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Microbiome series:

Microbial modulation of cardiovascular disease

J. Mark Brown[1] and **Stanley L. Hazen**[1]

Department of Cellular and Molecular Medicine, Cleveland Clinic Lerner Research Institute, 9500 Euclid Avenue, NC-10, Cleveland, OH 44195, USA.

Abstract

Although diet has long been known to contribute to the pathogenesis of cardiovascular disease (CVD), research over the past decade has revealed an unexpected interplay between nutrient intake, gut microbial metabolism and the host to modify the risk of developing CVD. Microbial-associated molecular patterns are sensed by host pattern recognition receptors and have been suggested to drive CVD pathogenesis. In addition, the host microbiota produces various metabolites, such as trimethylamine-*N*-oxide, short chain fatty acids and secondary bile acids, that affect CVD pathogenesis. These recent advances support the notion that targeting the interactions between the host and microorganisms may hold promise for the prevention or treatment of CVD. In this Review, we summarize our current knowledge of the gut microbial mechanisms that drive CVD, with special emphasis on therapeutic interventions, and we highlight the need to establish causal links between microbial pathways and CVD pathogenesis

Introduction

Although highly effective lipid-lowering drugs are available, cardiovascular disease (CVD) remains the number one cause of death in developed countries[1], and there remains a clear and unmet therapeutic need for the identification of new therapeutic targets for CVD. Genetic contributions have a strong role in disease pathogenesis; yet large scale genetic studies revealed that genetic variation is only a minor contributor (< 20%) to the risk of developing CVD [2,3]. Thus, environmental factors have a predominant role in CVD pathogenesis, and it has long been known that diet is a major contributor to the risk of developing CVD[4]. Resident microbial communities in the intestinal tract represent a key ‘metabolic filter’ of our diet, as they can convert common nutrients into metabolites, some of which have been linked to CVD[5,6]. Indeed, both epidemiological and animal model studies

Correspondence should be addressed to either author: brownm5@ccf.org; hazens@ccf.org.

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have provided strong support for the idea that the interplay between microorganisms and the host have a contributory role to atherosclerotic CVD[6–8]. Chronic infection with pathogenic bacteria, as well as intestinal dysbiosis, has been associated with the progression of CVD[11–19]. Bacterial molecular patterns can directly engage host pattern recognition receptors in the intestine, but also in the vasculature[5], promoting chronic inflammatory processes in the host. In addition to the direct stimulation of host immunity, the host microbiota can produce a wide variety of small-molecule metabolites that are sensed by host receptor systems to affect CVD pathogenesis in a manner that does not require direct microorganism-host interactions..

In fact, recent evidence suggests that both the susceptibility to atherosclerosis[7] and thrombosis[8] can be transmitted via gut microbial transplantation in mice. Research over the past decade has uncovered several key microbial metabolites such as trimethylamine N-oxide (TMAO), short chain fatty acids (SCFAs), and secondary bile acids that uniquely affect the progression of CVD. Although large antibiotic intervention trials have not shown clear benefit in CVD[20–22], drug discovery is entering a new and exciting phase where targeting gut microbial metabolites represents a new tractable therapeutic strategy.

In this Review, we discuss the relevant transmissible microbial communities and taxa that are associated with, and microorganism-derived metabolites directly involved with CVD pathogenesis. With the major technological advances in microbial profiling methods and other ‘omic’ analytic platforms, we are now uniquely positioned to discover candidate microorganisms and their derived metabolites of relevance to CVD-related phenotypes, and pursue studies aimed at understanding mechanisms by which our microbial inhabitants impact human disease. We review microorganism-driven pathways that were reported to have an impact on CVD pathogenesis, and discuss how gut microorganism-targeted therapeutics hold untapped promise as new therapies for the prevention and/or treatment of CVD.

Microorganism-associated molecular patterns

It is now well appreciated that diverse microbial communities reside within the intestinal tract, on the skin surface, and on nearly every exposed surface of the human body[5]. Although still controversial, there is also a growing body of literature suggesting that increased permeability of oral and intestinal epithelial barriers may enable a small number of bacteria to enter into the systemic circulation, where they can ultimately enter into host tissues to promote disease[9–11]. Whether residing in or on exposed surfaces, or when they access the systemic circulation, bacteria can directly engage the innate immune system to not only elicit appropriate bacteriocidal responses, but to also regulate host metabolism and inflammatory pathways relevant to CVD. This type of microorganism-host cross talk is driven by direct interaction of microorganism-associated molecular patterns (MAMPs) and host pattern recognition receptors (PRRs) (FIG.1). There are now a number of examples linking MAMP – PRR driven signaling pathways to heart disease, and in fact chronic infections. In addition, evidence of prior exposure to certain bacterial pathogens has been associated with increased CVD risk[11–19].

Several recent studies have analyzed microbiota communities in human oral, gut and atherosclerotic plaques from individuals with established atherosclerosis, and have found a reproducible correlation between CVD and bacterial pathogens, including *Chlamydia pneumoniae, Porphyromonas gingivalis, Helicobacter pylori and Aggregibacter actinomycetemcomitans*[12–15]. Importantly, some of these bacteria have been found in the intestinal tract as expected, but also within atherosclerotic plaques[13–15], which indicates the possibility of engagement of PRRs in highly distinct microenvironments. In a metagenome-wide association study (MWAS) for atherosclerosis it was recently reported that the genus *Collinsella spp*. is enriched in subjects with atherosclerosis, while *Eubacterium spp*. and *Roseburia spp*. are more abundant among healthy controls[16]. Whether these associations are causally linked to disease pathogenesis, or alternatively, are driven by the presence of disease or disease-associated comorbidities and/or therapeutics remains to be determined.

Initially described in the Helsinki Heart Study, the common microbial pathogen, *C. pneumoniae*, has repeatedly been associated with CVD[17–22]. In addition, *C. pneumoniae* has been shown in vitro to elicit cell autonomous proinflammatory effects in isolated macrophages, endothelial cells and smooth muscle cells, which would be consistent with a link to atherosclerotic CVD[18,19]. However, primary infection with *C. pneumonia* has inconsistently altered atherosclerosis phenotypes in mouse models, and antibiotic treatments effective at treating *C. pneumonia* have proven ineffective in clinical studies, leaving a causal role for the bacteria in disease pathogenesis still a matter of debate[20–23]. More evidence for a bacteria-CVD link was provided from studies investigating the pathogen *Porphyromonas gingivalis*, which has been investigated because of associations between periodontal disease and CVD risk[24–29]. Either oral or systemic infection with *P. gingivalis* accelerates atherosclerosis in animal models, in part by promoting macrophage foam cell formation and inducing platelet aggregration[24–29]. Also, there may be a link between *Helicobacter pylori* infection and CVD risk[30–32]. However, careful primary infection studies in mouse models of atherosclerosis have yielded conflicting results[30–32]. Collectively, there are a number of reports suggesting that pathogenic bacteria may be causally linked to atherosclerotic CVD. However, whether CVD can be viewed as an infectious disease is still matter of intense debate because, as noted above, a number of large randomized prospective human antibiotic and anti-viral trials have failed to show any benefit of treatment or prophylaxis therapy in CVD or mortality[20–23].

Even with the disappointing news with large antibiotic trials, there is ever expanding evidence that host PRRs are important regulators of CVD pathogenesis. For instance, single nucleotide polymorphisms (SNPs) in the host peptidoglycan receptor NOD1 are linked to early onset coronary heart disease[33], and Nod1$^{-/-}$ mice are protected against atherosclerosis on an apolipoprotein E null (apoE$^{-/-}$) background, which is a commonly used mouse model of atherosclerosis driven by severe accumulation of cholesterol in circulating remnant lipoproteins[34]. The heterodimeric complex of toll like receptors (TLR) 2 and TLR6 (TLR2-TLR6) is central to sensing bacterial di- and tri-acylated peptides[35,36]. A recently described SNP in the *Tlr6* gene has been linked to left ventricular wall thickening and markers of inflammation[37,38], and Tlr2$^{-/-}$ mice have strikingly reduced atherosclerosis, especially when challenged with synthetic TLR2 agonists[36]. Furthermore, transplantation of bone marrow from mice lacking the double-strand DNA receptor TLR3 into LDLr$^{-/-}$, which is a

commonly used mouse model of atherosclerosis driven by elevated low density lipoprotein cholesterol levels mice results in reduced atherosclerosis burden[39]. Among all of the CVD-associated MAMP-PRR pathways, the lipopolysaccharide (LPS) receptor, TLR4, has been most thoroughly investigated[40–44]. There have been a large number of reported TLR4 SNPs, with some having no association, some with increased, and some with decreased association to CVD risks[40–44]. However, genetic deficiency of TLR4 in either LDLr$^{-/-}$ or apoE$^{-/-}$ mice is reported to reduce atherosclerotic burden[41–43]. Importantly, many other non-bacterial 'endogenous' TLR4 agonists such as oxidized low density lipoproteins[42], saturated fatty acids[43], and high mobility group box 1 (HMGB1)[44] have been shown to promote atherosclerosis progression. Additionally, a SNP in TLR7, a receptor for single-strand RNA, has been linked to ischemic stroke[45], yet intriguingly, TLR7 deficiency in an apoE$^{-/-}$ background worsens atherosclerosis[53]. In a similar fashion, mice lacking the unmethylated CpG DNA receptor TLR9 in an apoE$^{-/-}$ background have exacerbated atherosclerosis[46]. Collectively, there is little doubt that activation of a number of host MAMP-PRR pathways can affect atherosclerosis progression. However, it remains doubtful that either activating or inhibiting PRRs is a viable option because of the central role of these receptors in innate immunity.

Microbial metabolites in CVD

In addition to surface antigens (MAMPs) on commensal and pathogenic bacteria, gut microorganisms enzymatically produce various metabolites that can both act locally in the gut, as well as travel systemically to affect host physiology in healthy and disease states. In fact, our gut microbiome represents a crucial filter of our diet, and effectively chemically diversifies each meal we consume. This simple idea, that microorganisms themselves, or products secreted from microorganisms, can cause disease or improve health (that is, probiotics), has been bolstered tremendously over the past decade. Recent advances in the fields of untargeted metabolomics in well-characterized clinical cohorts, coupled with mechanistic animal model studies have been used to discover several human disease-associated gut microbial metabolites. In fact, we now know that there are a large number of circulating metabolites that are derived solely or in part from bacterial metabolism of either dietary nutrients or diet-derived xenobiotics[47–50]. The rapidly expanding microorganism-dependent metabolome includes many methylamines, polyamines, polysaccharides, short chain fatty acids (SCFA), secondary bile acids, B vitamins, uremic toxins like *p*-cresol sulfate and indoxyl-sulfate, 4-ethylphenylsulfate, dihydrodigoxin, and a long list of xenobiotic-derived metabolites[47–50]. At this point, several microbial metabolites are recognized, the levels of which are associated with CVD-related phenotypes, but as association does not equal causation, the potential mechanistic participation of these metabolites in CVD remains unclear. A major limitation in progress on mechanistic studies, beyond their laborious nature, is that many of the circulating candidate analytes are yet chemically undefined. Moreover, even for those that are structurally resolved and available for study, we have almost no information about how microorganism-generated metabolites are sensed, whether it is through dedicated host receptors systems or whether they have non-receptor mediated mechanisms to affect CVD pathogenesis (for example, as allosteric modifiers).

Currently, there are three main classes of gut microorganism-dependent metabolites that have been linked to CVD risk either in humans or mice models - trimethylamines, short chain fatty acids and secondary bile acids. We focus on current mechanistic understandings through which these gut microorganism-dependent metabolites signal to the host and affect CVD pathogenesis.

The gut microbe-derived metabolite TMAO

Since its discovery and first reported link to CVD pathogenesis[51], the gut microbial co-metabolite trimethylamine-*N*-oxide (TMAO) has quickly gained traction as both a biomarker for human CVD risk, and as a promoter of atherothrombotic diseases[5–8,51–65]. In fact, numerous human and mouse studies have support the notion that the TMAO pathway is one of the first *bona fide* gut microbiome centered CVD drug targets[5,6,51–77]. The TMAO pathway is a meta-organismal metabolic pathway whereby nutrients that are present in high fat foods (phosphatidylcholine, choline, L-carnitine and likely other trimethylamine-containing nutrients) can be metabolized by several distinct gut microbial enzyme complexes (CutC/D, CntA/B, YeaW/X)to generate the primary gut microbial metabolite TMA. TMA can be synthesized by either the choline utilization TMA lyase system (CutC/D)[76], the carnitine Rieske-type oxgenase/reductase system (CntA/B)[77], or the YeaW/X system which can utilize multiple substrate sources[55]. TMA then enters the portal circulation and is further metabolized by host enzymes in the liver, called flavin-containing monooxygenases (predominantly the FMO3 isoform), to produce TMAO (FIG. 2). Several recent reviews have highlighted the clinical relevance and therapeutic potential of the TMAO pathway in CVD[5,6,50], so this will not be discussed in detail here. Briefly, a wealth of human and animal model data supports the conclusion that the gut microorganism-derived metabolite TMAO has both strong clinical prognostic value, and dietary provision of TMAO can promote atherosclerosis and thrombotic vascular disease in mouse models[5–8,51–65]. In addition to roles in atherosclerosis and thrombosis, the TMAO pathway has also been associated with other closely linked cardiometabolic diseases in humans including cardiac hypertrophy, cardiac fibrosis, chronic kidney disease, type 2 diabetes and obesity[66–75].

The gut microbiome-driven metabolite TMAO represents a promising gut microbiome-centered drug target [50] (Box 1). In fact, with the recent seminal discoveries of the microbial enzymes catalyzing the production of TMA[76,77], we are now poised to therapeutically intervene this pathway at the level of gut microbial enzymology. However, at this point molecular mechanisms whereby TMAO promotes atherosclerosis, thrombosis, heart failure, renal dysfunction, insulin resistance and obesity are still being elucidated. This is, in large part, due to the lack of understanding of the molecular sensor for TMAO (that is, the TMAO receptor). Although there is evidence that TMAO can function as an important osmolyte in biological systems[78,79], more recent evidence suggest that TMAO can rapidly signal to cells within minutes[8,80]. In isolated platelets, brief exposures to physiological levels of TMAO can potentiate thrombin-induced calcium release[8]. Likewise, treatment with TMAO in endothelial cells or smooth muscle cells, or direct injection in vivo, was shown to rapidly induce the activation of mitogen-activated protein kinases and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), or the upregulation of downstream adhesion proteins on aortic endothelial cells[80]. The kinetics of these TMAO-induced signaling

responses strongly suggest that host cells may possess a dedicated receptor or sensor system to transduce the TMAO signal to an appropriate cellular response (FIG 2). Likewise, elevated TMAO levels are associated with elevated phosphorylation of the protein Smad3, which is a key effector of transforming growth factor β (TGFβ) signaling[70] Identification of a host TMAO receptor is a strong possibility, given that structurally similar TMA has been shown to be a high affinity agonist for a cell surface G protein coupled receptor known as trace amine-associated receptor 5 (Taar5)[81,82]. In fact, there are now many examples of dedicated host receptor systems for metabolites originating from gut microbes[47–50]. Identification of a dedicated TMAO receptor would have broad implications, given the clear links of TMAO in human disease. It is tempting to speculate that TMAO receptor antagonists could be an effective therapeutic strategy to prevent or even treat established CVD.

As TMAO pathway drug discovery efforts advance, it will be imperative to understand the microbial taxa responsible for regulating flux through the TMA - TMAO pathway. For example, very little is known regarding which species or broader communities are the main producers of TMA in mouse and human ecosystems. In mouse studies in which dietary source nutrients for TMAO were specifically altered (for example, dietary provision of choline, L-carnitine or γ-butyrobetaine), a refined list of taxa has been associated with circulating TMAO levels, including the broad order of *RF39* (r=0,49; false discovery rate, FDR=0.04), families of *Erysipelotrichaceae* (r=0.40; FDR=0.01), *Lachnospiracea* (r=0.779; FDR=0.084), and at the genus levels *Prevotella* (r=0.44; FDR=0.001), *Anaeroplasma* (r=0.677; FDR=0.016), *Porphomonadaceae* (r=-0.581; FDR=0.052), and *Akkermansia muciniphila* (r=0.634; FDR=0.009)[51,52,55]. Follow-up studies in humans comparing vegetarians and omnivores showed that several taxa of fecal microorganisms are associated with circulating TMAO levels, including *Prevotella, Clostridiaceae, Incertae_Sedis_XII, Peptostreptococcaceae, Clostridium, Fusibacter, Lachospira*, and *Sporobacter*[52]. Such associations between bacterial taxa and circulating TMAO levels are only useful in hypothesis generation, but do not establish a direct link between the taxa and TMAO production. A more fruitful method of identifying *bona fide* TMA-producing bacterial species is to screen reference genomes to identify the genetic potential to generate TMA (for example, presence of TMA lyase enzyme operons that encode CutC/D, CntA/B and YeaW/X), followed by functional studies. An elegant example of such reference genome mining was recently described[83], supporting the power of genetics in predicting relevant metabolite-producing bacterial taxa. However, when it comes to metabolite production it is ultimately key to prove quantifiable biochemical potential. Coupling brute force microbial culture with biochemistry, a recent study screened 79 culturable human commensal bacteria for the potential to produce TMA from choline *in vitro*[84]. This important study identified eight distinct bacterial strains with the biochemical capacity to produce TMA from choline including *Anarococcus hydrogenalis*, *Clostridium asparagiforme*, *Clostridium hathewayi*, *Clostridium sporogenes*, *Edwardsiella tarda*, *Escherichia fergusonii*, *Proteus penneri* and *Providencia rettgeri*[84]. More importantly, low level colonization with a TMA producer (*C. sporogenes*) in the defined background of a core community that lacked TMA lyase activity (*Collinsella aerofaciens, Bacteroides caccae, Bacteroides ovatus, Bacteroides thetaiotaomicron, and Eubacterium rectale*) resulted in both TMA and TMAO generation *in*

vivo in recolonized gnotobiotic mice, as well as reductions in choline levels in the host[84]. Another recent study found that the choline utilization cluster (Cut) is widely distributed across many gut microbial phyla in human stool metagenomes[85]. In addition to human stool metagenomes, CutC can be found with similar frequency in oral mucosa and supragingival plaque[86], making it tempting to speculate that oral TMA production may be associated with the link between periodontal disease and CVD risk. Finally, a recent study has identified the human gut microbial taxa possessing the choline TMA lyase (CutC) and the carnitine oxygenase (cntA) using a multi-level screening approach[87]. Therefore, there has been rapid growth in our understanding surrounding which human commensals have the capacity to produce TMA from major source nutrients.

In addition to TMA producing bacteria, there is also mounting evidence that certain microbial species can metabolize TMAO and TMA for maintenance of trophic chains in both colonic and marine bacterial ecosystems[88–91], but the relevant microorganisms and operons involved are still being elucidated. One major pathway facilitating catabolism of TMAO is driven by the bacterial torCAD operon, which encodes a TMAO reductase (TorA), a c-type cytochrome (TorC), and a TorA-specific chaperone (TorD)[88]. Additionally, bacteria present in marine ecosystems can also degrade TMAO through a TMAO demethylase enzyme (Tdm)[89]. Certain marine bacteria from the *Roseobacter* clade can use both TMA and TMAO as energy sources, which is facilitated by importing these metabolites via a ATP-binding cassette transporter TmoXWV for subsequent catabolism[890,91]. Certain methanogenic bacteria present in the human colonic ecosystem such as Methanomassiliicoccus luminyensis can reduce TMA as a substrate for methanogenesis[92]. It remains possible that these bacterial TMA-TMAO catabolic pathways are another potential avenue of therapeutic intervention, and may be an ideal probiotic strategy. There is little doubt that identification of both TMA producing and TMA/TMAO catabolizing human gut commensals will be particularly informative in the area of prebiotics and probiotics.

Although the vast majority of studies support a mechanistic link between the TMAO pathway and CVD, It is important to note that not all studies have show clear association between dietary intake of TMAO source nutrients (choline or carnitine) intake and CVD risk[93–99]. One recent study showed that dietary L-carnitine supplementation, which modestly raised circulating TMAO levels, was associated with a ~15% decrease in aortic atherosclerotic lesion area without altering aortic cholesterol content in apoE$^{-/-}$ mice transgenically expressing human cholesteryl ester transfer protein[93]. Furthermore, a large (n=29,079) population study in Japan demonstrated that dietary choline and betaine intak, estimated by a semi-quantitative food questionnaire, was not associated with CVD mortality[94]. Likewise, in the large (n=14,430) American-based Atherosclerosis Risk in Communities (ARIC) study it was found that dietary choline intake was not significantly associated with CVD[95]. In agreement, a large (n=16,165) Dutch study showed that dietary intake of choline or betaine was not associated with CVD risk[96]. Unfortunately, none of these large population studies examining the relationship between dietary choline, betaine, or carnitine intake at CVD outcomes measured circulating TMAO levels. Therefore, additional studies are needed to determine whether TMAO itself correlates with CVD risk in these same large population studies. This is likely to be the case given that a recent large (n=19,256) meta-analysis of hard CVD endpoint studies showed that circulating TMAO

levels was a much stronger predictor of major adverse cardiovascular events (MACE) than its source nutrients (choline, betaine L-carnitine)[97]. Although the association between circulating TMAO levels and MACE are highly reproducible, some studies have suggested that this association in driven in part by impaired kidney function[98]. Also, a recent study showed that TMAO levels may not be equally associated with all types of vascular disease, especially in the case of large artery ischemic stroke and transient ischemic attack[99]. Collectively, although most studies suggest a strong association between TMAO levels and atherosclerotic coronary heart disease, these recent findings suggest that additional work is needed to understand which patient populations would benefit from TMAO lowering therapeutic strategies.

SCFAs and other fermentation products in CVD pathogenesis

The products of gut microbial fermentation are by far the most well-studied gut microbial metabolites, having key roles in both the maintenance of gut microbial ecology, and in fine-tuning host immunity and metabolic disease[100–104]. The major products of microbial fermentation of dietary fibers are SCFAs, with the most abundant metabolites being acetate, butyrate and proprionate. Although additional studies are needed to identify the most abundant human commensal producers, some information is available regarding which taxa are responsible for the majority of SCFA production. Acetate is produced by a large number of enteric bacteria, with major contributors such as *Ruminococcus spp.*, *Prevotella spp.*, *Bifidobacterium spp.*, *Bacteroides spp.*, *Akkermansia muciniphila, Blautia hydrogenotrophica, Clostridium spp., and Streptococcus spp.*[105,106]. Proprionate can be generated via three distinct biochemical pathways from *Bacteroides spp.*, *Phascolarctobacterium succinatutens*, *Dialister spp.*, *Veillonella spp.*, *Megasphaera elsdenii*, *Coprococcus catus*, *Salmonella spp., Roseburia inulinivorans*, *and Ruminococcus obeum*[105,107]. Butyrate can be generated from locally-generated acetate substrate by *Coprococcus comes*, *Coprococcus catus*, *Coprococcus eutactus*, *Anaerostipes spp.*, *Eubacterium rectale*, *Eubacterium hallii*, *Faecaibacterium prausnitzii and Roseburia spp.*[105,107]. Butyrate can also be generated via carbohydrate fermentation by several members of the *Lachnospiraceae, Ruminococcaceae, Acidaminococcaceae* families[108]. Undoubtedly, the major drivers of SCFA production in both human and rodent gut microbiomes remain completely understood, and reference genomes predict that many additional SCFA producers are yet to be discovered. SCFAs can function as a macronutrient energy source and hormone-like signaling molecules that enter the portal circulation to ultimately signal through dedicated host receptor systems to regulate innate immunity and host metabolism. Some of the currently identified host SCFA receptors include G protein receptor 41 (GPR41)[109], G protein receptor 43 (GPR43)[109], G protein receptor 109A (GPR109A)[110], and olfactory receptor 78 (OLF78)[111] (see below). Although SCFAs are the most well-studied gut microorganism-derived metabolites, it is still unclear whether SCFAs are causally linked to the development of human diseases, especially CVD, and their manipulation to modulate disease processes and susceptibility remains under investigation.

The vast majority of literature linking the gut microbiota to human disease implicate SCFAs as potential disease preventing or mitigating factors in obesity, diabetes, intestinal immunity, hypertension, kidney disease, cancer, and both alcoholic and non-alcoholic liver disease

(NAFLD)[100–104]. Although there is a substantial body of literature linking microorganism-derived SCFAs to CVD risk factors, there is a paucity of studies showing clear association with circulating SCFAs and CVD risk or mortality in humans. In fact, most of the links between SCFAs and CVD risk factors such as obesity, diabetes, hypertension, renal dysfunction, and liver disease have been established in animals models. Therefore, unlike TMAO, the potential for SCFAs to be a therapeutic target for CVD is not strongly supported by independently replicated clinical studies. Gut microorganism-derived SCFA and their links to metabolic disease in animal models have been recently covered in several excellent reviews[100–104]. Below, we therefore only highlight several SCFA intervention human trials, and we discuss recent results from studies using animal models.

In one of the first direct administration studies in humans, physiologically relevant doses of acetate or proprionate were given to examine acute effects on metabolism[112]. This study found no effect on the levels of blood glucose or insulin, but both SCFAs reduced the levels of circulating free fatty acids[112]. Moreover, independent studies showed that although acute (3 hours) gastric infusion of SCFAs had no effect on glycemia , the levels of free fatty acid ere reduced[113]. Follow-up studies demonstrated that SCFA administration increased serum glucagon levels, which may have indirectly altered the levels of circulating free fatty acid[114]. In a subsequent long-term study in which proprionate was supplemented for seven weeks, there was a modest decrease in fasting glucose and alterations in glucose-stimulated insulin secretion during an oral glucose tolerance test[115]. Furthermore, provision of dietary supplementation of non-digestible polysaccharides yielded contradicting results[116--122]. However, it is important to note that although these studies often claim the observed metabolic effects are driven by alterations in the levels of SCFA, fiber-rich diets have pleotropic effects that extend beyond SCFA biology. Recently, one of the first randomized double-blind placebo-controlled antibiotic trials was conducted in which the levels of SCFA and a number of metabolic parameters were carefully monitored[123]. As expected, the antibiotic (vancomycin) treatment group showed drug selective reorganization of gut microbial diversity and composition, which was associated with statistically significant reductions in the levels of SCFA[123]. Surprisingly, even with large effects on the levels of SCFA no apparent alterations in energy or glucose homeostasis, which indicates that the phenotypic effects of SCFA seen in mice are not so easily translated into humans[123]. Collectively, these studies highlight the critical need for additional clinical investigation into whether modulation of SCFAs would hold any benefit in patients with CVD.

Even with these seemingly negative results in humans, there are some emerging discoveries with SCFAs and other microbial fermentation products that deserve additional investigation. For instance, a recent report described a pathway by which SCFAs can engage the olfactory receptor OLF78 and GPR41 in the kidney to regulate renin secretion and blood pressure[97]. In addition, another study demonstrated that obstructive sleep apnea-induced hypertension is transmissible by gut microbial transplantation[111]. In addition to SCFAs, the gut microbial fermentation product lactic acid has been reported to activate the host G protein-coupled receptor GPR81 to suppress adipose tissue lipolysis[125]. Interestingly, inhibition of the lactate receptor GRP81 was shown to protect against ischemic brain injury in mice[126]. As noted above, another emerging biologically active non-SCFA bacterial fermentation product is succinate[127–129]. Succinate is produced in large quantities in mammalian cells during the

citric acid cycle, but it can also be generated by colonic microorganisms, including *Prevotella*[129]. *Prevotella*-generated succinate is presumed responsible for the observed beneficial effect on mice intestinal glucose production following provision of *Prevotella copri*[129]. Succinate can activate the host G protein-coupled receptor GPR91[127–1295], which indicates a potential new gut microbial metabolite-host receptor interaction that is relevant to CVD pathogenesis. With the exception of the butyrate receptor GPR109A[130], there have been no studies showing that SCFA, lactate or succinate receptor knockout mice exhibit alterations in atherosclerosis susceptibility, and therefore additional preclinical studies are needed.

The gut microbial bile acid metabolome in CVD

Gut microbial metabolism of bile acids represents one of the more intriguing stories of metaorganismal symbiosis, whereby an intimate bi-directional circuit produces essential detergents for intestinal fat absorption in the host, and provides a host-derived signal to maintain microbiome community structure[131–133]. Bile acids are now recognized to function as diverse signaling molecules that regulate host macronutient metabolism and energy expenditure (FIG. 3) (reviewed in REFs [131–140]). Once delivered to the colonic microenvironment, bile salts can elicit cytotoxic effects to certain members of the gut microbiome ecosystem[131–140]. Although not completely understood, primary bile salts and locally-produced secondary bile acids are thought to regulate microbial community structure via membrane detergent effects, and also by inducing DNA damage in certain members of microbial communities [141–144]. Interestingly, certain bacteria have evolved bile salt resistance mechanisms via alterations in efflux pumps[141,142], and alterations in membrane lipid and protein composition[143–147]. For example, *B. longum BBMN68* possesses a hemolysin-like protein that enables for selective resistance to taurine-conjugated bile salts[145]. Some bacteria such as *Camphylobacter spp*. and *Salmonella typhimurium* can be readily detected in gallbladder bile, where bile acid concentrations are extremely high[148]. The ability of bile salts and bile acids to dictate gut microbiome community structure likely has a major role in downstream effects on host metabolism. The microorganisms that are able to persist in the bile salt-rich colonic microenvironment can then chemically diversify bile salts into a number of biologically active species that can then be sensed by the host. One of the best-studied microorganism-driven biotransformations is the enzymatic hydrolysis of the C-24 *N*-acyl bond of glycine- or taurine-conjugated bile salts into free bile acids by bile salt hydrolase (BSH)[135–138]. BSH activity is widespread through both Gram-positive and Gram-negative species, including *Clostridium*, *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Bacteroides*, *Methanobrevibacter smithii*, *Methanosphera stadmanae*, and likely many others[135–138]. In addition to deconjugation, gut microorganisms are the sole source of 7α and 7β dehydoxylase activity, which generates ‘secondary’ bile acids such as deoxycholic acid (DCA), lithocholic acid (LCA), hyodeoxycholic acid (HDCA) and ursodeoxycholic acid (UDCA)[134–136]. Unfortunately, there is currently limited information in regards to the bacteria genera that possess 7α and 7β dehydoxylase activity, and additional studies are needed in this area. In fact, the vast majority of genetic and biochemical studies have focused primarily on substrains *Clostridium scindens*, in which the bile acid inducible (bai) operon has been well characterized[149]. In addition to deconjugation and 7α and 7β dehydoxylation reactions, gut microbes can oxidize or epimerize bile acids

via several distinct enzymes of the hydroxysteroid dehydrogenase (HSDH) family[150,151]. HSDH activity has been confirmed in a diverse variety of bacteria, including *Bacteroides*, *Clostridium*, *Escherichia*, *Egghertella*, *Eubacterium*, *Peptostreptococcus* and *Ruminococcus*. Formation of ethyl esters and long-chain fatty acid esters from bile acids can be driven by gut bacteria such as *Bacteroides, Eubacterium* and *Lactobacillus*[152,153]. However, the bacterial enzymes responsible for these esterification reactions are unknown. Finally, gut microorganisms can remove sulfate from either 3α or 3β-sulfated bile acids, and this sulfatase activity has been seen in *Clostridium*, *Peptococcus*, *Fusobacterium*, and *Pseudomonas*[154]. Collectively, through deconjugation, oxidation, epimerization, 7α/7β dehydoxylation, esterification and desulfation gut microorganisms chemically diversify the bile acid pool, and then the secondary bile acids can enter the portal circulation to functions as endocrine-like signaling molecules with potent effects on host physiology and disease.

Once bacterially modified bile acids enter the portal blood, typically in the postprandial state, they can engage an ever-growing number of recognized host bile acid receptors (FIG. 2). The best known host bile acid receptor is the nuclear hormone receptor farnesoid X receptor (FXR), which regulates the transcription of key genes involved in primary bile acid biosynthesis[155]. Importantly, polymorphisms in FXR have been linked with hyperglycemia and circulating free fatty acid levels[156], and genetic deletion of FXR in mice alters the progression of atherosclerosis in mice[157]. Recent studies have also shown that FXR is required for the metabolic reprogramming initiated by human microbial transplantation[158,159]. Interestingly, transplantation of human microbiota into conventional mice can alter mouse bile acid composition and induce FXR activation by reducing the levels of the FXR antagonist tauro-beta-muricholic acid[159] Another host bile acid receptor gaining attention in recent years is the G protein-coupled receptor TGR5[160,161]. TGR5 activation has been linked to increased energy expenditure[146], and TGR5 knockout mice are protected against atherosclerosis development[160]. Secondary bile acids such as lithocholic acid can activate the nuclear hormone receptor pregnane X receptor (PXR), which is a well known transcriptional regulator of xenobiotic and lipid metabolism[162]. Genetic deletion of the lithocholic acid receptor PXR in mice reduces the progression of atherosclerosis[163]. Interestingly, certain bacterially-modified bile acids (3-oxo-lithocholic acid and lithocholic acid) can also activate the vitamin D receptor (VDR)[164], and genetic studies in both humans and mice have shown a role for VDR activation in CVD-related disease[164–166]. Taurine-conjugated bile acids have been shown to activate specific muscarinic receptors[167], and polymorphisms in the muscarinic acetylcholine receptor M2 isoform have recently been associated with reduced heart rate and death by myocardial infarction[168]. Finally, all conjugated bile acids can activate signaling through the sphingosine-1-phosphate receptor 2 (S1PR2)[169], and S1PR2 knockout mice have been shown to have reduced atherosclerosis burden in the apolipoprotein E deficient background[170]. Collectively, microorganism-derived bile acid metabolites represent diverse endocrine signals that hold tremendous therapeutic potential. In fact, in May 2016, a semi-synthetic bile acid analogue, obeticholic acid (trade name Ocaliva) was approved by the United States Food and Drug Administration for the treatment of liver disease[171]. This success story is likely one of many to come at the microbial metabolite-host receptor interface.

Conclusions

Although drug discovery has historically focused on targeting human enzymes, we are entering a new era of microbial pharmacology as biomedical research is aiming to target the microorganisms that live within us to improve human health). In particular, CVD is uniquely positioned for success with several gut microbial metabolites clearly associated with CVD risk. Successes in drug discovery at the microbial metabolite-host receptor interface have already been realized, and preclinical proof of concept is now established for non-lethal small-molecule inhibitors of TMAO production[58]. In parallel, there are substantial efforts to develop prebiotic, probiotic and fecal transplantation strategies to interrupt microorganism-host pathways that are involved in CVD pathogenesis[50]. As we forge ahead to determine microbial contributions to the origin and progression of human diseases, it is imperative that we move away from the traditional microbiome profiling approaches that simply ask who is there. Instead, we need to ask what disease-causing microbial products can be identified. Once we identify relevant microorganism-derived molecules, we must adopt classic principals of endocrinology to identify the host receptor systems involved in disease pathogenesis. The diversity of chemicals and metabolites derived from our gut microbial symbionts is quite astounding, and provides an essentially untapped source of new drug targets for cardiometabolic disease.

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Glossary

pattern recognition receptor
host sensors which detect molecules typical for pathogens

atherosclerosis
a disease process in with the inside of an artery narrows due to the build up of plaque

thrombosis
the formation of a clot inside of a vessel

hyperlipidemic
a condition where blood lipids are elevated

ischemic stroke
a stroke that occurs when a blood vessel to the brain is blocked by a blood clot

metabolome
the complete set of small molecule chemicals found within a biological sample

transient ischemic attack

also called a “mini-stroke”a transient ischemic attack is a brief episode of neurological dysfunction cause by lack of blood flow to the brain

glycemia
the level of glucose in one’s blood

detergents
a surfactant or mix of surfactants that has cleaning or membrane disturbing properties

taurine
a major sulfur-containing amino acid

postprandial state
the state immediately following a meal

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Box 1: Therapeutic strategies

The majority of microbiome drug discovery efforts tend to focus on prebiotic or probiotic approaches, as the causative mediators and microbial species involved in disease pathogenesis remain to be determined for most host phenotypes under investigation. Given the mounting evidence linking the microorganism-dependent metabolite TMAO to CVD pathogenesis, there is strong likelihood that the TMAO pathway will be one of the first microbial pathways selectively targeted with non-lethal small molecules for the amelioration of human CVD. In strong support of this, a recent study showed the first proof of concept studies using a small-molecule inhibitor of gut microbial TMA lyase enzymes that led to a reduction in TMA and TMAO levels, and protection of mice against diet-induced atherosclerosis[58]. Regardless of which therapeutic approach is taken, because of the numerous mechanistic links between TMAO and the pathogenesis of disease, and strong corroborative associations from multiple human clinical studies, the gut microbial TMAO pathway holds tremendous potential as a therapeutic target for CVD treatment and prevention.

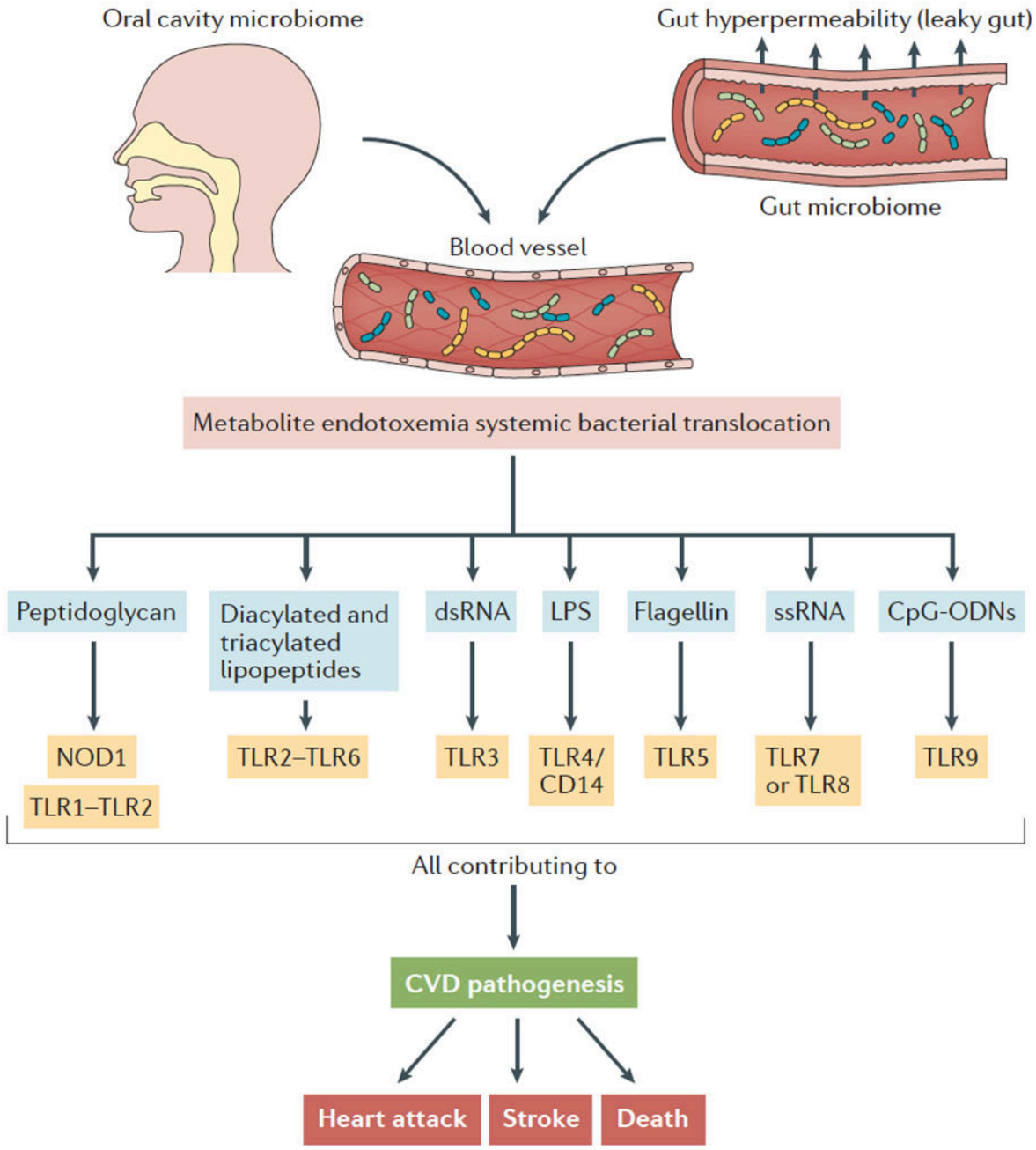

Figure 1. Direct engagement of pattern recognition receptors by microbial-associated molecular patterns driving CVD

Microbial-associated molecular patterns (MAMPs) can promote CVD via the direct engagement of host pattern recognition receptors (PRRs), promoting chronic inflammatory processes in the host. In the context of cardiovascular disease, dysbiosis in both the oral and gut microbiome can elicit local MAMP-PRR signaling within those microenvironments (not shown). In addition, systemic bacterial translocation can promote CVD by MAMP-PRR signaling in distant sites, including the liver and artery wall. Abbreviations: CD14, cluster of differentiation 14; CpG ODNs, CpG oligodeoxynucleotides; LPS, lipopolysaccharide; MI,

myocardial infarction; NOD1, nucleotide oligomerization domain-containing 1; TLR, toll-like receptor.

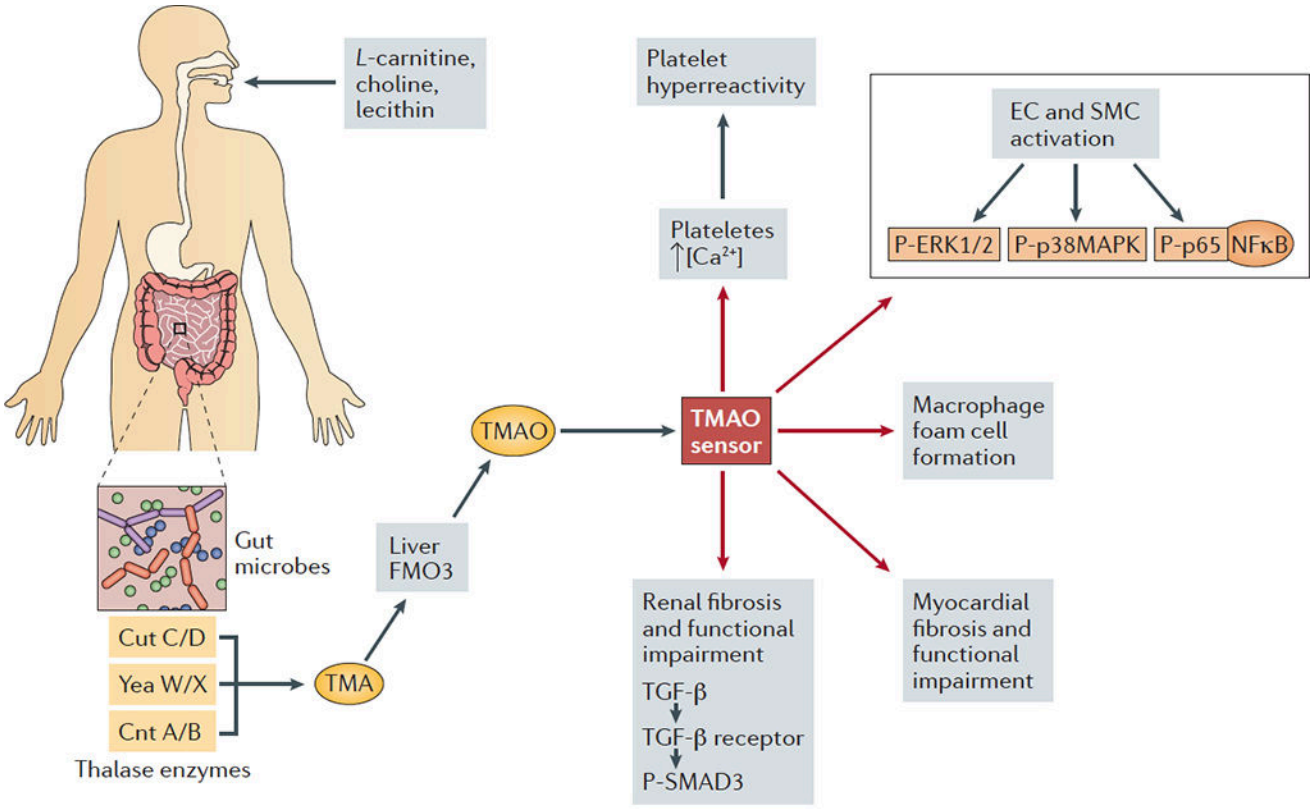

Figure 2. The metaorganismal TMAO pathway as a driver of CVD

Postprandial delivery of choline, phosphatidylcholine (PC), carnitine, γ-butyrobetaine, and likely other methylamine-containing source nutrient gut microbes provides substrate for the gut-microbial-mediated production of trimethylamine (TMA). Microbial TMA lyase enzymes (CutC/D, CntA/B, and YeaW/X) can then generate TMA, which enters the portal circulation and is ultimately delivered to the host liver. The host flavin-containing monooxygenase (FMO) family of enzymes, especially FMO3, can then convert TMA to TMAO. TMAO can then promote atherosclerosis, thrombosis, heart failure, insulin resistance, and kidney disease via tissue or cell type-specific reprogramming. Through an unknown receptor-mediated sensing mechanism (indicated as TMAO sensor), TMAO drives cell-specific signaling events that promote CVD pathogenesis. In platelets, TMAO rapidly enhances stimulus-induced calcium (Ca2+) release, which signals to drive pro-thrombotic programmes. In endothelial and smooth muscle cells TMAO rapidly activates mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-kB) to promote the expression of adhesion molecules such as ICAM and E-selectin. In addition, TMAO can signal through currently unidentified pathways to regulate increased macrophage foam cell formation. TMAO can also initiate profibrotic programmes in the heart and kidney via a transforming growth factor β (TGFβ) – phospho-SMAD3 signaling axis. Collectively, these cellular events converge to promote atherosclerosis, thrombotic vascular disease, and associated renal impairment.

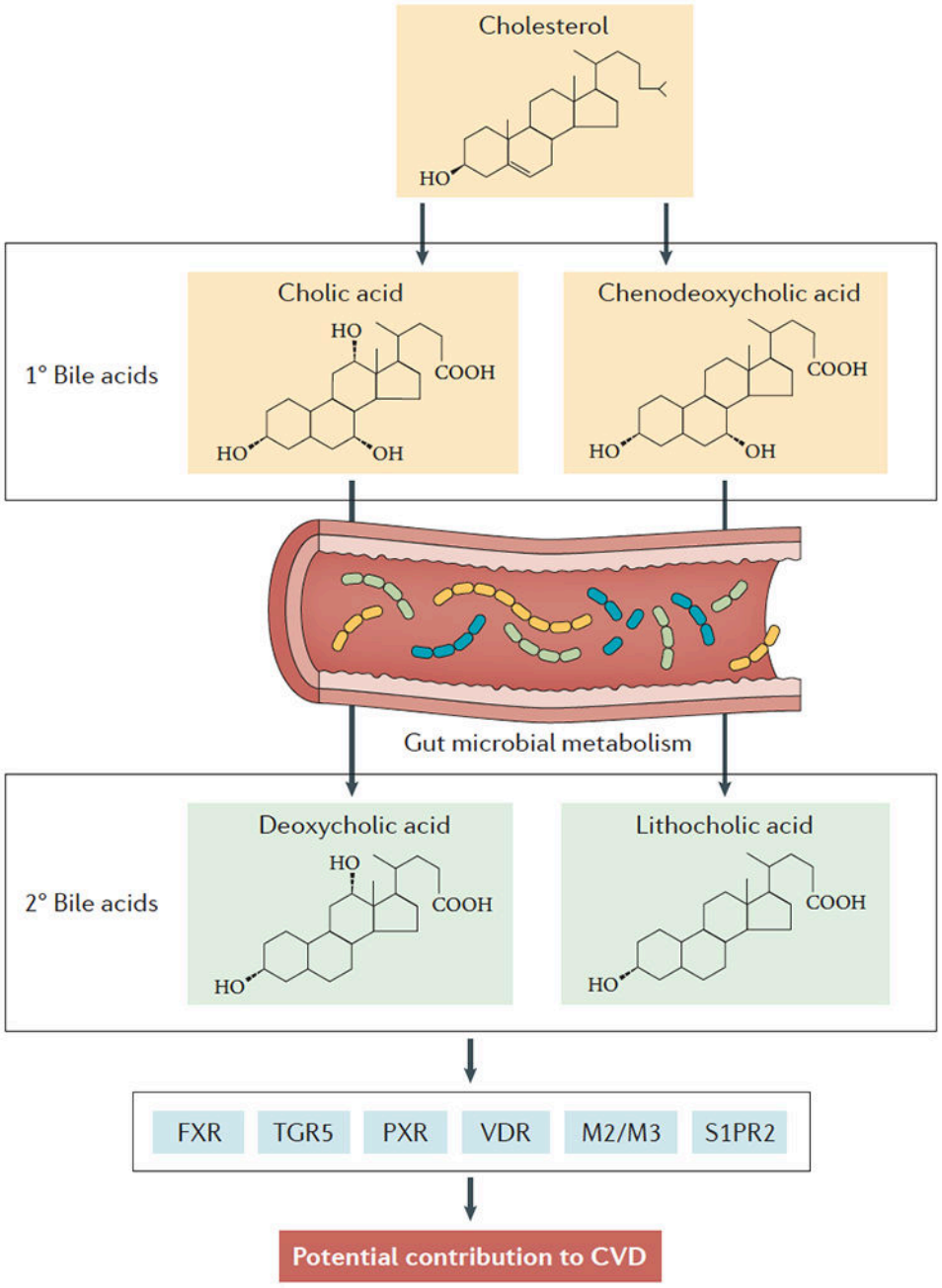

Figure 3. Microbial production of secondary bile acids in CVD

Initially, primary bile acids are synthesized in the host liver from cholesterol. *De novo* synthesized primary bile acids such as cholic acid, chenodeoxycholic acid (CDCA) and muricholic acid (MCA; only produced in rodents) are then conjugated with either glycine (humans) or taurine (humans and mice) at the C-24 carboxyl position. Following conjugation, resulting bile salts are secreted into bile along with cholesterol and phospholipids to form mixed micelles, which are transiently stored in the gall bladder. When a meal is ingested, the gall bladder contracts to release mixed micelles into the proximal intestine where they function as essential emulsifiers to enable proper absorption of

hydrophobic molecules such as fatty acids and fat-soluble vitamins (such as vitamin A, vitamin D, vitamin E and vitamin K) (not shown). Importantly, bile salts are left behind in the intestinal lumen where they ultimately traverse to the colon. Once in the colonic microenvironment, they participate in a bi-directional interplay regulating microbial community structure, and they are subsequent microorganism-driven metabolism of primary bile salts into secondary bile acids (deoxycholic acid and lithocholic acid), which can have an impact on host physiology and disease susceptibility. Importantly, after aiding in intestinal lipid absorption both primary bile salts and secondary bacterial metabolites are almost quantitatively re-absorbed (>95% recovered) in the ileum via dedicated host transporters in ileal enterocytes (not shown). This reabsorptive process provides newly diversified bile acid species, which can then signal to the host through dedicated receptor systems, including farnesoid X receptor (FXR), protein-coupled bile acid receptor 1 (TGR5), pregnane X receptor (PXR), vitamin D receptor (VDR), muscarinic receptors 2 and 3 (M2/M3), and sphingosine-1-phosphate receptor 2 (S1PR2).