exclusively through dietary sources. Only some food contain ergothioneine, with highest concentrations detected in boletus and oyster mushrooms (11.9–52.8 mg per 100 g), and, to a lesser extent, in chicken and pork liver (0.9–1.1 mg mL⁻¹), pork kidney (0.8 mg per 100 g), black and red beans (0.4–1.3 mg per 100 g), and oat bran (0.4 mg per 100 g).[60]

Recently, **δ-Valerobetaine** has been described as a constitutive metabolite of ruminant meat and milk. The presence of δ-valerobetaine has been reported in milk (30–60 μmol L⁻¹)

and meat (300–500 μmol kg^{-1}) from ruminant sheep, goat and cattle.[61] The levels of δ-valerobetaine were considerably higher in ruminant (sheep, goat and cattle) than in non-ruminant (horse, rabbit, chicken and pig) meat. δ-Valerobetaine was originated by the rumen microbiota from TML occurring in vegetable-derived ruminant diet. Moreover, the possible fate of δ-valerobetaine expected from dietary source by humans was assessed, and it was observed that increasing production of TMA over time was obtained when δ-valerobetaine was incubated with caecum content of a non-ruminat animal (rabbit) under anaerobic conditions.

Besides the aforementioned dietary precursors of TMA/TMAO (Fig. 1), active research is underway to evaluate and discover other novel dietary precursors and the role of gut microbiota to release TMA from these substrates.

4. The complex link between diet and endogenous TMAO levels

4.1. Association of TMAO levels with consumption of foods

Studies on the association of urine or plasma TMAO levels with consumption of foods of animal origin, rich in dietary trimethylamines have demonstrated that TMAO production shows wide inter- and intra-individual variations. Circulating TMAO is determined by a number of factors including type of diet, liver FMO activity and kidney function. Genetic factors appear to play only a marginal role in plasma TMAO levels suggesting that interrelationships between dietary precursors and the gut microbes are more likely determinants of variation in plasma TMAO levels in healthy population.[62]

TMAO is present in significant quantities in marine fish and shellfish and represent a significant source of dietary TMAO for humans. Consistent results across different nutritional intervention and observational studies have shown that TMAO in urine and/or plasma is a strong indicator of dietary exposure to fish. The intake of fish has been mentioned as a cause of higher urine levels of TMAO in some observational studies. For instance, after metabolomic analysis of urine obtained from different population samples (852 participants from China, Japan and USA), it was noted that high levels of TMAO were particularly dominant in the Japanese population, consistent with the high dietary intake of fish in this area.[63] Similarly, from cross-sectional data from the INTERMAP study, including 4680 participants from Japan, China, United Kingdom, and USA, urinary TMAO showed a stronger association with fish intake in the Japanese population sample.[64] In the cross-sectional KarMeN study in 2012/2013, Krüger et al. (2017) also observed that fish intake was associated with increased TMAO concentrations in both plasma and urine samples from German adult population (297 participants).[65]

Several nutritional intervention studies have reported high levels of TMAO in urine and plasma after consumption of fish. Consumption of single meal with salmon was followed by substantial increased postprandial urinary concentrations of TMAO,[66] suggesting that free TMAO present in fish can be readily absorbed without the involvement of gut microbiota. Likewise, longer-term intake of fish has also been associated with higher TMAO levels. For instance, 150 g seafood a day (salmon, cod, herring or shrimps) over a one-week period affected plasma and urine TMAO levels, which increased 16-fold and 6-fold, respectively, after the seafood-rich diet.[67]

When compared with other non-fish diets, it has been shown that TMAO is more closely associated with fish intake.[68,69] In crossover meal studies, fish-rich diets have been associated with significantly higher plasma TMAO levels than meat- and egg-rich diets. Thus, a major increase in TMAO concentration in urine was observed after fish intake but not after meat (chicken, red meat and processed meat) intake over 3 weeks;[68,70] also, urinary TMAO displayed a strong dose–response relationship with fish intake.[70] In a randomized, controlled crossover design with four arms comparing the effect of four different meals (fish, eggs, beef, and fruit control) taken in a single day, it was observed that fish consumption yielded ~50 times higher postprandial plasma concentrations of TMAO compared to eggs or beef intake.[69]

Unlike seafood, animal products do not contain free TMAO. Nevertheless, as mentioned above, there are a variety of TMA-containing nutrients, primarily found in foods of animal origin, and to a lesser extent in vegetable products, that can be potentially dietary precursors of TMAO.

As previously mentioned, eggs are one of the major sources of choline (and its esterified forms) in diets. One egg contains 250 mg of choline moiety per 100 g and one egg yolk contains 680 mg of choline moiety per 100 g.[53] Eggs are also a good source of cholesterol, hereby raising the debate about associations among egg consumption and risk of CVD. It has been shown that acute intake of choline from eggs may influence postprandial plasma TMAO levels in healthy subjects. Highlighting the role of gut microflora in this process, Tang et al. (2013) showed that subjects who consumed a dietary PC challenge (hard-boiled eggs and deuterium (d9)-labeled PC) had acute increases in plasma TMAO in a gut microflora-dependent manner.[71] Miller et al. (2014) also observed a dose-dependent increased plasma and urine TMAO concentrations at 24 h after a single meal exposure of 0, 1, 2, 4 or 6 egg yolks, with significant increases in TMAO concentrations from ≥2 egg yolks, and the highest TMAO concentrations achieved at 6 and 8 h after the ingestion of the eggs.[72]

However, when egg intake trials were conducted for longer periods of time, differing results were obtained. For instance, West et al. (2014) observed that regular egg consumption (8-week period, 6 eggs per week) did not increased post-intervention fasted plasma concentrations of TMAO (compared with an egg-free control diet) in lacto-ovo-vegetarian women.[73] These results agreed with data from the study reported by DiMarco et al. (2017), in which the intake of 1, 2, or 3 eggs per day for 4 weeks (n = 36) increased in a dose-dependent manner plasma choline concentrations while plasma TMAO remained unchanged for the duration of the dietary intervention.[74] In subsequent crossover dietary interventions carried out by the same research group, the effect of 3 eggs per day intake vs.

daily choline bitartrate supplementation (n = 30)[75] and consumption of 2 eggs per day *vs.* oatmeal for breakfast (n = 30),[76] were evaluated on plasma choline and TMAO levels. The obtained results confirmed that after a 4-week intervention period, choline was significantly increased in plasma when administered in the form of eggs or as dietary supplement; however, neither egg intake nor choline supplement increased fasting plasma TMAO levels when compared to baseline values. In an observational study, Obeid *et al.* (2017) examined the type of the vegetarian diet and it was reported that vegans and lacto-ovo vegetarians that followed stable dietary patterns for a mean of 10 years did not differ in plasma concentrations of TMAO.[77] Collectively, these results indicate that although acute egg ingestion increases postprandial plasma TMAO to some extent, there is not a clear association between egg consumption and increased fasting plasma levels of TMAO. Moreover, these works lack of gut microbiome information and the link between the microbiome and TMAO was not addressed, and thus, it should be further investigated.

It has also been found that depending on the structure of choline in different dietary sources and the foodstuffs in which they are present, absorption and metabolism may differ and impact the bioavailability of choline. This was exemplified by Mödinger *et al.* (2019) that compared the plasma kinetics of choline and its turnover into choline derivatives upon intake of two different choline sources: phosphatidylcholine from commercial krill oil and capsules containing choline bitartrate salt.[78] In this regard, higher TMAO concentrations were measured after intake of the choline bitartrate, compared to phosphatidylcholine from krill oil. Interestingly, Berge *et al.* (2015) also observed that dietary supplementation with krill oil resulted in increased choline and betaine but not plasma TMAO levels.[79]

Meat constitutes a significant part of human diet since it is an important source of high-quality proteins and important micronutrients.[80,81] Certain types of meats are also rich in fats and cholesterol as well as in ʟ-carnitine and choline.[50,53] A number of observational studies have investigated the effects of long-term dietary habits (*e.g.*, vegan/vegetarian *vs.* omnivore) on the generation of TMAO. Several studies observed that baseline plasma TMAO levels were lower among vegan and vegetarian subjects compared to omnivores.[14,82] In addition, urinary TMAO was found to be one of the most significant metabolites responsible for the differences observed between subjects following long-term (more than 5 years) lacto-vegetarian or omnivorous diets.[83] In another report, TMAO generation was also significantly lower in vegans/vegetarians after 2–3 months of continuous daily supplemental ʟ-carnitine ingestion.[84] It has also been reported that both vegan/vegetarian and omnivore alike showed increases larger than 10-fold in plasma TMAO levels after 1–2-month periods of choline supplementation.[82] In another cross-sectional study by Kruger *et al.* (2017) it was reported that red meat but not white meat intake was positively associated with plasma TMAO concentrations.[65]

Human dietary interventional studies, aimed at discovering nutrition markers in body fluids, have found an association between red meat consumption and increased plasma or urine levels of TMAO. A short-term (15 days) randomized crossover study showed that high red meat diet (420 g day^{-1}) increased urinary TMAO when compared to vegetarian diets; however, no difference in TMAO was observed between a low meat diet (60 g day^{-1}) and vegetarian diet.[85] In an intervention nutritional trial focused on various defined doses of meat (either chicken or beef) following a crossover design, TMAO was the only analyte that increased after beef intake (but not after chicken intake) and remained above baseline even after 24 h.[86] Moreover, after the ingestion of 0, 100, or 200 g beef, clear dose–response was observed for TMAO levels, with maximum plasma concentrations around 11 h after the intake. Similarly, in a randomized controlled crossover trial, the effect of 4-weeks intake of red meat, poultry, and non-meat protein sources as 25% of their daily calories, was investigated,[87] and it was observed that compared to the white meat or non-meat diets, increased plasma and urine levels of TMAO occurred after consumption of the red meat diet. Moreover, discontinuation of dietary red meat reduced plasma TMAO to the initial values within 4 weeks.

Other studies have not found an association between the intake of meat and TMAO levels. A dietary intervention study was carried out to discover exposure markers in urine after consuming different test meals prepared with three protein sources (meat, fish or vegetarian).[88] No clear difference in TMAO was observed between meals prepared with vegetarian or a meat source; conversely, TMAO was found as a fish consumption marker. In different cross-sectional study, Kühn *et al.* (2016) did not observe a clear association between meat consumption and fasting plasma levels of TMAO.[89] By contrast, TMAO concentrations showed clear intra-individual variation over time, presumably driven by a greater within-person than between-person variation. Similarly, Rohrmann *et al.* (2016) did not observe any associations between the consumption of meat (red, processed and white), fish, eggs and the plasma concentrations of TMAO.[90] However, participants with higher consumption of total milk and dairy products (but not cheese) showed higher plasma TMAO concentrations.[90] Concerning this issue, recently, it has been suggested that fermented dairy products have different effects to those of non-fermented dairy products on postprandial TMAO levels. Thus, Burton *et al.* reported that fermented milk products were associated with lower postprandial TMAO responses than non-fermented dairy consumption; on the other hand, daily consumption of dairy products did not differentially affect fasting TMAO.[91]

As can be appreciated, there is high heterogeneity in the described associations between meat intake and TMAO. This can be potentially explained due to differences in the study duration, quantity of food dosing, variability in ʟ-carnitine/choline content different types of tested meats, and the small number of participants included in some of the studies. Thus, careful attention should be paid when interpreting variations in urinary or plasma TMAO levels in diverse populations. Furthermore, the link between measured TMAO and dietary

TMA-rich foods could also be confounded by the large degree of inter-individual variability in gut microbiota composition. It seems reasonable to think that, considering the primary role of microorganisms in TMA generation, gut microbiota composition and community structure exert a strong influence in circulating TMAO levels. In line with this, some studies have suggested that gut microbiota composition influences the extent of TMAO production in dietary intervention studies.[69,92] Cho et al. compared TMAO response for eggs, beef, and fruit control taken in a single day, and a greater increase in postprandial plasma TMAO was detected for both eggs and beef compared to the fruit control.[69] They observed that high-TMAO producers, i.e. those subjects having ≥20% increase in urinary TMAO concentrations following the consumption of egg or beef meals, had more Firmicutes than Bacteroidetes and a less diverse gut microbiome. More specifically, high-TMAO producers were represented by Clostridiales at the Firmicutes phylum, within which Clostridiaceae, Lachnospiraceae, and Veillonellaceae were the most abundant. On the other hand, low-TMAO producers (<20% increase in urinary TMAO concentrations) were represented by Bacteroidales within the Bacteroidetes phylum, of which Bacteroidaceae and Prevotellaceae were predominant. These findings agreed with those obtained by Falony et al. (2015) suggesting that TMA production function is widespread in Firmicutes, Proteobacteria, and Actinobacteria, whereas it appears to be absent in Bacteroidetes.[40] In another randomized, controlled crossover design, changes in the gut microbiota composition were observed after 4 weeks of dietary intervention that varied in the protein source (lean-seafood or non-seafood).[92] A higher fecal level of TMA was observed after consumption of the lean-seafood diet compared to the non-seafood diet. Moreover, a trend toward a higher relative abundance of Firmicutes with an accompanying decrease in the relative abundance of Bacteroidetes was observed after the non-seafood diet. On the contrary, the ratio of Firmicutes to Bacteroidetes was maintained during the lean-seafood intervention.

Resistant starch has shown potential to exert an impact on the microbial ecosystem of the gut and its composition, since the gut microbiota uses dietary fiber as an energy source. Under this assumption, Bergeron et al. studied the effect of dietary resistant starch (RS) in the gut microbiota-derived metabolite TMAO in plasma. In a controlled, randomized, crossover dietary intervention, they observed that fasting plasma levels of TMAO were significantly increased by high vs. low RS intake, although this effect was dependent on total dietary carbohydrate intake.[93] Microbial composition of fecal samples showed that the proportions of certain taxa were correlated with plasma TMAO levels; however, results about microbiota composition were inconclusive.

4.2. Association of TMAO levels with dietary patterns

There is also a large interest in examining the effect of diet as a whole instead of intake of single TMA-enriched foods or nutrients. Some studies have attempted to associate the consumption of specific dietary patterns and health outcomes with plasma TMAO. Although there are still only few works, some of them have shown that dietary habits and patterns have the ability to influence circulating dietary trimethylamines as well as their microbial metabolites TMA and TMAO.

Western lifestyle, including diet, may predispose populations to chronic disease such as, CVD, colorectal cancer and type-2 diabetes (T2D). The Western diet frequently contains excessive saturated and trans fatty acids, animal protein content and simple sugars, and is low in fiber, vegetables and other plant-based foods. Moreover, some cross-sectional studies have shown evidence that high-protein and high-fat diets (related to Western lifestyle) are associated with gut microbial populations that are typified by a Bacteroides enterotype; whereas diets rich in carbohydrates and simple sugars are associated with a Prevotella enterotype.[94] Western-style diets or high-fat diets have shown to increase plasma TMAO levels. In a short-term (5 days) interventional trial, high-fat diet (55% fat) administered to healthy men did not increase fasting plasma TMAO concentrations, but led to increase TMAO in the postprandial period.[95] Slightly higher concentrations of L-carnitine, choline and TMAO were also found in those persons who had more Western-style dietary patterns.[96] O'Sullivan et al. (2011) pioneered the use of cluster analysis for the identification of dietary patterns.[97] In their work, three dietary clusters were identified on the basis of the energy contribution of different food groups. Cluster 3 (related to Western-style diet) had higher intakes of red meat, meat products and a lower contribution from vegetables, while Cluster 1 presented the highest intakes of vegetables, wholegrain breads, and fish. The key metabolites in urine responsible for the separation of dietary Clusters 1 and 3 were TMAO, glycine, O-acetylcarnitine, and phenylacetylglutamine.

Epidemiological studies have shown that the **Mediterranean diet (MD)** is associated with a lower risk of CVD and certain types of cancer.[98] MD is characterized by abundant plant-based foods (fruit, nuts, vegetables, legumes, cereals), olive oil as the principal source of fat, a high to moderate intakes of fish, a moderate or low consumption of eggs, poultry and dairy products (principally cheese and yogurt), a low consumption of processed meats, red meat and sweets, and also, wine consumed in low to moderate amounts, normally with meals.[99,100] Beneficial effects of MD have been attributed to the types of food consumed, total dietary pattern, as well as other aspects such as, eating and lifestyle behaviors.[98] Dietary interventions with MD have shown an inverse association between adherence to the MD and cardiovascular risk. The PREDIMED (PREvention with MEDiterranean DIet) was a pioneering multicenter, randomized, nutritional intervention trial, for the primary prevention of CVD with MD. The study was carried out in Spain from 2003 to 2011. In this study, participants who were at high cardiovascular risk (but with no CVD) received a dietary intervention with MD supplemented with extra-virgin olive oil (EVOO), a MD supplemented with nuts, or a control diet (with an advice on a low-fat content). Among persons at high cardiovascular risk (n

= 7447), MD supplemented with EVOO (n = 2543) or nuts (n = 2454) reduced the incidence of major cardiovascular events.[101] In a sub-population of the PREDIMED trial (n = 98), the effect of MD pattern on urinary metabolome was assessed by comparing subjects at 1 and 3 years of follow-up, after an MD supplemented with either EVOO or nuts, to those on advice to follow a control low-fat diet. By means of a non-targeted metabolomics approach after dietary intervention it was observed that a low-fat diet was associated to an increased 24 h urine hippurate and TMAO levels, compared with a MD supplemented with EVOO.[102] In a different work, 119 individuals from PREDIMED trial were studied, and according to their individual MD adherence based in PREDIMED 14-item questionnaire,[103] they were assigned to low (≤7 score) or high (≤10 score) adherence to MD.[104] Thirty-four metabolites were identified in urine as discriminant between low-MD and high-MD adherence. Surprisingly, among them, both dimethylamine and TMAO were higher in the high-MD adherence group, and authors associated this result to a higher fish intake in the high-MD adherence diet.

Other studies different from PREDIMED, have also evaluated the association between levels of TMAO and the adherence to the MD. In a cross-sectional observational study Barrea *et al.* (2019) used the PREDIMED 14-item questionnaire to assess the adherence to the MD,[105] and association between levels of TMAO and the MD in 144 healthy adults was found, with a clear gender difference in this association.[106] De Filippis *et al.* (2016) observed a cohort of 153 healthy volunteers comprising 51 vegetarians, 51 vegans and 51 omnivores, of which 30% had a high adherence to MD.[107] They found that the lower adherence to the MD group was related with a higher urinary TMAO levels. Associations were also found between TMAO levels and microbial genera that were more linked to the intake of animal proteins and fat. *Ruminococcus* genus from the *Lachnospiraceae* family was positively associated to TMAO. Interestingly, Griffin *et al.* (2019) showed that six months of dietary intervention with MD did not alter fasting TMAO concentration in a population of 115 healthy people at increased risk of colon cancer.[108] It was suggested that broad dietary pattern intervention over six months may not be sufficient for reducing TMAO concentrations. Also, Pignanelli *et al.* (2018) did not find any significant difference in the plasma levels of TMAO and other gut-derived uremic toxins associated with a MD score or with intake of dietary precursors among omnivorous vascular patients.[109]

5. Intervention strategies for targeting TMA/TMAO pathway

In last years, the recognition of mechanistic links between TMAO and pathogenic molecular processes associated with atherosclerosis and thrombosis has placed this microbial metabolite on the map as an emerging therapeutic target for the prevention and treatment of CVD. Considering the multifactorial nature of TMAO generation, various strategies may be envisaged to interfere with the different biochemical and physiological steps that constitute the metaorganismal TMA/TMAO pathway in order to reduce circulating TMAO levels and prevent or reduce the risk of CVD (Fig. 2). In this context, limiting the dietary consumption of food enriched in dietary trimethylamines, such as some animal source foods, could be a straightforward point of intervention; however, this would entail adverse clinical consequences since, as discussed above, they contain nutrients that are required for optimal health. Another option for intervention is the inhibition of hepatic FMO3 activity. It has been pointed out that this might not be a suitable approach, as impaired FMO3 activity gives rise to the inherited disorder trimethylaminuria (TMAU), a condition called Fish odor syndrome. Affected individuals cannot produce TMAO and, consequently, excrete large amounts of malodorous TMA in their urine, sweat, and breath.[110] On the contrary, hepatic FMO3 is increased in insulin-resistant mice and humans,[111] and recent research by Chen *et al.* (2019) suggests that FMO3 inhibition could be a suitable target to control TMAO production.[34] Their study provided interesting data showing that FMO3 inhibition by dietary 3,3′-diindolylmethane or indole-3-carbinol (phytochemicals in cruciferous vegetables such as cauliflower and Brussel sprouts) supplementation reduced hepatic TMAO in insulin-resistant mice leading to a concomitant decrease in activated PERK and FoxO1, and improved glucose tolerance. It was reasoned that partial inhibition of FMO3 activity could be a tradeoff for having the beneficial TMAO-lowering effects without causing odor.

Alternatively, the discovery of microbiota as a major factor determining the amount of TMA generated in the gut has raised the possibility for devising strategies to modulate gut microbiota composition and its metabolic function. A large body of literature has demonstrated that administration of antibiotics can markedly decrease circulating TMAO levels. However, the transient nature of the changes in circulating TMAO levels, the various undesirable side effects and the serious risk for antibiotic resistance associated with the use of antibiotics are some of the reasons for dismissing their use as a therapeutic strategy to modulate circulating TMAO levels. In line with the microbiota obliged role in TMA formation, remodeling gut microbiota composition or its metabolic function by repopulating gut with other microorganisms could be a feasible alternative to decrease circulating TMAO levels. In this regard, fecal microbiota transplantation (FMT) has been successfully used to prove the transmissibility of atherosclerosis susceptibility[112] and elevated platelet reactivity and thrombosis potential[113] in animal models. By contrast, in a double-blind randomized controlled pilot study in metabolic syndrome patients, the FMT from single lean vegan-donor did not altered TMAO levels or parameters related to vascular inflammation, in spite of changes in intestinal microbiota composition were detected.[114] These contrasting outcomes reflect that FMT as therapy for treating CVD is still in its infancy and further research is needed to test its efficacy and safety for reducing CVD risk.[115]
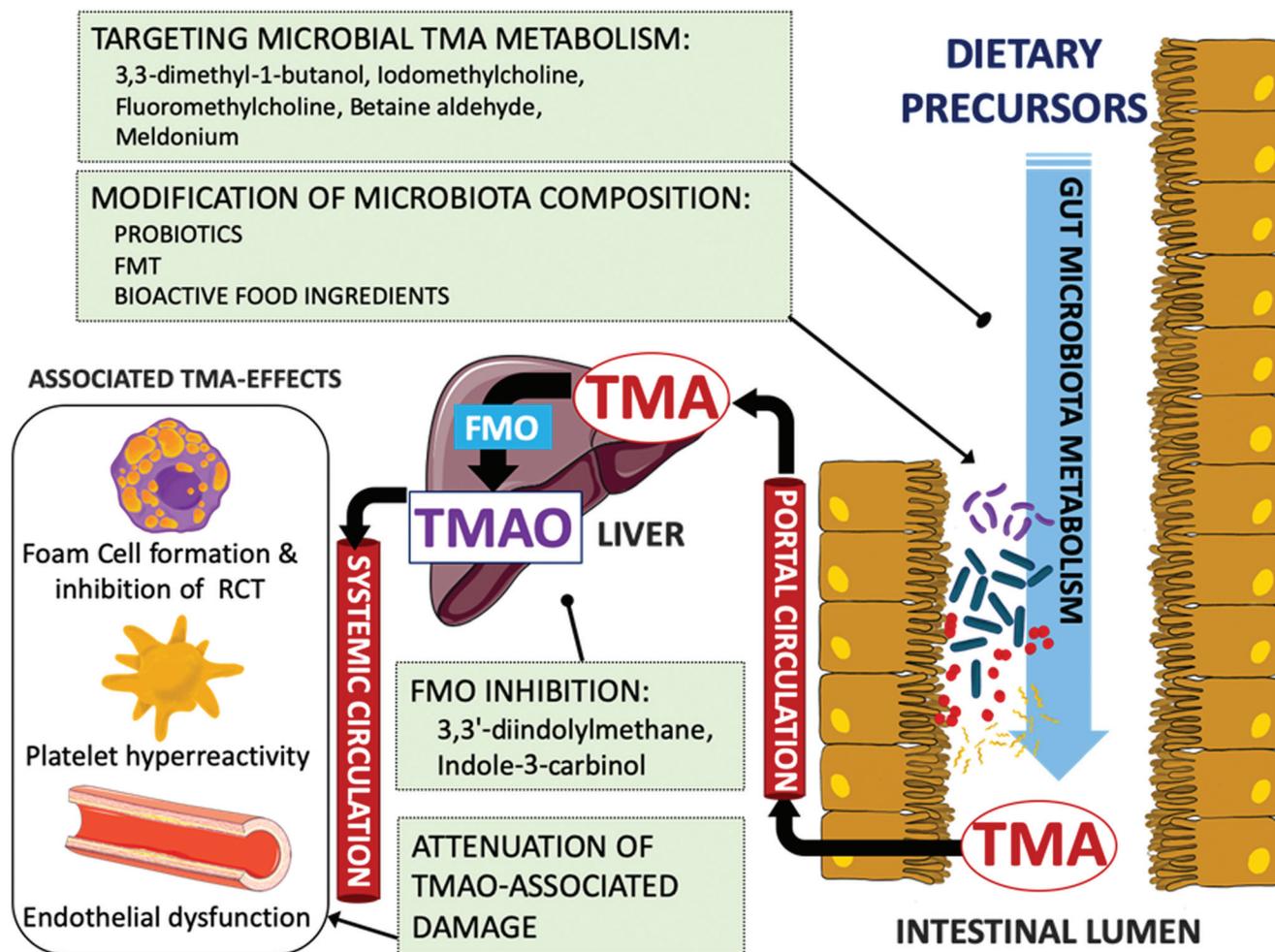
Fig. 2 Scheme of TMA/TMAO metaorganismal pathway and suggested intervention targets (green boxes).

Another strategy to alter microbiota composition is oral administration of probiotics. The idea of using living microorganisms that have a beneficial effect on health to repopulate the gut microbiota and reduce the extent of atherosclerosis is very attractive since it is thought to be non-invasive and to have little or no side effects. The various possible mechanisms by which probiotics could modulate the microbial metabolism and antagonize other strains to attenuate CVD have been recently reviewed by Din *et al.* (2019).[116] Various oral probiotics have been evaluated for their ability to modulate the circulating TMAO levels in different cohorts including healthy subjects or patients with a CVD-related pathology.[117–120] However, none of them seemed to significantly alter TMAO levels in the treated groups compared to the respective placebo groups. Other strategies involving the oral intervention with bacteria have been explored for the ability of specific strains to target TMA biosynthesis in the gut. For instance, Matsumoto *et al.* (2017) investigated the effects of *Bifidobacterium animalis* subsp. *lactis* LKM512 supplementation during 12 weeks in the fecal TMA content of healthy volunteers.[121] Authors observed a reduction in fecal TMA concentration in the group sup-

plemented with the probiotic that was coincident with a lower relative abundance of specific bacterial groups, including TMA-producing bacteria. Qiu *et al.* (2017) have explored another interesting strategy aimed at depleting TMA levels in the gut using TMA-degrading bacteria. In their report, oral administration of a TMA-metabolizing strain (*Enterobacter aerogenes* ZDY01) reduced the levels of TMA in cecum and TMAO in serum, and altered microbial community composition in mice.[122] Despite these encouraging results, human intervention studies showing the efficacy of probiotics to reduce plasma TMAO are still lacking.

A specially promising strategy is represented by the oral administration of compounds that are able to specifically inhibit microbial TMA biosynthesis in the intestinal lumen. This targeted approach has been explored to discover structural analogs of choline to inhibit choline TMA lyase, a major player in TMA biosynthesis. The 3,3-dimethyl-1-butanol (DMB), naturally found in olive oil and grapeseed oil, was the first molecule to specifically inhibit choline TMA lyase *in vitro* and *ex vivo* on fecal suspensions without exhibiting lethal effects on targeted bacteria.[41] In addition, co-feeding experi-

ments with 1% choline supplement and 1% (v/v) DMB in the drinking water showed that exposure to DMB significantly reduced the circulating levels of TMAO in C57BL/6J mice, attenuating macrophage foam cell formation and atherosclerosis induced by high-choline diet. Further advances of the same group in this research line were directed to explore other possible benefits of targeting microbial TMA biosynthesis for reducing CVD risk, such as for instance the attenuation of thrombosis potential.[123] In this case, various novel choline TMA lyase inhibitors, named halomethylcholines, were designed and developed to fulfill various criteria to act as suicide-substrates. The rationale behind this was that upon recognition and cleavage by the enzyme, the analog would be transformed in a highly reactive product that could lead to the irreversible inactivation of the enzyme. Using this approach, iodomethylcholine and fluoromethylcholine were found to exhibit the most potent inhibitory activity as determined in lysates from *Proteus mirabilis* with reported EC_{50} values of 1.3 nM and 900 pM, respectively, and greatly exceeding the observed EC_{50} for DMB under similar experimental conditions (*i.e.*, 100 μM). It was also shown that choline TMA lyase inhibition in mice fed a high-choline diet reduced circulating TMAO levels and platelet aggregation. In a recent report, a structure-guided approach has been applied to discover choline and betaine analogues able to inhibit choline TMA lyase.[124] In their interesting report, authors detailed the mechanism of interaction between the enzyme and the inhibitor betaine aldehyde, a molecule found in celery and other foodstuffs. The inhibition of TMA formation was demonstrated *in vitro* with a recombinant choline TMA lyase from *Desulfovibrio alaskensis* G20, in whole cell assays with various choline-metabolizing human gut isolates, and *ex vivo* with human fecal suspensions. Besides choline TMA lyase, other enzyme activities are known to take part in the microbial production of TMA in the gut, and thus, could also be susceptible for inhibition. In this regard, the anti-ischemic drug meldonium has been suggested to inhibit endogenous L-carnitine biosynthesis and enhance renal excretion of TMAO.[67] Further *in vitro* studies have shown that this analog of L-carnitine and γ-butyrobetaine is also able to suppress L-carnitine transformation into TMA by the intestinal TMA-producer *Klebsiella pneumoniae* without affecting bacterial uptake of L-carnitine and growth, which may be suggestive of Rieske-type carnitine monooxygenase inhibition.[125] These findings pave the way for the discovery of new candidate molecules that may serve in interventions to interfere with TMA biosynthesis in the gut lumen, and subsequently, reduce circulating TMAO levels and its linked deleterious effects on health.

As discussed previously, some investigations about the influence of dietary TMA precursors (and their rich food sources) on TMAO levels have resulted in mixed evidence from observational and intervention studies (summarized in Table 1). However, the generalized idea is that high intake of food enriched in TMA precursors has the potential to increase circulating TMAO levels. Also, the frequent observation that certain dietary patterns that include abundant intake of plant-based foods may reduce circulating TMAO levels suggests the possibility that plant-derived dietary constituents, other than TMA precursors, may also interfere with the TMA/TMAO metaorganismal pathway and affect the TMAO levels. Sustaining the hypothesis that certain dietary compounds may modulate the gut microbial composition and metabolism, the search for novel food ingredients that target the gut microbiota components and/or its TMA metabolic capacity offers great opportunities to devise novel functional foods to control TMA production and circulating TMAO levels.

6. Dietary intervention with bioactive food ingredients and natural extracts

Given the demonstrated relationship between diet, microbial TMA metabolism, and atherosclerosis promotion, the possibility that some dietary constituents with proved cardioprotective activities may also modulate circulating TMAO levels cannot be totally dismissed as these aspects have been poorly investigated. In this area, *in vitro* studies evaluating the potential of different dietary phytochemicals to interfere with microbial TMA generation have provided interesting data that bring to light the difficulties to investigate the complex link between diet and gut microbial metabolism.[126] As it will be discussed next (and summarized in Table 2), a number of studies have been directed to investigate the effects of food constituents or plant-based extracts on circulating TMAO levels and/or the associated damage without examining possible implications in gut microbiota composition and metabolism. Alternatively, other studies on food ingredients and phytochemicals with TMAO-reducing activity have also included interesting data on different taxa of gut microbiota associated with the observed TMAO changes. In most of the cases, such associations do not establish a causative role of those taxa in TMAO changes, although such findings might offer great opportunities for hypothesis generation. In addition, most studies are currently carried out in animal models, and therefore, more translational research will be required to corroborate the efficacy of candidate dietary constituents in humans, and their observed beneficial impact.

6.1. Evidence from animal studies

Connected with the idea that intervention with specific dietary constituents could be a feasible option to interfere with TMAO, and possibly contribute to prevent atherosclerosis, various published studies investigating the potential effects of different food constituents, including dietary fibers, oils, and phytochemicals individually or in extracts, on circulating TMAO levels and/or its effects in animal models, are next discussed.

Food ingredients and natural extracts that attenuate TMAO-related damage. Several studies have reported the ability of various food supplements and natural extracts to ameliorate or suppress the plasma TMAO-induced deleterious effects in animal models. To facilitate functional interpretation, various

Table 1 Dietary intervention studies in healthy population with foods rich in TMAO and/or TMA precursors. Associations of TMAO levels and gut microbiota composition

Food or dietary pattern	Study design	Participant characteristics	Duration of intervention	Dietary intervention	Effect on TMAO level[a,b]	Changes in gut microbiota[c]	Ref.
Fish	Controlled trial	N = 24	1 day	Single meal	↑ urine (P)	n.r.	66
Fish and shellfish	Controlled trial	N = 8 (4♀ + 4♂)	1 week	150 g seafood per day	↑ urine ↑ plasma	n.r.	67
Fish or red meat	Randomized, controlled trial	Adult population (60 ± 3 years) N = 20 (9♀ + 11♂)	3 weeks	Meal for 3 consecutive days per week. Fish diet: week 1 88 g day⁻¹, week 2 222 g day⁻¹, week 3 412 g day⁻¹ Red meat diet: week 1 80 g day⁻¹, week 2 158 g day⁻¹, week 3 283 g day⁻¹	Fish: ↑↑ urine (P) Red meat: ↑ urine (P)	n.r.	70
Fish, chicken, red meat, processed meat	Randomized, controlled trial	Adult male population (51–64 years) N = 40♂	3 weeks	Meal for 3 consecutive days per week: Fish diet: week 1 98 g day⁻¹, week 2 259 g day⁻¹, week 3 483 g day⁻¹ Chicken diet: week 1 88 g day⁻¹, week 2 187 g day⁻¹, week 3 290 g day⁻¹ Red meat diet: week 1 80 g day⁻¹, week 2 158 g day⁻¹, week 3 283 g day⁻¹ Processed meat: week 1 80 g day⁻¹, week 2 158 g day⁻¹, week 3 283 g day⁻¹	Fish: ↑ urine (P) Fish: ↑ plasma (P,F)	n.r.	68
Fish, egg, red meat	Randomized, controlled crossover trial	Adult population (27.8 ± 1.0 years) N = 40 (20♀ + 20♂)	1 day	Test meal 1: fish (6 ounces cod fillet) Test meal 2: beef (6 ounces beef patties) Test meal 3: eggs (three whole hard boiled) Test meal 4: control fruit (natural apple sauce)	Fish: ↑↑ plasma (P) Egg: ↑ plasma (P) Beef: ↑ plasma (P)	High TMAO producers: ↑Firmicutes ↓Bacteroidetes	69
Eggs	Longitudinal, double-blind, randomized	Adult male population (28–53 years) N = 6 (4♀ + 2♂)	1 day	Egg doses: 0, 1, 2, 4, or 6 egg yolks Volunteers received the 5 egg doses with each dose separated by 2-week washout period.	≥2 yolks: ↑ plasma ≥2 yolks: ↑ urine	n.r.	72
Fish, meat	Randomized, controlled trial	Adult population (20–50 years) N = 17 (13♀ + 4♂)	3 weeks	Nine test meals with three different protein sources: fish, meat and vegetable	Fish meal: ↑ urine	n.r.	88
Seafood, non-seafood	Randomized, crossover trial	Adult population (18–65 years) N = 19 (12♀ + 7♂)	4 weeks	Seafood diet (cod, pollack, saithe, scallops) Non-seafood diet (chicken, lean beef, Turkey, pork, egg, milk products) Volunteers received two dietary treatments for 4 weeks separated by 5-week washout periods	↑ TMA in feces	Non-seafood diet: ↑Firmicutes ↓Bacteroidetes	92
Eggs	Single blind, randomized, crossover trial N = 15	Lacto-ovo-vegetarian women (35.7 ± 12.9 years) N = 15♀	8 weeks	Diet 1: 6 eggs per week (n-3FA-enriched) Diet 2: 6 eggs per week (n-3FA-nonenriched) Diet 3: egg-free (control) Volunteers received three dietary treatments separated by 4-week washout periods	NC in plasma (F)	n.r.	73

Table 1 *(Contd.)*

Food or dietary pattern	Study design	Participant characteristics	Duration of intervention	Dietary intervention	Effect on TMAO level[a,b]	Changes in gut microbiota[c]	Ref.
Eggs	Non-randomized, crossover trial $N = 38$	Young population (24.1 ± 2.2 years) $N = 38$ (19♀ + 19♂)	4 weeks	Diet 1: 1 egg per day; Diet 2: 2 eggs per day; Diet 3: 2 eggs per day. Volunteers received three dietary treatments for 4 weeks separated by 2-week washout periods	NC in Plasma (F)	n.r.	74
Eggs, choline	Non-randomized, crossover trial $N = 30$	Young population (25.6 ± 2.3 years) $N = 30$ (16♀ + 14♂)	4 weeks	Diet 1: 3 egg per day; Diet 2: 400 mg choline per day. Volunteers received two dietary treatments for 4 weeks separated by 3-week washout periods	NC in plasma (F)	n.r.	75
Eggs	Randomized, crossover trial	Young population (18–30 years) $N = 50$ (25♀ + 25♂)	4 weeks	Diet 1: 2 egg per day; Diet 2: 384 g oats per day	NC in plasma (F)	n.r.	76
Krill oil (PC-rich), choline	Randomized, crossover trial	Population aged 18–65 years $N = 18$ (9♀ + 9♂)	1 day	Treatment 1: 8 g krill oil; Treatment 2: 620 mg choline; Treatment 3: 6 g fish oil (control). Volunteers received a single dose of the three treatments separated by 2-week washout periods	Choline: ↑↑ plasma (P); Krill oil: ↑ plasma (P)	n.r.	78
Krill oil (PC-rich)	Controlled trial	Middle-aged population (18–36 years) $N = 17$ (15♀ + 2♂)	4 weeks	Treatment: 9 capsules krill oil (500 mg per each)	NC in plasma	n.r.	79
Red meat	Randomized, crossover trial	Adult male population (25–74 years) $N = 12♂$	2 weeks	Diet 1 (low-meat): 60 g day^{-1}; Diet 2 (high-meat): 420 g day; Diet 3 (non-meat sources): 420 g day^{-1}. Volunteers received three dietary treatments for 2-week separated by 1-week washout periods	High-meat diet: ↑ urine	n.r.	85
Red meat, chicken	Randomized, controlled trial	Middle-aged population (22–37 years) $N = 18♂$	1 day	Test meal 1: red meat (100 g and 200 g); Test meal 2: chicken (100 g and 200 g); Test meal 3: rice control (125 g)	Red meat: ↑ plasma	n.r.	86
Red meat, poultry	Randomized, controlled crossover trial	Adult population (21–65 years) $N = 113$ (44♀ + 69♂)	4 weeks	Diet 1: red meat-rich diet (beef/pork); Diet 2: white meat-rich (poultry); Diet 3: non-meat control diet (legumes, grains). Volunteers received three 4-week dietary treatments separated by 2-week washout periods	Red meat: ↑ plasma (F); Red meat: ↑ urine (F)	n.r.	87
Fermented, non-fermented dairy products	Randomized, crossover trial	Young male population (18–39 years) $N = 14♂$	2 weeks	Diet 1: probiotic yogurt per day; Diet 2: acidified milk per day. Volunteers received two dietary treatments for 2 weeks separated by 3-week washout periods	Ac. milk: ↑ urine (P); Ac. milk: ↑ plasma (P)	n.r.	91
Milk, cheese	Randomized, crossover trial	Young population (18–39 years) $N = 10$ (5♀ + 5♂)	1 day	Test meal 1: milk; Test meal 2: cheese; Test meal 3: soy	NC in plasma/urine	↑ Uncharacterised genus (*Lachnospiraceae*); ↓ *Eisenbergiella* (*Lachnospiraceae*)	91

Table 1 *(Contd.)*

Food or dietary pattern	Study design	Participant characteristics	Duration of intervention	Dietary intervention	Effect on TMAO level[a,b]	Changes in gut microbiota[c]	Ref.
Resistant starch	Controlled, randomized, crossover trial	Middle-aged population (mean 44 years) $N = 52$ (32♀ + 20♂)	2 weeks	Diet 1: high-resistant starch diet (high RS) Diet 2: low-resistant starch diet (low RS) The study consisted of two arms: higher and lower total carbohydrate (CHO) intake with comparation of high RS vs. low RS intake in random order in each arm	Lower-CHO/high RS: ↑plasma (F)	Not relevant	93
Dietary patterns							
Western diet (high-fat)	Controlled trial	Young population (18–30 years) $N = 10$♂	5 days	Following a 2-week eucaloric control diet (baseline testing), participants consumed a eucaloric, high-fat diet (55% fat) for 5 days.	NC in plasma (F) ↑plasma (P)	n.r.	95
Mediterranean diet	Parallel, multicenter, randomized, controlled trial	Non-diabetic subjects at high CVD risk from PREDIMED Adult population (mean 66.7 years) $N = 98$ (70♀ + 28♂)	1 and 3 years	Diet 1: mediterranean diet plus EVOO Diet 2: mediterranean diet plus nuts Diet 3: control low fat diet	Low-fat diet: ↑urine (F)	n.r.	102
Mediterranean diet	Randomized, controlled trial	Healthy people at increased risk of colon cancer (mean 52 ± 12 years) $N = 115$ (83♀ + 32♂)	6 months	Diet 1: mediterranean diet (higher fish intake) Diet 2: healthy eating diet (higher red mead intake)	NC in serum (F)	n.r.	108

[a] Increase (↑), decrease (↓), no changes (NC). [b] Changes in TMAO levels refer to plasma, urine or fecal samples. Fasting (F), postprandial (P). [c] Non reported (n.r.).

Table 2 *In vivo* studies investigating links between bioactive food and natural ingredients on circulating TMAO levels

Food or natural source	Intervention	Duration	Animal model/diet	Main findings	Effect on TMAO[a,b]	Effect on FMO[a]	Changes in gut microbiota[a]	Ref.
Fish	2% fish oil (triglyceride phenotype, DHA + EPA)	12 weeks	Male C57BL/6 mice/high-fat diet + 0.2% TMAO	Amelioration of TMAO-aggravated adipose tissue inflammation and impaired glucose tolerance	—	n.r.	n.r.	128
Fish	1.5% fish oil (triglyceride phenotype, DHA > 70%)	8 weeks	Male C57BL/6 mice/rodent chow	FMO3 mRNA level was significantly higher in fish oil diet	↑	↑	n.r.	129
Ginger	1% extract enriched in gingerol and shogaol isomers	12 weeks	Male C57BL/6 mice/high-cholesterol diet + 0.2% TMAO	Alleviation of the TMAO-aggravated elevation in plasma TC and vascular inflammation	—	n.r.	n.r.	130
Tartary buckwheat (Fagopyrum tataricum)	200–800 mg flavonoid-rich extract per kg bw	8 weeks	Male Kunming mice/rodent chow + 1.5% TMAO	Reduction of TMAO-induced vascular dysfunction and hepatic injury	n.r.	n.r.	n.r.	131
Apple peel	100–400 mg phloretin per kg bw	10 weeks	Male Kunming mice/rodent chow + 3% choline	Alleviation of vascular endothelial dysfunction and liver injury induced by choline	n.r.	n.r.	n.r.	132
Hovenia dulcis	200–800 mg myricetin purified extract per kg bw	8 weeks	Male Kunming mice/rodent chow + 3% choline	Amelioration of choline-induced vascular endothelial dysfunction and liver injury	n.r.	n.r.	n.r.	133
Fruits and herbs	200 and 400 mg ursolic acid per kg bw	8 weeks	Male Kunming mice/rodent chow + 3% choline	Protective against high-choline diet-induced endothelial damage and liver injury	n.r.	n.r.	n.r.	134
Red Fuji apple peel	300–900 mg polyphenol extract per kg bw	8 weeks	Male Kunming mice/rodent chow + 3% choline	Amelioration of choline-induced vascular endothelial dysfunction and liver injury	n.r.	n.r.	n.r.	135
Gynostemma pentaphyllum	200–800 mg saponins-enriched extract per kg bw	8 weeks	Male Kunming mice/rodent chow + 3% choline	Protection against endothelial dysfunction and liver injury in high choline-treated mice	n.r.	n.r.	n.r.	136
Lentinus eodes and *Flos lonicera*	0.675% lentinan and F. lonicera polysaccharides	8 weeks	Male Sprague-Dawley rats/high-fat diet	Attenuation of obesity symptoms	↓ urine	n.r.	n.r.	138
Lonicera caerulea berry	75–300 mg polyphenol-enriched extract per kg bw	12 weeks	Male Sprague-Dawley rats/high-cholesterol diet	Improvement of plasma lipid profile and promoted greater neutral and acidic sterol excretion	↓	n.r.	n.r.	139
Garlic	10 mg allicin per kg per day for a 25 g mouse	6 weeks	Male C57BL/6 (B6) mice/chow + 0.02% L-carnitine	During a challenge test with carnitine, plasma TMAO levels in C57BL/6 mice fed with dietary carnitine were 4–22 times greater than that in the control chow diet group	↓	n.r.	↓ *Robinsoniella peoriensis* ↑ *Clostridium* sp. Culture Jar-13	140

Table 2 (Contd.)

Food or natural source	Intervention	Duration	Animal model/diet	Main findings	Effect on TMAO[a,b]	Effect on FMO[a]	Changes in gut microbiota[a]	Ref.
Grapes, berries	0.4% resveratrol	1 month	Female C57BL/6J mice/chow + 1% choline	Attenuation of TMAO-induced atherosclerosis by decreasing TMAO levels and increasing hepatic BA neosynthesis via gut microbiota remodeling	↓	↑ mRNA & activity	↑ Bacteroidetes/↓ Firmicutes	141
			Female ApoE⁻/⁻ mice/chow + 1% choline				↑ Bacteroides, ↓ Lactobacillus, ↑ Bifidobacterium, ↑ Akkermansia, ↓ Prevotella, ↓ Ruminococcaceae, ↓ Anaerotruncus, ↓ Alistipes, ↓ Helicobacter, ↓ Peptococcaceae	
Fish/flaxseed	20% energy from fish oil or from flaxseed oil	12 weeks	ApoE⁻/⁻ mice under a typical western diet + 0.2% TMAO	Fish oil was more effective in inhibiting TMAO-induced atherogenesis by lowering plasma cholesterol and inflammatory cytokines. Both oils could reverse TMAO-induced decrease in fecal acidic sterols	—	n.r.	Fish oil: ↓ Firmicutes, ↑ Bacteroidetes, ↑ Actinobacteria, ↓ Desulfovibrionaceae	142
Grape, grape wine	250 mg pterostilbene per kg per day	6 weeks	Female C57BL/6 (B6) mice/chow + 1.3% L-carnitine	Amelioration of carnitine-induced vascular inflammation after consumption of pterostilbene is partially mediated via modulation of gut microbiota composition and hepatic enzyme FMO3 gene expression	↓	↓ mRNA	↑ Bacteroides ↓ Erysipelotrichia	143
Oolong tea and citrus peel	1% tea water extract/1% polymehtoxyflavones-enriched preparation	6 weeks	Female C57BL/6 (B6) mice/chow + 1.3% L-carnitine	Attenuation carnitine-feeding-induced vascular inflammation	↓	↓ mRNA	OT: ↑ Bifidobacteriales, ↑ Lactobacillus	144
Decaisnea insignis seeds	6 g omega-7 enriched oil per kg bw	12 weeks	Male Kunming mice/rodent chow + 3% L-carnitine	Inhibition against the elevation of hepatic injury biomarkers activities and dyslipidemia, as well as liver inflammatory cytokine release in L-carnitine-fed mice	↓	n.r.	CP-PMF: ↑ Akkermansia muciniphila ↓ Firmicutes, ↓ Proteobacteria, ↓ Erysipelotrichaceae ↑ Lactobacillus, ↑ Akkermansia	145
Red bell pepper	200 mg capsanthin extract per kg bw	12 weeks	Male C57BL/6J mice/high-fat diet	Reversed glucose tolerance, effectively decreased the serum triglycerides, total cholesterol, low-density lipoprotein cholesterol induced by high-fat diet	↓	n.r.	↑ Bacteroidetes, ↑ Bifidobacterium, ↑ Akkermansia, ↓ Ruminococcus ↓ Firmicutes/Bacteroidetes	146
Lycopersicon esculentum, Amaranthus tricolor, and Sorghum bicolor	200 mg lycopene, amaranth, or sorghum red per kg bw	12 weeks	Male C57BL/6J mice/high-fat diet	Improvment of the liver profiles of TG and TC, and effectively ameliorated lipid metabolism	↓	↓ activity	↓ Clostridium; LC and SR: ↑ Akkermansia, AR and SR: ↓ Prevotella	147

Table 2 *(Contd.)*

Food or natural source	Intervention	Duration	Animal model/diet	Main findings	Effect on TMAO[a,b]	Effect on FMO[a]	Changes in gut microbiota[a]	Ref.
Chicory roots, others	GI: 7.5% GOS + 7.5% inulin PF: polydextrose + insoluble dietary fiber GI/PF: 7.5% GI + 7.5 PF	3 weeks	Male BALB/c mice/rodent chow	A single type of oligosaccharides or dietary fiber caused the reduction of bacteria species and an increase in TMAO. The flora diversity was improved and the TMAO values were unaltered when the addition of nutritionally balanced GI/PF mixture	GI: ↑ PF: ↑ GI/PF: —	n.r.	GI: ↑ *Bacteroides*, ↓ *Alloprevotella* species PF: ↓ *Bacteroides*, ↑ *Alloprevotella* species GI/PF: ↑ *Bacteroides*, ↑ *Alloprevotella*, ↑ *Parabacteroides* species	148
Natural, fermented, and steam-exploded wheat bran	7.5% and 15% arabinoxylan-enriched fiber	4 weeks	C57BL/6 mice/western-style diet (+20% red meat)	Decreased energy intake, weight gain, intestinal pH values, and serum lipid and cholesterol levels. Soluble dietary fiber-diet also enhanced the production of short chain fatty acids with activation of the intestinal epithelial AMPK	→	n.r.	*Enterococcus* significantly positively associated with plasma TMAO *Candidatus stoquefichus* significantly negatively associated with plasma TMAO and TMA	149
Ligustrum lucidum dried ripen fruit	4.9 g extract per kg per day	65 days	Aged male ICR mice/rodent chow	Preserved non-osteoporotic bone phenotype and an improvement on the cognitive function	→	↓ protein	Prevented alterations induced by chemical aging treatment in the relative abundance of *SMB53, Clostridium, Sutterella, Coprococcus, Desulfovibrio, Allobaculum, Lactobacillus,* and *Bifidobacterium*	151
Herb leaves, seeds, roots, flowers, and animal parts	0.5 ml Luhong granules per kg per day	6 weeks	Wistar rats/rodent chow	Improvement of intestinal flora and intestinal barrier function to delay ventricular remodelling after myocardial infarction	→	n.r.	↓ *Bacteroidales*, ↓ *Alistipes*, ↓ *Phascolarctobacterium*	152
Scutellaria baicalensis	25–200 mg baicalin (flavonoid) per kg per day	1 week	Male C57BL/6J mice/rodent chow	Ameliorates neuropathology in a repeated cerebral ischemia-reperfusion model mice by remodeling the gut microbiota	→	—	↑ *Halomonas smyrnensis* ↑ *Citromicrobium sp WPS32*, ↓ *Parabacteroides johnsonii*, ↓ *Bacteroides uniformis*, ↓ *Lactobacillus plantarum*	153

[a] Increase (↑), decrease (↓), unaltered (—), non-reported (n.r.). [b] Otherwise stated, changes in TMAO levels refer to plasma or serum.

models of TMAO-induced damage have been utilized. Elevating plasma TMAO by direct oral intake of TMAO or supplements enriched in choline or L-carnitine are the most common examples used to induce the development of different lesions. For instance, Gao et al. (2014) reported that 0.2% TMAO supplementation in high-fat diet-fed C57BL/6 mice for three weeks exacerbates impaired glucose tolerance, inflammation in adipose tissue and obstructs the hepatic insulin signaling.[127] These observations were further confirmed by the same authors in another report, and suggested that the same dietary regimen but supplemented with 2% fish oil (DHA + EPA, 55%) for 12 weeks can mitigate TMAO-aggravated glucose intolerance and also decrease adipose tissue inflammation.[128] Interestingly, further research by the same authors suggested that 8-weeks supplementation with fish oil can transiently increase plasma TMAO levels via increased liver FMO3 activity after administration of TMAO precursors in mice.[129] In a similar approach, He et al. (2018) reported that the exacerbated vascular inflammation and hypercholesteromic effects of adding 0.2% TMAO in high-cholesterol diet-fed C57BL/6J mice were ameliorated with the addition of 1% ginger extract enriched in gingerol and shogaol isomers.[130] The cholesterol-lowering effect observed with dietary ginger extract was hypothesized to be the result of an increase in the synthesis and excretion of fecal bile acids. Although the report did not include gut microbiota data, it was speculated that the extract may reduce the number of coprostanol-producing bacterial species that could explain the observed changes of specific fecal neutral sterols. In a separate report, a 1.5% TMAO was administered in drinking water to Kunming mice, allowing them free access to water and rodent chow for 8 weeks.[131] Besides the control and TMAO-treated mice groups, three more mice groups under TMAO drinking water intake regime were administered intragastrically with different doses of a purified flavonoid-rich tartary buckwheat extract (enriched in rutin and quercetin). TMAO intake was reported to cause severe liver injury and vascular dysfunction that the flavonoid extract could prevent. In this study, the plasmatic TMAO were not reported, and authors suggested that the protective effect exerted by the tartary buckwheat extract may be mediated by inhibition of ROS generation and elevation of vascular nitric oxide production.

Although it is well known that lack of dietary choline can lead to non-alcoholic fatty-liver disease and muscle damage, a growing number of reports have demonstrated that ingestion of some choline-enriched diets can effectively induce TMAO-associated damage in mice, such as endothelial dysfunction and liver injury. In a series of works, Yang et al. have investigated the ability of various phytochemical-enriched natural extracts and food ingredients to reverse the harmful effects of chronic high-choline diet. More precisely, healthy male Kunming mice fed with regular chow supplemented with 3% dietary choline in water for 8 weeks served as experimental model for testing the protective potential of flavonoids, such as phloretin[132] and myricetin purified extract from Hovenia dulcis Thunb.;[133] other phenolic compounds such as the triter-

pene ursolic acid[134] and those found in red Fuji apple peel extract;[135] and the saponin-enriched extract from Gynostemma pentaphyllum[136] against vascular dysfunction and hepatic damage. These co-feeding studies with the mentioned bioactive compounds and choline suggest that such phytochemicals have the potential to ameliorate the plasma lipid profile, hepatic function and biomarkers for endothelial function in mice. However, in spite of these observed beneficial effects, their underlying mechanism remain unclear and a link between them and TMAO damage cannot be directly established since TMAO levels in plasma and tissues were not reported. With regards to bioactive saponins, an in vitro study with HUVECs has recently demonstrated that an asparagus saponin-enriched extract reverses the inhibitory effect of TMAO on cell proliferation.[137] Such effect appears to be mediated by Clock and Baml1 molecules, that play relevant roles in circadian cycle and are under control of NEAT1 and MAPK pathways. In contrast to other reports, TMAO exposure seemed to have a negative effect on MAPK signaling; however, such inhibitory activity was observed at TMAO concentrations that are well above the physiological circulating levels reported in humans and mice.

Accumulated evidence suggests that high-fat diet induces obesity concomitant with the rise of circulating TMAO levels in animal models. In a study with high-fat diet fed Sprague-Dawley rats, this model was established to evaluate the anti-obesity effects of supplementing a mixture of lentinan β-glucan and Flos Lonicera polysaccharide after a 8-weeks period.[138] In this case, untargeted NMR metabolomic analysis combined with multivariate analysis indicated that polysaccharide mixture intervention reduced the TMAO levels in urine compared to the levels found during the endogenous metabolic response to high-fat diet, suggesting that polysaccharides may also affect the structure of intestinal flora; however, further research would be necessary to verify such hypothesis. In male Sprague-Dawley rats, high-cholesterol diet also induced high levels of plasma TMAO in addition to hypercholesterolemia.[139] Supplementing the diet with a polyphenol-enriched extract from Lonicera caerulea berry attenuated such effects and increased fecal excretion of neutral and acidic sterols. Authors discussed that a plausible underlying mechanisms to explain the cholesterol-lowering effect of the extract involved inhibition of cholesterol absorption, mediated by downregulation of NPC1L1, ACAT2 and MTP, and upregulation of ABCG5/G8 transporters. However, the mechanism by which the extract induced plasma TMAO levels are unclear since gut microbiota data and hepatic FMO3 activity were not reported.

Bioactive ingredients that alter TMAO levels and affect gut microbiota. Collectively, the aforementioned studies provide encouraging results about the ability of food ingredients and natural extracts for alleviating TMAO-related harmful effects. However, our knowledge about the link between the observed effects by those individual compounds or mixtures on endogenous TMAO and the gut microbiota is very limited. In the last decade the investigation of the effects of dietary constituents, other than TMA precursors, on microbiota compo-

sition, structure and metabolism has attracted much more attention; however, in the last three years is when a significant number of reports, directed to study potential microbiota-mediated mechanisms underlying the observed effects of bio-active dietary constituents (individually or in extracts) on TMAO levels have been published. One of the first reports aimed at investigating these aspects is the one related with dietary allicin and its potential to reduce the transformation of ingested L-carnitine into TMAO through modulation of gut microbial ecosystem in mice.[140] Although in their study authors used synthetic allicin as supplement, this compound is naturally present in crushed garlic and it has a recognized broad spectrum antimicrobial activity. To test their hypothesis, authors performed experiments in male C57BL/6 mice with an amount of allicin, given *via* gastric gavage, equivalent to the amount that an adult human would acquire from 5 cloves of freshly blended garlic per day. Allicin supplementation was tested with two chow diets, the normal control and 0.02% L-carnitine in the drinking water, as calculated to be equivalent to the intake of an adult human following an omnivorous diet. After feeding mice for 6 weeks, a L-carnitine challenge test indicated that plasma TMAO increased up to 22 times greater in mice fed with dietary L-carnitine than that in the control chow diet, and more interestingly, allicin gavage in the L-carnitine supplemented diet fed mice counteracted the elevation of TMAO levels in plasma during the challenge. A comparison of microbiota composition in cecum in the different mice groups revealed differences in the relative abundance of some bacterial species, and the analysis of data suggested positive correlation of several species, including *Robinsoniella peoriensis*, with elevation of TMAO in plasma. Although, authors hypothesized that allicin may play as a Rieske protein inhibitor to reduce L-carnitine conversion to TMA by gut microbiota, various questions may remain opened, including a possible inhibitory effect of allicin of FMO3 activity.

The anti-atherosclerotic effects of resveratrol and their underlying mechanisms associated with microbiota composition have been investigated by Chen *et al.* (2016).[141] In their study, authors found that addition of 1% choline in chow diet during 30 days significantly increased the plasmatic levels of TMA and TMAO in female C57BL/6J mice, whereas the same diet supplemented with 0.4% resveratrol reversed the elevation of both metabolites. However, this effect was transient since a washout with a chow diet for 1 month after the treatment failed to reduce TMA and TMAO in the same mice. Interestingly, resveratrol supplementation appeared to increase hepatic FMO3 activity, indicating that the observed resveratrol-induced decrease in TMAO levels was not mediated by FMO3 inactivity in the liver, and therefore, suggesting a possible involvement of gut microbiota as mediator of the resveratrol effects. Reinforcing that hypothesis, *ex vivo* experiments with cecal content from resveratrol-treated mice showed a lower capacity to produce TMA from choline than in the control non-supplemented mice. Analysis of gut microbiota composition revealed that the choline-enriched diet supplemented with

this phytochemical induced significant changes at the phylum level showing an evident decrease of the Firmicutes to Bacteroidetes ratio, and at genus level, resveratrol increased the relative abundance of genera, *Bacterioides*, *Akkermansia*, *Lactobacillus* and *Bifidobacterium*, the two latter being related to bile salt hydrolase activity, bile acids deconjugation and fecal excretion. Connected to this, bile acid content in ileum was reduced, an effect that was coincident with the down-regulation of the gut-liver FXR-FGF15 axis and the activation of bile acids synthesis.

More recently, a comparison of the effects of fish oil and flaxseed oil on gut microbiota of ApoE^{−/−} mice under a typical western diet supplemented with TMAO for 12 weeks has recently been reported.[142] The experimental design included various diets, including a Western diet, a WD with added 0.2% TMAO, and two experimental WDs with added TMAO and with half of the lard being replaced by either flaxseed oil or fish oil for 12 weeks. In agreement with previous studies, results showed that addition of 0.2% TMAO to WD increased by 2-fold circulating plasma TMAO, and significantly aggravated atherosclerotic lesions. Neither flaxseed oil nor fish oil significantly altered the plasma TMAO concentration; however, both flaxseed oil and fish oil could reverse the reductions in fecal acidic sterol excretion and CYP7A1 expression induced by the TMAO addition to WD. On the other side, only fish oil feeding had a significant effect facilitating the fecal excretion of neutral sterol and could significantly lower the plasma concentration of proinflammatory cytokines. Amplicon sequencing analysis of 16S rDNA gene suggested that TMAO addition into Western diet did not lead to an obvious shift in overall microbial profile, whereas both flaxseed oil and fish oil had great impacts on the microbial profile composition. For instance, feeding fish oil led to a sharp decrease in the Firmicutes to Bacteroidetes ratio, and featured an increased population of *Actinobacteria* compared with feeding western diet with TMAO. Fish oil was more effective than flaxseed oil in promoting the growth of short-chain fatty acid-producing bacteria and lowering microbial generation of lipopolysaccharides. Thus, it could be concluded that, in general, fish oil exerted more potent cardioprotective effects than flaxseed oil in counteracting TMAO-exacerbated pro-atherosclerotic effects.

More recently, Koh and colleagues reported the effect of pterostilbene, a dimethoxylated analog of resveratrol found in a wide variety of dietary sources, on alleviating TMAO generation and its harmful consequences in vascular function.[143] In this case, addition of 1.3% L-carnitine water to chow diet increased plasma TMAO up to eight-fold in C57BL/6 mice. Supplementation of the high-carnitine diet with 0.05% pterostilbene partially suppressed TMAO elevation compared to the observed in the high-carnitine control group. Pterostilbene also significantly down-regulated FMO3 gene expression compared with the FMO3 gene expression level observed in mice fed high-carnitine diet, a finding that may also explain the reduced plasma TMAO observed in mice fed pterostilbene supplemented high-carnitine diet. In addition, 16S rRNA sequencing analysis revealed that supplementing diet with this poly-

phenol increased the relative amount of *Bacteroides* and decreased *Turicibacter*, a genus belonging to *Erypsipelotricia*, recently associated with elevated TMAO. To note, authors discussed that, in agreement with the recent report by Wu *et al.* (2019),[137] measurements of *CntA* gene abundance in fecal samples did not provide a good correlation with TMAO levels, an observation that reflects the multifactorial nature of TMAO generation.

In another work, same researchers utilized the same experimental protocol to study the activities of Oolong tea water extract and citrus peel polymethoxyflavones-enriched preparation against TMAO formation and vascular inflammation induced by high-carnitine feeding.[144] In this case, the experimental high-carnitine diet was supplemented with either 1% Oolong tea extract or 1% polymethoxyflavone preparation and utilized in mice feeding for 6 weeks. Similar results with regard to plasma metabolites and FMO3 gene expression as previously discussed with pterostilbene were obtained. More precisely, plasma TMAO levels in Oolong tea extract- and polymethoxyflavone-treated mice were significantly lower than those in the high-carnitine control diet group, but the plasma L-carnitine levels were more elevated. In addition, both supplements suppressed the FMO3 gene up-regulation observed in high-carnitine diet fed mice. Interestingly, cecum microbiota analysis suggested that carnitine group increased the relative abundance of Proteobacteria and Tenericutes and reduced the relative abundance of Deferribacteres, and at genus level, both supplements increased the relative abundance of *Bacteroides* compared to the high-carnitine group. In addition to this common effect to both supplements, in polymethoxyflavones-treated group the relative abundance of *Akkermansia* increased, whereas those of *Lactobacillus* and Bifidobacteriales increased only in the Oolong tea-treated mice, changes that were similar to the observed effects exerted by resveratrol, as reported by Chen *et al.* 2016.[141]

Decaisnea insignis seed oil mainly consists of monounsaturated fatty acids, and it has been reported to have a similar fatty acid profile as olive oil. Zhang *et al.* (2019) have recently investigated the effectivity of this oil in alleviating liver dysfunction induced by high-carnitine diet in mice.[145] *D. insignis* seeds were extracted using supercritical fluid extraction, and the obtained oil was used to supplement normal diet with 3% L-carnitine in drinking water in Kunming male mice for 12 weeks. Chronic administration of such high-carnitine diet elevated plasma and urinary TMAO and TMA levels and induced abnormal liver lipid metabolism compared with normal diet. On the other side, diet with *D. insignis* seed oil decreased TMAO levels and improved hepatic serum lipid and liver injury biomarkers in high-carnitine diet fed mice. Moreover, *D. insignis* seed oil consumption affected the total SCFA content in high-carnitine diet fed mice. Another interesting counteracting effect of the seed oil extract treatment were observed in microbiota composition. For instance, it restored the unbalance exerted by high-carnitine diet at the phylum level, specially decreasing the Firmicutes to Bacteroidetes ratio. Also the relative abundance of Proteobacteria in high-

carnitine diet was significantly decreased by seed oil administration. Interestingly, at the genus level, seed oil treatment increased proportions of *Akkermansia*, *Bacteroides*, and *Lactobacillus* in high-carnitine diet-fed mice, whereas *Erypsipelotrichaceae* abundance was reduced. In addition, the latter genus showed significant positive correlation with increased serum TMAO levels.

As mentioned above, compelling evidence suggests that TMAO regulates cholesterol and sterol metabolism in various tissues and processes, thus having a putative role in obesity. Connected with this, Wu and colleagues investigated whether the anti-obesity effect of various food ingredients and extracts are associated with gut microbiota and TMAO using high-fat diet-induced obese C57BL/6J mice.[146,147] In one report, authors extracted red bell pepper using supercritical fluid extraction to obtain a product containing 92.5% capsanthin, a carotenoid pigment with reported anti-inflammatory activity.[146] Capsanthin-enriched extract was added to high-fat diet and administered for 12 weeks. Capsanthin extract alleviated the high-fat diet-induced high blood glucose level and dyslipidemia, decreased by 31% serum TMAO level and reduced body weight by 27.5% compared with untreated high-fat diet-fed mice. Furthermore, capsanthin modestly reduced the relative abundance of Firmicutes and increased that of the Bacteroidetes in the high-fat diet group. More importantly, capsanthin-enriched extract consumption increased some genera including *Allobaculum*, *Akkermansia*, *Bifidobacterium*, *Blautia*, *Coprobacillus*, whereas the relative abundance of some other declined, including *Lactobacillus*, *Prevotella*, *Helicobacter*, *Bacteroides*, *Ruminococcus*, *Parabacteroides*, *Odoribacter* and *Bilophila*, suggesting that capsanthin-enriched extract modulated the gut microbial environment of high-fat diet-fed mice. Authors hypothesized that observed decrease in serum TMAO level with capsanthin treatment could be due to changes in the intestinal microbiota environment caused by the weight loss in mice; however, as in other studies the influence of the extract on the liver FMO3 activity was not reported. In further research by the same group, the same experimental setting was utilized to study anti-obesity activity and association with gut microbiota structural changes and TMAO generation of other three food pigments. Thus, lycopene, amaranth, and sorghum red pigments were obtained in the laboratory from *Lycopersicon esculentum* (M.), *Amaranthus tricolor*, and *Sorghum bicolor*, respectively.[147] The three pigments provided similar results as with capsanthin extract in terms of serum TMAO, glucose level and dyslipidemia, being shorgum red the least potent in lowering TMAO levels. After the administration of high-fat diet supplemented with lycopene, amaranth, or sorghum red, the intestinal microbial environment improved in terms of increased bacterial diversity. Lycopene and sorghum red favored the proliferation of *Akkermansia*, whereas amaranth and sorghum red limited the proliferation of *Prevotella*. Thus, similar conclusions and hypothesis with regard to TMAO metabolism were drawn; however, in this study, data indicated that supplementation with these phytochemicals significantly reduced liver FMO3 activity, suggesting

that lower hepatic capacity to metabolize microbial TMA into TMAO may be possible.

Dietary fibers are known to provide direct physical benefits, including increased fecal bulking and laxation. A growing body of evidence suggests that certain dietary fibers have the ability to modify microbiota structure and function, and decrease CVD risk, which made them suitable supplements for functional food development. Among dietary fibers, water-soluble dietary fiber has been recognized to exert beneficial effects on health by different mechanisms. The effects of supplementation of soluble fibers on TMA/TMAO metabolism and the related gut microbiota have recently gained great attention. For instance, Cheng *et al.* (2017) observed that galacto-oligosaccharides plus inulin and ordinary dietary fiber, supplemented individually or mixed for three weeks, had different effects on the gut microecology in male BALB/c mice.[148] Results revealed that functional oligosaccharides (galacto-oligosaccahrides and inulin) and ordinary dietary fiber supplementations gradually elevated TMAO levels over the experimental period, while the combination of both types of fibers favored reduction of TMAO levels in serum. That observation was coincident with the higher flora diversity found in supplementing diet with both fibers compared to individual supplementation and non-supplemented diets.

Li *et al.* (2017) investigated the effects of soluble dietary fibers on TMA and TMAO metabolism in C57BL/6 mice fed 20% red meat supplementation according to the Western-style diet.[149] In this report, chemically diverse arabinoxylan-enriched fiber fractions obtained from natural, fermented (inoculating *Rhizopus oryzae*), and steam-exploded wheat bran, were added to Western-style diet at two different concentrations (7.5% and 15%). The effects of the different supplements were investigated on parameters affecting host metabolism and gut microbiota structure after dietary intervention for 4 weeks. Results demonstrated that soluble fiber supplemented-diets could reduce TMA and TMAO metabolism by 40.6 and 62.6%, respectively. Dietary fiber feeding, specially fermented wheat bran, changed gut microbial ecology and promoted the growth of certain beneficial bacterial species. Soluble fiber-diet improved the serum lipid profile, decreased energy intake, weight gain, and intestinal pH values. The characterization of microbial communities revealed some interesting associations, such as for example the occurrence of phyla that are negatively associated with TMA and TMAO production. Also, in one of the diets supplemented with fermented dietary fiber, the phyla positively associated with cholesterol metabolism was found to predominantly belong to *Turicibacter* and *Bifidobacterium*, which coincided with lower TMA and TMAO plasma concentrations. Other interesting findings were that diets supplemented with soluble fibers also increased the production of SCFAs with activation of the intestinal epithelial adenosine monophosphate-activated protein kinase (AMPK). Further research of this topic suggested that compared with red meat consumption, fiber consumption favored a shift in the mice gut microbiome towards increased production of butanoate, which was concomitant to up-regu-

lation of genes that depend on AMPK signaling in the host intestinal epithelium.[150]

Fructus Ligustri Lucidi, coming from the dried ripe fruit of *Ligustrum lucidum* possesses a recognized anti-aging property to treat osteoporosis for a long time in Asia. A *Fructus Ligustri Lucidi* aqueous extract (enriched in flavonoids, terpenoids, phospholipids, and a long list of other classes of compounds) could have anti-osteoporotic effects and improve the cognitive function as observed in studies with chemically-aged male ICR mice with D-gal and NaNO$_2$ for three months.[151] Among other deleterious effects, aging mice presented significantly higher serum TMAO levels compared to those found in the normal controls. Interestingly, oral administration of the extract reduced serum TMAO to levels close to control diet, but also decreased hepatic FMO3 activity. In order to better understand the potential role of gut microbiota in the extract's efficacy against TMAO elevation and osteoporosis, authors compared normal control, aged control, and extract-induced gut microbiota changes in aged mice. Results revealed that gut microbiota in aged mice was characterized by abnormal changes in the Firmicutes to Bacteroidetes ratio, that were restored by the treatment with the fruit extract. Also, species of several bacterial taxa, including *Sutterella*, *Unclassified_Clostridiales*, *Coprococcus*, *Clostridium*, *Aldercreutzia*, *Oscillospira*, *Desulfovibrio*, *Bifidobacterium*, and *Lactobacillus* were associated with hepatic FMO3 activity and/or serum TMAO concentrations.

Yang *et al.* (2019) found that oral administration of Luhong granules, a complex mixture of various herb leaves, seeds, roots, flowers, and animal parts, for 6 weeks in acute myocardial infarction-induced Wistar rats could partially reverse ventricular remodeling and improve intestinal barrier function.[152] To note, the serum TMAO and lipopolysaccharides levels in the untreated acute myocardial infarction-induced rats was increased, but the levels of both bacteria-derived products were decreased in the Luhong granules-treated rats. Amplicon sequencing of fecal material of untreated acute myocardial infarction-induced rats showed changes in *Desulfovibrionaceae*, *Escherichia*, *Akkermansia*, Bacteroidales, *Alistipes*, and *Phascolarctobacterium*, in which there are potential TMAO producers. Furthermore, Luhong granules-treated rat presented reduced relative abundance of Bacteroidales, *Alistipes*, and *Phascolarctobacterium*, three genera that can promote TMAO generation.

The flavonoid baicalin have shown to improve glycolipid metabolism and the production of SCFAs by remodeling the gut microbiota. Recently, the neuroprotective mechanism of orally administered baicalin has recently been explored in the mouse model of repeated cerebral ischemia-reperfusion injury, including the effects of baicalin on the gut microbiota.[153] The reported results suggested that plasma TMAO levels were elevated in mice subjected to repeated cerebral ischemia-reperfusion, and baicalin supplementation suppressed cognitive and memory decline induced by repeated cerebral ischemia-reperfusion, restored functional connectivity and suppressed neuroinflammation. Interestingly, the results indicated that elev-

ated plasma TMAO levels correlated with behavioral and electrophysiological deficits in the model group mice compared to non-ischemic control mice. The gut microbial composition in ischemic mice featured a decrease in the Bacteroidetes and Firmicutes relative abundances in ischemic compared to non-ischemic control mice. In this experimental model, baicalin treatment restored the levels of Firmicutes and Bacteroidetes, and reduced TMA and TMAO synthesis without altering the levels of liver FMO3 protein and the activity of hepatic FMO. Also, the effects of baicalin were diminished when ischemic mice were pretreated with broad-spectrum antibiotics to deplete gut microbial populations, suggesting the involvement of gut microbes in the neuroprotective activity of baicalin.

Taking collectively the studies mentioned above, it seems reasonable to suggest that oral administration of food ingredients, including certain dietary fibers, animal and seed oils, and phytochemicals such as phenolic compounds, carotenoids, and other constituents naturally present in plant-based foods may influence the levels of circulating TMAO in animal models and counteract some of its deleterious effects. To note, a number of structurally diverse compounds or extracts seem to affect hepatic FMO activity which may also explain the observed changes in circulating TMAO levels. In spite of the great effort made in the available studies, only very few of them have successfully demonstrated a possible involvement of lower TMAO levels and gut microbiota as part of the underlying mechanism of the observed attenuating effects of the bioactive compounds under study. Some of the reported changes in microbiota by administration of bioactive food compounds affect community structure and composition. As discussed above, some dietary interventions were able to reduce the abundance of microorganisms that can promote TMAO generation, providing a plausible explanation of the observed decrease in TMAO levels. Although various hypotheses can be envisaged to explain such changes in the gut microbial ecology, the mechanistic insights of such community-induced changes have not been thoroughly explored yet. With regard to the link between microbiota and TMAO levels, the potential effects of bioactive food compounds in microbiota metabolism and function is another aspect that remains scarcely investigated. Considering the recent studies reporting on the inhibitory activity of naturally occurring compounds in food against microbial choline TMA-lyase, it can be expected further research aimed at exploring new food sources to attenuate gut microbial TMA generation.

6.2. Evidence from human studies

Evidence from human studies is required to better understand the role of the impact of certain dietary components on the composition and metabolism of gut microbiota for CVD prevention. As reported in previous sections, various human intervention studies reporting the effects of TMA precursors-enriched foods or diets on circulating TMAO levels have been published (summarized in Table 1). However, a very limited number of studies have reported the circulating TMAO-lower-

ing effects of specific foods, individual food constituents and natural extracts supplementations in humans.

Several of the published studies in humans have detected unexpected changes in TMAO levels in urine as part of the observed data in metabolomic studies. For instance, untargeted metabolomics studies have revealed changes in urinary TMAO levels after interventions with *Origanum dictamus* tea and *Curcuma longa* extract as supplements in humans.[154,155] Although effects on microbiota composition or function was a potential explanation for the observed decrease and increase in urinary TMAO after respective interventions, a possible alteration of kidney function was not excluded. An article by Hernández-Alonso *et al.* (2017) reported the results of a randomized, controlled, crossover trial with two 4-month dietary intervention arms separated by 2-week washout period conducted in prediabetic subjects.[156] During the intervention, two diets were provided, the control diet and a diet supplemented with 57 g day^{-1} of pistachio. In the study, 24 h urine samples, collected at baseline and at the end of each dietary intervention period, were subjected to NMR-based untargeted metabolomic analysis. Interestingly, although pistachios are rich in choline, comparison of the detected urinary metabolites before and after pistachio supplementation revealed that TMAO levels were significantly decreased following pistachio supplemented diet. Also, pistachio supplementation decreased the level dimethylamine compared to that found in the control diet. Other interesting findings in that study were the observed reduction of other two microbial metabolites, namely hippurate and *p*-cresol sulfate, in the urine from subjects after supplementation with pistachio compared to those under control diet.

Besides its role in CVD, TMAO is considered a uremic toxin, and it usually reaches maximum circulating (and urine) levels in individuals with CKD. A report by Hill *et al.* (2019) explored the feasibility of consuming a high β-glucan oat supplement and its effect after 12 weeks on uremic toxins and markers of mineral metabolism in CKD patients.[157] Almost one third of the enrolled patients did not tolerate the β-glucan oat supplement and withdrew from the study. Serum TMAO level decreased by 17%, and four weeks after discontinuation of the β-glucan oat supplement, TMAO levels were raised. However, the reduction in TMAO levels was statistically significant when only those patients who had TMAO levels above average healthy levels were included in the analysis, which could be indicative that β-glucan exerts its greatest effects in CKD patients with high baseline TMAO concentrations. Obeid *et al.* (2016) reported a study aimed at investigating the effects of dietary supplements other than TMA precursors on plasma TMAO levels in humans.[158] Their study was based on a randomized single-blinded non-placebo-controlled design in which volunteers without prevalent obesity and diabetes were supplemented with either vitamin D and calcium, or vitamin D, calcium, folic acid, and vitamins B5 and B12 for 12 months. Plasma homocysteine, TMAO and choline metabolites were measured at baseline and at the end of the intervention. Although a 67% reduction of plasma fasting TMAO was observed after vitamins D and B supplementation, authors dis-

cussed that the change did not exceed the expected biological variations of TMAO.

As exemplified previously, some of the studies, aimed at investigating the effects of individual phenolic compounds or enriched extracts in animal models, recognize the alteration of microbiota composition or function as a putative mechanism to alter the circulating TMAO levels. The rationale behind the claimed suitability of polyphenols to modulate microbiota composition or function stems from the poor bioavailability of these compounds, which varies depending on its chemical structure. This fact makes some phenolic compounds excellent candidates to reach the distal intestine and exert a significant impact on gut microbiota ecology and metabolism. However, an emerging hypothesis proposes that a potential mechanism of action for the TMAO-lowering effect of certain polyphenols could be based on the ability of such phenolic compounds to donate electrons to TMAO, which acts as an electron acceptor, resulting in reducing TMAO and generation of TMA. It has been speculated that such chemical events could occur at blood level, provided that polyphenols reach blood at physiological relevant levels. These ideas have been proposed in a series of papers reporting randomized trials to study polyphenol-enriched matrices with enhanced characteristics for bioaccessibility of phenolic compounds.[159–161] In the first study, a microencapsulated grape pomace polyphenolic extract with maltodextrin in acid-resistant capsule registered as Taurisolo® was evaluated in terms of the intestinal bioaccessibility of resveratrol, one of the representative polyphenol in the extract.[159] The efficacy of the supplement was also assayed in a randomized, placebo-controlled, crossover clinical trial on healthy subjects. More precisely, after a 7-day washout period, volunteers were subjected to a 28-day intervention period in which capsules containing 300 mg Taurisolo® were administered twice daily. Results indicated that supplementation with grape pomace polyphenolic extract capsules had a lowering effect on TMAO serum levels at the end of the intervention of about −63.5%. In addition, the reported values of resveratrol in blood after oral administration suggested that the polyphenol was absorbed at the intestinal level and reach the bloodstream. In a subsequent study, authors carried out a monocentric, double-blind, randomized, placebo-controlled, 2-arm parallel-group trial.[160] In this case, the cohort was constituted of overweight/obese subjects that were subjected to a run-in period lasted 4 weeks, whereas the intervention period and a follow-up period lasted 8 and 4 weeks, respectively. Two intervention groups were established, one supplemented with 300 mg Taurisolo® twice daily and the other supplemented with same dose of Taurisolo® and 300 mg pectin twice daily. Authors found TMAO serum levels significantly decreased after both interventions, but no significant differences were found between the two interventions, suggesting that pectin does not alter the effect exerted by grape pomace polyphenols. To note, authors discussed that the observed reduction of ox-LDL (a circulating oxidative stress biomarker) after polyphenol supplementation reinforces the hypothesis of an antioxidant effect of the supplement exerted at blood level. Despite these

relevant observations, the potential effects of the extract on gut microbiota are lacking and mechanistic studies remain to be conducted to confirm the exact underlying mode of action by which Taurisolo® polyphenols would reduce TMAO to TMA.

In a separate report, the effect of lactic fermentation on the bioaccessibility of polyphenols in Annurca apple (an apple variety with high oligomeric procyanidins) was evaluated.[161] To test its bioactivity, a comparative study of the effects of lactofermented apple puree (inoculated with *Lactobacillus rahmnosus* and fermented for 24 h), the unfermented puree counterpart and the probiotic alone (*L. rahmnosus*), on plasma lipid profile and TMAO was performed. To achieve this, individuals with CVD risk factors were randomized to three arms, according to a four-weeks run-in period, an eight-weeks intervention, and a four-weeks follow up study. Results indicated that fermentation increased individual free polyphenols from a minimum of 20% up to 45%. Also, intervention with the lactofermented puree exerted a higher variation of serum parameters, including the elevation of HDL cholesterol by +61.8% and the reduction in TMAO levels by −63.1%. In addition, the three interventions induced a strong increase in *Bifidobacterium* and *Lactobacillus* population. Interestingly, unfermented puree supplementation determined the greatest effects on these genera, followed by the intervention with *L. rhamnosus*, whereas lactofermented puree supplementation induced a significantly minor influence, making reasonable the hypothesis that the supplement richer in intact pectic compounds (unfermented puree) would provide with more substances of prebiotic interest which are well-known to stimulate the growth of fermentative bacteria.

Apart from these encouraging results about the potential of food ingredients and natural extracts to reduce circulating TMAO levels, some other articles can be found in the scientific literature reporting ineffective dietary interventions in humans or interventions that have a TMAO-elevating effect. For instance, Angiletta *et al.* (2018) evaluated the potential for commonly consumed dietary polyphenols administered at nutritionally relevant doses to reduce TMAO and γ-butyrobetaine in obese subjects, a population with generally high TMAO levels.[162] Authors performed a secondary analysis on samples from a previous human clinical study on the impacts of green tea and cocoa supplementation for a period of five days. As the study guidelines involved controlled feeding, it was assumed that the intake of TMAO precursors choline, L-carnitine and betaine were consistent. On the one hand, no significant differences were detected in circulating levels of TMAO and γ-butyrobetaine across the interventions. On the other hand, authors found great heterogeneity in the intra-individual variability of TMAO concentrations between subjects and interventions. Interestingly, a closer look into the data allowed the identification of "responders" and "non-responders" to the green tea and cocoa beverage with high-flavonoid content; however, no quantitative characteristics, including BMI, body mass, age, fat mass or lean mass were significantly different between responders and non-responders. In this article, authors raised many interesting questions such as the reliability of plasma TMAO measured at a single time point as

biomarker, and the importance of considering the factors that might influence TMAO variability, including gut microbiota composition, hepatic FMO3 expression and activity, dietary compliance, *etc.* Baugh *et al.* (2018) reported another non efficacious dietary intervention intended to reduce circulating TMAO levels.[163] In this case, a randomized clinical trial to evaluate inulin supplementation in controlled feeding for 6 weeks was proven to be ineffective in reducing either fasting or postprandial TMA and TMAO levels in overweight/obese adults at risk for T2D compared to those detected in placebo group.

7. Conclusions and future prospects

It has been demonstrated that diet plays a key role in the production of TMAO since it provides with the nutritional precursors to produce TMA and TMAO. In general, there is association in the levels of circulating TMAO after the consumption of foods rich in dietary trimethylamines; however, some results are conflicting, and therefore, it would be necessary to address, for instance, the different bioavailability of dietary precursors and their effect on gut microbiota composition and its TMA metabolic capacity. In last years, TMA/TMAO metabolism has emerged as an attractive target for prevention and treatment of cardiovascular disease. Targeting microbiota and host metabolic processes involved in TMA and TMAO generation through diet is an approach that holds promise for future intervention. The ability of specific foods, food constituents and phytochemicals found in herbs to lower the levels of circulating TMAO has been mostly demonstrated in animal models. In very few cases, the association between changes in TMAO levels and gut microbiota has been clarified, and the exact mechanisms underlying the effects of the dietary elements object of study remain to be elucidated. More importantly, studies showing that reducing circulating TMAO levels exerts a positive effect in humans are scarce. On this point, as most of the studies have been conducted in animals, the findings are difficult to translate to humans. Future research on this area should face current challenges in microbial research and others more inherent to the study of TMA/TMAO metabolism, as for instance, the high intra-individual variability of plasma TMAO levels observed in some individuals. With the increasingly growing refinement and affordability of next-generation sequencing and other omics technologies, it is expected a shift from studies focusing on describing microbial community composition to more function-oriented research on gut microbiota. Thus, it can be anticipated that shot-gun metagenomics, *meta*-transcriptomics, *meta*-proteomics, metabolomics and bioinformatics will be key to disentangle the complex links among diet, microbial metabolism and host health.

Abbreviations

AMPK	Adenosine monophosphate-activated protein kinase
CD36	Cluster of differentiation 36
CDP-choline	Cytidine-5-diphosphate-choline
CKD	Chronic kidney disease
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DMB	3,3-Dimethyl-1-butanol
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
EVOO	Extra-virgin olive oil
FMO	Flavin-containing monooxygenase
FMT	Fecal microbiota transplantation
GPC	Glycerophosphocholine
HMGB1	High-mobility group B1
HUVEC	Human umbilical vein endothelial cell
IL	Inflammatory cytokines interleukin
IOM	American Institute of Medicine
LDL	Low-density lipoprotein
MD	Mediterranean diet
NLRP3	Pyrin domain-containing-3
PC	Phosphatidylcholine
PChol	Phosphocholine
PERK	Protein kinase R-like endoplasmic reticulum kinase
PREDIMED	PREvention with MEDiterranean Diet
rDNA	Ribosomal RNA
RS	Resistant starch
SPM	Sphingomyelin
T2D	Type-2 diabetes
TF	Tissue factor
TLR4	Toll-like receptor 4
TMA	Trimethylamine
TMAO	Trimethylamine *N*-oxide
TMAU	Trimethylaminuria
TML	*N*6,*N*6,*N*6-Trimethyl-L-lysine

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There are no conflicts of interest.

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