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Dietary metabolism, the gut microbiome, and heart failure

W. H. Wilson Tang, MD[1,2,3,4,5], **Daniel Y. Li, MD**[5], and **Stanley L. Hazen, MD PhD**[1,2,4,5]

[1]Center for Microbiome & Human Health, Lerner Research Institute, Cleveland Clinic

[2]Department for Cellular & Molecular Medicine, Lerner Research Institute, Cleveland Clinic

[3]Center for Clinical Genomics, Heart and Vascular Institute, Cleveland Clinic

[4]Department of Cardiovascular Medicine, Heart and Vascular Institute, Cleveland Clinic

[5]Cleveland Clinic Lerner College of Medicine at Case Western Reserve University, Cleveland, Ohio.

Abstract

The contribution of gut microbiota to human health and diseases has expanded our insights into how microbial composition and function impacts the human host. Heart failure has long been associated with splanchnic circulation congestion, leading to bowel wall edema and impaired intestinal barrier function. This is thought to heighten the overall inflammatory state via enhanced bacterial translocation and the presence of bacterial products in the systemic circulation. Recently, several metabolites produced by gut microbes from dietary metabolism have also been linked to pathologies such as atherosclerosis, hypertension, heart failure, chronic kidney disease, obesity and type 2 diabetes mellitus. These findings suggested that the gut microbiome functions like an endocrine organ by generating bioactive metabolites that can directly or indirectly impact host physiology. We will discuss several newly discovered gut microbial metabolic pathways, including the production of trimethylamine (TMA)/ trimethylamine N-oxide (TMAO), short-chain fatty acids, and secondary bile acids, that appear to participate in the development and progression of cardiovascular diseases, including heart failure. We will also discuss the gut microbiome as a novel therapeutic target for the treatment of cardiovascular disease, and potential strategies for targeting intestinal microbial processes.

Keywords

Gut microbiota; diet; heart failure

Introduction

Heart failure is the final common process that results from a variety of initial cardiac insults and subsequent imbalances in compensatory mechanisms and pathogenic processes[1,2]. It has long been recognized that heart failure is associated with altered intestinal function[3,4]. While this “gut hypothesis” in heart failure has prevailed over the years, the emphasis has focused

Address for correspondence: W. H. Wilson Tang, MD, 9500 Euclid Avenue, Desk J3-4, Cleveland, OH 44195., Phone: (216) 444-2121, Fax: (216) 445-6165, tangw@ccf.org.

on enhanced gut bacterial translocation and resultant heightened inflammatory responses as well as oxidative stress as a consequence of heart failure-induced ischemia and congestion within the intestines. However, research studies supporting the “gut hypothesis” thus far are associative in nature and have not demonstrated a causal link between gut microbial processes and heart failure pathogenesis. Also, the role of nutrition and diet in the causation, prevention, and management of heart failure remains poorly understood.

At birth, the human intestinal tract is relatively sterile, but as we are abruptly and continuously exposed to our environment, the gut becomes rapidly colonized with trillions of typically non-pathogenic bacteria (10^{13}–10^{15})[5–8]. The stomach, due to its low pH, contains relatively few organisms, while the small intestines contain aero-tolerant microbes, mainly streptococci, and lactobacillus[5,9]. The colon, with a microbial density of 10^{11} to 10^{12} organisms per milliliter, is one of the most densely populated bacterial habitats on Earth[5,7]. In fact, gut microbiota contributes between 0.2 and 2 kg of the weight of a typical adult and constitutes nearly 50% of the dry weight of feces[5,8]. Gut microbiota remain in a symbiotic relationship within us, their human hosts, and provide crucial contributions that directly shape our health. For example, gut microbiota synthesize vitamin K and biotin, facilitate absorption of key nutrients, promote and shape the development of the mucosal immune system, and influence oral tolerance to ingested antigenic foods[10–13]. Gut microbiota also promote an essential barrier function by repressing the overgrowth or colonization of potentially harmful bacteria through competitive exclusion and production of diverse chemical mediators, such as quorum-sensing compounds and bacteriocins, which collectively keep the microbiota environment in check[14–16].

A typical healthy human gut is colonized by over 500 different microbial species, and an even larger number of microbial strains[17,18]. Humans thus inherently possess an extended genome of microbial genes and functions that are collectively known as the microbiome. The number of genes within the gut microbiome is estimated to exceed the number in the human genome by two orders of magnitude. Also, while many gut community structural features are “inherited” early in life, especially from one’s mother during vaginal delivery[19–21], the composition of gut microbiota varies considerably among individuals and host genetic variations may predispose individuals towards altered microbiota composition[8,22]. Due to this diversity in composition, the microbiome, in addition to host genetics, plays a significant role in individualized responses to major environmental exposures including our diet, xenobiotics and drug treatments[23–25]. The gut microbiome thus plays a role in person to person variations in disease susceptibility, and in responses to our diet[25]. Gut microbiota also impact how one metabolizes drugs, and can contribute to the production of pharmacologically active secondary metabolites that can foster adverse side effects or chemotherapeutic drug toxicities[26,27].

In this Review, we discuss the long-standing hypothesis of how the modulation of inflammation by the gut barrier participates in the development of heart failure. We examine the emerging evidence for an altered gut microbiome environment in heart failure. Moreover, we discuss several notable gut microbiota mediated metabolic pathways and products that have recently been revealed to be involved in heart failure. These pathways and products include the trimethylamine (TMA)/ trimethylamine Noxide (TMAO) pathway,

short-chain fatty acids pathway, and bile acid pathways. Finally, we consider potential therapeutic strategies targeting the intestinal microbiota to influence the susceptibility and progression of cardiovascular disease.

Impaired gut barrier function and inflammation in heart failure

Typically, a “healthy” gut microbial community maintains gut barrier function, including intact, tight junctions in the mucosa and normal mucosal immunity. Mice lacking adherens junction proteins will spontaneously develop colitis, highlighting the importance of cell-to-cell adhesion in maintaining a healthy mucosal barrier[28]. The current “leaky gut” hypothesis of heart failure[3,4] posits that bowel wall edema and impairment in barrier function during heart failure leads to translocation of gut microbiota components into the host circulation, endotoxemia, and consequently, a heightened systemic inflammatory state[29] (FIG. 1). Evidence exists wherein heightened bacterial translocation occurs during heart failure as a consequence of one or more mechanisms including structural and functional alterations of the gastrointestinal tract that occur with splanchnic congestion, as well as with abnormalities of host immunological defense.

An altered intestinal barrier function can arise from multiple factors, including as a consequence of a hypoperfused gut that allows gut microbiota components to be translocated into the bloodstream[30]. Decreased cardiac output and the resulting sympathetic vasoconstriction in heart failure stimulates adaptive re-distribution of the systemic circulation, leading to congestion in multiple end organs, including the bowel wall[31]. A decrease in perfusion particularly affects the structure of villi in the intestinal mucosa, which is susceptible to ischemia due to the countercurrent circulation present in the villus: the artery and vein run in parallel, thereby creating a descending tissue gradient in oxygen pressure and/or tension from the base to the tip of the villus[32]. As a result, intramucosal acidosis can be observed in approximately half of patients with decompensated heart failure[33]. Moreover, even light activity is observed to increase intragastric pCO_2 in those with chronic heart failure[34]. Structurally, intestinal wall thickening with edema can be observed in patients with heart failure[29]. The collagen content in their mucosal walls is also increased, in proportion to the severity of heart failure[35]. Of interest, bowel wall thickening has been directly correlated with circulating levels of C-reactive protein, blood leukocytes, and increased markers of intestinal permeability[29]. Thus, a combination of both structural and functional intestinal alterations directly contribute to worsened enterocyte health, and as a result, the intestinal barrier integrity. Therefore, increased paracellular transport can be observed in heart failure[29].

Compromise of the intestinal barrier can allow endotoxin, also known as lipopolysaccharide (LPS), to readily enter the bloodstream. LPS is found on the outer membrane of Gram-negative bacteria. It is a classic pathogen-associated molecular pattern molecule and can induce the expression of a wide array of inflammatory downstream products primarily through the Toll-like receptor 4 (TLR4) pattern recognition receptor[36]. Moreover, this effect occurs through multiple tissues and cell types, such as macrophages, dendritic cells, cardiomyocytes and cardiac fibroblasts[37]. Patients with decompensated heart failure have higher blood levels of endotoxin[38]. Moreover, targeted sampling studies have shown that

LPS levels are higher in blood samples recovered from the hepatic vein as opposed to the systemic circulation (direct sampling from the ventricles), providing direct evidence that LPS can be translocated from the gut[39]. Additionally, LPS itself can further promote deterioration of mucosal barrier function[40].

There is evidence that the intestinal absorption of endotoxin is a significant stimulus for systemic increases in inflammatory cytokines during heart failure[41,42]. In heart failure subjects, heightened circulating levels of multiple cytokines (e.g., TNF, IL-1, and IL-6) are associated with more severe clinical symptoms and can predict poorer patient survival[43–45]. Interestingly, while endotoxin levels in patients with heart failure have been shown to be reduced with diuretic or antibiotic therapy, plasma cytokine levels have not always been observed to change, suggesting a sustained effect as the disease process is triggered[38,46]. Strong preclinical evidence exists for these inflammatory mediators to participate in cardiac apoptosis, hypertrophy, and fibrosis[47]. For example, in mice, administration of TNF at pathologic concentrations is reported to result in a dilated cardiomyopathy phenotype[48]. Furthermore, transgenic overexpression of TNF in another mouse study resulted in cardiac hypertrophy, ventricular dilation, and fibrosis[49].

Increased serum concentrations of TNF, IL-1β, and IL-6 have also been shown to be potent inducers of intestinal permeability[50–53]. This increased permeability complements the effects of endotoxin in the circulation and promotes a vicious feedforward cycle of increased endotoxin translocation and inflammatory cytokine accumulation. However, the results of clinical studies that tested this hypothesis (including numerous anti-TNF trials) reveal additional complexity (e.g., The Randomized Etanercept North American Strategy to Study Antagonism of Cytokines (RENAISSANCE), the Research into Etanercept Cytokine Antagonism in Ventricular Dysfunction (RECOVER), and Anti-TNF in Congestive Heart Failure (ATTACH))[54,55]. In brief, these studies revealed negative results and perhaps even increased cardiovascular events in the treatment group. By contrast, the Advance Chronic Heart Failure Clinical Assessment of Immune Modulation (ACCLAIM) trial utilized nonspecific immunomodulation therapy[56]. In this study, patient blood was stressed (via a combination of heat, ozonation and ultraviolet irradiation) to induce cell death, and the resulting material is re-injected into the patient to produce an anti-inflammatory stimulus. Although the primary endpoint of reduction in all-cause mortality or cardiovascular admission in this RCT was not met, the subgroup of patients with NYHA functional class II chronic heart failure (n=919) appeared to have significant reductions in all-cause mortality and hospital admissions for cardiovascular causes.

Although not in a heart failure cohort, groundbreaking findings from the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial, which involved the use of canakinumab, anIL-1β receptor antagonist, demonstrated for the first time in a large randomized clinical trial that targeting inhibition of the inflammatory pathway (through at least the IL-1β pathway) can reduce the risk of incident adverse cardiovascular events independently of lipid lowering[57]. In addition to an anti-inflammatory effect, it is conceivable that inhibiting the IL-1β receptor can also reduce intestinal epithelial tight junction permeability, leading to cardiovascular benefits due to its modulation of the microbiota. Additional studies (initiated after positive proof of concept findings[58–61])

involving both canakinumab in a subgroup of CANTOS patients, as well as anakinra (which blocks both IL-1α and IL-1β receptors), are underway to explore alternative anti-inflammatory targets that may benefit heart failure patients[62,63].

In the gut hypothesis of heart failure, much focus has been placed on understanding how inflammation triggers heart failure and other related comorbidities. However, as we described, investigations of inflammatory cytokines have resulted in mixed evidence from clinical studies. The range of clinical findings demonstrate that targeting the inflammatory process through cytokine-driven pathways yields varied results; however, these undoubtedly complex inflammatory pathways are not discussed further as they have been thoroughly reviewed elsewhere[64,65]. These studies raise further interest in investigating the role of endotoxin-induced inflammation and the microbiota in the pathogenesis of heart failure. Indeed, we hope that the knowledge gained about the gut microbiome and its links to heart failure will lead to better risk stratification of heart failure subjects, as well as helping to identify novel pharmacologic strategies that target the microbiome and improve human health.

Dysbiosis in heart failure

The intestine is unique in its constant exposure to trillions of microbial antigens; this creates an environment constantly at risk of barrier dysfunction. The microbiota might be able to directly modify the intestinal barrier[66,67]. Therefore, a better understanding of the gut microbiota (a primary contributor to underlying inflammation) will help to tease out beneficial net effects for heart failure in this complex multi-level network.

Changes in the proportions of specific taxa of gut microbiota have been observed in various disease states, including obesity[68,69], inflammatory bowel disease[70], type 1 and type 2 diabetes mellitus[71,72], biliary disease[73], and intestinal cancers[74,75] among many others. Animal studies have shown that the intestinal microbial environment can affect the efficiency of harvesting energy from the diet and affect obesity[69]. It has been demonstrated that the gut microbiota can play a role in the development of metabolic abnormalities such as insulin resistance and nonalcoholic fatty liver disease[76,77]. Furthermore, psychiatric disorders, including autism and depression, have also been associated with alterations in gut microbiota in what is known as the “gut-brain hypothesis.”[78–80]

Research has now gone further by demonstrating a mechanistic link via direct microbial transplantation, and evidence of transmission of disease susceptibility. Turnbaugh et al. demonstrated in a seminal study that adiposity could be transferred by microbiota transplantation[69]. In the setting of cardiovascular disease, both atherosclerosis and increased platelet activation were shown to be transmittable with microbial transplantation and linked to elevated trimethylamine N-oxide (TMAO) production, a novel microbiota-obligate metabolite that we will review in detail in later sections[81,82]. Moreover, several studies have demonstrated that microbes can transmit alterations in vascular tone and blood pressure. Pluznick et al. demonstrated that microbiota could produce SCFA to modulate blood pressure through short-chain fatty acid interaction with the host G protein-coupled receptors (GPCRs) Olfr78 and Gpr41; antibiotic treatment can then alter blood pressure in Olfr78

knockout mice[83]. A salt-sensitive rat model with cecal microbial transplant demonstrated altered blood pressure and longevity in the recipient[84]. Similarly, an obstructive sleep apnea (OSA) model also demonstrated that cecal microbial transplantation from the OSA mice transmitted elevated blood pressure to the recipient[85]. Finally, a probiotic therapy (Goodbelly) study reported that delivery of *Lactobacillus plantarum* 299v reduced circulating leptin and improved ventricular function and remodeling after left anterior descending artery ligation[86]. However, there have only been a limited number of studies examining the role of gut microbiota in heart failure.

Alterations in gut microbial composition

Several small cohort studies have demonstrated that alterations in microbial communities are present in heart failure patients (Table 2). In addition to correlations with inflammation and intestinal permeability, Sandek et al. also observed bacterial overgrowth consisting of mucosal biofilm and increased bacterial adhesion in heart failure patients[29,87]. Newer studies showed that more pathogenic microbes such as *Candida*, *Campylobacter*, *Shigella,* and *Yersinia* could be detected in the stools of heart failure patients and correlated with heart failure severity[88]. Supporting these findings, using a national inpatient data registry, Mamic et al. observed significant increases in C*lostridium difficile* infections amongst patients with a heart failure diagnosis at discharge after controlling for relevant patient and hospital characteristics[89]. Moreover, across these hospitalizations, patients admitted with urinary tract infections, pneumonia, or sepsis with concomitant *C. difficile* infection had higher in-hospital mortality compared to those without[89].

The use of 16s rRNA gene sequencing is now a standard methodology to identify proportions of bacterial taxa within the gut. However, such analyses rarely delve deep enough to identify individual species, and certainly cannot provide strain-specific data. Metagenomics and deep sequencing provide greater depth of classification of the microbial community members present and has provided new insight into our study of the microbiota as we can now better detect species that were previously not able to be cultured. These methods have only recently been utilized in heart failure studies. Using 16s rRNA sequencing, Luedde et al. showed in a small cohort of age and sex-matched patients that heart failure patients tended towards decreased intra-individual taxonomic diversity (alpha-diversity) whereas the inter-individual diversity was able to distinguish between heart failure and control patients[90]. Extending Luedde et al's findings, Cui et al. analyzed the metagenomic data of 53 heart failure patients and was also able to distinguish patients with congestive heart failure from controls[91]. However, in this small study, additional differentiation between ischemic and dilated cardiomyopathy based on gut microbiota composition was not found. In the heart failure patients, the authors observed enrichments in gut microbial genes involved in LPS synthesis as well as genes necessary for the generation of the pro-atherogenic gut metabolite, TMAO (discussed in detail below). Furthermore, they observed decreases in *Faecalibacterium prausnitzii* that have previously been characterized in elderly congestive heart failure patients[92]. This bacterium is an abundant butyrate-producing species, and these findings could be demonstrative of this bacterium's importance in anti-inflammatory functions. Finally, a more comprehensive analysis of microbiota from Kummen et al. used discovery and validation cohorts (n=44 and n=40 respectively) along

with 266 population-based controls and adjustment for multiple risk factors to create a more robust heart failure microbiota signature[93]. They reaffirmed the decreased microbial diversity in disease, identified changes in fifteen core taxa, and emphasized a depletion of the *Lachnospiraceae* family (of which several members are butyrate producers) that was inversely associated with increased levels of soluble CD25, a marker for T-cell and macrophage activation. This once again highlights the potential importance of the microbial modulation of inflammation through SCFA production in disease states. Lastly, in their small cohort, the authors found that depletion of *Eubacterium hallii*[94], another important commensal SCFA producer, and increased soluble CD25, was associated with death or heart transplantation.

These studies are important first steps into categorizing the microbiota profile in heart failure. However, it is important to note that studies examining the microbial diversity in heart failure is still a budding field. Multiple discrepancies can be noted when comparing major bacterial groups identified in studies summarized in Table 2. For example, *Eubacterium Rectale*, a gut symbiont and butyrate producer was found to be increased by Sandek et al. using fluorescent in situ hybridization in gut mucosal biofilms but decreased by Kamo et al[29,92]. It can be noted that this bacteria has also been associated w/ decreases in atherosclerosis[95]. Similarly, Luedde et al. observed that *Collinsella* was decreased in the HF cohort[90] while this genus has been found to be enriched in atherosclerosis[95]. Lastly, Kummen et al. observed multiple genus belonging to the Lachnospiraceae family to be decreased in HF[93], but interestingly, this family has also consistently demonstrated positive associations with plasma TMAO levels[82,96]. As methodologies become more uniform, reproducibility must be affirmed in future studies with adequate power and more importantly, clinically homogenous heart failure diagnoses.

Environmental and treatment-related factors

One limitation of the current studies seeking to link specific gut microbial taxa to disease presence, susceptibility, or severity is the confounding that is always present from both environmental and intrinsic factors related to the presence of the disease and its treatment. Regarding environmental factors – diet, medications, and the surrounding milieu play substantial roles in shaping our gut microbiome[103]. Similarly, the intrinsic physiology of heart failure, in addition to the multi-factorial etiologies which lead to this state, also require scrutiny. However, these potential confounders can also lead us to novel questions in the investigation of microbiome differences. For example, determining how medication-microbiome interactions alter or perhaps drive specific changes in microbial composition is of relevance[104]. In addition to the focus on diet and the microbiota, numerous new studies have already demonstrated dramatic effects driven by widely used medications such as metformin and statins on the bile acid pool, as well as on microbial signatures associated with disease[105,106]. The reverse can also occur wherein alterations in gut microbiota influence drug metabolism, with potential impact on the host. For example, digoxin may be inactivated by the actinobacterium *Eggerthella lenta*[107]. Similarly, in a recent study of the human gut microbiome in 1,135 Dutch individuals, the metabolism of heart failure management drugs (such as angiotensin inhibitors, β-blockers, and angiotensin II receptor antagonists) was associated with inter-individual variations in gut microbiota

composition[108]. Gut microbiota can even alter levels of host hepatic drug metabolism enzymes, thereby modulating an individual's circulating drug levels and responses to therapeutics[109–111]. In an intriguing recent study examining nearly 1,000 clinically used medications, an estimated 24% of the drugs were found to suppress or impact the growth rate of at least one human commensal in culture[104]. These studies raise significant new questions about the enormous potential interplay between our gut microbiome and inter-individual differences in drug metabolism, as well as the potential development of antibiotic resistance[104,112]. It remains to be seen whether further understanding of how the gut microbiome may influence the inter-individual variability of drug responses, impact personalized medicine and the management of heart failure patients.

Gut microbiota-generated metabolites known to impact cardiometabolic processes in the host

Short-chain fatty acids—Gut microbiota within the distal intestinal tract foster fermentation processes that often produce short-chain fatty acids (SCFAs), a major nutrient source for the ceco-colonic epithelium[113]. These organic fatty acids, defined by being from one to six carbons in length, are important end-products of colonic fermentation and are derived from macronutrients such as dietary fiber, resistant starches, as well as sugars or proteins that escape digestion in the upper intestinal tract. While many branched chain and hydroxylated SCFA of various structures can be made, the majority have yet to be fully characterized. Notably, the vast majority of SCFAs produced in the colon appears to be the simple aliphatic two, three and four carbon SCFAs acetate, propionate and butyrate, with an assumed approximate 60%, 20%, and 20% distribution, respectively[69,113]. A significant proportion of the SCFAs produced in the distal bowel is reabsorbed from the gut lumen by facilitated apical solute transport via multiple different transporters[114–117] as well as via passive (concentration-dependent) diffusion[118]. Because of their abundance within the distal intestinal tract, SCFAs also play a role in colonic acid-base status[119,120]. Furthermore, myriad different SCFA products have been readily measured in blood[121]. In recent studies, SCFAs have been mechanistically linked via specific host receptor recognition (including Gpr41, Gpr43, Olfr78) to development of hypertension through alterations in blood pressure in the host (FIG. 2)[83,122,123]. Moreover, acetate-producing bacteria have also recently been identified in preclinical studies as a potential protective intervention for the treatment of hypertension, adverse cardiac hypertrophy, and fibrosis development[124]. It was demonstrated that both high fiber and acetate feeding in a deoxycorticosterone acetate-salt hypertension mouse model resulted in decreased blood pressure, decreased cardiac and renal fibrosis, and improved cardiovascular function. This study also concluded that SCFA receptors, including Gpr43 and Olfr78, were not readily detectable in the cardiac transcriptome. Therefore, additional independent mechanisms by which SCFA modulate cardiac function likely exist and require further investigation. In addition, it is important to note that SCFA pharmacokinetics (such as production and tissue utilization) vary significantly between humans and smaller animals which limits the direct extrapolation of SCFA peripheral effects from animal models to human disease[125].

Bile acids—Bile acids are traditionally viewed as emulsifiers of fats and fat-soluble vitamins to aid intestinal absorption. The primary bile acids, such as cholic and

chenodeoxycholic acid (CDCA), are synthesized through the oxidation of cholesterol in the liver[126]. These primary bile acids are typically reabsorbed (>95%)[127]. However, microbiota in the colon can further metabolize any unrecycled bile acids to produce secondary bile acids such as deoxycholate (DCA), lithocholate (LCA), ursodeoxycholate (UDCA), and numerous (at least 50) others[128,129]. Nearly all of the bile acid pool in the large intestine comprises secondary bile acids with concentrations up to 1,000μM. These secondary bile acids also enter the enterohepatic circulation with any remaining excretion occurring through the feces[130].

Studies suggest that both the composition and pool size of bile acids are altered in subjects with heart failure. For example, in a recent small cohort study of heart failure patients and sex-matched controls (n=142 and n=20, respectively), it was reported that the ratio of primary to secondary bile acids was decreased in heart failure subjects[131]. The difference in bile acid ratios was driven mainly by decreased levels of primary bile acids. Moreover, there was a shift in the secondary bile acid profile, though the total secondary bile acid level remained similar. There is no doubt that bile acids and microbiota are inextricably linked as primary bile acids are biochemically modified by the microbiota while the resulting composition of the bile acid pool can conversely affect the microbiota profile. This raises questions of how these shifts in profile may affect host physiology in general, as well as how they affect processes relevant to cardiac physiology and disease susceptibility. The interactions between bile acids and the microbiota can be both direct and indirect. As an example, in disease states such as cholestasis, bile acids will reach supraphysiologic levels. Early studies of primary bile acids have been shown to exert direct negative myocardial chronotropic effects in a dose-dependent fashion[132,133]. Though at lower concentrations relative to primary bile acids even in cholestatic states, secondary bile acids can also exert similar effects. It has been shown that at increased concentrations of bile acids (10^{-3}M), significant reductions in both receptor density and affinity of beta-adrenoreceptors were observed[134]. Although it was not specifically compared in this adrenoreceptor study, the reductions in receptor density and affinity appeared greater for DCA, a secondary bile acid. Moreover, there are many mechanistic connections between bile acids, lipid signaling and biochemical pathways as outlined below.

Our understanding of bile acid physiology was greatly expanded by the discovery of bile acid responsive receptors, such as Farnesoid nuclear receptor (FXR)[135,136] and various G-protein coupled receptors (GPCRs) including muscarinic receptors, TGR5, and sphingosine phosphate receptor[137–139]. Among these receptors, FXR is the most well-studied example of a bile acid signaling receptor that modulates metabolism and inflammation[140]. FXR responds to CDCA as the most potent endogenous agonist (amongst major bile acids) followed by DCA and LCA. Furthermore, FXR mediates a negative feedback control on the synthesis of bile acids through ileal increases of FGF15[141]. The majority of literature on FXR is focused on its metabolic effects in obesity and liver pathophysiology[140]. Nonetheless, the role of FXR has been explored in atherosclerotic disease, and its activating ligands have been shown to antagonize inflammatory responses through repression of nuclear factor-kappaB (NF-kB)[142,143]. Given the potential for NF-kB to cause cardiac hypertrophy independent of processes such as hypertension, this presents indirect evidence for the potential of FXR-targeted therapies to improve heart failure[144,145]. However, the

converse has also been observed. Antagonism of FXR has also been suggested to improve cardiac function. For example, in vitro studies on cardiomyocytes pinpoint FXR as a mediator of apoptosis through mitochondrial signaling[146]. Moreover, FXR knockout mice demonstrated better recovery following myocardial infarction through reduced apoptosis and fibrosis[146].

Because of the many mechanistic links between bile acids and processes relevant to cardiovascular disease, various bile acids have been explored for their potential clinical applications. However, like the physiological role of bile acids in cardiovascular disease, the therapeutic studies have thus far experienced mixed results. A prominent example is obeticholic acid (OCA), a synthetic bile acid derived from CDCA and the first FXR agonist utilized in human intervention studies. OCA is currently FDA approved for the treatment of primary biliary cholangitis[147–149]. Moreover, its ability to reverse fibrosis in patients with nonalcoholic steatohepatitis (NASH) was demonstrated in a proof-of-concept study followed by a randomized control trial (RCT)[150,151]. These findings give confidence for speculation that targeting FXR for optimization of metabolic parameters might then also reduce cardiovascular complications. However, in the OCA trial for NASH, additional metabolic derangements such as increased blood lipid levels were also observed, in contrast to the findings of other proof of concept studies[152,153]. In a divergent study, the effect of supplementation with another secondary bile acid, UDCA, on endothelial function and inflammatory markers in chronic heart failure patients was investigated in a double-blind RCT[154]. As UDCA is capable of forming mixed micelles around LPS, it was hypothesized that decreased endotoxin levels and a corresponding reduction in inflammation would result, improving peripheral blood flow in subjects with chronic heart failure[155]. The results showed limited but positive improvements in peripheral blood flow. However, these effects appeared to occur independently from inflammation, as markers such as tumor necrosis factor (TNF) and IL-6 were unchanged. Together, these findings not only show a need for additional validation of currently studied pathways to advance bile acid therapies, but they also highlight the numerous gaps in our understanding of bile acid physiology.

Trimethylamine and trimethylamine N-oxide

Recent work from our group has demonstrated, for the first time, that the trimethylamine (TMA)/trimethylamine N-oxide (TMAO) pathway represents a novel union between diet, gut microbiota, and both atherosclerosis and thrombosis risks[82,96,156–159]. TMAO is generated from dietary precursors that contain the TMA moiety such as phosphatidylcholine, choline, and L-carnitine. These nutrients, found in high-fat foods, yield TMA through the action of multiple microbial enzyme complexes (described in a later section) and enters the portal circulation where further metabolism by host enzymes in the liver, called flavin-containing monooxygenases (FMO and primarily the FMO3 isoform), produce TMAO (FIG. 3). Initial studies revealed a strong association between gut-microbiota-dependent phospholipid metabolism and atherosclerosis risks mediated by generation of the pro-atherosclerotic metabolite TMAO.

Using untargeted metabolomics as a discovery platform, increased plasma levels of small-molecule metabolites, including TMAO, were shown to predict an increased risk of CVD in

stable patients undergoing elective cardiac evaluation who subsequently experienced a myocardial infarction, stroke or death[158]. The top candidate metabolites were subjected to confirmation studies in an independent validation cohort of 1,876 patients. Subsequent animal model studies showed that this pathway was mechanistically linked to atherosclerosis[156,158], and heart failure susceptibility[160]. For example, it was demonstrated that direct supplementation of TMAO or its dietary precursors, choline[158] or carnitine[156,159], both resulted in enhanced macrophage foam cell formation and atherosclerotic plaque development in aortic roots of atherosclerosis-prone apolipoprotein $E^{-/-}$ mice. These findings were accompanied by observations of increases in both the scavenger receptor CD36 and scavenger receptor A1 (SRA1) protein, correlating with the findings of increased atherosclerosis burden[156,158]. A relationship between heightened TMAO and increased risk for myocardial infarction, stroke or death was then validated in an independent cohort of 4,007 patients, which showed a 2.5-fold increased risk for major adverse cardiovascular events in patients in the highest quartile of TMAO[157]. To prove causality, antibiotic abrogation of gut microbiota was shown to result in improved phenotypes, and microbiota transplantation studies with low versus high TMA generating community confirmed that one could successfully transmit TMA production potential and correspondingly, transmit enhanced diet dependent pro-atherogenic phenotype, thereby, satisfying a fundamental criteria of Koch's postulates[81]. Similarly, microbial transplantation studies have confirmed that heightened TMAO generation, platelet responsiveness, and thrombosis potential may be transmitted from donor to recipient[82].

Commonly discussed confounders to the TMAO story concern dietary intakes of fish (which can contain TMAO) and L-carnitine. We note that much work needs to be done in this field as we pick apart these dietary links to TMAO and determine what macronutrients may be beneficial or harmful. Despite commentary on this factor, few studies have directly investigated the relationship between fish, TMAO, and cardiovascular disease. First, TMAO content correlates with high salinity and deep, cold environments[161,162]. In fact, most fish do not contain TMAO, just as most fish do not contain significant amounts of omega-3 fatty acids[163,164]. Thus, broad sweeping generalizations about the health benefits or otherwise of eating fish need to be more nuanced. Nonetheless, high dietary fish intakes, especially of deep sea (and cold water) varieties of fish, which are more likely to contain TMAO, is associated with increases in urinary TMAO excretion[165]. However, it is also clear that in people with adequate renal function, this rise in TMAO is transient. Of note, a recent study supplementing fish protein to a western diet in Apolipoprotein $E^{-/-}$ mice showed increases in atherosclerotic plaque area and calcification when compared to the matched soy or casein protein fed counterparts[166]. The fish protein in this study was derived from the turbot, a highly consumed fish in Europe; its fatty acid profile contained high levels of monounsaturated fats, and a low omega-6 to omega-3 ratio, while the TMA level was 20-fold higher than that in the comparison diets. It was observed that although blood lipid levels in the mice remained similar between all diet groups, TMAO was significantly elevated in the fish protein group.

Also, fish oil itself may counteract the harmful effects of TMAO. It appears that in co-feeding experiments with fish oil and TMAO, the inflammatory effect of elevated TMAO feeding was ameliorated with the addition of fish oil[167]. The debate regarding the

relationship between TMAO levels and eating fish have mostly been encapsulated to the macro scale. However, it is more challenging to ascertain micronutrient contents in such capacities. Moreover, individuals at significant risk for plasma TMAO retention, namely the CKD cohort, are advocated to reduce their protein intake[168,169].

L-carnitine is a popular supplement that has been studied for its potential to improve lipid profiles and metabolism[170]. In a study conducted by investigators associated with manufacturers of L-carnitine, their findings suggested that L-carnitine feeding resulted in increased TMAO but a lower atherosclerotic burden. This finding has often been cited as opposing evidence to the numerous studies which have associated TMAO with poor clinical outcomes. In their study, male mice were supplemented with Lcarnitine or vehicle controls. A surprising inverse correlation between TMAO level and lesion area in the thoracic aorta was observed. It is interesting that the model of choice was male mice since significant sexual dimorphism exists for trimethylamine monooxygenase (encoded by *Fmo3*) expression in mice: male mice display 100-fold decreased liver *Fmo3* mRNA compared to females, resulting in far lower natural levels of plasma TMAO[171]. Given this understanding, it is unlikely that L-carnitine feeding in male mice will induce significant changes in plasma TMAO levels. If one converted the parts per million units from this study to the more conventionally utilized micromolar to report plasma TMAO, we observe very similar values of TMAO in this study to that of a typical C57BL6 male mouse[171]. In fact, the highest subgroup cutoff of 0.2ppm converts to 2.6μM. These low levels of TMAO have never been implicated in disease. Although critical commentaries are essential for discussion, criticism must be met with an objective evaluation of the data at hand.

Overall, in the discussion of microbial metabolites, TMAO is likely only the tip of the iceberg. In the untargeted metabolomics study by Wang et al., there were a total of 18 unidentified peaks that were found to be associated with major adverse cardiovascular events (*refer to the supplement in ref. 153*)[158]. Moreover, beyond the three metabolites found to be in the TMAO biochemical pathway, the remaining unknown metabolites also demonstrated strong hazard ratios in unadjusted analysis. Similar discovery approaches can conceivably be undertaken for heart failure patients as well, though careful patient selection is a must as heart failure has a broad definition and significant technical hurdles exist in the chemical derivatization of unknown metabolites. Nonetheless, there likely exists additional microbial generated metabolites with potential links to heart failure and cardiometabolic disease.

The meta-organismal TMA/TMAO pathway

Since the first studies demonstrating that the TMA/TMAO microbial-human metabolic pathway can generate disease-relevant metabolites that contribute to atherogenesis[156–158], a multitude of pathophysiologic associations have now been linked with TMAO (FIG. 1). Here, we summarize basic and clinical findings, namely revolving around thrombosis, inflammation, and chronic kidney disease, which are entwined in the upstream etiologies that contribute to heart failure of ischemic or non-ischemic origin. Furthermore, new data also suggest that even in acute settings, cardiovascular function, specifically hemodynamics, may be altered by TMAO, as well.

Atherogenesis and altered platelet function.

Extending the original findings of the relationship between TMAO and atherogenesis, we have also demonstrated that plasma TMAO levels are associated with incident thrombotic event risk[82,172]. Mechanistic studies show that TMAO acts to potentiate platelet reactivity through alterations in stimulus-dependent calcium signaling. Increased calcium release from platelets and enhanced activation was observed following stimulation from sub-maximal levels of multiple different agonists such as ADP, thrombin, and collagen[82]. This finding suggests that TMAO-dependent enhancement in stimulus-dependent platelet activation occurs via a target down-stream from these platelet agonists but proximal to stimulus-dependent release of intracellular calcium stores. Finally, fecal transplantation further demonstrated that choline diet-mediated heightened thrombosis potential in vivo is a transmittable trait[82].

The mouse findings were replicated in both a large scale (n=4,007) clinical observational study showing circulating TMAO levels are strongly associated with incident thrombotic event risks[157], as well as within a small prospective human cohort study involving the taking of choline supplementation[172]. In both omnivores and vegans alike, choline supplementation resulted in heightened TMAO levels and increased ADPdependent aggregation responses. Moreover, a dose-dependent association was observed between plasma TMAO levels and heightened platelet responsiveness[172]. Surprisingly, TMAO dependent enhancement in platelet responsiveness was shown to attenuate low-dose aspirin-induced reduction in platelet aggregation[172]. Thus, in subjects taking low-dose aspirin, elevation in TMAO levels by choline supplementation overcame the aspirin-induced reduction in platelet responsiveness, suggesting that socalled aspirin resistance may in part be attributed to heightened generation of prothrombotic factors by the gut microbiota. These findings also are consistent with clinical studies showing that elevated TMAO levels can help to explain the residual elevation in cardiovascular disease risk in subjects on traditional preventive cardiovascular disease medications, including antiplatelet agents. The findings of platelet hyper-reactivity with elevated TMAO levels are also complemented by studies discovering that TMAO is capable of inciting a robust inflammatory response in aortic tissue in vivo[173]. This inflammatory response is believed to be mediated in part by endothelial cell NF-kB activation, with small-molecule inhibitor studies suggesting the involvement of a GPCR. A pro-inflammatory signaling effect of TMAO has been independently replicated in studies utilizing human umbilical vein endothelial cells, as well as in studies exploring additional mechanisms such as the NLRP3 inflammasome[174,175].

Despite overwhelming clinical and animal model data supporting a link between TMAO and CVD, many questions remain. Most importantly, the nature of the “TMAO receptor,” if one indeed exists, remains unknown. TMAO is known to function as a protein conformation altering small molecule (i.e., a protein chaperone mimetic)[176]. It may, therefore, impact signaling pathways not only via a classic receptor-ligand interaction but as an allosteric modifier. Thus, detailed molecular understanding of the exact mechanism of action by which TMAO exerts its pro-inflammatory and prothrombotic effects remains undiscovered. It is known that the TMAO precursor, TMA, is detected through the GPCR TAAR5 chemosensory receptor, to which TMA binds with high affinity, though TAAR5 is known

not to recognize TMAO[177]. Therefore, there is optimism for the discovery of a potential receptor-mediated effect of TMAO, as well. It is of interest that TMAO has been reported to potentiate alternative signal transduction processes such as intracellular calcium signaling and platelet reactivity, or sustained increases in blood pressure after angiotensin infusion[82,178].

Direct participation in heart failure susceptibility.—In the heart failure setting, animal experiments from Organ et al. found that either choline supplementation (which raises circulating TMAO), or direct TMAO feeding in mice, resulted in higher systemic TMAO levels, enhanced myocardial fibrosis and worsened hemodynamic and anatomic parameters after trans-aortic constriction (TAC)[160]. Furthermore, in separate studies, it is reported that removal of the TMAO diet after TAC resulted in a greater myocardial recovery in the mice[179]. These findings were corroborated in a different system to induce cardiovascular dysfunction by Chen et al[180]. Mice fed a western-diet were given 3,3-dimethyl-1-butanol, a TMA-lyase inhibitor, to reduce the production of TMA and hence, conversion to TMAO. In this study, it was found that inhibition of TMA and TMAO production resulted in significantly improved ventricular remodeling and hemodynamic parameters of mice on a western diet[180].

Beyond animal model studies showing heightened TMAO is associated with worse adverse ventricular remodeling and function in heart failure models, our group has reported findings further exploring clinical links between TMAO and heart failure by examining sequential subjects with a history of heart failure who underwent cardiac evaluations[181]. We observed that subjects with heart failure have significantly higher levels of TMAO compared to age- and sex-matched non-heart failure subjects. We further showed that elevated plasma TMAO in subjects with heart failure was associated with worse long-term survival, even after adjusting for cardio-renal parameters (FIG. 4), and regardless of underlying etiology[181]. These findings suggest that understanding why TMAO levels are elevated in the setting of heart failure may provide valuable insights into how gut microbiota may contribute to disease progression in heart failure. In a separate study, we observed similar findings in chronic systolic heart failure patients[182]. The most intriguing aspect of this association was that heightened TMAO levels correlate with adverse diastolic indices such as increased left atrial volume index, suggesting that perhaps an accumulation of this metabolite may affect tissue mechanics. Similar findings have subsequently been reported in an independent Norwegian cohort where plasma TMAO levels were elevated in the heart failure cohort and predictive of transplant-free survival[183]. The authors observed associations between TMAO and increased pulmonary artery pressure, as well as wedge pressure, which represent indirect estimates of stress on the left atrium. Most recently, Schuett et al. independently validated the association of TMAO with poor heart failure outcomes, specifically in heart failure with reduced ejection fraction patients[184]. Moreover, they found that TMAO was able to better predict outcomes compared to BNP in this population, after adjusting for traditional risk factors and renal function. One can speculate that in the setting of volume overload, TMAO, as an organic osmolyte, could be drawing fluid into spaces where they usually do not reside, or through a yet unelucidated receptor-mediated mechanism.

Multiple studies in the chronic setting thus suggest that TMAO impacts heart failure susceptibility and incident risks, in part through increased myocardial fibrosis, and accompanying diastolic dysfunction. One obvious question then is whether there is a similar relationship between TMAO and adverse outcomes in the acute decompensated heart failure (ADHF) setting. In support of this, recent clinical data in a study of ADHF patients from Suzuki et al. show that inclusion of TMAO into risk models provided improved risk stratification for in-hospital mortality in combination with clinical scores[185]. Additionally, the authors also found that utilizing TMAO levels concurrently with elevated NT-proBNP improved outcome prediction. These clinical studies are summarized in Table 1. Novel evidence from Savi et al. observed worsened cardiomyocyte contractility in the presence of TMAO *in vitro*[186]. However, different findings were observed in a CD1 mouse model from Oakley et al. which showed that high levels TMAO has direct effects on increases in myocardial contractility and calcium flux in a dose-dependent manner[187]. These findings suggest that TMAO could potentially impact myocardial contractility in the acute setting. However, it is difficult to reconcile the two studies given the substantial differences in TMAO dose used (20100uM vs. 300–3000uM) as well as the experimental settings (*in vitro* vs. *ex vivo)*. Therefore, additional studies are necessary to elucidate these mechanisms further.

Renal disease and heart failure susceptibility.—Beyond a myocardium centered focus on heart failure pathogenesis, a contributory and synergistic cardio-renal connection has long been recognized[188]. Importantly, a substantial proportion (40–50%) of individuals with heart failure also demonstrate some extent of kidney dysfunction[189,190]. Many pathophysiologic processes contribute to this connection, including altered fluid status, neuro-hormonal responses, and metabolic dysregulation. Therefore, appropriate management of heart failure also requires paying close attention to any underlying renal dysfunction.

In our initial studies of TMAO, we hypothesized that an efficient potential mechanism for conferring protection from cardiovascular disease and heart failure development could be via prevention in the accumulation of TMAO[157]. Subsequent studies showed that TMAO is rapidly cleared from the body after ingestion[157,165,191]. The strong relationship between kidney function and TMAO has been recognized in multiple early studies on rat models showing primarily renal excretion greater than 95% after radiolabel ingestion[192]. More detailed renal physiology evidence so far only exists in models of deep-sea fish or chicken from decades ago, and have demonstrated tubular secretion, reabsorption, or passive transport as potential mechanisms of renal excretion[193–195]. However, it is difficult to extrapolate these findings to a mammalian system. TMAO was incidentally found but not further characterized in several studies investigating broad-scale metabolites associated with CKD. This association was first seen in small cohorts of dialysis, transplant or late-stage CKD patients[196]. Then in a larger cohort from the Framingham Heart Study, elevated circulating levels of both choline and TMAO were observed to predict the incident development of CKD in subjects with normal renal function at the time of enrollment and sample collection[197].

To investigate the relationship between TMAO and CKD development, our group observed that high levels of TMAO in patients with CKD (defined as eGFR <60mL/min) portended a significantly increased mortality risk with HR 1.93 (95%CI 1.13–3.29)[198]. Translating findings to mice models, we observed that both chronic TMAO and choline feeding resulted in striking development of renal fibrosis, along with increased levels of tubular injury markers such as cystatin C, pSMAD, and KIM-1/tubulin ratio[198]. These observations have since been replicated in multiple independent observational cohorts across a spectrum of CKD patients[199–203]. Furthermore, in the past two years, multiple large observational studies have been published that recapitulate our initial observations which we have reviewed in detail.

In a small comparative GWAS study involving n=1,892 subjects with relatively preserved renal function, it was suggested that genetic variant play a relatively weak role in determining TMAO levels[204]. Interestingly, in a clinical cohort from the Seattle Kidney Study involving n=339 subjects, it was reported that specific polymorphisms of FMO3 were associated with elevated circulating TMAO. In this latter study, the recruited patients also had impaired kidney function and thus clearance, which may have heightened the sensitivity for the detection of genetic variants associated with variations in circulating levels of TMAO, despite its smaller sample size. Furthermore, another recent study also observed increased FMO3 expression associated with decreased kidney function in mice, suggesting genetic impact on plasma TMAO variability in specific settings such as decreased renal function[205].

In addition, questions exist whether genetic differences in ethnicity may also play a role in the relationship between TMAO levels and observed genetic variants associated with plasma levels – Shafi et al. reported that black CKD populations tended to have better survival than their white CKD counterparts within the same quintile of TMAO levels[201].

Overall, beyond CKD and heart failure patients, the observational clinical data relating elevated TMAO to poorer prognosis has been robust. Most recently, several independent meta-analyses arrived at similar conclusions of elevated TMAO being prognostic for cardiovascular events or mortality, independent of renal function and cardiovascular comorbidities[206–208].

Gut microbiota-relevant interventions for heart failure

Prebiotic and probiotic therapy

Prebiotics are non-digestible food products (non-culturable) that can alter microbial composition and function, or induce the growth of select micr[209]oorganisms. Probiotics, on the other hand, represent live microorganisms. These supplements are thought to be able to improve the intestinal microbial ‘balance’ by altering the microbial composition and community structure. Some preliminary evidence suggests that administration of select probiotics can be cardioprotective. For example, treatment of rats with a beverage containing *L. plantarum* 299v 24 hours before coronary ligation reportedly produced reduced infarct sizes and improved left ventricular function[86]. In a separate study, administration of *Lactobacillus rhamnosus* GR-1 also demonstrated similar results in a rat myocardial

ischemia model[210]. A decrease in the adipokine leptin was attributed as a potential mechanism of the probiotic benefits. However, it is notable that the 16s sequencing in the GR-1 study showed that the treatment strain did not appear to colonize or alter the gut community structure/composition. Of interest, a randomized control pilot study with *Saccharomyces boulardii* was reported to show benefits in heart failure patients, with left ventricular ejection fraction being improved in the short term, along with decreases in serum creatinine and inflammatory markers[211]. These findings are very preliminary and must be balanced with the concerns of probiotic administration in a population that tends to become more critically ill. Though probiotics have an excellent safety record, the lack of regulation across supplements could potentially raise the risk of probiotic translocation and associated sepsis[212,213].

Dietary intervention

Dietary interventions to alter the progression of heart failure have primarily focused on the reduction of salt, to prevent hypertension and maintain volume homeostasis[1]. A formally adopted strong recommendation from the American College of Cardiology/American Heart Association guidelines is the Dietary Approaches to Stop Hypertension (DASH) eating plan consisting of a diet that is rich in fruits, vegetables, whole grains, and low-fat dairy foods; includes meat, fish, poultry, nuts, and beans; and is limited in sugar-sweetened foods and beverages, red meat, and added fats. Multiple observational studies suggest that a DASH diet reduces heart failure incidence[214–217]. A small randomized, controlled clinical trial of 48 patients assigned to either a DASH diet (n=24) or general guidelines for HF management (n=24), those in the DASH group demonstrated better 6 minute walk performance, quality of life, and trended towards increased arterial elasticity after a three month intervention. Interestingly, there appeared to be little difference in the salt intake between groups, which suggests that other dietary components require further investigation[218]. Moreover, biomarker and cardiac morphometry data from another small pilot study named DASH-Diastolic Heart Failure (DASH-HF), showed that the 13 females patients on the DASH diet had significant weight loss, improved cardiac function along with improvements in multiple surrogate markers such as blood pressure, BNP, urinary isoprostanes and acylcarnitines[219–221]. Beyond the DASH diet, there is still a gap in the literature for additional dietary interventions as a definitive therapeutic route for heart failure. Proper nutrition such as the Mediterranean diet, can certainly aid the prevention of cardiovascular events and the subsequent development of heart failure. Despite the recent retraction and revision, the benefits of the Mediterranean diet for primary prevention of cardiovascular diseases are still supported by the Prevención con Dieta Mediterránea (PREDIMED) study, and there are also data showing that adherence to a Mediterranean diet is more likely to result in lower levels of TMAO[222]. This may be because of the presence of 3,3-dimethyl-1-butanol (DMB) (which we will discuss later), which inhibits TMAO production and is found in extra virgin olive oil, a major component of the Mediterranean diet. Two recent studies explored the potential of the Mediterranean diet to improve the prognosis of acute heart failure. A pre-specified secondary outcome for heart failure incidence was investigated in PREDIMED, but there was no association between diet and heart failure incidence[223]. However, this population was not powered to provide validated conclusions. In the MEDIT-AHF study, which prospectively followed emergency department acute heart failure admissions, the authors did

not see long-term benefits in mortality[224]. However, the patients did have decreased risk for rehospitalization.

Microbial TMA-lyase inhibitors

With strong associations in heart failure, we look ahead and discuss strategies for targeted inhibition of TMAO production as a framework for other potential microbiota targets. Much attention has been focused on the reduction of TMAO levels, with an emphasis focusing on the meta-organismal production pathway[225]. Thus, both microbial and host liver enzymes represent potential targets for reducing TMAO production, either as a therapeutic target or as scientific proof of principal in understanding the importance of the TMAO meta-organismal pathway to host pathophysiology. Following ingestion of a TMA containing nutrient (the choline moiety being the most abundant in a typical diet), distinct microbial enzyme systems participate in the generation of TMA, the precursor of TMAO[158,171] (FIG. 5). The choline utilization gene cluster contains catalytic and regulatory polypeptides, CutC/D, which appear to serve as the primary microbial catabolic enzyme system for choline conversion into TMA (i.e., choline TMA lyase) within the human gut microbiome[226]. Additional microbial enzyme systems that contribute to TMA generation are now known to include CntA/B and yeaW/X for conversion of carnitine into TMA[226–230]. In a study by Wang et al., DMB was developed as a competitive inhibitor against CutC catalyzed choline TMA-lyase activity to reduce the production of TMAO[96]. This inhibitor was found to be non-lethal to human commensals possessing CutC and fostered suppression of both TMA production in cultured human commensals and TMAO lowering in mice on a choline supplemented diet. Most importantly, exposure to DMB was shown to also effectively reduce atherosclerosis progression in the apolipoprotein E−/− mouse model without significant adverse toxicity[96]. Interestingly, DMB was also observed to be present in some cold-pressed extra-virgin olive oils[96], a major component of the Mediterranean diet. Hence, there may be some mechanistic link between microbial inhibition of TMAO production and the benefits of Mediterranean diet. However, it is unclear if the reduction in TMA as a result of TMA-lyase inhibition may contribute to as of yet unrecognized or unintended effects. Other less validated examples of potential TMA generating inhibitors include meldonium, an analog of γ-butyrobetaine and carnitine, that is reported to be able to reduce carnitine to TMA conversion, or resveratrol, which while not an inhibitor, is suggested to reduce TMAO levels by reorganization of microbial community structure[231,232].

Future directions

The realization that gut microbiota can contribute to both normal health and disease susceptibility provides a novel perspective and permits expansion in our approach to disease research and therapeutic opportunities. The next steps in future investigations include teasing out, at a mechanistic level, how the gut microbiota contribute to heart failure and cardiovascular disease. This includes defining chemical mediators, microbemicrobe as well as microbe-host interactions within the complex microbiota ecosystem. Much work remains beyond the swath of association studies regarding understanding host receptors which perpetuate signaling processes that lead to altered host physiology. Moreover, are there specific genetic factors which may predispose individuals towards disease susceptibility in

the combination of both specific nutrient depletion/excess and microbiota functional capability? Finally, translation of “microbiome-centred” findings will be necessary through well-designed prospective, longitudinal studies.

The most relevant phenotypes must be considered to bring microbial information to clinical practice. Although sequencing of the microbial genome is becoming more common in research practice, the utility of this methodology for clinical practice is still unclear. Because sequencing information, unless the more expensive meta-genomic option, is performed, cannot estimate at all the phenotype pattern of the microbiome but rather only the “potential” phenotype based on the genes present. Rather, measurement of metabolic profiles in the blood or urine to guide appropriate dietary recommendations and to provide targeted interventions will result in more translational findings. These interventions in the future can take numerous forms. Beyond dietary recommendations, one can imagine sequestrants or binding resins as one form, intercepting the microbial metabolites of interest before absorption into the host. Bile acid sequestrants are an early lipid-lowering medication, and thus this approach, albeit for an alternative class of intestinal luminal metabolites, has precedence. Yet another potential approach will be non-lethal microbial inhibitors, such as DMB for TMAO. Other more potent TMA-lyase inhibitors are under investigation, and the use of a non-lethal microbial enzyme inhibitor has the potential theoretical benefit of being less likely to foster the development of microbial resistance because the selective pressure for developing resistance is reduced compared to an antibiotic. Finally, the use of pre or probiotics – to result in “terraforming” of the gut microbial community and function to achieve changes in microbial metabolites is an alternative approach and an area of active investigation.

Conclusions

Undoubtedly, there is a complex association between gut microbiota and the cardiovascular system that can influence and even determine cardiovascular diseases. Understanding the role of the gut microbiota is of paramount importance--- chronic inflammation, underlying comorbidities, antibiotics, diet, and probiotics can drastically alter the long-term composition of the gut microbiota and thereby impact our health. Similarly, increasing clinical use of pre- and probiotics may have unappreciated effects. Future research must elucidate the mechanisms behind these complex associations to determine whether a causal link exists between this “forgotten organ” and heart failure. Because the gut microbiota represents a relatively uncharted ground for novel pharmacologic targets, we need to develop a solid understanding of these underlying mechanisms to pave the way for targeted therapeutic strategies in heart failure.

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Competing interests

Drs. Tang and Li have no relationships relevant to the contents of this paper to disclose. Dr. Hazen is named as co-inventor on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics. Dr. Hazen is a paid consultant for Proctor & Gamble. Dr. Hazen has received research funds from Proctor & Gamble and Roche Diagnostics. Dr. Hazen is eligible to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland Heart Lab/Quest Diagnostics or Proctor & Gamble. The other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

Glossary

Meta-organism	Polygenomic organism consisting of a community of living beings of different species (e.g. microbiota and host) associated by interspecies symbiosis.
Metagenomics	The study of the collective genome of microorganisms from an environmental sample
Metatranscriptomics	The study of the collective transcripts of microorganisms from an environmental sample

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Text Box 1

Categorization of the Gut Microbiota -

Several approaches that attempted to classify bacteria as “beneficial” or “harmful” have been highly cited despite being over-simplified, such as the three enterotypes paradigm (designated as enrichment in *Bacteroidetes* phylum*, or Prevotella, or Ruminococcus* genus based on multidimensional cluster and principal component analysis of 61 fecal samples) or the Firmicutes to Bacteroidetes ratios[97,98]. Although these approaches provided an initial paradigm for the study of microbiota effects on health, such broad classification schemes were difficult to replicate, as gradients rather than distinct groups arose as a more accurate descriptor[99,100]. Furthermore, such characterizations belie an appreciation of the nuances that an individual microbial species or strain can under certain circumstances be considered healthful and essential in supporting host health, yet in other situations (e.g., an extreme increase or decrease in level) be considered a potential contributor to disease.

Endeavors such as the Human Microbiome Project and MetaHIT aim to create a catalog of bacteria in the healthy human gut microbiome[6,101]. Through deep sequencing, it is possible to predict functional groups of the bacteria within our gut. This knowledge is essential as it has been shown that functional genes may not be present in bacteria of the same species from different individuals (though they may be shared amongst communities)[102]. Further, such analyses only relay relative functional capacity and thus may not reliably predict global functional capacity. Therefore, progression to direct functional interrogation (e.g. metabolomics, peptidomics, and direct microbial transplantation studies) beyond any sequence-dependent measurement (metagenomics or metatranscriptomics) is needed to move beyond quantification of microbe copy number, or predicted gene presence, to better demonstrate function, disease associations, and participation.

Key points

- The “gut hypothesis” of heart failure postulates that bowel wall edema and impaired intestinal barrier function in heart failure can promote bacterial translocation and heightened inflammation
- Changes in the composition and diversity of gut microbiota have been observed in patients with heart failure
- Microbial metabolites, especially those derived from dietary nutrients, can generate paracrine and endocrine effects that can also lead to an increased susceptibility to heart failure
- Novel therapeutic strategies targeting microbial metabolic pathways and/or metabolites, as well as altering microbial composition, have the potential to modulate cardiovascular disease susceptibility and prevent progression to heart failure

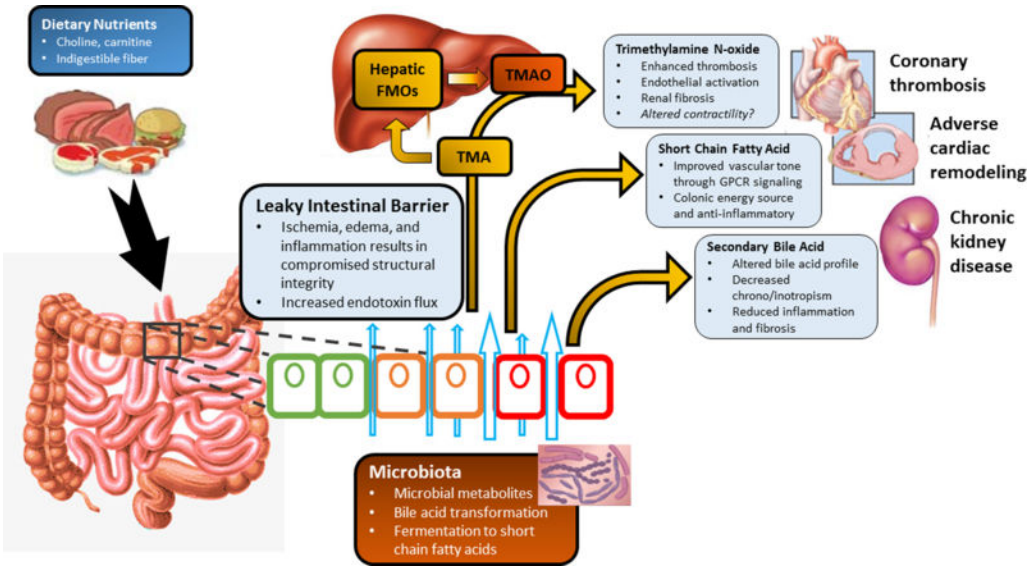

Figure 1. Microbial-host meta-organismal pathway linking dietary metabolism, gut microbiota, and cardio-renal disease progression.

Legend: Poor cardiac output in heart failure results in intestinal ischemia, edema, and inflammation which leads to a “leaky” intestinal barrier. This allows for increased passage of inflammatory bacterial products to enter the bloodstream causing chronic low-grade inflammation. Furthermore, this alters the intestinal environment and impacts both the normal microbial community which resides in the gut and subsequently, the metabolic products from these bacteria. The metabolic pathways include fermentation of indigestible fiber to short chain fatty acids which have protective properties reducing inflammation and improving vascular tone. Dietary sources including choline, phosphatidylcholine, l-carnitine and other methylamine-containing nutrients provide substrates for microbiota mediated production of trimethylamine (TMA). TMA then enters the portal circulation and is converted by the hepatic host flavin-containing monooxygenase (FMO) family of enzymes to trimethylamine n-oxide (TMAO). TMAO can promote the development of atherogenesis, thrombosis, kidney disease, and heart failure. Additionally, the bacterial transformation of bile acids can result in altered bile acid profiles which then can affect systemic inflammatory and fibrotic processes. Collectively, these processes can influence the individual susceptibility, severity of heart failure.

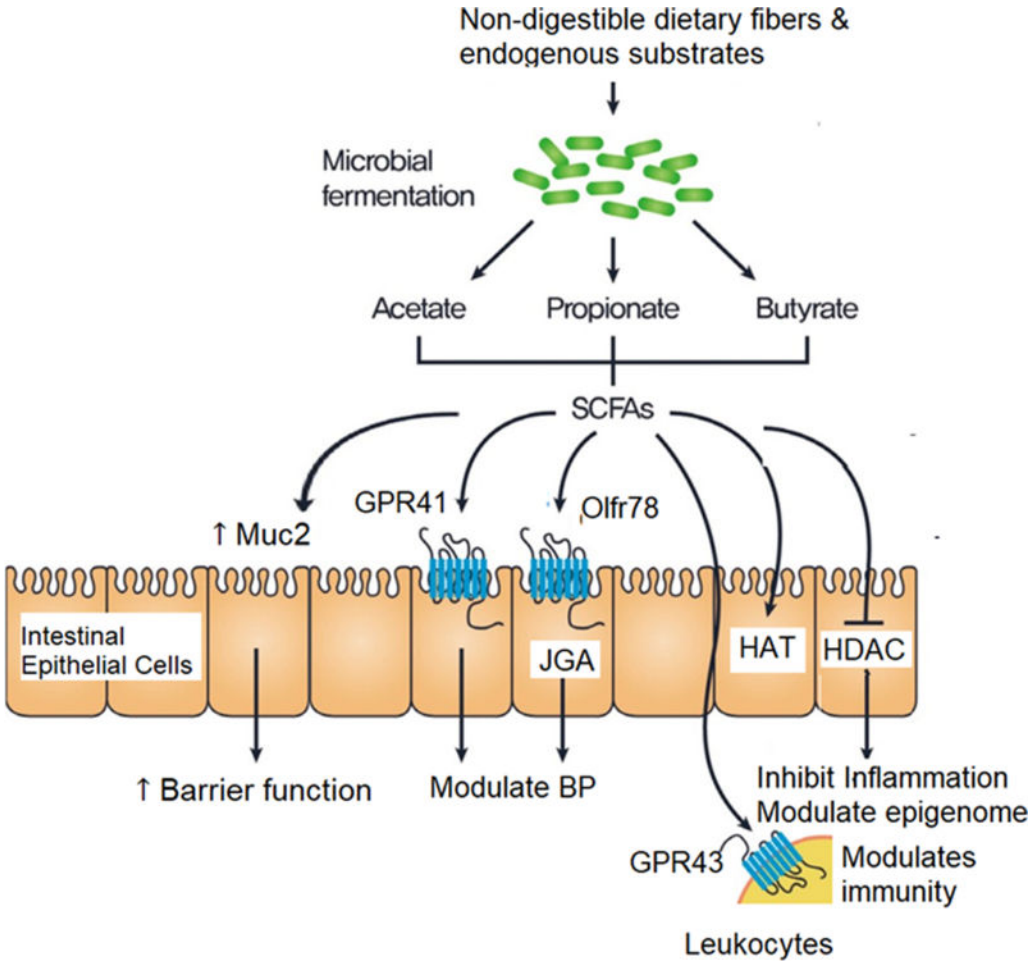

Figure 2. Downstream Effects of Gut Microbiota-generated Short-chain Fatty Acid (SCFA) in Cardiovascular System

Legend: SCFAs produced by gut microbiota exert their cardiovascular effects by 1) indirectly improving intestinal barrier function by promoting mucous production; 2) activate Olfr78 in mouse (or hOR51E2 in humans) in the renal juxtaglomerular apparatus (JGA) and peripheral vasculature, that leads to increased renin release, and raised blood pressure thereby counteracting hypotensive responses mediated by GPR41; and 3) activate histone acetyltransferases (HAT) and inhibit histone deacetylases (HDACs) thereby inhibiting inflammation, balancing gene regulation (epigenome) and modulates immune cell activation.

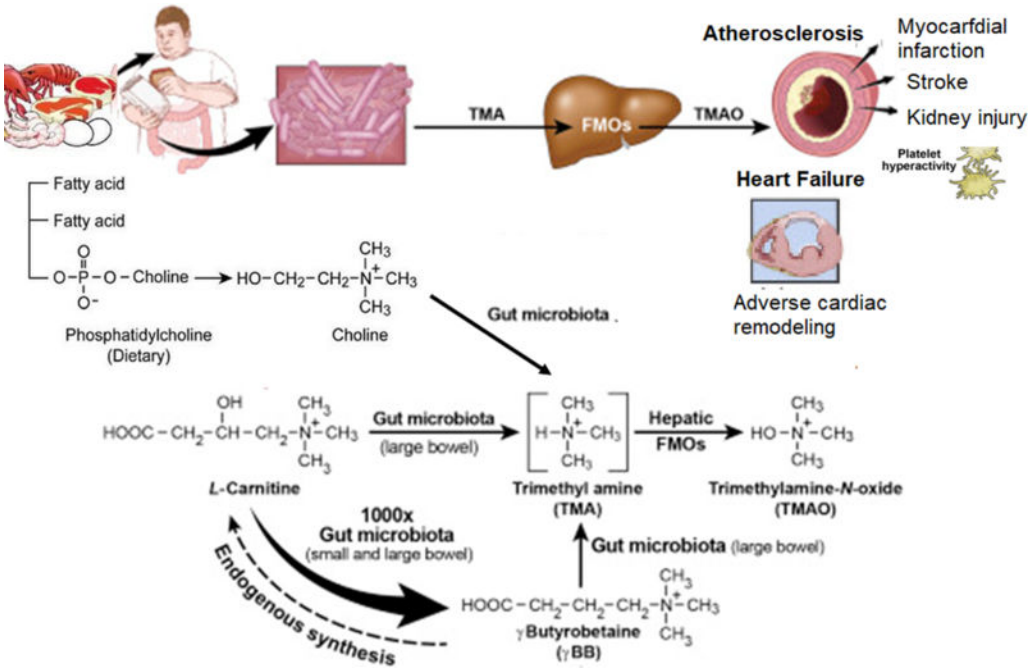

Figure 3: Microbial Generation of Dietary-Induced Trimethylamine (TMA) and Trimethylamine N-oxide (TMAO)

Legend: Diets rich in choline/phosphatidylcholine and carnitine can be metabolized by certain species of gut microbiota to TMA that is then converted to TMAO by hepatic flavin mono-oxygenase (FMO, especially FMO3) and excreted via the kidneys from the circulation. Accumulation of TMAO levels has been associated with atherogenesis, platelet hyperactivation, and future development of adverse cardiac events such as myocardial infarction, stroke, and death. Adverse cardiac remodeling is also associated with elevated TMAO levels. Gamma butyrobetaine, a precursor for endogenous synthesis of carnitine, can also be converted to TMA (Modified from Wang et al, Nature 2011; Koeth et al, Nat Med 2013; Koeth et al, Nat Med 2014)[156,158,227]

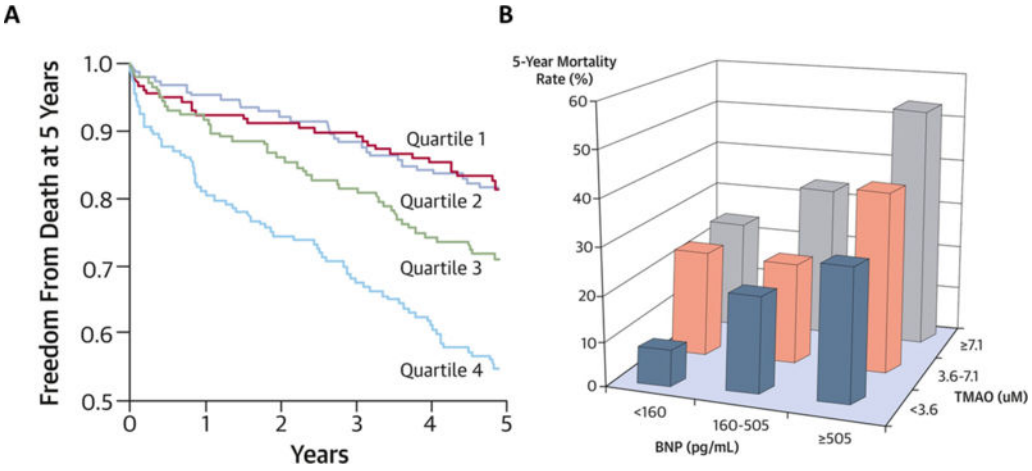

Figure 4. The relationship between plasma levels of TMAO and 5yr mortality risk in Patients with Chronic Heart Failure.

Legend: Patients with a history of heart failure stratified according to quartiles of fasting TMAO levels and circulating B-type natriuretic peptide (BNP) levels showing an increased incidence of 5-year mortality with rising TMAO levels, independent of BNP levels (from Tang WH et al., *J Am Coll Cardiol* 2014)[181]

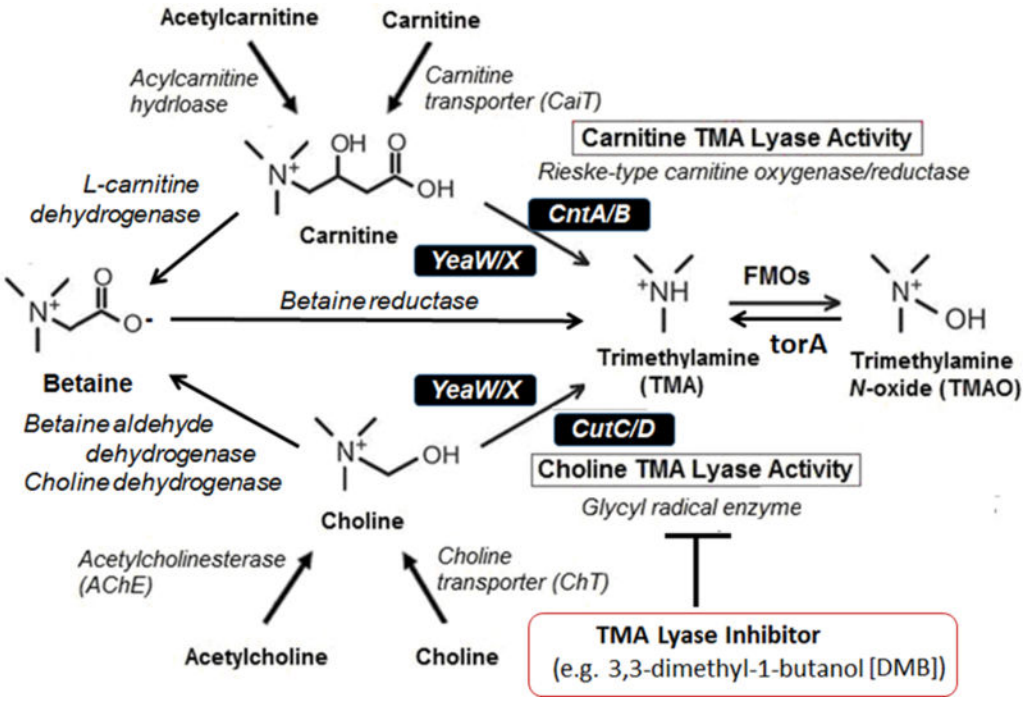

Figure 5. Microbial Metabolic Pathways as Potential Druggable Targets.

Caption: Carnitine (and gamma butyrobetaine) can be converted by gut microbiota to trimethylamine (TMA) via carnitine TMA lyases such as CntA/B or YeaW/X, whereas choline can be converted to TMA via choline TMA lyases such as CutC/D or YeaW/X. Betaine conversion to TMA is in much lower quantities. Many of these enzymes are unique in some microbial species, making them attractive druggable targets for inhibition to reduce TMA production (such as 3,3-dimethyl-1-butanol [DMB] as choline TMA lyase inhibitors (Wang et al., Cell 2015).

Table 1.

Summary of Clinical Investigations Between TMAO and Cardiovascular Related Outcomes in Heart Failure Cohorts

Group	Subjects	Study Population Criteria	Endpoint of Interest	Results
Tang et al.[181]	720	Stable cardiac patients with a history of heart failure undergoing elective coronary angiographic evaluation	All-cause mortality over 5-year of highest quartile TMAO	Adjusted HR 1.75, CI 1.07–2.86, P < 0.001
Tang et al.[182]	112	Stable but symptomatic, chronic systolic heart failure (LVEF ≤ 35%) with comprehensive echocardiographic evaluation	Composite endpoint all-cause mortality and cardiac transplantation over 5 years per SD ln TMAO	Adjusted HR 1.46, CI 1.03 2.14, P=0.03
Suzuki et al.[185]	972	Patients with AHF based on progressive worsening or new onset of shortness of breath along with clinical signs of pulmonary edema, peripheral edema, or elevated jugular venous pressure	All-cause mortality; Composite of death or rehospitalization due to HF at 1 year of highest TMAO tertile	Adjusted HR 1.16, CI 1.01–1.33, P=0.037; non-significant after inclusion of renal function
Trøseid et al.[183]	155	Patients with stable HF for >6 months in NYHA functional class II–IV	Transplant free survival over median 5.2 years of highest TMAO tertile	Unadjusted HR 2.25, CI 1.28–3.92, P=0.005; non-significant after adjustment HR 1.79, CI 0.90–1.79, *P* = 0.097
Schuett et al.[184]	823	Patients referred for coronary angiography with heart failure diagnosis in accordance with European guidelines	Death and CV death over mean follow-up of 9.7 years of highest TMAO tertile	Adjusted death HR 1.84, CI 1.44–2.36; CV death HR 1.98, CI 1.47–2.68; remained significant after additional adjustment

AHF – Acute Heart Failure; CAC – Coronary Artery Calcium; cIMT – carotid intima-media thickness; CI – 95% Confidence Interval; COXPH – Cox Proportional Hazard; HR - Hazard Ratio; LVEF – Left Ventricular Ejection Fraction; OR- Odds Ratio; RR – Rate Ratio

Table 2.

Summary of Contemporary Studies Investigating Heart Failure and Alterations in Microbiota

Author	Sample Groups and Measurements	Results Summary
Sandek et al.[29]	FISH of mucosal biofilm samples (20 CHF, 22 Ctrl) collected by sigmoidoscopy	Elevated bacterial concentration and increased strain diversity of mucosal biofilm in CHF patients; Increased detection frequency (from 38 FISH probes) of *Bacteroides/Prevotella*, *Eubacterium Rectale*, *Faecalibacterium Prausnitzii* in CHF; Increased gut permeability as measured by lactulose-mannitol test.
Sandek et al.[87]	FISH of mucosal bacterial film (22 CHF; 20 Ctrl) collected by sigmoidoscopy; Stool samples (21 CHF; 17 Ctrl)	Concentrations and proportions of both anaerobic and aerobic bacteria in the stool were not significantly different; Trend of positive correlation between increased anaerobic juxtamucosal bacteria and decreased intestinal blood flow.
Pasini et al.[88]	Stable CHF patients with NYHA I-II (n=30), NYHA III-IV, (n=30), and matched healthy control (n=20) had stool samples collected to measure bacteria and *Candida* species using traditional culture techniques.	The CHF population (NYHA III-IV in particular) had large increases in detected pathogenic bacteria including *Campylobacter*, *Shigella*, *Salmonella*, *Yersinia enterolytica*, and *Candida* species; Increased gut permeability as measured by cellobiose sugar test
Mamic et al.[89]	2012 Healthcare cost and utilization project National Inpatient Sample data	*Clostridium difficile* infection rates were higher in hospitalizations with discharge diagnosis of HF compared with those without HF after controlling for patient demographics and comorbidities and hospital characteristics.
Luedde et al.[90]	Bacterial 16S rRNA gene sequencing of fecal samples from 20 patients with heart failure with reduced ejection fraction due to ischemic or dilated cardiomyopathy and matched controls selected from the PopGen study.	Tendency for decreased bacterial diversity in HF with significant differentiation between HF and control patients; Those with HF showed significant decreases in *Coriobacteriaceae*, *Erysipelotrichaceae* and *Ruminococcaceae* on the family level and decreases in *Blautia*, *Collinsella*, uncl. *Erysipelotrichaceae* and uncl. *Ruminococcaceae* on the genus level.
Cui et al.[91]	Bacterial 16S rRNA gene sequencing of fecal samples from consecutive recruitment of 53 ischemic and dilated cardiomyopathy CHF patients (94% of NYHA III-IV) and 41 controls.	Significant differentiation between CHF and controls but similar between ischemic and dilated cardiomyopathy patients; *Faecalibacterium prausnitzii* decrease and *Ruminococcus gnavus* increase were the essential characteristics identified in this CHF patient cohort.
Kamo et al.[92]	Bacterial 16S rRNA gene sequencing of fecal samples from 12 HF patients and 12 age-matched controls; 12 HF patients younger than 60 and 10 HF patients 60 or older.	Significant differentiation between HF and controls; Those with HF showed significant decreases in *Clostridium* and *Dorea* at the genus level and decreases in *Eubacterium Rectale* and *Dorea longicatena* at the species level; Older HF patients had depletion of *Faecalibacterium* and enriched *Lactobacillus* genus compared to younger HF patients.
Kummen et al.[93]	2 independent cohorts of stable HFrEF cohorts (discovery, n = 40; and validation, n = 44; NYHA II–IV) and population-based control subjects (n = 266, randomly allocated to HF cohorts for comparison).	Decreased bacterial diversity in HF even after risk factor adjustments; Increase in genus *Prevotella, Hungatella, Succinclasticum*; Decrease in multiple genera belonging to *Lachnospiraceae* family - *Anaerostipes, Blautia, Coprococcus (3), Fusicatenibacter, Lachnospiraceae FCS020, NCS2004, ND3007, Pseudobutyrivibrio, Eubacterium Hallii group*; Decrease in *Rumminococcaceae Faecalibacterium* and *Bifidobactericeae Bifidobacterium*

CHF-Congestive Heart Failure; FISH – fluorescent in situ hybridization; HF – Heart Failure; NYHA – New York Heart Association; Uncl. - unclassified