The emerging role of gut microbial metabolism on cardiovascular disease

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The gut microbiome has been implicated in the progression of cardiovascular diseases (CVD) including hypertension, dyslipidemia, atherosclerosis, thrombosis, heart failure, and ischemic stroke. Metabolomics studies in humans and diverse mouse populations have revealed associations between diet-derived gut bacterial metabolites, including trimethylamine-*N*-oxide, short-chain fatty acids, and intermediates of aromatic amino acid breakdown, with progression of CVD. Functional studies in animals fed diets of defined composition have been instrumental for establishing causal links between these metabolites, the microbes that produce them, dietary substrates and disease. The purpose of this review is to discuss recent progress in our understanding of how gut microbial metabolism of food influences the development of CVD and to outline experimental approaches that can be useful for addressing crucial knowledge gaps in the field. Together, this body of work supports the notion that the gut microbiomes mediate many of the effects of diet.

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Introduction

A major role of the intestinal microbiota is to aid in the harvest of nutrients from diet. Dietary components that are not readily absorbed in the small intestine serve as growth substrates for members of the gut microbiota, which in turn generate myriad metabolites, many of which impact host development and physiology. Epidemiological studies have revealed a number of associations between alterations in the organismal and functional composition of gut microbiome and cardiovascular traits [1–5,6•,7,8] (Table 1). While the interpretation of these studies is complex due to the many factors that contribute to gut microbiome composition (e.g. subjects with different diseases may have unique diets and take medications that modify the microbiome), a number of consistent patterns are starting to emerge among these reports; for example, subjects with coronary artery disease (CAD), obesity and diabetes harbor lower levels of taxa involved in the production of the short-chain fatty acid butyrate [3,6•,7] and they also tend to harbor higher levels of pro-inflammatory proteobacteria and lower overall bacterial diversity [1,4,5,6•]. Although in most cases, it is not clear whether these microbial changes precede or cause disease, these alterations likely impact the metabolic potential of the gut microbiome and how diet is metabolized.

It has been long known that diet is a major contributor to the risk of CVD; however, recent well-controlled animal studies connecting the gut microbiota and CVD have broadened our understanding of how diet may affect cardiovascular health and disease. These studies support the notion that the gut microbiome mediates many of the beneficial and detrimental effects of diet on CVD. There are now several examples for which microbial-derived diet-dependent metabolites have been associated with CV phenotypes and causal links between the metabolite (s) and disease established. In this review, we summarize recent findings from clinical cohorts showing associations between gut microbes and cardiovascular health (Table 1), and discuss the microbial-derived molecules generated from dietary components that have been linked with CVD pathogenesis, such as methylamines, short-chain fatty acids (SCFAs), and aromatic amino acids catabolites. From these studies, a discovery pipeline for identifying novel microbial players in CVD emerges. This experimental approach starts with hypothesis-generating screen of human/rodents with cardiovascular phenotypes of interest (Figure 1a). Microbial features that show strong correlation with disease are further validated in gnotobiotic mice harboring communities that vary in specific microbes and/or microbial functions (Figure 1b). Key experimental reagents used for follow-up validation studies include culture collections of well-characterized microbes, diets of defined composition and gnotobiotic mouse models of disease.

Bacterial modulation of methylamines; production of trimethylamine-*N*-oxide

Humans studies have shown that levels of circulating trimethylamine-*N*-oxide (TMAO) are associated with CVD development and increased risk of adverse cardiovascular events [9–11]. The pathway resulting in TMAO

Table 1

Clinical studies showing associations between the gut microbiome and cardiovascular traits

Cohort	Size	Sequencing	Main Findings	Ref
LifeLines-DEEP population cohort	893 subjects	16S rRNA	Healthy lipid levels (HDL↑, TG↓) are associated with increased microbial diversity.	[1]
LifeLines-DEEP population cohort	1264 subjects	Metagenome	CVD-related proteins are associated with the gut microbiome.	[2]
LifeLines-DEEP population cohort	978 subjects	Metagenome	Bacterial L-methionine biosynthesis is associated with atherosclerosis.	[3]
300-Obese cohort	297 subjects	Metagenome	A *Ruminococcus* species is associated with liver fat content.	[3]
TwinsUK cohort	617 subjects	16S rRNA	Arterial stiffness is inversely correlated with microbial diversity.	[4]
BSH (Bogalusa Heart Study) cohort	55 high versus 57 low CVD risk subjects	16S rRNA	*Prevotella* and *Tyzzerella* are associated with high CVD risk subjects. *Alloprevotella* and *Catenibacterium* are associated with low CVD risk subjects.	[5]
A cohort in China	187 controls versus 218 CAD patients	Metagenome	Bacterial pathways for transport of simple sugars and amino acids, synthesis of the O-antigen of LPS, metabolism of glycerolipids and degradation of fatty acids are enriched in patients. Bacterial pathways for biosynthesis of vitamins and host glycan degradation are enriched in controls.	[6●]
Göteborg Atheroma Study Group Biobank	13 controls versus 12 CVD patients	Metagenome	*Eubacterium*, *Roseburia*, and *Bacteroides* are enriched in controls,	[7]
A cohort in Japan	30 controls versus 30 CAD patients	16S rRNA	*Bacteroides vulgatus* and *Bacteroides dorei* are reduced in patients.	[8]

generation is initiated upon ingestion of foods rich in phosphatidylcholine, choline, and carnitine, which are converted into trimethylamine (TMA) by enzymes encoded by the gut microbiota including choline-TMA lyase system (CutC/D) and the carnitine Rieske-type oxygenase/reductase system (CntA/B and YeaW/X)). Once TMA is absorbed from the gut lumen into the portal vein, it is further metabolized in the liver by flavin monooxygenases (FMO), mainly FMO isoform 3, to generate TMAO. Studies in mice have shown that TMAO enhances development of atherosclerosis at least in part by inhibiting reverse cholesterol transport [9], which results in increased accumulation of lipids in vessel wall macrophages. TMAO also causes platelet hyperre-activity and increases thrombosis risk through augmented Ca^{2+} release from intracellular stores [12]. Platelet thrombi are central to the development of most vascular ischemic events and substantial evidence has accumulated linking platelet hyperreactivity with vascular thrombotic diseases, including CAD, peripheral artery disease and strokes.

The best characterized bacterial system for TMA production is the choline-TMA lyase system (CutC/D) that involves a radical C–N bond cleavage of choline to generate TMA and acetaldehyde [13]. The genes encoding this system are part of a cluster that includes *cutC*, which encodes a glycyl radical enzyme with choline trimethylamine-lyase activity; *cutD*, which encodes a glycyl radical-activating protein; and genes encoding proteins involved in the assembly of microcompartments which may sequester the acetaldehyde generated as a by-product during TMA production. Genome analyses of sequenced isolates plus *in vitro* measurements of TMA production from choline revealed that (i) the *CutC/D* genes are present in many human gut bacteria, (ii) they are predictive of bacterial choline metabolism, and (iii) they are widely but discontinuously present across diverse bacterial phyla, including Firmicutes, Actinobacteria, Proteobacteria. Remarkably, neither these genes nor TMA-lyase activity has not been detected in members of the Bacteroidetes [13,14].

Identification of bacterial strains and the genes involved in TMA production from choline has been helpful for dissecting the impact of bacterial choline metabolism on host biology. Gnotobiotic mice colonized with microbial communities that vary only in their capacity to make TMA from choline showed that (i) TMAO accumulates in the serum of mice colonized with TMA-producing species [13], (ii) exacerbates metabolic disease [15], (iii) induces epigenetic changes [15] and (iv) enhances platelet reactivity and thrombosis formation [16●●].

In addition to the effects of TMAO on metabolic disease, atherosclerosis and arterial thrombosis, its effects on heart failure (HF) are emerging. In a prospective, observational study including 155 consecutive patients with chronic HF, TMAO levels were elevated in patients with HF,

Figure 1
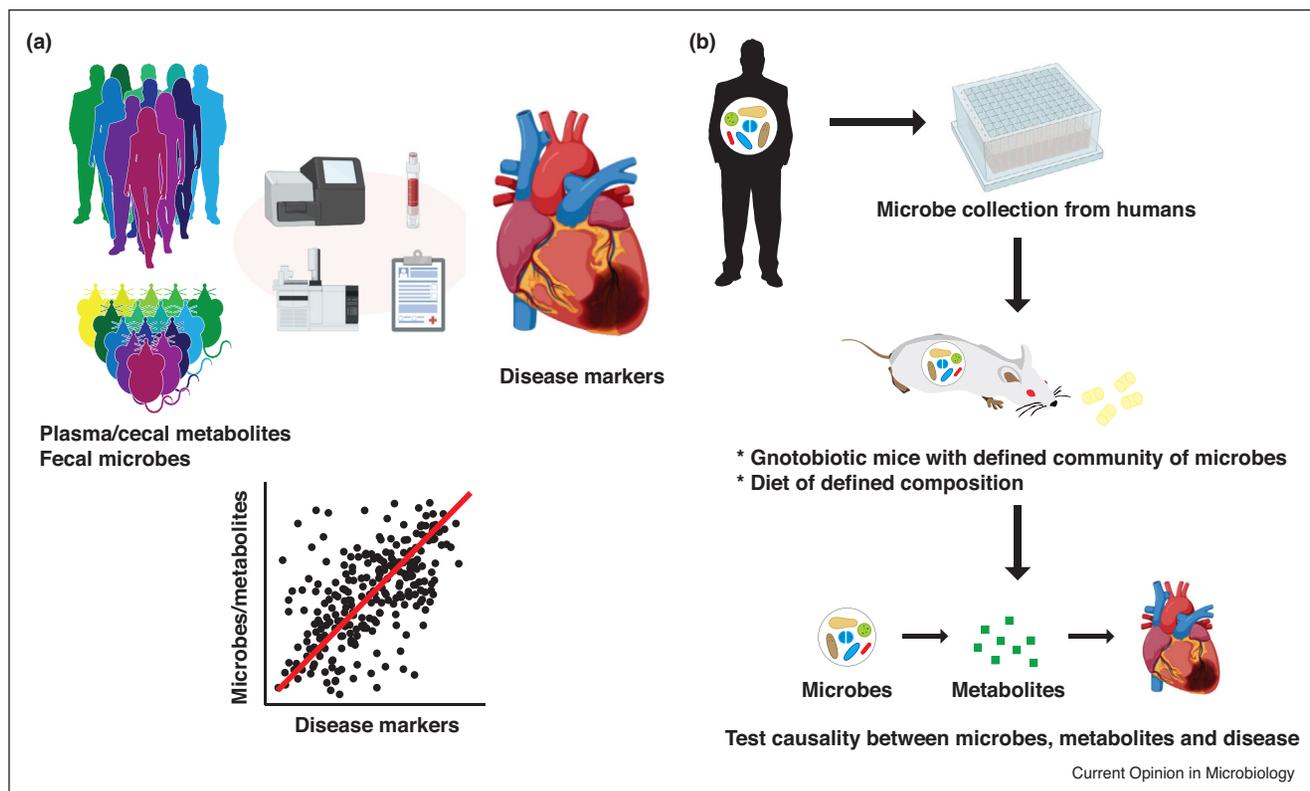
Hunting microbes and metabolites that contribute to CVD. **(a)** Discovery pipeline starts with hypothesis-generating screen (plasma/cecal metabolites, fecal microbes) of humans/rodents with desired cardiovascular phenotypes. **(b)** Features that show strong correlation with disease are further tested/validated in humanized gnotobiotic mice harboring communities that vary in specific microbes and/or microbial functions. Diets used in animal studies are carefully designed to provide relevant substrates needed for colonization of taxa of interest and production of desired metabolites. Access to collections of diverse, sequenced, well-characterized microbes is key for successful implementation of this approach. Using this pipeline we discovered that butyrate-producing bacteria interacts with dietary components to generate butyrate, which in turn inhibits atherosclerosis. Created with BioRender.

and levels were correlated with the severity of adverse outcomes [17]. Consistent with this, mice subjected to transverse aortic constriction — a cardiac pressure overload model that elicits HF in mice — develop greater myocardial fibrosis in response to either a high choline diet or supplementation with TMAO, compared to animals subjected to the same model but receiving low choline or no TMAO respectively [18]. TMAO is cleared by the kidney and subjects with chronic kidney disease (CKD) accumulate higher levels of this metabolite compared to healthy controls. Furthermore high levels of dietary choline or TMAO supplementation contribute to progressive renal fibrosis and dysfunction in mice [19]. Remarkably, patients with CKD have an enhanced risk of cardiovascular events and mortality when compared with age-matched and gender-matched individuals with normal kidney function and traditional risk factors (e.g. dyslipidemia, age, obesity) do not adequately predict those at risk for cardiovascular events. In these patients TMAO is independently associated with risk of adverse cardiovascular events [20].

Although the vast majority of studies support a link between the TMAO pathway and CVD (reviewed elsewhere [11]), not all studies have shown association between plasma TMAO levels and CVD risk or have demonstrated causal link with atherosclerosis in mice [21,22], and additional work is needed to understand the basis of these discrepancies (e.g. are there any yet uncharacterized modulators of TMAO effects?). Additionally, there are several key questions that remain unanswered including the mechanism(s) by which TMAO acts on the host and whether there is receptor for TMAO in mammals. Nonetheless, development of inhibitors targeting bacterial choline-TMA lyase has shown promising results in preventing TMAO accumulation and ameliorating CVD in mouse models [23,24••].

Fermentation of carbohydrates: production of short chain fatty acids (SCFAs)

Mammals produce a small number of enzymes involved in the breakdown of dietary carbohydrates, mainly targeting sucrose, lactose and starch. This is in contrast with the

gut microbiome which collectively encodes thousands of predicted carbohydrate-active enzymes that enable gut microbes access myriad carbohydrates present in the diet and intestinal mucus. Bacterial metabolism of carbohydrates results in the production of SCFAs — mainly acetate, propionate and butyrate, hydrogen gas, and other organic acids including succinate and lactate. SCFAs generated in the intestine can in turn be used by other microbes (e.g. butyrate-producers use acetate and lactate, methanogens use acetate, sulfate-reducing bacteria use acetate and lactate) or absorbed and metabolized by the host [25]. Upon absorption, SCFAs exhibit a wide range of physiological functions including inhibition of histone deacetylases and modulation of immune responses and intestinal barrier function. Some of these effects are mediated via activation of G-protein-coupled receptors (GPRs), including GPR41 (propionate > butyrate > acetate) [26,27], GPR43 (propionate = butyrate = acetate) [26,27], GPR109A (butyrate, β-hydroxybutyrate) [28,29], and olfactory receptor 78 (propionate, acetate) [30]. SCFAs are the best well-studied gut microbiota-derived metabolites, and it has recently become evident that some SCFAs modulate development of CVD.

High levels of fermentable fiber in the diet prevented the development of hypertension and HF in the deoxycorticosterone acetate-salt mouse model [31]. Remarkably these effects were mimicked by acetate supplementation in drinking water. Protective effects caused by fiber and acetate were associated with downregulation of cardiac and renal *Egr1* (early growth response protein 1), a master regulator involved in cardiac hypertrophy, cardiorenal fibrosis, and inflammation. Similarly, oral propionate supplementation to mice reduced cardiac hypertrophy and fibrosis, and atherosclerotic lesion burden and beneficially influenced T helper cell homeostasis [32]. Moreover, supplementation of a mixture of SCFAs (acetate, propionate, and butyrate) was also shown to improve the host immune response and cardiac repair capacity after myocardial infarction in a mouse model [33]. It is important to note that increased levels of SCFAs, particularly acetate and propionate, have also been associated with negative effects of metabolic health [34–36]. It is possible that the effects of these metabolites may vary depending on tissue, concentrations, and context (e.g. diet, host genetics). Furthermore, many animal studies rely on oral administration of SCFAs (e.g. in drinking water or by oral gavage); this route of administration causes pronounced increases in SCFA levels in proximal small intestine and only a small fraction of it reaches large intestine. In contrast, SCFAs are endogenously generated mainly in large intestine and their production is sustained as long substrates are available, thus caution needs to be taken when interpreting these supplementation studies.

As mentioned above several studies indicate that patients with type 2 diabetes and CVD harbor lower levels of butyrate-producing bacteria in their gut, including *Faecalibacterium* sp.

and *Roseburia* sp. [6•,37,38]. We showed that the genus *Roseburia* sp., was inversely correlated with atherosclerotic lesion development in a genetically diverse mouse population called Hybrid Mouse Diversity Panel [39•]. We used germ-free (GF) *apolipoprotein E* (*ApoE*)-deficient mice colonized with synthetic microbial communities that differ in their capacity to generate butyrate to demonstrate that *Roseburia intestinalis* interacts with dietary plant polysaccharides to (i) impact gene expression in the intestine, directing metabolism away from glycolysis and toward fatty acid utilization, (ii) improve intestinal barrier function and circulating levels of pro-inflammatory lipopolysaccharide, (iii) increase circulating levels of anti-inflammatory ketone body β-hydroxybutyrate and (iv) ameliorate atherosclerosis. Furthermore, intestinal administration of butyrate via tributyrin (ester composed of butyrate and glycerol) reduced endotoxemia and atherosclerosis development. However, the signaling cascade and/or the receptors that mediate these effects have not been identified. Additionally, studies in humans (i.e. clinical trials) are needed to test the feasibility of introducing butyrate-producing species combined with specific dietary substrates to selectively enhance production of butyrate and reduce CVD. This idea of combining probiotics and prebiotics — known as synbiotics — to enhance human health has been tested in pilot studies for the treatment of non-alcoholic fatty liver disease with moderate success [40]. By providing the probiotic organism with selectively fermentable substrates, the invading species is in principle given a competitive advantage. Nonetheless, successful implementation of this approach will require a clear understanding of the realized niche of the probiotic species and the metabolic potential of the recipient microbiota, so the right prebiotic is administered. Additionally, having an understanding of the individual mechanisms underlying colonization resistance will be necessary to develop personalized strategies to counteract this resistance.

Bacterial metabolism of aromatic amino acids

Microbial metabolism of aromatic amino acids, including tyrosine, phenylalanine, tryptophan, and histidine results in the production of compounds that may impact CVD as well. Microbial metabolism of tryptophan is well-studied and results in the production of several catabolites including indole, tryptamine, indole ethanol (IE), indolepropionic acid (IPA), indolelactic acid (ILA), indoleacetic acid (IAA), skatole, indole aldehyde (IAld), and indole acrylic acid (IA). Microbes and pathways involved in the production of these metabolites have recently been reviewed [41]. Tryptophan catabolites are absorbed through the intestinal epithelium and enter the circulation where they act [41]. Some of these metabolites are beneficial and have anti-inflammatory activity, for example, IPA, IE, and IA have anti-inflammatory effects, which in principle could have protective effects against CVD. For example, the mucin-degrading bacterium *Peptostreptococcus* sp. metabolizes tryptophan to produce IA, which has beneficial effects on intestinal barrier function and

inflammatory responses [42]. *Lactobacillus* species are also known to metabolize tryptophan to indole metabolites. Mice monocolonized with *L. murinus* exhibited much higher fecal ILA and IAA in contrast to GF controls, suggesting that *L. murinus* is capable of producing these indoles in the intestine, [4]. In conventionally raised (ConvR) animals a high salt diet affected the gut microbiome in mice by depleting *Lactobacillus murinus* and reduced fecal levels of ILA and IAA, which was associated with increased number of proinflammatory Th17 cells [43••]. Oral administration of *L. murinus* to these mice increased fecal indole levels, reduced Th17 cells, and ameliorated salt-sensitive hypertension, highlighting a novel connection between high salt intake and the gut-immune axis.

While indole has beneficial effects, liver metabolism of this compound results in the production of indoxyl sulfate (IS), which is a toxin that is excreted by the kidneys. Patients with CKD accumulate this compound which in turn exacerbates this condition [44]. In CDK patients IS has been linked with (i) increased aortic calcification, (ii) increased arterial stiffness, and (iii) higher mortality from cardiovascular events [45]. Interestingly, deleting the gene encoding tryptophanase — enzyme that converts tryptophan into indole — in *Bacteroides thetaiotaomicron* reduces the levels of IS *in vivo* [46], suggesting that inhibition of this gut bacterial enzyme could be a therapeutic option for CKD.

Tryptophan is also an important precursor for the production of serotonin (5-hydroxytryptophane; 5-HT), an important regulatory factor in the gastrointestinal tract and other organs. More than 90% of 5-HT is synthesized from tryptophan in the gut; circulating platelets uptake 5-HT produced in the GI tract and release it at sites of vessel injury where it promotes blood coagulation. Serum concentrations of 5-HT were substantially reduced in GF mice compared to ConvR mice [47]. Colonization of GF mice with spore-forming bacteria is sufficient to modulate metabolites that promote colon 5-HT biosynthesis and restore platelet activation to normal levels [47].

Bacterial metabolism of tyrosine and phenylalanine results in the accumulation of phenols as phenyl acetic acid and p-cresol. p-cresol is absorbed in the gut and metabolized to p-cresyl-sulfate (pCS) in enterocytes or to p-cresyl-glucuronide in the liver. High levels of pCS cause vascular endothelial and smooth muscle cell damage by inducing oxidative stress [48]. Serum pCS levels are associated with CVD not only in hemodialysis patients but in patients with CKD without dialysis as well [49]. A recent screen identified 36 phenol-producing strains and 55 p-cresol-producing strains [50]. Genome analyses of these taxa indicate that the phenol producers encode genes with high homology to tyrosine phenol-lyase and/or hydroxyarylic acid decarboxylase, whereas the p-cresol-producers encode p-hydroxyphenylacetate decarboxylase and/or tyrosine lyase homologues. Manipulating the abundance of the p-cresol-producing strains or these enzymes may represent potential useful therapies for CVD and CKD.

Bacterial metabolism of histidine has also been linked with metabolic disease. A recent study revealed that the bacterial metabolite imidazole propionate is present at higher concentrations in patients with type 2 diabetes relative to healthy subjects [51••]. Follow-up mouse studies showed that imidazole propionate impaired glucose tolerance and insulin signaling at the level of insulin receptor substrate. Additional studies are needed to understand how diet-microbe interactions modulate the production of imidazole propionate and whether its levels are associated with CVD.

Conclusions

The rapid advancement of culture-independent sequencing approaches as well the implementation of discovery-based (i.e. untargeted) metabolomics, followed by functional studies in animals including gnotobiotic mouse studies have helped broadened our understanding of how diet-microbiome interactions affect CVD. Altogether these studies support the notion that the gut microbiome mediates many of the beneficial and detrimental responses to diet on CVD. This body of work has also identified novel potential therapeutic opportunities, some of which are currently being explored. Furthermore, this is likely the tip of the iceberg: as our understanding of the chemistry of microbe-diet interactions advances and novel compounds are incorporated in clinical studies, novel microbial metabolites relevant to CVD will emerge. Using the discovery pipeline shown in Figure 1 we will be able to move from correlations between microbial features and CVD to establishing causal relationships.

Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest
•• of outstanding interest

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