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Intestinal Microbiota in Cardiovascular Health and Disease JACC State-of-the-Art Review



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ABSTRACT

Despite major strides in reducing cardiovascular disease (CVD) burden with modification of classic CVD risk factors, significant residual risks remain. Recent discoveries that linked intestinal microbiota and CVD have broadened our understanding of how dietary nutrients may affect cardiovascular health and disease. Although next-generation sequencing techniques can identify gut microbial community participants and provide insights into microbial composition shifts in response to physiological responses and dietary exposures, provisions of prebiotics or probiotics have yet to show therapeutic benefit for CVD. Our evolving understanding of intestinal microbiota-derived physiological modulators (e.g., short-chain fatty acids) and pathogenic mediators (e.g., trimethylamine *N*-oxide) of host disease susceptibility have created novel potential therapeutic opportunities for improved cardiovascular health. This review discusses the roles of human intestinal microbiota composition and metabolism as a novel therapeutic target for CVD. (J Am Coll Cardiol 2019;73:2089-105) © 2019 by the American College of Cardiology Foundation.

utrition is one of the key modifiable risk factors for cardiovascular health. However, the prevalence of ideal levels of diet have remained low, reaching 0.6% in children and 1.5% in adults (1). In contrast, 50% of young adults (ages 20 to 49 years) and 30.9% of older adults (ages 50 years or older) have reported poor levels of diet (1). Recent studies have highlighted dietary nutrient intake as a key contributor to global health and disease susceptibility (2). What we eat also provides nutrients for intestinal microbial metabolism. Thus, a more holistic view of metabolism is evolving—the combination of both intestinal microbiota and host metabolic transformations contribute to our overall metabolism and interindividual variations in our metabolic profiles. Intestinal microbiota serve as a filter of our largest environmental exposure—what we eat. Because numerous intestinal microbiota—generated metabolites are biologically active and affect host phenotypes, the intestinal microbiome also functions as a



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ABBREVIATIONS AND ACRONYMS

CAD = coronary artery disease CKD = chronic kidney disease
CKD = chronic kidney disease
CVD = cardiovascular disease
FMO = flavin monooxygenase
FXR = farnesoid X receptor
HF = heart failure
γ BB = γ-butyrobetaine
SCFA = short-chain fatty acid
TMA = trimethylamine
TMAO = trimethylamine N-oxide

TML = trimethyllysine

major endocrine organ that is responsive to dietary intake. It communicates with distal organs in the host through complex pathways via intestinal microbiota-generated metabolites and has been shown to affect phenotypes relevant to cardiovascular disease (CVD), ranging from inflammation, obesity, and insulin resistance, to more direct processes like atherosclerosis and thrombosis susceptibility (3). This review discusses the roles of human intestinal microbiota in normal physiology, their associations with disease susceptibilities, and the potential of modulating intestinal microbiota as novel therapeutic targets for CVD.

NORMAL INTESTINAL MICROBIOME

The human intestine harbors trillions of microbial cells as an essential part of our healthy physiological ecosystem. These communities of bacteria, fungi, archaea, and viruses are often collectively referred to as "microbiota," and their genome as the "microbiome." Most of the known intestinal microbial community is composed of bacteria in the phyla Bacteroidetes, Firmicutes (especially Clostridia species), Actinobacteria, Proteobacteria, and Verrucomicrobia (Figure 1) (4). Understanding or defining what constitutes a normal microbiome is challenging and may encompass considerations of the functional core, the healthy community ecology, and the resistance, resilience, and stability perspectives of the microbial ecology and related metabolites (5). Nevertheless, taxa in these phyla are relatively stable over time within an individual, and relatively consistent among family members while varying widely between unrelated individuals living in different households (6). However, the microbial communities in a household can be substantially altered, especially in those with physical contact (7). The full extent of how much the microbiota is altered over time within an individual remains to be determined. Beginning at childbirth, there are substantial environmental influences on an individual's intestinal microbial composition, function, and metabolism that can directly or indirectly affect host metabolism (8). Under physiological conditions, intestinal microbiota continue to stimulate the immune system, especially via intestinalassociated lymphoid tissues. In addition, intestinal microbiota are involved in activating and differentiating a wide range of T and B lymphocytes, as well as modulating the mucosal production of immunoglobulins (especially immunoglobulin A) (4).

HIGHLIGHTS

- Intestinal microbiota are mechanistically linked to physiological processes that affect cardiovascular health.
- Dietary nutrients serve as key environmental influences to intestinal microbiota and human host metabolism.
- Modulating intestinal microbiota composition and metabolism may serve as targets for cardiovascular disease prevention.

NEXT-GENERATION SEQUENCING METHODS TO ASSESS MICROBIAL DIVERSITY. Historically, microbial composition was investigated by traditional culture-based methods, which can be tedious and only permit sampling of a small proportion of intestinal microbial inhabitants. Culture-based approaches provide a valuable opportunity to directly obtain information regarding bacterial metabolism and growth requirements, as well as the potential future use of such cultured strains for experimental investigations. Accordingly, significant effort has been placed on isolating culture strains from the human gut (9,10). culture-independent, next-generation Recently, sequencing focused on taxonomic assignments via DNA sequences, which allowed previously unculturable bacteria to be identified with targeted "culturomics" to gain new mechanistic insights (11). The term "metagenomics" refers to a collective genome of microorganisms from an environmental sample that informs the microbial ecosystem. Microbial nucleic sequences are then used as a proxy for estimation of organism identity and the relative abundance of complex microbial communities. As shown in Figure 2, 2 common strategies (12) were used to obtain this sequence information:

- Targeted amplicon sequencing: one common sequencing method detects the sequence differences of the hypervariable region of the bacterial 16S ribosomal RNA (rRNA) for the taxonomic identification of the bacteria present. Although facile and informative, regions of variability with 16S analyses are typically insufficient to provide species-level resolution. Furthermore, different results can be generated based on the different regions analyzed.
- 2. Whole genomic shotgun sequencing: using highthroughput genomic sequencing combined with advanced computational bioinformatics, identification of taxonomic and potential functional



profiles of both known and unknown microbial communities can be achieved without the need to culture them in the laboratory (many are yet to be explored) (13). Although theoretically comprehensive and insightful, many studies are still underpowered.

Despite advances in next-generation microbial sequencing, there are some limitations in their clinical and research applications (14). Quality and quantity of recovered nucleic acids may vary depending on the time of sample collection (sample types, source location, and collection and/or processing methods), storage, and processing techniques (from microbial genome extraction to library preparation, sequencing, quality filter, or sequence identification steps). Technically, amplification bias, inadequate internal sequencing controls (positive and negative), or contamination may lead to changes in microbial composition that do not reflect the true changes. Meanwhile, tissue samples are dominated by host DNA, and high sequencing depth or approaches are necessary to enrich the microbial DNA. In addition, results of such analyses are typically



presented as a proportion, rather than an absolute level, and the presence of specific microbes in a specimen may not equate to pathogenicity.

SPECTRUM OF MICROBIAL DIVERSITY ACROSS SPECIES. Beyond interindividual variabilities, humans have intestinal microbiota that are distinct from other species. However, most intestinal microbiota mechanistic research has used the murine model with its well-defined phenotypes and genotypes, as well as carefully controlled environmental variations such as diets and housing conditions in experiments. Mice can vary widely across inbred

TABLE 1 Attered Intestinal Microbiota Composition Associated with Cardiovascular Diseases					
First Author (Ref. #), Year	Population	Technique	Increase in CVD	Decrease in CVD	
Karlsson et al. (145), 2012	12 MI/stroke Swedish patients vs. 13 age/sex-matched control subjects	Gut metagenome	Collinsella	Eubacterium Roseburia	
Dinakaran et al. (146), 2014	80 Indian CVD (valvular, ischemic, congenital heart) patients vs. 40 healthy control subjects	16S rRNA and metagenome in blood	Proteobacteria Actinobacteria Propionibacterium phages Pseudomonas phages Rhizobium phages Lymphocystis virus Torque Teno viruses		
Yin et al. (147), 2015	141 stroke/TIA Chinese patients vs. 94 asymptomatic control subjects	16S rRNA V4 region	Enterobacteriaceae Proteobacteria Escherichia/Shigella	Bacteriodetes Bacteriodales Bacteroidaceae Bacteriodes	
Emoto et al. (32), 2016	39 CAD Japanese patients vs. 30 age/ sex-matched control subjects with risk factors vs. 50 healthy control subjects	Terminal RFLP	Firmicutes/bateriodetes ratio Lactobacillales	Bacteroides + Prevotella	
Feng et al. (148), 2016	59 CAD Chinese patients vs. 43 healthy control subjects	Gut metagenome	Streptococcus sp. M334 and M143 Clostridium sp. HGF2		
Jie et al. (33), 2017	218 ACVD (stable/unstable angina, acute MI) Chinese patients vs. 187 healthy control subjects	Gut metagenome	Enterobaceriaceae Escherichia coli	Butyrate-producing bacteria Roseburia intestinalis	
			Klebsiella spp	Faecalibacterium cf. prausnitzii	
			Enterobacter aerogenes Oral cavity microbiome Streptococcus sp	Common microbiome members Bacteriodes spp	
				Prevotella copri	
			Lactobacillus salivarius	Alistipes shahii	
			Solobacterium moorei		
			Atropobium parvulum Other microbiome members Ruminococcus gnavus		
			Eggerthella lenta		

ACVD = atherosclerotic cardiovascular disease; CAD = coronary artery disease; CVD = cardiovascular disease; MI = myocardial infarction; RFLP = restriction fragment length polymorphism; TIA = transient ischemic attack.

strains and can be distinctly different from humans in anatomy, genetics, and physiology, as well as dietary intake and environmental exposures. Different mouse models can also lead to diverged shifts in intestinal microbiota composition and may have different crosstalks between intestinal microbiota and the host. Nevertheless, many proof-of-concept demonstrations have used gnotobiotic (all microorganisms are either known or excluded [as in germfree]) mouse models. Some of the strongest data that have shown potential participation of intestinal microbiota in CVD susceptibility have used germ-free mice during microbial transplantation studies to demonstrate transmission of a phenotype or disease, which fulfils an essential element of the "Koch's postulate" for microbial pathogenesis (15,16). Examples in which microbial transfer mice studies have shown transmission of phenotypes relevant to CVD include obesity and/or adiposity (17,18), atherosclerosis (19,20), hypertension (21-23), thrombosis (24), renal insufficiency (25), and insulin resistance (26,27). The introduction of human fecal samples to demonstrate transmissibility of disease phenotypes (e.g., features of metabolic syndrome) have been recently reported (28). Discussions regarding the contributory role of intestinal microbiota on obesity and inflammatory diseases have recently been extensively reviewed elsewhere (29).

ALTERATIONS IN COMPOSITION OF INTESTINAL MICROBIOTA: DYSBIOSIS

The term "dysbiosis" refers to the condition of having an imbalance in the microbial communities either in or on the body. Intestinal microbial compositional changes associated with the presence of numerous diseases and/or phenotypes have been the focus of most human microbiome research studies over the past decade. The ability to identify specific compositional patterns of microbiota that are associated with enhanced disease susceptibilities over time is an attractive concept. In the healthy intestines, Bacteroidetes and Firmicutes contribute most of the total bacterial species, and their ratio is often considered a



relative estimate of intestinal microbial health. Although intriguing and potentially insightful, Bacteroidetes and Firmicutes represent diverse phyla, and such analyses are thus relatively coarse and also associative in nature. purely Furthermore, host-microbial interactions are often dynamic and depend on local nutrient availability, oxygen tension, pH, gastric motility, and many other parameters. Therefore, microbial communities are often unique and distinct across regions, and along the entire alimentary tract. This poses challenges in interpreting microbial composition based on analyses on fecal materials (30). Viruses, fungi, and archaea also contribute to the non-host DNA sequence data obtained during deep sequencing analyses beyond bacteria, thereby contributing to further complexity in analyses and interpretations.

DYSBIOSIS IN ATHEROSCLEROSIS AND CORONARY **ARTERY DISEASE.** Distinct microbial compositional changes have been described in the setting of atherosclerotic coronary artery disease (CAD) in various case-control studies that used fecal samples from patients with different phenotypes (Table 1). Whether these represent microbiota taxa associated with CAD versus medications or risk factors that contribute to CAD development has not been fully characterized. The earliest study that explored microbial composition changes associated with atherosclerotic plaques, as well as oral and intestinal microbiota, was reported by Koren et al. (31). This study used pyrosequencing of 16S rRNA genes to survey bacterial taxa whose proportions were associated with CAD. More contemporary studies reported characteristic changes in CAD patients who had a significant increase in

Lactobacillales (Firmicutes) coupled with a decrease in Bacteroidetes (Bacteroides and Prevotella), which was not observed in a comparative cohort of patients with diabetes (32). In one of the largest metagenome-wide association studies to date, Jie et al. (33) observed an increased abundance of Enterobacteriaceae and oral cavity-asscuated bacteria and relatively depleted butyrate-producing bacteria in patients with atherosclerotic CVD versus those in healthy control subjects (33). The potential influence of dysbiosis in atherosclerotic CVD pathogenesis should not be confused with earlier investigations that focused on microbial pathogens implicated in epidemiological associations. Mechanistic studies implicated that specific microbial pathogens such as Chlamydia pneumonia, Helicobacter pylori, and Porphyromonas gingivalis could directly invade vascular cells and leukocytes, and promote inflammation of the gums or lungs. However, randomized controlled trials using antibiotics to target these microbial pathogens have not yielded any clinical benefits in reducing morbidity and mortality in patients with CAD. The pathogenic contributions of these microbes remain unclear (34). Careful examination in early studies revealed that Chryseomonas was identifiable in atherosclerotic plaque samples, and the combined abundances of Veillonella and Streptococcus in atherosclerotic plaques were correlated with their abundance in the oral cavity, which is another indirect link between periodontal disease and atherosclerosis (31). A larger cohort with available endarterectomy samples revealed taxa that belonged predominantly to Proteobacteria and Actinobacteria, with no difference between asymptomatic and symptomatic patients, or plaque regions, which suggested less influence on plaque vulnerability (35).

DYSBIOSIS IN HEART FAILURE. Alterations in intestinal microbial composition have been well described in patients with heart failure (HF), especially with reduced diversity and depletion of core intestinal microbiota (36). As a potential result, patients with HF have long been observed to be more prone to Clostridium difficile infections (37). Careful characterization of intestinal luminal surfaces have revealed significantly increased bacterial overgrowth with mucosal biofilm and increased bacterial adhesions in patients with HF versus non-HF control subjects (38). With bowel wall edema during splanchnic congestion accompanying HF, intestinal barrier function is impaired, and structural components of microbiota may have enhanced interaction with host intestinal mucosa (Central Illustration). When such interactions occur with surface intestinal epithelial cells via



Increase in proportional hazards with fasting trimethylamine *N*-oxide (TMAO) levels in a cohort of 2,235 patients who underwent undergoing elective coronary angiography. The fasting TMAO fourth quartile cutoff of 6.2 μ M represented a heightened 5-year, all-cause mortality risk.

pattern recognition receptors, it can stimulate host immune responses and lead to vascular inflammation. This was observed in cases of HF in which acute exacerbations were associated with increased circulating lipopolysaccharide detection and downstream inflammatory responses (39). Enhanced abundance of pathogenic microbial colonies of fungi (Candida spp) and bacteria (Campylobacter, Shigella, and Yersinia) were isolated from fecal samples of HF patients, especially in those with elevated right atrial pressure and impaired intestinal barrier function (40). Three independent HF cohorts that used sequencing techniques to characterize intestinal microbial compositions reported a consistent decrease in microbial diversity and a depletion of several butyrate producers (Faecalibacterium prasusnitzii, Lachnospiraceae family, Eubacterium hallii) that were inversely associated with inflammatory biomarkers (36,41-43). DYSBIOSIS AND HYPERTENSION AND DIABETES

MELLITUS. Although there have been varying reports about intestinal microbiota profiles, patients with type 2 diabetes have distinct intestinal microbiota

signatures compared with subjects without diabetes (44). Specifically, lower concentrations of butyrateproducing microbes such as Roseburia intestinalis and Faecalibacterium prausnitzii, and higher concentrations of Lactobacillus gasseri, Streptococcus mutans, and some Clostridiales, Desulfovibrio, and Proteobacteria species have been observed (45). Metformin administration increased Akkermansia muciniphila and resulted in a relative increase in some Escherichia species that might have had beneficial effects on glucose homeostasis (46,47). Meanwhile, insulin-resistant patients had increased levels of branched chain amino acids, which were especially associated with the presence of Prevotella copri and Bacteroides vulgatus, which might drive insulin resistance and increased branched chain amino acid levels in mice (48). Human studies revealed that postprandial glucose responses to dietary intake could be modulated by intestinal microbiota (49).

High blood pressure is a leading cause of CVD, and high dietary salt intake has been implicated in the pathophysiology of hypertension. Alterations of the intestinal microbiome in response to high salt intake were recently observed in an experimental study in mice (50). High salt intake resulted in a depletion of *Lactobacillus murinus*. Consequently, treatment of mice with *Lactobacillus murinus* prevented saltsensitive hypertension partly by modulating TH17 cells (50). These results warrant further study in humans.

PROBIOTIC AND DIETARY INTERVENTIONS TARGETING INTESTINAL MICROBIOTA. Insights gained from intestinal microbiota and its associated metabolic pathways provided an opportunity to explore the contributory role of intestinal microbiota in generating the variability of physiological responses to dietary nutrients. By leveraging the ability to monitor continuous glucose levels, an individualized glycemic pattern emerged when carefully curated dietary information with machine learning algorithms was based on metagenomic information (49). Microbiome responses were measured in >800 people using 16S rRNA and shotgun metagenomic profiling to assess taxonomy and function, respectively; therefore, tailoring dietary intake to an individual's intestinal microbiome could potentially minimize an increase in post-prandial glucose (49). These results underscored how food advice and/or interventions might have the potential to be individually tailored to each person (because of the huge variation in post-prandial glucose response to "bad" and "good" food). These observations might allow a greatly advanced accuracy of nutritional advice in the future, as we enter the era of "personalized medicine." However, rigorous prospective studies to determine whether such approaches can affect cardiovascular risk are still warranted.

Theoretically, direct modulation of microbial composition has the potential to restore healthy microbial communities and promote cardiovascular health. In a rat myocardial infarction model, administration of broad spectrum antibiotics was associated with changes in leptin levels and aromatic amino acid catabolites, as well as a reduction in infarct sizes (51). Furthermore, administration of either Lactobacillus plantarum or Lactobacillus rhamnosus GR-1 was associated with attenuation of post-infarction cardiac remodeling in rats (51,52). Interestingly, foodborne microbes only transiently colonized the intestines. Unlike antibiotic-associated and Clostridium difficile--associated diarrhea, whether probiotics and prebiotics can directly influence the overall microbial distributions has not been established in human studies (53,54). Although there are varying reports of lipid and blood pressure lowering effects with probiotics, human intervention studies showing their efficacies are limited, and there are currently no clinical recommendations for their prescription (55).

MICROBIAL METABOLITES AS PHYSIOLOGICAL MODULATORS: SHORT CHAIN FATTY ACIDS AND BILE ACIDS

One key role for intestinal microbiota is to support every day physiological functions in food digestion via various fermentation processes in response to dietary intake of substrates (56). Some metabolites can even be directly absorbed into the host circulation and serve as hormones to distant organs as sites of action. Other metabolites may be further metabolized by host enzymes similar to prohormones, which serve as downstream mediators or signaling molecules. It is likely that most microbial-generated metabolites can provide synergistic effects that promote health. However, toxic metabolites can also accumulate, especially when pathogenic species are colonizing or when normal host clearance mechanisms (e.g., renal function) of these metabolites are compromised. Detection of this "food metabolome" provides a unique opportunity to gain insight not only for the quality and quantity of food intake, but also for the functional consequences as a result of complex microbial-host metabolism (57).

PHYSIOLOGICAL EFFECTS OF SHORT-CHAIN FATTY

ACIDS. Anaerobic fermentation of undigested nutrients, such as resistant starch, dietary fiber, and various complex polysaccharides, produce fatty acids ranging from 1 to 6 carbon chains that are commonly

referred to as short-chain fatty acids (SCFAs). Examples include acetate, propionate, and butyrate, which are actively and passively absorbed at the colonic epithelium into the portal vein (58). Although they provide approximately 5% to 10% of energy for the human host, SCFAs serve as signaling molecules to bodily systems, including modulation of autonomic systems and systemic blood pressure, as well as inflammatory responses and other cellular functions. SCFAs exhibit a wide range of physiological functions, including histone deacetylases inhibition, chemotaxis and phagocytosis modulation, reactive oxygen species induction, cell proliferation, and intestinal barrier integrity alteration (58). Patients with type 2 diabetes mellitus have less abundance of butyrate-producing bacteria and more Lactobacillus spp (44,45,59). SCFAs, in particular, butyrate, can serve as energy substrates for epithelial cells of the intestines (60,61). Furthermore, vancomycin treatment reduces the abundance of butyrate-producing bacteria in patients with metabolic syndrome, which highlights their important role in maintaining insulin sensitivity (62).

Recent mechanistic demonstrations also revealed that SCFAs can directly activate specific distinct Gprotein-coupled receptors. Some of the G-protein-coupled receptors identified to interact with SCFAs from genetic and mouse model studies include G-protein receptor 41 (GRP41) and olfactory receptor 78 (Olfr78) (Figure 2) (63). In particular, Olfr78 is highly expressed in the renal juxtaglomerular apparatus, where it mediates renin secretion in response to SCFAs. In addition, both Olfr78 and GRP41 are expressed in smooth muscle cells of small resistance vessels, where they differentially mediate vascular tone. Interestingly, Olfr78 knock-out mice are hypotensive (64), whereas GRP41 knock-out mice are hypertensive (65), which implies that these pathways may be physiologically important links between SCFAs and host blood pressure control. The 3-carbon SCFA propionate may stimulate Olfr78 to raise blood pressure, whereas stimulation of GRP41 can lower blood pressure (64,66). The obligatory role of intestinal microbiota in generating SCFAs was demonstrated by antibiotic treatment that raised blood pressure in Olfr78 knock-out mice, thereby further supporting the involvement of these receptors in blood pressure control. Recent animal studies demonstrated that intestinal microbiota-derived SCFAs are critical for the host immune response and cardiac repair capacity after myocardial infarction in a mouse model with or without antibiotics (67). However, direct demonstration of such effects in human CVD remains limited.

PHYSIOLOGICAL EFFECTS OF BILE ACIDS AND **MICROBIAL MODULATIONS.** Bile acids facilitate the absorption of dietary fat and fat-soluble molecules. Several bile acids can regulate energy metabolism through activation of nuclear receptors such as Gprotein-coupled bile acid receptor 1 (TGR5) and the farnesoid X receptor (FXR) (68). Intestinal FXR appears to regulate hepatic cholesterol 7*a*-hydroxylase (CYP7A1), the rate-limiting enzyme in bile acid synthesis, through a fibroblast growth factor (FGF)-15/19-dependent mechanism (69). Therefore, humans produce a large conjugated hydrophilic bile acid pool, which is maintained through positive feedback antagonism of FXR in the intestine and liver. Through bile salt hydrolysis and bile acid 7α -dehydroxylation, intestinal microbiota are capable of producing secondary bile acid hormones that affect host physiology by agonism of FXR in the intestine and liver, which results in a smaller, unconjugated hydrophobic bile acid pool (68). Bile acids such as deoxycholic acid can serve as a direct antimicrobial agent because of its hydrophobicity and detergent properties on bacterial membranes (70). Therefore, a dynamic equilibrium exists between diet-intestinal, microbiome-bile acid pool size and composition. Hydrophilicity of the bile acid pool can be associated with disease states, whereas reduced bile acid levels in the gut can be associated with bacterial overgrowth and inflammation. A semisynthetic bile acid analogue and a potent FXR agonist that was recently approved for treatment of non-alcoholic steatohepatitis, obeticholic acid, may reduce bacterial translocation and intestinal inflammation (71).

THERAPEUTIC POTENTIAL OF DIETARY INTERVENTIONS TO MODULATE SCFAs. The many links among the altered intestinal microbial community, metabolites, and susceptibility for CVD and metabolic diseases have placed a spotlight on the intestinal microbiome as a potential novel target for therapeutics. Currently, diet modulation is the major therapeutic tool used in clinical practice to affect chronic metabolic diseases, and although lifestyle interactions can clearly affect intestinal microbial community structure and function, there are few studies that have explored the effects of dietary interventions on the intestinal microbiome in humans. Existing studies of diet on the intestinal microbiota in humans have generally seen modest effects over the short term (72). Nevertheless, extreme shifts from animal-based to plant-based diets can modify regional and systemic productions of SCFAs, thereby potentially contributing to some of the proposed beneficial effects of these diets. In one study, an animal-based diet was associated with increases in the abundance of bile-tolerant microorganisms (Alistipes, Bilophila, and Bacteroides) and decreases in the levels of Firmicutes that metabolize dietary plant polysaccharides (Roseburia, Eubacterium rectale, and Ruminococcus bromii) (53). As a result, there were significant reductions in fecal acetate and concentrations when butyrate subjects were switched from plant- to animal based-diets (53). Fecal microbiota transplantation from lean donors to insulin-resistant patients with metabolic syndrome led to improved insulin sensitivity and was associated with increased abundance of butyrateproducing bacteria such as Roseburia (28). By colonizing germ-free apolipoprotein E-deficient (Apoe^{-/-}) mice with intentional microbial communities with or without Roseburia intestinalis revealed that microbe-diet interactions are crucial to understanding the interplay between microbiota and CVD (73). In the presence of plant polysaccharides Roseburia intestinalis could produce butyrate and confer protection against atherosclerosis, whereas no protection was observed in those on low-plant polysaccharide diets (73). Similarly, the microbiota protected *Apoe^{-/-}* mice against atherosclerosis when fed a chow diet rich in plant polysaccharides but not when fed Western style diets (74).

MICROBIAL METABOLITES AS PATHOGENIC MEDIATORS: TRIMETHYLAMINE *N*-OXIDE

Discoveries of potential pathogenic mediators that directly or indirectly modulate disease susceptibilities have provided a valuable window into microbial-host interactions that can modulate cardiorenal risks. In an initial discovery based on untargeted metabolomic analysis, Wang et al. (75) identified 18 small-molecule analytes that in subsequent validation case-control cohorts (approximately, 2,000 subjects) repeatedly distinguished between patients with and without future development of major adverse cardiovascular events (death, myocardial infarction, and stroke). Some of these metabolites have now been identified as known predictors of CVD risk that are not associated with intestinal microbiota (such as L-citrulline) (76). Three of the analytes (m/z 76, 104, and 118) were closely correlated with each other, which suggested participation in a common pathway. One in particular (m/z 76) seemed to be driving the association with incident CVD risks and was subsequently shown to be trimethylamine N-oxide (TMAO), an intestinal microbiota dependent by-product of dietary choline and phosphatidylcholine (75). Another unknown metabolite whose levels were strongly associated with incident CVD risks was also identified as the amino acid trimethyllysine (TML), which was shown to serve as a nutrient precursor for intestinal microbiota–dependent TMAO generation (77).

MICROBIAL GENERATION OF TML AND HOST **PRODUCTION OF TMAO.** Microbial catabolism of dietary nutrients that possess a trimethylamine (TMA) [N(CH3)3] moiety, such as choline, phosphatidylcholine, and L-carnitine, can serve as precursors for TMA generation by specific microbial enzymes ("TMA lyase") residing in the intestines (75,78,79). TMA, an odorous gas with a rotten fish smell, is then absorbed by the host, and following delivery to the liver via portal circulation, is rapidly converted into TMAO by hepatic flavin monooxygenase (FMOs; particularly FMO3) (80). Although female mice appeared to have greater FMO3 activity than male mice, human genome-wide association studies have yet to identify any sex differences in FMO3 variants (81,82). Patients with genetic polymorphisms of FMO3 experienced a metabolic disorder of excessive TMA called fish malodor syndrome (or trimethylaminuria) (83). TMAO is eventually predominantly excreted by the kidneys (Central Illustration) (84).

Much of the chemistry of TMA as a toxic metabolite was originally studied because of its accumulation as a result of purification in sewage (85). Choline is an abundant chemical moiety in bile, and is continuously delivered into the intestines in omnivores and vegans alike. Carnitine is an abundant nutrient in meat, especially red meats. Both choline and carnitine within the gut are absorbed within the small bowel via specific transporters, but absorption is incomplete, particularly with large meals that can saturate the uptake systems. Consequently, both dietary choline and carnitine ingestion can lead to significant elevations in TMA and TMAO, which has been shown to have many adverse effects on host metabolism, particularly those that affect cardiovascular health (86).

DIETARY-INDUCED TMAO GENERATION AND ATHEROGENESIS. After observing that plasma levels of TMAO are dose dependently associated with CAD in subjects, initial functional studies sought to determine if the associations observed were mechanistically linked to disease causation. To directly demonstrate a pro-atherogenic contribution of the meta-organismal (i.e., involving both microbe and host) TMAO pathway, initial studies fed mice with a choline- or carnitine-rich diet, and demonstrated increases in plasma TMAO levels, cholesterol-laden macrophage foam cell formation, and enhanced aortic atherosclerotic plaque development (75,79).

Conversely, germ-free mice (lacking intestinal microbes) or short-term broad spectrum antibiotic suppression of intestinal microbiota eliminated TMAO-generating capacity, and suppressed diet (choline or carnitine) dependent atherosclerotic plaque enhancement (75,79). Microbial transplantation experiments using cecal microbial communities recovered from a high TMA/TMAO-producer in-bred strain of mice (C57BL/6J) compared with a low TMA/ TMAO producer (NZW/LacJ mice) were shown to transmit choline diet-dependent enhancement in atherosclerosis (19). Similarly, Zhu et al. (24) demonstrated that microbial transplantation of a high TMA producing community could transmit TMAO generation and enhanced thrombosis potential into recipient germ-free mice. In further studies that used germ-free mice as recipients and synthetic microbial communities capable of producing TMA versus not producing TMA, it was firmly established that the atherogenic metabolite TMAO and choline metabolism were integrally linked to intestinal microbiota and its adverse functional consequences (20,87).

HUMAN STUDIES OF PROGNOSTIC VALUE OF CIRCULATING TMAO LEVELS. When translated to humans, suppression of TMAO production was observed in healthy individuals taking a short course of poorly absorbed antibiotics, which further illustrated the obligatory role of intestinal microbiota in TMA/TMAO generation (79,88). In a cohort of >1,800 subjects, plasma TMAO levels were positively associated with CAD, peripheral artery disease, and history of myocardial infarction, independent of traditional risk factors (75). In a subsequent study that combined data from >4,000 subjects who underwent elective coronary angiography, elevated TMAO predicted major adverse cardiac events, such as death, myocardial infarction, and stroke over a 3year period, even in the presence of elevated choline and betaine levels (nonmicrobial metabolites) (88,89). These prognostic effects were also observed in subsets of patients with a history of HF (90), diabetes mellitus (91), peripheral artery disease (92), chronic kidney disease (CKD) (93), high atherosclerotic burden (94), acute coronary syndrome or myocardial infarction (95-97), and even non-CVD patients (98), all independent of traditional risk factors. In addition, elevated TMAO levels were associated with a degree of atherosclerotic burden as defined by the number of vessels with CAD by angiography, and with SYNTAX (Synergy Between PCI With Taxus and Cardiac Surgery) scores (99). These findings were validated by independent cohorts on different continents, and reviewed recently in several meta-analyses (100-102), which suggested an estimated cutoff value of >6 μ M (approximately the third and/or fourth quartile cutpoint of many cohorts) was predictive of the highest risk of adverse cardiac events (**Figure 3**). However, in those with excessive circulating TMAO (such as those with end-stage kidney disease on hemodialysis, with the first quartile in excess of 25 to 50 pM), the incremental prognostic value of TMAO appeared to be diminished (103,104), and probiotics had no effects on TMAO levels in this population (105).

Although TMAO in animal models exerts numerous atherosclerosis and/or thrombosis and inflammation promoting effects, the precise receptor or chemical sensor that detects TMAO remains unknown. Animal studies indicated that TMA/TMAO production leads to modulation of cholesterol and sterol and/or bile acid metabolism, as well as alterations of bile acid pool size, composition, and transport (79). Additional global effects of TMAO include impairment of reverse cholesterol transport and promotion of forward cholesterol transport. In vitro and in vivo experimental evidence of TMAO also showed modulating vascular dysfunction and inflammatory responses (106), including in the NLRP3 inflammasome pathways (107,108). In older mice, antibiotics reversed endothelial dysfunction and arterial stiffening accompanied by lower TMAO levels and oxidative stress, as well as greater antioxidant enzyme expression (109).

Recently, TMAO was shown to have direct effects on platelets, altering stimulus-dependent intracellular calcium signaling in response to multiple agonists, and fostering enhanced platelet reactivity and thrombotic risks. For example, direct injection of TMAO or dietary choline and intestinal microbiota-dependent TMAO elevation was shown to heighten platelet responsiveness and to promote faster clot formation in vivo using multiple different thrombosis models in mice (24). Similarly, genetic manipulation of host hepatic FMO3 expression levels in mice (e.g., reduction via targeted antisense oligonucleotides, or over-expression as a transgene) were shown to alter systemic TMAO levels, and correspondingly, both altered platelet responsiveness and in vivo thrombosis potential (110). Enhancement in platelet responsiveness with dietary choline supplementation and TMAO elevation was also observed in recent interventional studies that examined platelet aggregation responses in humans (111). These latter studies also showed that low-dose aspirin mildly reduced TMAO levels in subjects, and attenuated the pro-thrombotic (heightened platelet aggregation responses) effect observed with elevated TMAO levels (111).

High levels of TMAO in subjects with normal renal function in the Framingham cohort were reported to

herald increased risk for development of CKD (112), and in animal model studies, chronic elevation of TMAO was shown to foster both renal functional impairment and tubulointerstitial fibrosis, along with activation of the pro-fibrotic transforming growth factor- β pathways (93). In an adenine-induced mice model of CKD, concomitant increase in hepatic FMO3 expression occurred with increased circulating TMAO levels (113). Similarly, human subjects with HF had elevated levels of TMAO and experienced worse outcomes (90,114-116). In murine models, heightened TMAO levels exacerbated cardiac remodeling with pressure overload via transaortic constriction (117).

OTHER RELATED INTESTINAL MICROBIOTA-GENERATED METABOLITES. The same nutrient may be processed by various metabolic pathways and at different regions of the intestines; thus, the food metabolome detected from plasma samples can only represent the sum total of their balance. In the complex carnitine biosynthesis pathway, precursors of carnitine, γ -butyrobetaine (γ BB), and TML were also thoroughly investigated as potential substrates for TMA/TMAO production (77,118). Specifically, dietary L-carnitine is converted into TMAO via 2 sequential intestinal microbiota-dependent transformations in humansan initial rapid generation of the atherogenic intermediate yBB, followed by transformation into TMA via low-abundance microbiota communities rather than single species in response to omnivorous diet patterns (119). Elevated levels of both metabolites were associated with adverse long-term outcomes in patient cohorts, yet mechanistic studies revealed that they might exert somewhat different physiological and/or pathogenic effects on cardiovascular health than TMAO (77,79). For example, although dietary carnitine in the Apoe^{-/-} model accelerates atherosclerosis, this effect appears to be mediated via intestinal microbiota-generated TMA (and thus, TMAO) because the dietary effect of carnitine is no longer observed when intestinal microbiota are suppressed with oral poorly absorbed antibiotics. Human clinical studies showed that the prognostic value of carnitine in incident CVD risk prediction was attenuated in statistical models in which TMAO was included (79). Studies that examined the association between circulating levels of yBB and incident adverse cardiovascular risks have not yet been adequately assessed. Similarly, examination of the clinical prognostic value of yBB before adjustments for TMAO versus following adjustments for TMAO in future work is needed, because dietary provision of yBB to atherosclerosis-prone mice enhanced aortic root atherosclerosis, but only in the presence of intact intestinal microbiota and TMAO generation (118). It is likely that we have only observed the tip of the iceberg of microbially produced metabolites that modulate CVDs. The recent identification of imidazole propionate as a microbially produced metabolite that is enriched in type 2 diabetes and induces insulin resistance when injected in mice suggests that other classes of metabolites should be considered as disease modulators (120).

DIETARY AND DRUG INTERVENTIONS TARGETING TMAO LEVELS. Like SCFAs, it is clear that TMA/ TMAO formation is largely dependent on nutrient sources. Dietary sources of choline and/or phosphatidylcholine and carnitine can clearly influence systemic levels. Overall, vegans and vegetarians have lower circulating TMAO levels and fecal TMA/ TMAO-generating capacities than their omnivores counterparts (79). Specifically, diets rich in red meat are associated with higher levels of circulating TMAO and significantly reduced fractional renal excretion of TMAO compared with diets with white meat or no meat (121). Long-term exposure to oral L-carnitine supplementations can also induce TMA/ TMAO-generating capacities in humans. This same effect of dietary L-carnitine supplementation has been observed in mice (118) and in humans (122,123). Because these are common over-the-counter nutritional supplements and serve as food additives both for human and livestock dietary consumption, their overall long-term impact on cardiovascular health is unknown and needs to be investigated. Plasma TMAO levels can also increase with a high fat content (124), although the effects are less clear in isocaloric diets (121). It is also of clinical interest that cardioprotective drugs like statins may also have effects on TMAO (125). Healthy individuals who receive choline or carnitine supplementation can have increased circulating TMAO levels, yet such levels can be partially attenuated by concomitant ingestion of oral aspirin (111,119,126). In a recent clinical interventional study, fecal microbial transplantation from lean vegan donors to metabolic syndrome recipients did not alter TMAO levels, despite improving indexes of glycemic control (127). However, the baseline TMAO levels in the subjects with metabolic syndrome enrolled in the study were generally low at baseline, making the study more powerful for observing an improvement abnormalities in glucose metabolism than in reduction in microbiota-dependent TMAO а generation (127).

A minority of fish have elevated TMAO levels (especially deep sea fish), in which the metabolite is

used as part of the freeze avoidance mechanism. Thus, fish intake can generate high levels of TMAO in humans, although the specific fish and even the time of year (temperature of water the fish are harvested from) can affect TMAO levels in fish (128,129). Therefore, it is not surprising that questions have been raised as to whether TMAO is merely a marker of CVDs because decades of epidemiological research have shown that high fish consumption may lower risk of CVDs (130,131). There have been reports of variable acute or short-term effects of food groups such as eggs (rich with phosphatidylcholine) and red meat (rich with carnitine) on circulating TMAO levels (129). However, caution should be used about making causal assumptions that counting food groups equates to modulating microbial and/or host metabolic pathways. From a scientific perspective, TMA concentrations are high in fish compared with equivalent protein quantities from soy or casein (milk). Yet when equivalent amounts of proteins from these sources were fed to atherogenic-prone mice, those fed fish proteins had the highest quantity of atheroma formation (132). It is also important to highlight that not all fish are equivalent. Most fish do not have omega-3 fatty acids. In addition, it has not been shown directly that changes in fish consumption are beneficial in all patients (130), nor in those who are vulnerable to TMAO accumulation (e.g., in patients with CKD).

With the availability of TMAO as an in vitro clinical diagnostic test to identify those at increased CVD risk, it remains unknown if dietary recommendations can be tailored to monitor TMAO, much in the same way that triglycerides or blood glucose are monitored, and to provide dietary advice. Whether current dietary recommendations that promote cardiovascular health have potential beneficial impact on the intestinal microbiome in general, or TMAO specifically, remains to be determined. It is important to recognize that most dietary advice recommends approaches that would be expected to lower TMAO, including lowering of caloric and fat consumption, and focusing on reduction of high-fat, high-cholesterol foods (e.g., generally, animal products). A recent European panel took into account the potential impact of intestinal microbiota-generated TMAO for their dietary reference recommendations for choline (133). Preliminary data that support the TMAO-lowering effects of hypocaloric diet and exercise (134), as well as intermittent fasting (135), are promising.

POTENTIAL OF THERAPEUTIC INTERVENTIONS TO DIRECTLY MODULATE TMAO PRODUCTION. Approaches to manipulate the intestinal microbiome and its metabolic pathways hold some promise, although they have yet to be realized in clinical practice. Identification of microbial enzymes that work in tandem to convert nutrient components such as choline or carnitine into TMA have spawned the discovery of several small molecule inhibitors that can modulate TMA/TMAO levels and cause adverse cardiovascular effects in experimental studies (79,136-138). The prototype of such a TMA lyase inhibitor is called 3,3-dimethyl-1-butanol, which in microbial cell cultures and in vivo mouse models can reduce TMA/TMAO production without compromising microbial cell survival (138). Mice that received 3,3-dimethyl-1-butanol appeared to have a reduced atherosclerotic burden, lower macrophage foam cell formation, and to have attenuated cardiorenal disease progression (138). Surveying common dietary sources have identified 3,3-dimethyl-1butanol in some samples of cold-pressed, extra-virgin olive oil, a major component of the Mediterranean diet that has been associated with improvement in cardiovascular health (138). However, the impact of Mediterranean diet on changes in TMAO have been somewhat mixed (139-141). Nextgeneration TMA lyase inhibitors have recently been reported to be highly potent, suppressing TMAO levels in animal models. These agents have also shown the potential to selectively target and accumulate within intestinal microbiota, which permits sustained inhibition of TMA-producing microbial enzymes with limited systemic exposure within the host (142).

CONCLUSIONS AND FUTURE PERSPECTIVES

Over the last several years, accumulating data have suggested an important link between the intestinal microbiome and CVD. It has become clear that the microbiome plays an important role at the intersection of diet and CVD, that is, by metabolizing dietary components that lead to the release of SCFAs, some of which likely promote important beneficial cardiovascular effects. However, a major knowledge gap exists because most studies have focused on characterizing the microbial composition rather than their functional alterations and downstream consequences. We now recognize that microbiomedependent metabolism may also lead to production of metabolites with potential adverse cardiovascular effects, such as TMAO, which may promote atherosclerosis and heightened thrombosis risks. These observations provide an excellent opportunity for the development and testing of novel therapeutic strategies that target the intestinal microbiome for prevention and treatment of CVD. Approaches may

include personalized dietary interventions, probiotics and/or prebiotics, or nonlethal microbial inhibitors that target specific pathways once they are identified (e.g., TMA production). Agents that target the TMAO pathway would also be expected to have multiple additional potential therapeutic benefits, including reducing the progression of renal functional decline, HF progression, and adverse outcomes in numerous high-risk cohorts (those with type 2 diabetes, CKD, and HF). However, well-powered prospective intervention studies are needed to validate this novel therapeutic approach. It is also important to stress that cardiometabolic diseases likely result from several metabolites, which may contribute to a variable extent in different individuals with high or low susceptibility, and that TMAO is likely only the "tip of the iceberg." Future identification of microbially produced metabolites and investigation of whether they are causally linked to cardiometabolic disease will provide exciting potential novel opportunities to improve cardiovascular health and prevention.

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