The contributory role of gut microbiota in cardiovascular disease

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Our group recently discovered that certain dietary nutrients possessing a trimethylamine (TMA) moiety, namely choline/ phosphatidylcholine and L-carnitine, participate in the development of atherosclerotic heart disease. A meta-organismal pathway was elucidated involving gut microbiota–dependent formation of TMA and host hepatic flavin monooxygenase 3–dependent (FMO3–dependent) formation of TMA–N–oxide (TMAO), a metabolite shown to be both mechanistically linked to atherosclerosis and whose levels are strongly linked to cardiovascular disease (CVD) risks. Collectively, these studies reveal that nutrient precursors, gut microbiota, and host participants along the meta-organismal pathway elucidated may serve as new targets for the prevention and treatment of CVD.

Introduction

Cardiovascular disease (CVD) remains the leading cause of death in both the United States and industrialized societies, with growing incidence in developing countries (1). Factors contributing to CVD arise from genetic sources, environmental sources, or a combination of genetic and environmental sources (2). Despite extensive investigations in search of causal genetic variants, such as large-scale GWAS, less than one-fifth of attributable cardiovascular disease (CVD) risk has been accounted for from genetic determinants (3, 4).

At the same time, major advances in the treatment of atherosclerosis beyond high-potency lipid-lowering agents has not yet materialized. Indeed, even with high-potency statin therapy, at least a 50% residual risk remains (6), with the majority of events unchecked. Therefore, there is still ample room for improving our understanding of the processes contributing to CVD pathogenesis, and for improved prevention and treatment of CVD. While the recognizable contribution of genetics to CVD will no doubt significantly increase with time, a reassessment of environmental contributions to CVD pathogenesis and risks for adverse events is certainly worthwhile.

Our largest environmental exposure is what we eat. Technically speaking, food is a foreign object that we take into our bodies in kilogram quantities every day. From the latest National Health and Nutrition Examination Survey (NHANES, 2009–2010), the majority of individuals sampled achieved an intermediate or poor Healthy Eating Index (1). However, dietary composition is often difficult to assess, and even precise quantification of dietary intake may not necessarily reveal the many known and unknown factors that may influence the contributions of specific dietary nutrients to disease susceptibility (7). There has also been an overwhelming lack of appreciation at the bedside regarding the intricate and complicated processes that transform ingested food into the myriad metabolites that enter the circulation and fulfill or adversely affect various functional and metabolic processes in the body.

Over the past decade we have increasingly begun to appreciate the ecological diversity of microbes living symbiotically with us, a large proportion of which reside within our intestines. We now know that the human gut harbors more than 100 trillion microbial cells, far outnumbering the human host cells of the body (8). Indeed, a sobering fact is that Homo sapiens DNA is estimated to represent less than 10% of the total DNA within our bodies, due to the staggeringly large numbers of microbes that reside in and on us, primarily within our gut (9). Our microbial symbiont guests have coevolved with us and affect a wide range of physiologic and metabolic processes of the body. The major taxon present in gut microbiota consist primarily of 2 major bacterial phyla, Firmicutes and Bacteroidetes, whose proportions appear to remain remarkably stable over time within individuals and their family members (9, 10). However, the composition of the remaining gut microbiota is remarkably diverse and dynamic, with both acute and chronic dietary exposures significantly affecting the overall microbial community (8, 11). Hence, our metabolism and absorption of food occurs through the stable yet dynamic filter of trillions of bacteria that populate our digestive system, making the gut microbiota a filter of our largest environmental exposure. The composition of our gut microbial community is affected not only by dietary exposures, but also by genetic variants within the host — changes in the “terrarium” in which the gut microbes reside. Thus, there is a complex and somewhat dynamic interplay among acute and chronic dietary exposures, a fluid yet remarkably resilient gut microbial community, host genotype, and other external environmental exposures that collectively influence our meta-organismal metabolism, global physiology, and disease suscep-
This Review summarizes many recent developments in our understanding of the contributory role of gut microbiota as an active participant, through this endocrine function, in the development of atherosclerosis and its adverse complications of CVD. It also details strategies for targeting gut microbiota and the recently discovered meta-organismal pathway participants that contribute to trimethylamine–N-oxide (TMAO) formation for the potential prevention and treatment of CVD.

Discovery of the association between TMAO and CVD

Initial hypothesis-generating studies used untargeted metabolic analyses of plasma samples to identify novel metabolites and their connecting pathways potentially associated with cardiovascular risk (25). An iterative process of case-control studies was performed using an initial learning cohort, a subsequent independent validation cohort, and then a third, larger independent validation cohort (n = 1,876 subjects), which identified a number of metabolites whose levels in plasma were reproducibly correlated with CVD risks (25). Structural validation revealed 3 of the metabolites were linked to phosphatidylcholine (PC; lecithin) metabolism — choline (m/z 104), betaine (m/z 116), and TMAO (m/z 76) (25). Betaine is a known direct oxidation product of choline (26, 27). TMAO had previously been suspected to arise from bacterial metabolism of choline via an intermediate, trimethylamine (TMA), and subsequent hepatic oxidation via flavin monooxygenase 3 (FMO3), thus forming TMAO (28–31). PC is the major dietary

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source of choline in omnivores (32–34), and direct ingestion of PC was shown to result in rises in choline, betaine, and TMAO levels (25). Further, our studies suggested that plasma levels of TMAO showed the strongest positive correlation with CVD risk (25). Thus, our initial metabolomics studies suggested that plasma levels of 3 metabolites of dietary PC and gut microbiota might be linked to CVD in humans (25).

Choline is an essential dietary nutrient. While we can synthesize much of our requirements, we still need to consume some choline in our diet or else develop a deficiency state, which is characterized by fatty liver, altered one-carbon methyl donor metabolic pathways, and neurologic disorder (19, 32, 35, 36). An obligatory role for gut microbiota in both TMA and TMAO formation from ingested PC was confirmed in animal model studies, which included germ-free mice (25), as well as human clinical investigations involving ingestion of egg yolk, isotope-labeled PC, and a cocktail of oral antibiotics (37). Recently, the association between acute egg yolk ingestion and increased plasma and urine TMAO concentrations was independently confirmed in humans (38). Additional studies have shown that L-carnitine, an alternative TMA-containing nutrient found almost exclusively in red meat, similarly serves as a dietary precursor to gut microbial production of TMA and TMAO in both mice and humans (ref. 39 and Figure 1). It is thus remarkable that the highest levels of choline and L-carnitine are often found in foods rich in cholesterol and fats, such as red meat, liver, and egg yolk. While numerous large-scale epidemiologic studies have linked red meat ingestion with heightened mortality and CVD risks, the relationship between egg ingestion and cardiovascular risks has shown conflicting results (40–49). Since other dietary nutrients possess a TMA moiety (e.g., glycerophosphocholine, betaine, various short- and long-chain acyl carnitines, and sphingomyelin), there are likely other gut microbiome-dependent pathways that lead to the formation of TMA and TMAO, though these remain largely unexplored.

TMAO is not a widely recognized metabolite in mammals, although its role as a marine bacterial nutrient is emerging (50). TMAO is abundant in some fish, in which it is used in the freeze avoidance response as a form of “antifreeze” (51). It is also known to possess small-molecule protein chaperone mimetic behavior (52), presumably owing to its small size and a combination of both hydrophobic and polar characteristics. Originally believed to be an inert nitrogenous waste product of protein degradation excreted in urine (53), more recent studies revealed that TMAO may have direct biological activity that facilitates the development or propagation of atherosclerosis and its adverse CVD events. TMAO supplementation of apolipoprotein E-null mice was observed to foster enhanced macrophage foam cell formation in both the artery wall and the peritoneal cavity, as well as to promote aortic root atherosclerotic plaque development (25). Moreover, enhanced protein surface levels were observed of previously implicated scavenger receptors (CD36 and SR-A1) on macrophages involved in cholesterol accumulation and foam cell formation (25). Direct dietary exposure to TMAO or its precursors, choline or L-carnitine, (the latter 2 only in the presence of intact gut microbiota, which are required for TMAO formation), elicited significant reductions in reverse cholesterol transport in vivo in mouse models, as well as alterations in cholesterol and sterol metabolic pathways in multiple compartments including the artery wall, the liver, and the intestines (25, 39). Alterations were also observed in bile acid pool size and composition induced by TMAO (39). Further investigations revealed that TMAO levels help to explain a significant portion of atherosclerosis aortic root development across multiple different inbred strains of mice, and that TMAO levels are influenced by hepatic Fmo3, which is under complex control, including regulation by the farnesoid X receptor (FXR), a bile acid–activated nuclear receptor (54).

While plasma levels of all 3 metabolites (choline, betaine, and TMAO) identified in the original metabolomics study are associated with increased risk of CVD phenotypes in subjects presenting for cardiac risk evaluation (n = 1,876) (25), subsequent analyses in larger cohorts revealed that the prognostic value was largely confined to the formation of TMAO, especially from choline and L-carnitine (39, 55). In an alternative subsequent expansion study of over 4,000 subjects undergoing elective coronary angiography, elevated TMAO levels predicted major adverse cardiac events such as death, myocardial infarction (MI), and stroke over a 3-year period. Specifically, patients in the upper quartile for TMAO levels (compared with the lowest quartile) had a significant, 2.5-fold increased risk of experiencing a major adverse cardiac event (MI, stroke, or death), independent of traditional cardiovascular risk factors, renal function, and medication use, as well as overall poorer event-free survival (37).

In additional studies, dietary L-carnitine was similarly shown to foster accelerated atherosclerosis in mouse models, but only in the presence of intact gut microbiota and TMAO generation (39). Further, in a study examining plasma carnitine levels in sequential subjects undergoing elective diagnostic cardiac evalu-
we observed a remarkably strong adverse prognostic value associated with elevated plasma TMAO levels among a cohort of stable patients with heart failure (n = 720) that was incremental to traditional risk factors, cardio-renal indices (B-type natriuretic peptide and glomerular filtration rate), and markers of systemic inflammation (C-reactive protein) (56). On the other hand, TMAO has been known to accumulate in the plasma of patients with impaired renal function, such as those with chronic kidney disease (CKD) and end-stage renal disease (57). Exciting new metabolomics data from the Framingham Heart Study indicated that TMAO was one of the few metabolites in the plasma of healthy subjects whose levels predicted incident development of CKD (58). Furthermore, results from recent studies in mice fed a high-fat diet also suggest that dietary TMAO may exacerbate impaired glucose tolerance, obstruct hepatic insulin signaling, and promote adipose tissue inflammation (59). The scope of biological processes affected by both TMAO and the TMAO production pathway is just starting to be appreciated, with multiple newly recognized links to chronic cardiometabolic disorders.

A diet/meta-organismal pathway is involved in TMAO formation

The precise mechanisms through which TMAO leads to CVD and adverse events are still under investigation. The striking associations between circulating TMAO levels and CVD risks observed in several large-scale clinical cohorts, coupled with the growing body of animal model data demonstrating direct mechanistic links with atherosclerosis and alterations in cholesterol/sterol metabolism (25, 37, 39, 54, 55, 60), collectively suggest that the meta-organismal pathway involving diet, gut microbes, TMA, liver FMO3, and TMAO may be an important new paradigm to consider for an improved understanding of atherosclerotic heart disease and perhaps other cardiometabolic disease processes. There may also exist other metabolites downstream of or in parallel with this pathway that might contribute to the observed findings with TMAO. More global approaches are warranted to study the “meta”-metabolome, and deciphering the functional participation, if any, of gut microbiota in diseases. Indeed, gut microbiota may influence disease susceptibility and responses to dietary exposures, as well as the magnitude of host genetic variant influences on disease phenotypes. The broader and more complex interrelationships between intestinal microbiota and their human host under differing environmental exposures needs to be considered as a potential contributor to the CVD process.

The studies described above thus reveal new potential dietary changes, gut microbial composition and enzyme pathways, and host enzymes (e.g., hepatic FMO3) as potential treatment targets to reduce cardiovascular risk (Figure 3). We will examine each step in this meta-organismal pathway and discuss potential interventions suggested for reducing TMAO generation.

**Figure 3. Examples of microbial enzymes that generate TMA, using choline or carnitine as substrates.** Thus far, 2 distinct microbial enzyme systems have been identified in vitro that can produce TMA from either choline or carnitine as substrate (75, 77). The choline-utilizing enzyme cutC (catalytic polypeptide) and its partner cutD (regulatory polypeptide) make a complex that selectively uses choline as substrate and releases TMA (choline TMA lyase activity). Similarly, a microbial oxygenase (CntA) complexes with the microbial reductase (CntB) to form an active complex that selectively uses carnitine as substrate and forms TMA (carnitine TMA lyase activity). In theory, inhibition of microbial choline and/or carnitine TMA lyases may serve as a potential therapeutic target for CVD through the reduction of microbially generated TMAO.
Modifying dietary substrate. Modifying dietary nutrient intake is an obvious potential intervention that may influence the TMAO-producing meta-organismal pathway. Altered dietary exposure may potentially affect TMAO production by either reduction in the direct precursor substrate for TMA production or by altering gut microbial community composition, reducing the synthetic capacity to produce TMA from the different TMA-containing nutrients. TMA has long been recognized as an odorous byproduct of choline, PC, and L-carnitine during the decomposition of plants and animals, often by enterobacteria (31). Choline (free and esterified forms) and L-carnitine are the most common dietary nutrients ingested that produce TMA and TMAO (30, 37–39, 55).

Choline is present in most animal and some plant products, but is particularly abundant in egg yolk, meats, liver, fish, high-fat dairy products, and some nuts (34). The major dietary source of choline is PC (or lecithin), the primary phospholipid in membranes. Choline can be metabolized into betaine and function as a methyl donor that produces S-adenosylmethionine, influencing DNA and histone methylation (26, 32, 35). Severe choline deficiency causes neurologic impairment, and the targeting of choline metabolism enzymes is under investigation to promote tumor growth arrest (36, 61). In vivo studies in which trimethyl-d9-labeled betaine or PC were ingested showed a direct generation of d9-TMA and d9-TMAO within plasma and urine (37, 39, 55). Interestingly, only choline (and not betaine or lecithin) was found to produce a detectable rise in urine TMAO excretion in one small human study employing a variety of dietary exposures (30). More recent studies examining ingestion of either hard-boiled egg or egg yolk (37, 38, 62) or ingestion of trimethyl-d9-PC (37) have confirmed direct generation of TMAO from PC ingestion in humans. L-carnitine is a TMA nutrient that, in contrast to choline, is not required in our diets, since it is endogenously produced in mammals from dietary lysine, the single most abundant amino acid in plant and animal proteins (30, 63, 64). Omnivores obtain most L-carnitine from their diet. In cells, carnitine functions in the transport of fatty acids into mitochondria (65).

Epidemiologic studies have assessed the contributions of these dietary nutrients and CVD risks. High intake of choline and betaine was associated with diminished inflammation in one study (66, 67). However, an alternative report noted only a modest association with choline and betaine intake and CVD (68), which lost significance following adjustments for CVD risk markers (67, 69). Nevertheless, any nutritional study has a confounding factor of evaluating a broad mix of nutritional factors that may have opposing effects. Egg whites, for example, are large sources of immunomodulatory and antioxidant proteins (70), which are often ingested together with egg yolks if eggs are consumed.

As choline, PC, and carnitine are primary sources of gut microbiota-associated TMAO production, dietary modulation is a logical intervention strategy. We previously showed that vegetarians and vegans have markedly reduced synthetic capacity to make TMA and TMAO from dietary L-carnitine and have lower plasma TMAO levels than omnivores (39). Similarly, studies from our group (39) and others (71) show that gut microbial communities differ in vegetarians and vegans compared with omnivores. In animal model studies, chronic exposure to dietary L-carnitine increased TMA synthetic capacity by 10-fold (as monitored in a defined oral challenge of trimethyl-d9-carnitine), with a concurrent shift in gut microbial composition (39). Thus, chronic dietary exposure (e.g., omnivore vs. vegan/vegetarian among humans, or normal chow vs. chow plus L-carnitine in mouse studies) shifts gut microbial composition, with a selective advantage for bacterial species that prefer L-carnitine as a carbon fuel source to increase in proportion within the community and amplify the potential to produce TMA. Chronic dietary exposure can produce a profound effect on TMA synthetic capacity, whereas single (acute) dietary exposures affect TMA and TMAO production predominantly via modulation of precursor substrate availability.

While elimination of L-carnitine from the diet is a potentially attainable goal that may reduce some TMAO formation, because choline is an essential nutrient, its complete elimination from the diet is unwise. Further, bile has a very high total choline (PC) content, and the rapid turnover and sloughing of intestinal epithelial cells results in significant exposure of distal gut segments (and hence metabolites) to choline, independent of dietary intake. Absorbent removal of TMA from the intestines by specific oral binders is a challenging but potentially feasible therapeutic approach for lowering TMA and TMAO levels. Such a strategy has been tested by the removal of gut microbiota–associated uremic toxins (72), and in animal models of renal dysfunction, the oral charcoal absorbent AST-120 significantly decreased necrotic areas and lessened aortic deposition of the uremic toxin indoxyl sulfate without affecting lesional macrophage or collagen content (73) while attenuating monocyte inflammation (74).

Regulating microbial metabolism: contributions of microbial enzymes. Significant progress has recently been made in identifying some of the microbial enzymes that likely contribute to TMAO formation from different dietary substrates. Thus far, 2 distinct classes of enzymes have been reported that differ in their overall catalytic strategy for cleaving the C-N bond in choline and carnitine (Figure 3 and refs. 75–77). TMAO production by these enzymes can actually be considered a bacterial waste product released by the microbial enzyme systems, with the remaining carbon fuel source liberated from the parent substrate (choline/PC or carnitine) being used. By identifying known microbial enzymes that can use the predicted cleavage product as substrate after TMAO release, bioinformatics and genome mining approaches were used to identify a gene cluster responsible for anaerobic choline degradation within the genome of the choline-degrading bacterium Desulfovibrio desulfuricans (75, 76). Biochemical characterization of the microbial enzyme revealed a choline-specific TMA lyase that used an unusual glycol radical enzyme intermediate. The microbial enzyme was found within a “choline utilization” (cut) gene cluster that contains several tightly clustered genes, including a catalytic unit (cutC) and a regulatory polypeptide (cutD), both of which were required for anaerobic TMAO production from choline (75, 76). In other studies that used a similar bioinformatics approach with Acinetobacter baumannii as the model, an alternative microbial enzyme cluster (catalytic and regulatory protein CntA and CntB) specific for carnitine TMA lyase activity was also recently reported (77). Characterization of recombinant isolated CntA/B showed they formed a 2-component Rieske-type oxygenase/reductase complex (77).

The relative quantitative contributions of cutC/D- or CntA/B-type microbial enzyme clusters to TMA and TMAO production in humans is not yet known. Indeed, the existence of additional micro-
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Inhibiting host metabolism: contributions of hepatic FMOs. The conversion from TMA to TMAO requires an oxidation step that is mediated by host enzyme machinery in the form of FMOs (54, 78–81). Gut microbe–produced TMA reaches the liver rapidly via the portal circulation, where a cluster of hepatic FMO enzymes efficiently oxidizes TMA into TMAO (54, 82). Previous studies have shown that subjects with a genetic defect in FMO3 can have markedly elevated TMA levels, leading to a noxious body odor that characterizes the condition (fish odor syndrome or trimethylaminuria [TMAU]) (83, 84). Given that TMAO, and not TMA, appears to be the culprit metabolite that fosters a proatherogenic effect, one might expect that subjects with this genetic disorder are protected from CVD. However, its rarity has thus far precluded report of any known cardiovascular phenotype. Interestingly, other than TMA, little is known about the endogenous substrates of the FMOs; rather more is known about the ability of FMOs to oxidize a wide range of xenobiotics and toxicants, including drugs such as barbiturates. Collectively, the FMOs are thought to oxidize a large number of endogenous amines (including TMA) as well as structurally diverse sulfur-, phosphorus-, and selenium-containing compounds. In recent studies each of the FMO family members were cloned and expressed, to determine which possessed synthetic capacity to use TMA as a substrate to generate TMAO. FMO1, FMO2, and FMO3 were all capable of forming TMAO, though the specific activity of FMO3 was at least 10-fold higher than that of the other FMOs (54). Further, FMO3 overexpression in mice significantly increased plasma TMAO levels, while silencing FMO3 decreased TMAO levels (54). In both humans and mice, hepatic FMO3 expression was observed to be reduced in males compared with females (25, 54) and could be induced by dietary bile acids through a mechanism that involves FXR (54). Recent studies among multiple different inbred strains of mice in a “mouse diversity panel” linked variations in strain and sex to hepatic expression/activity of FMO3, TMAO levels, and atherosclerosis (54). Hence, further investigations using FMO3 loss- and gain-of-function experimental models to more directly probe this aspect of the TMAO pathway and cardiometabolic phenotypes are warranted.

In recent studies we examined natural genetic variants and hepatic expression levels of genes among multiple inbred strains of mice and humans to identify potential genetic determinants of TMAO production and their associations with CVD phenotypes (60). FMO3 and TMAO were significantly correlated, and TMAO levels accounted for 11% of the variation in atherosclerosis across the inbred strains of mice examined (60). We used a compara-
tive GWAS approach to discover loci for plasma TMAO levels in mice and humans, and identified a locus for TMAO levels on chromosome 3 (P < 0.001) that co-localized with a highly significant (P < 0.001) cis-expression quantitative trait locus for solute carrier family 30 member 7 (Slc30a7) (60). Although this zinc transporter might represent a positional candidate gene responsible for the association signal at this locus in mice (60), these are weak associations in GWASs. Furthermore, GWASs for plasma TMAO levels in 1,973 humans identified 2 loci with suggestive evidence of association (P < 0.001) on chromosomes 1q23.3 and 2p12; however, genotyping of the lead variants at these loci in 1,892 additional subjects failed to replicate their association with plasma TMAO levels (60). The relatively limited genetic signals observed for TMAO levels in humans thus far is consistent with the concept that interpersonal differences in diet and the repertoire of gut microbial species, moreso than host genetic variants, likely serve as the primary determinants of plasma TMAO levels (60).

Pharmacologic inhibition of the FMOs in general and FMO3 specifically is expected to reduce TMAO production and potentially serve as a therapy for CVD risk reduction. However, given the known adverse effects of FMO3 inhibition from sufferers of fish odor syndrome, the untoward odorous side effects of inhibiting this enzyme make it a less attractive target.

Concluding remarks

Results of the studies described above suggest that a broader view of human metabolism, physiology, and disease must be considered. We are walking communities comprised not only of a Homo sapiens host, but also of trillions of symbiotic commensal microorganisms within the gut and on every other surface of our bodies. Gut microbiota serve as a filter for our largest environmental exposure — what we eat — and the microbial community within each of us significantly influences how we experience a meal. The global meta-metabolome within is remarkably complex and is influenced by dietary inputs, microbial composition, host genetic factors, and other external exposures. We need to appreciate that our gut microbial communities make up a large and plastic endocrine organ that may influence multiple metabolic and physiological processes. Simply cataloging the microbes within is not sufficient. Indeed, simply sequencing the microbial genes is insufficient because, as with eukaryotic cells, the presence of DNA alone does not necessarily translate into protein synthesis and function. Future studies need to focus on discovery and understanding at the functional level of specific microbial pathways and products that contribute to our physiology and that may contribute to disease processes. Gut microbiota represent a new target for therapeutic manipulation and targeting for the treatment and prevention of complex cardiometabolic diseases. The exploration of gut microbiota contributions to human host physiology and diseases represents an incredibly exciting and potentially fertile territory in biomedical research.

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