GUT MICROBIOTA AND INFLAMMATION: RELEVANCE IN CANCER AND CARDIOVASCULAR DISEASE

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GUT MICROBIOTA AND INFLAMMATION: RELEVANCE IN CANCER AND CARDIOVASCULAR DISEASE

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Editorial: Gut Microbiota and Inflammation: Relevance in Cancer and Cardiovascular Disease

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Editorial on the Research Topic

Gut Microbiota and Inflammation: Relevance in Cancer and Cardiovascular Disease

The chronic inflammatory process is the mutual starting factor of numerous non-communicable diseases, including cancer and cardiovascular, neurologic, respiratory, and metabolic disorders. Directly or indirectly, the inflammatory mediators can favor the cancer development, inducing mutations in different loci, especially in tumor-suppressor genes, or inferring with post-translational modifications and favoring aberrant DNA methylation.

Increasing and recent evidence link the mammal inflammatory status and correlated immune response with the microbiota, a complex ecosystem of microorganisms, such as bacteria, archaea, fungi, viruses, and protozoans (Amedei, 2019; Boem et al., 2020; Niccolai et al., 2020). The gastrointestinal tract shows the greatest microbial diversity and density, named gut microbiota (GM), that exerts crucial nutritional (e.g., carbohydrates fermentation) and metabolic functions, including the xenobiotic metabolism (Rowland et al., 2018). In addition, GM is essential for the correct development of the gut-associated lymphoid tissue (GALT) and the regular evolution of the innate and specific immune system (Cebra, 1999). Instead, the immune system itself has evolutionarily developed to favor a symbiotic relationship with different microorganisms (Belkaid and Hand, 2014).

Having made these premises, the aim of our research topic has been to gather new information regarding the mechanisms regulating the early phases of these disabling inflammation-based human diseases.

In eubiosis, the microbiota-immunity axis facilitates the ideal orchestration of both innate and adaptive immune response in order to modulate the most appropriate reaction (Zitvogel et al., 2016). In contrast, dysbiosis can change the immune status, rendering the host susceptible to endo/exogenous alterations, breaking the tolerance vs. self-components, and triggering immune responses deficient or excessive (the so-named chronic inflammation). In turn, this might support the beginning of cancer and autoimmune diseases, and the GM could represent a non-negligible link between these two contrasting disorders.

In line with this, Cao et al. explored the hypothesis that *Fusobacterium nucleatum*) could intensify the intestinal inflammation, promoting the intestinal mucosal barrier damage. In fact, increasing data suggest that GM bacteria, especially *F. nucleatum*, are associated with Crohn's disease (CD), but it is not clear how the *F. nucleatum* supports the CD pathogenesis. The authors documented that *F. nucleatum* was enriched in 41.21% of CD tissues and, of note, was correlated with the clinical course and activity, and refractory CD behavior. In addition, they showed that *F. nucleatum* infection is linked to the activation of the endoplasmic reticulum stress (ERS) pathway in the CD progression to

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promote the devastation of the intestinal mucosal barrier. To define the specific mechanisms involved, they used *in vitro* (human normal epithelial cell line NCM460 and the FHC cell line ATCC) and the mouse model of Crohn's disease and showed that the *F. nucleatum* targeted caspase activation and recruitment domain 3 (CARD3) to activate the ERS pathway and promote *F. nucleatum*–mediated mucosal barrier damage. Therefore, *F. nucleatum* coordinates a molecular network involving CARD3 and ERS to control the CD process. In conclusion, the authors suggest that quantifying and targeting *F. nucleatum* (and its associated pathways) could provide valuable insight into both the prevention and treatment of Crohn's disease.

As previously reported, and recently well-reviewed (Novakovic et al., 2020; Oikonomou et al., 2020), different studies link the interplay microbiota-immune response with cardiovascular diseases, for which hypertension represents the main risk factor. Hypertension is a clinical condition, resulting from a multifaceted interplay of endogenous and environmental factors, where the GM role has been strongly supposed but is still poorly understood. To clarify this scenario, Silveira-Nunes et al. evaluated the fecal microbiota of hypertensive patients, investigating at the same time the serum cytokines' signature as immunological profile.

In the enrolled patients, they documented a clear intestinal dysbiosis characterized by reduced biodiversity compared with the normotensive counterpart. In fact, along with a reduction in *Bacteroidetes* members, hypertensive patients showed an increased number of *Lactobacillus* and *Akkermansia* and decreased relative abundances of butyrate-producing bacteria, such as *Roseburia* and *Faecalibacterium,* within the Lachnospiraceae and Ruminococcaceae families. In addition, in the same hypertensive patients, the authors documented an altered inflamed status characterized by an amplified TNF and IL-6 production and increased TNF/IFN-γ ratio. In other words, Silveira-Nunes et al. document, for the first time, an evident association of hypertension with altered GM composition and inflammation status pointing to ignored bacteria as potential contributors to intestinal homeostasis loss and thus the high vulnerability of hypertensive patients to inflammation-related pathologies.

Several studies have shown that the development of atherosclerosis, the dominant cause of cardiovascular diseases, is associated with trimethylamine N-oxide (TMAO) levels (Koeth et al., 2013; Illiano et al., 2020). Specifically, Yang et al. reported how TMAO may affect inflammation, immune response, and cholesterol metabolism as well as atherothrombosis processes, and therefore the development of atherosclerosis. In addition, the authors described clinical studies demonstrating that increased plasma levels of TMAO are a risk factor for i) major adverse cardiovascular events (stroke, myocardial infarction, or death) in atherosclerotic patients and ii) subsequent cardiovascular events among patients with recent prior ischemic stroke (Tang et al., 2013; Haghikia et al., 2018). The gut microbiota is one of the crucial factors in the TMAO generation, and changes in its composition have marked effects on TMAO levels. Indeed, Yang et al. reported that the administration of *Lactobacillus plantarum* ZDY04, *Enterobacter aerogenes* ZDY01, and *Enterococcus faecium* WEFA23 decreased serum TMAO concentrations by remodeling gut microbiota in mice. The authors suggest that TMAO could be used as a novel approach for the prevention and treatment of atherosclerosis.

In line with these data, Alushi et al. describe the potential relationship between gut microbiota metabolites, including TMAO, and the calcification of the aortic valve. The authors reviewed the calcific aortic valve disease (CAVD) pathophysiology, highlighting that inflammation and immune system activation are the common factors between this disease and atherosclerosis (Hulin et al., 2018). They hypothesize a role of the microbiota in the development and progression of CAVD, through a direct valvular damage caused by specific bacterial taxa or a stimulation of immune response and valve calcification. The former mechanism seems to be related to the oral microbiota because increasing evidence supports the presence of oral bacteria in the valvular tissue, and it has been demonstrated that specific strains of *Streptococcus* mutants have selective virulence for infectious endocarditis (Cohen et al., 2004). For the second indirect mechanism, as stated above, TMAO levels appear to be related to calcification degree, whereas short-chain fatty acids (generated by bacterial fermentation of dietary fiber) seem to promote the shifting of the immune response between pro- and anti-inflammatory pathways (Russo et al., 2016). Altogether, these data suggest that the microbiota could play a role in the development of CAVD.

Of note, Mendelian randomization analyses have strengthened the linear correlation between the concentration of low-density lipoprotein cholesterol (LDL-C) and the incidence of cardiovascular events. The LDL-C concentration has been identified as a primary causative and modifiable risk factor for the development of atherosclerosis (Parolini, 2020). The review by Villette et al. describe the gut microbiota's impact on cholesterol levels. Recent data from epidemiological studies reported associations between phylum, bacteria taxa, and cholesterolemia. Bile acid biosynthesis is the predominant metabolic pathway for cholesterol catabolism in the human body (Hofmann, 1999); however, cholesterol is actively metabolized by intestinal bacteria, mainly in coprostanol, which is very poorly absorbed by the intestine (Gérard, 2013). The intestinal bacteria are also responsible for the conversion of primary bile acids to secondary bile acids (Gérard, 2013). In humans, antibiotic treatment causes cholesterol reduction mainly through the inhibition of the above-mentioned process determining a decrease in the hydrophobicity of bile acids. Interestingly, gut microbiota can affect the efficacy of statins, the leading pharmaceutical class in hyperlipemia therapeutic care.

In summary, the papers contained in this special issue provide new data supporting the role played by the intestinal microbiota in Crohn's disease and hypertension, as well as in the development of atherosclerosis, CAV disease, and serum cholesterol levels.

AUTHOR CONTRIBUTIONS

The authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Gut Microbiota-Dependent Marker TMAO in Promoting Cardiovascular Disease: Inflammation Mechanism, Clinical Prognostic, and Potential as a Therapeutic Target

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Cardiovascular disease (CVD) is the leading cause of death worldwide, especially in developed countries, and atherosclerosis (AS) is the common pathological basis of many cardiovascular diseases (CVDs) such as coronary heart disease (CHD). The role of the gut microbiota in AS has begun to be appreciated in recent years. Trimethylamine N-oxide (TMAO), an important gut microbe-dependent metabolite, is generated from dietary choline, betaine, and L-carnitine. Multiple studies have suggested a correlation between plasma TMAO levels and the risk of AS. However, the mechanism underlying this relationship is still unclear. In this review, we discuss the TMAO-involved mechanisms of atherosclerotic CVD from the perspective of inflammation, inflammation-related immunity, cholesterol metabolism, and atherothrombosis. We also summarize available clinical studies on the role of TMAO in predicting prognostic outcomes, including major adverse cardiovascular events (MACE), in patients presenting with AS. Finally, since TMAO may be a novel therapeutic target for AS, several therapeutic strategies including drugs, dietary, etc. to lower TMAO levels that are currently being explored are also discussed.

Keywords: trimethylamine N-oxide, atherosclerosis, cardiovascular disease, inflammation mechanism, clinical prognostic stratification, therapy

INTRODUCTION

Atherosclerosis (AS) and resulting cardiovascular diseases (CVDs) are serious threats to human health (Anderson et al., 1991; Vilahur et al., 2014). Multiple studies have identified a strong link between the gut microbiota and AS (Wang et al., 2011; Koeth et al., 2013; Jonsson and Backhed, 2017; Ma and Li, 2018), Trimethylamine N-oxide (TMAO), an important gut microbe-dependent metabolite, is generated from dietary choline, betaine and L-carnitine which are metabolized to trimethylamine (TMA) through gut microbiota metabolism and further converted to TMAO by hepatic flavin monooxygenases (FMOs) (Lang et al., 1998; Wang et al., 2011; Koeth et al., 2013; Jonsson and Backhed, 2017; Ma and Li, 2018). The mechanism of TMAO participating in AS is still under further investigation. It is undeniable that AS is a chronic inflammatory disease, and inflammation is constantly induced throughout the course of the disease (Ross, 1999; Libby et al.,

2002; Tuttolomondo et al., 2012). Studies showed that increased TMAO level induced the activation of NF-kappa B (NF-κB) pathway and increased the expression of pro-inflammatory genes including inflammatory cytokines, adhesion molecules and chemokines (Seldin et al., 2016; Ma et al., 2017). Oxidative stress and NLRP3 inflammasome activation could also be triggered by TMAO, whereat inflammatory cytokines such as IL-18 and IL-1β were increased released (Sun et al., 2016; Boini et al., 2017). In addition, TMAO contributed to platelet hyperreactivity and thrombosis (Zhu et al., 2016; Zhu et al., 2017). In human clinical studies, elevated TMAO levels were associated with increased risk of AS and CVD (Wang et al., 2011; Koeth et al., 2013; Stubbs et al., 2016). Moreover, prospective cohort studies have shown that increased plasma TMAO levels predicted an elevated risk of major adverse cardiovascular events (MACE) such as MI, stroke or death (Tang et al., 2013; Tang et al., 2014; Wang et al., 2014; Li et al., 2017; Li et al., 2019). This article reviews the relationship between gut microbe-dependent TMAO and AS from the perspective of the mechanism including inflammation, inflammation-related immunity, cholesterol metabolism, and atherothrombosis, and its potential for clinical prognostic and as therapeutic target.

TMAO METABOLISM

Choline, L-carnitine, betaine, and other choline-containing compounds are the major nutrient precursors of gut microbe-dependent TMAO, a pro-atherogenic metabolite (Wang et al., 2011; Liu et al., 2016). These precursors are present in the human diet and are metabolized to TMA by the gut microbiota and various enzymes (al-Waiz et al., 1992; Koeth et al., 2013). TMA can be absorbed in the intestines and delivered to the liver through the portal circulation, where it is converted to TMAO by hepatic FMOs (Lang et al., 1998; Bennett et al., 2013; Zhu et al., 2018). In addition, natural preformed TMAO is remarkably high in fish, which can be directly absorbed after consumption and then excreted in the urine (Landfald et al., 2017).

Diet plays a key role in the generation of TMAO. L-carnitine and choline are mainly present in animal-origin foods, such as meat (especially red meat), meat products, eggs, and shellfish, while betaine is found mostly in plants (Jonsson and Backhed, 2017; Janeiro et al., 2018). Dietary L-carnitine is abundant in red meat and chronic supplementation was shown to accelerate AS by altering the microbial composition and increasing the production of TMA and TMAO (Koeth et al., 2013; Ferguson, 2013). Data from a prospective follow-up of 84,136 women over 26 years indicated that high intakes of red meat significantly elevated risk of coronary heart disease (CHD) (Bernstein et al., 2010). In addition, chronic consumption of red meat increased TMAO levels produced from carnitine, but not choline, and decreased renal TMAO excretion, and plasma TMAO levels decreased within 4 weeks after discontinuation of red meat consumption (Wang et al., 2019). γ-Butyrobetaine (GBB), a metabolite of dietary L-carnitine, is involved in the transformation of L-carnitine to the pro-atherogenic metabolite TMAO (Koeth et al., 2014). Koeth et al. (2013) found that omnivorous human subjects had higher TMAO levels than vegans or vegetarians after chronic L-carnitine supplementation. Their recent research further indicated that both omnivores and vegans or vegetarians could rapidly convert carnitine to GBB, while the subsequent gut microbial-dependent conversion of GBB to TMA was diet induced, especially through omnivorous dietary patterns and chronic L-carnitine exposure (Koeth et al., 2019). However, association between dietary choline/betaine, which are essential nutrient for health (Zeisel and Da Costa, 2009; Ueland, 2011), and risk of incident CVD was not be supported in meta-analysis, and plasma choline/betaine levels predicted risk of future MACE only when TMAO was elevated (Wang et al., 2014; Meyer and Shea, 2017). In addition, a high-fat or western-like diet increased plasma TMAO in human and animal studies (Boutagy et al., 2015; Chen et al., 2017), whereas the Mediterranean diet (MD) showed beneficial effects (De Filippis et al., 2016; Pignanelli et al., 2018).

The gut microbiota is another crucial factor in the generation of TMAO (Wang et al., 2011; Koeth et al., 2013; Ma and Li, 2018), as it has been shown to be essential for converting dietary compounds into TMA in gnotobiotic mice and human studies (Wang et al., 2011; Tang and Hazen, 2014; Zeisel and Warrier, 2017), and changes in the gut microbiota have marked effects on TMAO levels. For example, patients with large-artery atherosclerotic stroke and transient ischemic attack displayed obvious intestinal dysbacteriosis and reduced blood TMAO levels (Yin et al., 2015). In C57BL/6J mice, chronic exposure to the fungicide propamocarb induced significant changes in the gut microbial community structures, resulting in a significant increase in TMA levels in the feces (Wu et al., 2018). The microbial capacity for generating TMA appears to be important for the development of AS. Nine strains that can produce TMA from choline *in vitro* have been identified in the human gut. Low levels colonization of TMA-producing bacteria resulted in a significant accumulation of plasma TMAO in germ-free mice (Romano et al., 2015). It was also shown that TMA and TMAO levels were initially higher and the numbers of aortic lesions were increased in choline diet-fed ApoE$^{-/-}$ mice transplanted with microbiota from high TMAO-producing C57BL/6J strains than in mice transplanted with low TMAO-producing NZW/LacJ strains (Gregory et al., 2015).

The final step in TMAO formation is the oxidation of TMA, which is mediated by the flavin-containing monooxygenase (FMO) family members FMO1 and FMO3. FMO3 exhibited 10-fold higher specific activity than FMO1, and thus plays a major role in TMAO formation (Bennett et al., 2013). Gender is another important factor in the oxidation of TMA. In female mice, most TMA N-oxygenation was catalyzed by FMO3, and in both genders, 11–12% of the TMA was converted to TMAO by FMO1 (Veeravalli et al., 2018). FMO3 was significantly down-regulated by testosterone in mice, suggesting the mechanism why in both humans and mice, the expression of hepatic FMO3 was lower in males than in females (Bennett et al., 2013). Consistent with these results, sterile female mice colonized with TMA-producing bacterial strains had higher plasma TMAO levels and hepatic FMO3 activity than male mice (Romano et al., 2015). Furthermore, FMO3 is regulated by farnesoid X receptor (FXR), a nuclear receptor activated by bile acid (Lefebvre et al., 2009), and injection of FXR ligands induced the expression of FMO3 and the production of TMAO in mice (Bennett et al., 2013).

TMAO levels were significantly associated with body mass index (BMI) in healthy adults with different risk factors (Wang et al., 2016), and studies in humans and mice have shown that plasma TMAO levels increased with ageing (Wang et al., 2014; Li et al., 2017.

POSSIBLE ROLE OF TMAO IN PROMOTING AS

Below, we discuss the TMAO-involved mechanisms of AS from the perspectives of immunity, inflammation, cholesterol metabolism, and atherothrombosis (**Figure 1**).

TMAO and Inflammation-Immunity

AS is a chronic inflammatory disease in which the innate and adaptive immune systems respond to many endogenous and exogenous molecules and highly diverse challenges (Ross, 1999; Belkaid and Hand, 2014). A large number of studies on the mechanism of AS have revealed that the net effect of immune activation is pro-atherogenic. Therefore, at least to some extent, AS should be considered as an autoimmune disease (Shoenfeld et al., 2001; Hansson, 2009). The role of TMAO in the immune mechanism of AS is summarized below (**Figure 1A**).

During the course of disease, autoantigen modification activates innate and adaptive immune responses. Heat shock protein (HSP) is an autoantigen found in atherosclerotic plaques and the blood circulation (Rodriguez-Iturbe and Johnson, 2018). Georg Wick et al. showed that the autoimmune reaction against HSP60 is the initial event of AS (Wick et al., 1995; Wick et al., 2004). Classical risk factors for AS trigger the cellular immune response in macrophages *via* the expression of stress-induced HSP (Wick et al., 2014). HSP shows a high degree of homology among species from microbes to humans (Khandia et al., 2017). Microbial or autologous HSP60 can be bound to endothelial cells *via* Toll-like receptors and serve as targets for autoimmune responses (Ohashi et al., 2000; Wick et al., 2004). Recent studies have demonstrated that gut microbe-dependent TMAO could induce changes in the expression or conformation of HSP. For example, studies performed in murine J774A.1 macrophages showed that TMAO induced stress and led to increased protein expression of GRP94 and HSP70, which may participate in the abnormal activation of macrophages involved in foam cell

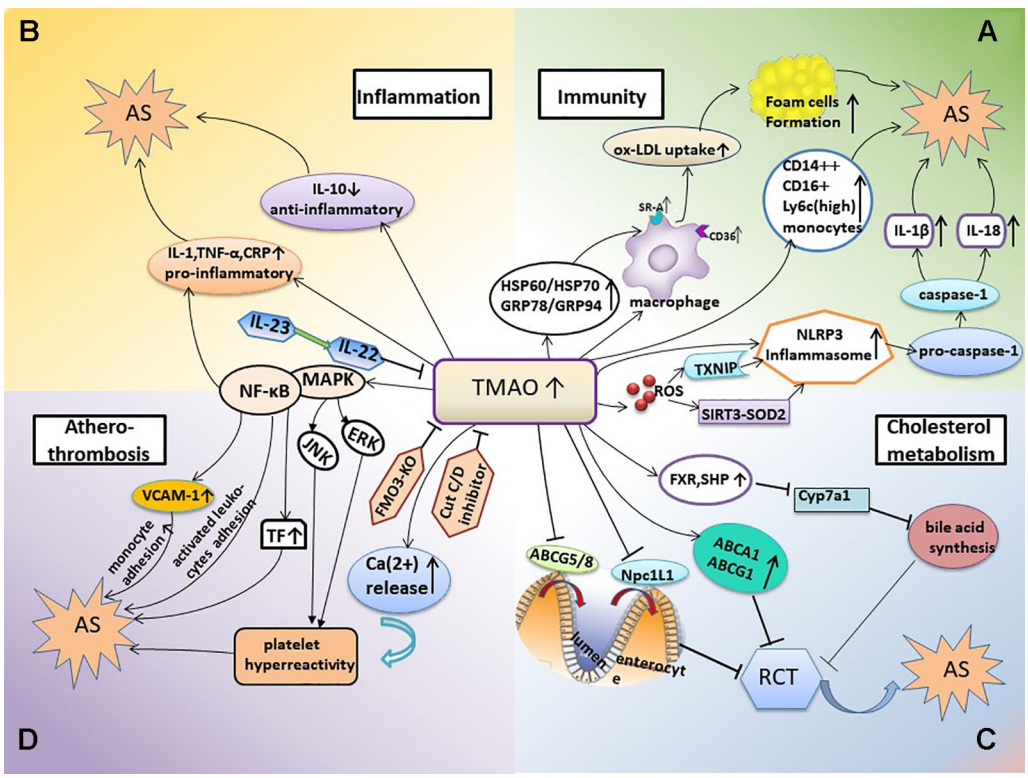

FIGURE 1 | TMAO-involved mechanisms promoting AS. **(A)** TMAO and inflammation-immunity. In this mechanism, elevated TMAO activates the expression of SR-A1 and CD36 in macrophages, thus stimulating the uptake of ox-LDL and foam cell formation; the TMAO-induced increase in HSP expression is also involved in this process. TMAO levels are positively associated with monocyte activation and inflammation. Elevated TMAO levels also induce NLRP3 inflammasome activation and subsequently trigger inflammatory and immune responses. **(B)** TMAO and inflammation. Elevated TMAO levels lead to inflammation, accompanied with increased expression of pro-inflammatory cytokines and decreased expression of anti-inflammatory cytokines. **(C, D)** TMAO also inhibits bile acid synthesis and RCT, contributes to platelet hyperreactivity, and enhances the potential for thrombosis; all of which promote the occurrence of AS. AS, atherosclerosis; HSP, heat shock protein; RCT, reverse cholesterol transport; FXR, farnesoid X receptor; SHP, small heterodimer partner; Npc1L1, Niemann-Pick C1-like1; CRP, C-reactive protein; FMO3-KO, FMO-3 knockout; TF, tissue factor; VCAM-1, vascular cell adhesion molecule-1; JNK, c-JUN NH2-terminal protein kinase; ERK, extracellular signal-regulated kinase.

formation (Mohammadi et al., 2018). Similarly, TMAO increased the mRNA expression of the stress-induced heat shock proteins HSP60 and GRP78, a hallmark of endoplasmic reticulum stress induction, which was related to an increased risk of AS (Mohammadi et al., 2015).

The innate immune system recognizes the conserved structures of pathogens *via* a series of pattern recognition receptors (PRRs), prompting immune cells to respond and secrete inflammatory cytokines (Rock et al., 2010; Schroder and Tschopp, 2010; Takeuchi and Akira, 2010). Scavenger receptor (SRs), which include SR-A and CD36, are a group of typical PRRs on the surface of macrophages that can recognize and engulf oxidized low-density lipoprotein (ox-LDL) (Endemann et al., 1993; Boullier et al., 2001; Getz, 2005; Collot-Teixeira et al., 2007), another important autoantigen involved in AS (Maiolino et al., 2013). This engulfing is not regulated by the negative feedback of intracellular cholesterol. Therefore, lipid-overloaded macrophages turn into foam cells, which is one of the earliest cellular hallmarks of the atherosclerotic process (Bobryshev, 2006; Jin et al., 2018). TMAO activated the expression of SR-A1 and CD36 in macrophages, thus stimulating the uptake of ox-LDL and foam cell formation (Febbraio et al., 2000; Wang et al., 2011). When the production of TMAO was inhibited by antibiotics, the number of macrophages and the formation of foam cells in aortic lesions of ApoE$^{-/-}$ mice were reduced (Wang et al., 2011). Other studies reported that TMAO increased the expression of CD36 and the formation of foam cells induced by ox-LDL, which could be reduced by siRNA-mediated knockdown of CD36 as well as inhibitors of MAPK (SB230580) and JNK (SP600125). This suggested that the CD36/MAPK/JNK pathway may play a key role in TMAO-induced foam cell formation (Geng et al., 2018). Targeted destruction of CD36 also prevented the development of atherosclerotic lesions in mice (Febbraio et al., 2000). However, *in vitro* study found that different concentrations of TMAO have no effect on the formation of foam cells in mouse macrophages (Collins et al., 2016).

The NLRP3 inflammasome is a polyprotein complex formed by the activation of PRRs that was recently reported to be crucial for the development of AS (Duewell et al., 2010; Hoseini et al., 2018). The activated NLRP3 inflammasome converted pro-caspase-1 to active caspase-1, which promoted the maturation and secretion of IL-18 and IL-1β and triggered the inflammatory and immune responses (Martinon et al., 2002; Martinon and Tschopp, 2007; Takahashi, 2014). Notably, studies have shown that elevated TMAO levels induce NLRP3 activation. For example, experiments using carotid artery endothelial cells (CAECs) and wild-type mice with partially ligated carotid arteries showed that TMAO significantly induced NLRP3 inflammasome activation and increased caspase-1 activity, IL-1β production, and cell permeability, which contributed to the endothelial injury that initiates AS (Koka et al., 2016; Boini et al., 2017). This mechanism may be related to both lysosomal dysfunction and redox regulation (Boini et al., 2017). Moreover, the essential of intracellular ROS in activating NLRP3 inflammatory was verified in hyperhomocysteinemia (HHcy) mice model (Wang et al., 2017). Activation of the TMAO-induced endothelial NLRP3 inflammasome was reduced by a mitochondrial ROS scavenger or SIRT3 overexpression in human umbilical vein endothelial cells (HUVECs), which suggested that the activation was mediated in part by inhibition of the SIRT3-SOD2-mtROS signaling pathway (Chen et al., 2017). Other studies proposed that this activation was mediated by the ROS-TXNIP pathway (Zhou et al., 2010; Sho and Xu, 2019). TXNIP is the most widely studied protein which links ROS and NLRP3 inflammasome (Zhou et al., 2010; Lane et al., 2013; Ye et al., 2017). Oxidative stress and activation of the TXNIP-NLRP3 inflammasome were triggered by TMAO, whereat inflammatory cytokines IL-18 and IL-1β were released in a dose- and time-dependent manner (Sun et al., 2016). In addition, studies performed in fetal human colon cells (FHCs) showed that TMAO caused dose- and time-dependent increases in NLRP3 inflammasome activation and ROS production (Yue et al., 2017).

TMAO levels were significantly correlated with the percentage of pro-inflammatory intermediate CD14^{++}CD16^{+} monocytes in ischemic stroke patients (Haghikia et al., 2018). TMAO was also positively correlated with two biomarkers of monocyte activation and inflammation (sCD14 and sCD163) in HIV patients with carotid AS (Shan et al., 2018). More specifically, sCD14 was independently associated with TMAO in untreated HIV-infected subjects (Haissman et al., 2017). Moreover, the numbers of pro-inflammatory murine Ly6C^{high} monocytes were higher in mice fed a choline-rich diet which increased TMAO synthesis than in chow-fed control mice (Haghikia et al., 2018).

TMAO and Inflammation

AS is a chronic inflammatory disease, and inflammation is constantly induced throughout the course of the disease (Ross, 1999; Libby et al., 2002; Tuttolomondo et al., 2012). Several studies have shown increased expression of pro-inflammatory cytokines when plasma TMAO levels were increased. For example, obese mice induced by feeding a western diet, which is a risk factor for AS (Boutagy et al., 2015; Chen et al., 2017), had higher plasma TMAO levels as well as increased expression of pro-inflammatory cytokines, including TNF-α and IL-1β, and decreased expression of the anti-inflammatory cytokine IL-10 (Chen et al., 2017) (**Figure 1B**). Moreover, a study of 271 German adults (Rohrmann et al., 2016) revealed a positive association between the plasma concentration of TMAO and low-grade inflammation. Subjects with elevated plasma TMAO levels had higher plasma levels of TNF-α, sTNF-R p75, and sTNF-R p55; however, there were no differences in the plasma levels of IL-6 and C-reactive protein (CRP). Another study suggested that TMAO levels were positively associated with IL-1β and hsCRP levels in 81 patients with stable angina (Chou et al., 2019). CRP is an inflammatory biomarker that is best validated currently and levels of CRP prospectively assess the risk of AS and atherosclerotic complications and CV risk stratification (Burke et al., 2002; Libby et al., 2002; Libby and Ridker, 2004). Therefore, the relationship between TMAO and CRP needs to be further determined. *In vitro* studies performed in cultured endothelial progenitor cells (EPCs) showed that TMAO promoted cellular inflammation and increased oxidative stress (Chou et al., 2019).

The NF-κB pathway plays a regulatory role in the expression of many AS-related pro-inflammatory genes (Tak and Firestein, 2001;

Baker et al., 2011) (**Figure 1B**). Seldin et al. (2016) demonstrated that TMAO enhanced the expression of inflammatory genes in the aortic endothelium and smooth muscle cells and promoted the adhesion of activated leukocytes to endothelial cells in Ldlr$^{-/-}$ mice fed a choline-rich diet. Pharmacological inhibition suggested that activation of mitogen-activated protein kinase (MAPK) and NF-κB signaling were required for these processes. In addition, TMAO induced several pro-atherogenic inflammatory proteins including cyclooxygenase 2, E-selectin, IL-6 and intracellular adhesion molecule 1 by activating NF-κB (Seldin et al., 2016). NF-κB is also a crucial regulator of atherosclerotic thrombosis. Studies on HUVECs demonstrated that TMAO increased monocyte adhesion, which was partly attributed to upregulation of vascular cell adhesion molecule-1 (VCAM-1) expression by activated protein kinase C (PKC) and p-NF-κB (Ma et al., 2017). Moreover, TMAO increased the expression and activity of tissue factor (TF) *via* activation of the NF-κB signaling pathway in primary human coronary artery endothelial cells (HCAECs), thereby promoting atherothrombosis (Cheng et al., 2019).

Inactivation of the IL-23–IL-22 signal led to a systemic increase of TMAO levels. Fatkhullina et al. (2018) proposed that IL-23 and its downstream target IL-22 repressed AS by inhibiting pro-atherogenic microbiota and microbial metabolites such as TMAO. IL-22 is a characteristic cytokine of Th17 myriad and IL-23 controls the function of Th17 subsets (Buonocore et al., 2010; Peshkova et al., 2016). Though Wang et al. revealed that IL-22 and TH17 pathway could alleviate metabolic disorders in diabetes (Wang et al., 2014), the role of IL-22 and IL-23 in AS remains to be determined.

TMAO and Cholesterol Metabolism

TMAO plays a key regulatory role in lipid metabolism (**Figure 1C**). Studies in mice revealed that dietary supplementation with TMAO, choline, or carnitine decreased reverse cholesterol transport (RCT) (Janeiro et al., 2018), a mechanism that counteracts excess cholesterol deposition in peripheral tissues by transporting excess cholesterol to the liver and small intestine (Chistiakov et al., 2015). Ross and Glomset first proposed that atherosclerotic lesions developed when there was an imbalance between arterial cholesterol deposition and removal after endothelial injury, suggesting a relationship between RCT and AS (Glomset, 1968; Ross and Glomset, 1973).The major pathway for cholesterol elimination is the metabolic synthesis of bile acids in the liver (Zarate et al., 2016), and it was reported that the size of the total bile acid pool was significantly smaller in mice administered TMAO (Koeth et al., 2013). Other studies in ApoE$^{-/-}$ mice demonstrated that TMAO repressed hepatic bile acid synthesis by inhibiting Cyp7a1 expression in the classical pathway of bile acid synthesis and that this inhibition might be mediated by activation of the nuclear receptor FXR and small heterodimer partner (SHP), thus accelerating the formation of aortic AS (Makishima et al., 1999; Ding et al., 2018). In peritoneal macrophages derived from C57BL/6J mice that were exposed to TMAO *in vitro*, the expression of ABCG1 and ABCA1 was modestly increased and cholesterol efflux was detected. Moreover, dietary supplementation with TMAO significantly decreased the expression of both enteral cholesterol transporters Niemann-Pick C1-like1 (Npc1L1), which transports cholesterol into enterocytes from the intestinal lumen, and ABCG5/8, which transports cholesterol out of enterocytes into the gut lumen (Jia et al., 2011; Koeth et al., 2013). However, it is not clear whether the changes in these transporters are involved in the observed systemic decrease in RCT induced by TMAO.

The TMA/FMO3/TMAO pathway driven by gut microbiota is also an important regulator of lipid metabolism (Bennett et al., 2013; Warrier et al., 2015). The TMAO-generating enzyme FMO3 reduced RCT, decreased the intestinal absorption of cholesterol, and changed the composition and size of the bile acid pool (Warrier et al., 2015; Al-Rubaye et al., 2019). Previous studies showed that mice lacking LXR were unable to induce the transcription of gene encoding Cyp7a, confirming the important role of LXR in dietary cholesterol metabolism (Peet et al., 1998; Chiang et al., 2001). In cholesterol-fed mice, knockdown of FMO3 induced macrophage RCT stimulated by liver X receptor (LXR), thus improving the cholesterol balance and protecting against AS (Warrier et al., 2015).

TMAO and Atherosclerotic Thrombosis

In animal models, TMAO has been shown to directly cause AS and thrombosis (Koeth et al., 2013; Chen et al., 2016). The risk of platelet hyperreactivity and thrombosis is increased in many conditions associated with atherosclerotic CVD, such as oxidative stress and hyperlipidemia (Podrez et al., 2007; Chen et al., 2008). Evidence from human and animal model studies suggested one possible mechanism being that TMAO contributed to platelet hyperreactivity and enhanced the potential for thrombosis (Zhu et al., 2016; Zhu et al., 2017) (**Figure 1D**), which were correlated with prospective risk of coronary events and death and the extent of terminal organ injury such as myocardial injury (Trip et al., 1990; Kabbani et al., 2001; Kabbani et al., 2003; Frossard et al., 2004). Direct exposure of platelets to TMAO increased stimulus-dependent activation of platelets *via* multiple agonists by increasing the release of intracellular Ca^{2+} (Zhu et al., 2016). In addition, in pathological conditions of AS and hyperlipidemia, oxLDL activated platelets through a specific CD36-dependent platelet signaling pathway, in which increased activation of MAPK JNK2 and MAPK ERK5 were regard as critical mediators (Chen et al., 2008; Yang et al., 2017; Yang et al., 2018). Studies have confirmed that TMAO plays an important role in inducing foam cell formation and vascular inflammation by up-regulating the MAPK/JNK pathway and MAPK/ERK pathway (Geng et al., 2018; Wu et al., 2019). However, contrary to studies in the general populations, no evidence for TMAO-induced platelet hyperreactivity was detected in HIV-infected individuals (Haissman et al., 2017). Furthermore, as mentioned above, TMAO promoted atherothrombosis by increasing TF expression (Cheng et al., 2019), monocyte adhesion (Ma et al., 2017), and endothelial cell adhesion by activated leukocytes (Seldin et al., 2016), which were mediated by activation of the NF-κB signaling pathway.

Studies of microbial colonization in sterile mice have shown that increased platelet reactivity and thrombosis potential were sufficient to be delivered *via* microbial CutC-dependent TMA/TMAO

production in a host (Skye et al., 2018) (**Figure 1D**). Consistently, oral administration of a CutC/D inhibitor markedly reduced plasma TMAO levels and rescued diet-induced platelet hyperreactivity and thrombosis without significant toxicity or increased bleeding risk (Roberts et al., 2018). Moreover, FMO3-knockout mice showed markedly reduced systemic TMAO levels and thrombosis potential (Shih et al., 2019). Recent studies proposed reducing platelet aggregation and arterial thrombosis *via* targeted suppression of gut microbial proteins associated with TMAO production as a promising therapeutic target (van Mens et al., 2019).

UTILITY OF TMAO FOR PREDICTING CLINICAL RISK AND PROGNOSTIC STRATIFICATION IN AS

TMAO may be a novel predictive biomarker for AS (**Table 1**). It was identified strongly associated with AS in a large independent clinical cohort for CVD (N = 1,876) (Wang et al., 2011). In CHD cohorts, Zhong et al. (2019) found that the plasma concentrations of TMAO, creatinine, choline, and carnitine were notably higher in CHD patients (n = 302) than in those with normal coronary arteries (n = 53). In addition, plasma TMAO was an independent predictor in CHD patients (n = 423) with or without type 2 diabetes mellitus (T2DM) (Dong et al., 2018). It was shown that urinary TMAO, but not its precursors, was correlated with the risk of CHD (n = 275) and may accelerate the development of CHD (Yu et al., 2019). In patients with ST-segment elevation myocardial infarction (STEMI; n = 335), elevated plasma TMAO levels predicted both a high SYNTAX score and the presence of multivessel disease, which were used to quantify the coronary atherosclerotic burden. Thus TMAO was associated with higher coronary atherosclerotic load in STEMI patients (Sheng et al., 2019). In HIV-infected individuals (n = 520), plasma TMAO levels were related to carotid AS progression, and higher TMAO levels were correlated with an enhanced risk of carotid plaques (Shan et al., 2018). Randrianarisoa et al. (2016) reported that serum TMAO levels had positive correlation with carotid intima-media thickness (cIMT), independent of established cardiovascular (CV) risk markers. However, there was no significant association between changes in TMAO levels and changes in cIMT across the population over 10-year follow-up (Meyer et al., 2016). In addition, TMAO levels was similar in subjects with or without carotid atherosclerotic plaques (Yin et al., 2015). TMAO may not significantly promote the risk of early atherosclerotic disease in healthy adults (Meyer et al., 2016).

Prospective cohort studies have shown that increased plasma TMAO levels predicted an elevated risk of MACE in patients with pre-existing AS (**Table 1**). A 3-year follow-up of 4,007 patients undergoing elective coronary angiography revealed a significant association between elevated plasma TMAO levels and increased risk of MACE (stroke, myocardial infarction (MI), or death) (Tang et al., 2013). In patients with chest pain (n = 530) and acute coronary syndromes (ACS) (n = 1683), plasma levels of TMAO and its nutrient precursor TML could predict both the near-term (30-day/6-month) and long-term (1–7-year) risks of CV events (Li et al., 2017; Li et al., 2019). Among patients with stable coronary artery disease (n = 2235) (Senthong et al., 2016), heart failure (n = 720) (Tang et al., 2014) and peripheral artery disease (PAD) (n = 935) (Senthong et al., 2016), elevated TMAO levels could predict 5-year mortality and increased plasma TMAO levels were respectively associated with a 4-fold, 3.4-fold and 2.7-fold increased mortality risk. Moreover, TMAO independently predicted all-cause mortality or reinfarction (death/MI) at 2 years but was not able to predict death/MI at 6 months in patients hospitalized for acute MI (n = 1079). However, TMAO improved risk stratification for death/MI at 6 months by down-classifying the risk of some patients (Suzuki et al., 2017). Another study also demonstrated a graded relationship between plasma TMAO levels and the risk of subsequent CV events among patients presenting with recent prior ischemic stroke (Haghikia et al., 2018). In addition, higher plasma L-carnitine, choline and betaine levels portended increased risks for CVD and incident MACE, but only among individuals with high TMAO levels simultaneously (Koeth et al., 2013; Wang et al., 2014). However this association was not be supported in meta-analysis (Meyer and Shea, 2017). Furthermore, several studies also showed no remarkable association between TMAO and history of CHD or incident CV events or CV mortality (Kaysen et al., 2015; Mueller et al., 2015; Skagen et al., 2016).

In summary, current researches suggest that TMAO levels could be potentially combined with existing risk stratification tools and may offer a novel approach for the prevention and treatment of atherosclerotic disease. Further studies are needed to define the plasma TMAO levels that represent increased risk and establish the correlation between metabolite concentrations and increased CV/MACE risk.

POTENTIAL OF TMAO AS A THERAPEUTIC TARGET IN AS

Several therapeutic strategies to lower TMAO levels that are currently being explored are summarized below (**Figure 2**).

Dietary Control of TMAO and AS

Diet is an important factor affecting TMAO levels and the progression of AS (Bennett et al., 2013). Elevated TMAO levels were observed over a 4-week interval in individuals consuming a high-fat diet (HFD) that is predominantly animal based, compared to individuals consuming a low fat and the MD (Boutagy et al., 2015; Park et al., 2019), but there was no difference in fasting TMAO levels during a 2-week HFD intervention (Boutagy et al., 2015). The MD, a nearly vegetarian diet, was proved to be the strongest evidence for the dietary prevention of major CV events (De Lorgeril et al., 1999; Estruch et al., 2013; Spence, 2018). Greater adherence to MD showed a beneficial role on reducing TMAO levels, CV and overall mortality (Trichopoulou et al., 2003; Sofi et al., 2008; De Filippis et al., 2016). It is noteworthy that gender has a significant influence on MD adherence. In 144 healthy adults with MD, males presented lower adherence to the MD, higher energy intake and higher TMAO levels than females (Barrea et al., 2019). However, another study found no significant changes in fasting TMAO levels after a 6-month intervention with the MD in 115 healthy adults with an elevated risk of colon cancer (Griffin et al., 2019). These findings showed that

TABLE 1 | Human studies of TMAO as a potential novel and independent risk factor for predicting clinical risk of atherosclerosis (AS) and prognostic stratification.

	Study	Patient population	Main findings/outcomes
Positive results	Wang et al., 2011	Subjects undergoing selective cardiac evaluations (N = 1,876)	Elevated levels of fasting choline, TMAO and betaine were dose-dependent associated with the risk of CVD
	(Zhong et al., 2019)	302 with CHD and 59 with NCA in southern China	Plasma concentrations of TMAO, creatinine, choline, and carnitine were notably higher in CHD patients than in those with NCA
	(Dong et al., 2018)	132 controls, 243 with CHD, and 175 with CHD and T2DMv	Plasma TMAO levels were remarkably higher in CHD patients than in controls and were significantly elevated in CHD patients with T2DM; TMAO was an independent predictor in CHD patients with or without T2DM
	(Yu et al., 2019)	275 with CHD and 275 controls	Urinary TMAO, but not its precursors, was correlated with a risk of CHD and may accelerate the development of CHD
	(Sheng et al., 2019)	335 with STEMI and 53 healthy controls	TMAO levels were higher in STEMI; elevated plasma TMAO levels predicted both a high SYNTAX score and the presence of multivessel disease and were associated with higher coronary atherosclerotic load
	(Shan et al., 2018)	520 HIV-infected and 217 uninfected (112 incident plaque cases)	In HIV-infected individuals, higher TMAO levels were correlated with an enhanced risk of carotid plaques
	(Randrianarisoa et al., 2016)	220 subjects in the Tübingen lifestyle intervention program	Newly demonstrated that elevated serum TMAO levels had positive correlation with increased cIMT
	Tang et al., 2013	4007 patients undergoing elective coronary angiography	Elevated plasma TMAO levels were associated with an increased risk of incident MACE.
	(Li et al., 2019)	530 with chest pain (suspected ACS) and 1683 with ACS	Elevated TMAO/TML levels were correlated with MACE over both 30 days and 6 months of follow-up and were also relevant to incident long-term (1-year and 7-year) all-cause mortality
	Tang et al., 2014	720 patients with stable heart failure	Elevated plasma TMAO levels were associated with a 3.4-fold enhanced mortality risk and predicted 5-year mortality risk.
	(Senthong et al., 2016)	2235 with stable CAD; 935 with PAD	Higher plasma TMAO levels were respectively associated with a 4-fold and 2.7-fold enhanced mortality risk in a 5-year follow-up period and could predict 5-year all-cause mortality risk.
	(Suzuki et al., 2017)	1079 with acute MI	TMAO independently predicted death/MI at 2 years, but was not able to predict death/MI at 6 months, and was superior to currently used biomarkers
	(Haghikia et al., 2018)	78 and 593 with recent prior ischemic stroke	Both cohorts showed that higher plasma TMAO levels were related to an increased risk of subsequent CV events
Negative results	Yin et al., 2015	322 patients with atherosclerotic ischemic stroke and TIA and 231 asymptomatic AS controls	Stroke and TIA patients had significantly lower TMAO levels than asymptomatic group, rather than higher. And there was no significant change in blood TMAO levels in asymptomatic atherosclerotic controls.
	Meyer et al., 2016	817 participants	TMAO was not associated with cIMT, a measure of AS, during10-year follow-up.
	(Skagen et al., 2016)	264 with carotid artery AS and 62 healthy controls	No remarkable association between TMAO and CV mortality was found
	Kaysen et al., 2015	235 patients receiving hemodialysis	No obvious association between serum TMAO levels and hospitalizations or CV death and all-cause mortality.
	Mueller et al., 2015	339 patients of suspected CAD.	Plasma TMAO or betaine levels were not associated with the presence of CHD or MI history or incident CV events during 8-year follow-up.

CHD, coronary heart disease; NCA, normal coronary arteries; T2DM, type 2 diabetes mellitus; STEMI, ST-segment elevation myocardial infarction; MI, myocardial infarction; cIMT, carotid intima-media thickness; MACE, major adverse cardiovascular events; ACS, acute coronary syndromes; CAD, coronary artery disease; PAD, peripheral artery disease; TIA, transient ischemic attack.

fasting TMAO levels are not strongly regulated by a moderate modification of the diet (Randrianarisoa et al., 2016).

Lifestyle interventions can also affect plasma and urine levels of TMAO. For example, among 16 obese adults, compared to a eucaloric diet and exercise, a 12-week hypocaloric diet and exercise decreased the percent change in TMAO (Erickson et al., 2019). Moreover, TMAO levels were reduced in 34 prepubertal obese children after a 6-month lifestyle intervention; notably, changes in TMAO concentrations after intervention were not related to choline intake but were negatively correlated with fiber intake (Leal-Witt et al., 2018). However, another study confirmed that the mean TMAO levels did not change during the lifestyle intervention (Randrianarisoa et al., 2016). Recent studies have demonstrated the effect of dietary supplements on TMAO and AS. Samulak et al. (2019) reported that oral L-carnitine supplementation markedly elevated plasma TMAO levels, but did not induce variations in the lipid profile or other adverse CV event markers in healthy older women within 24 weeks. Dietary sphingomyelin (SM) supplementation also remarkably decreased the atherosclerotic lesion area in the aortic arch in chow-fed ApoE$^{-/-}$ mice, reduced serum TMAO levels in C57BL/6 mice, and did not affect circulating SM levels or increase AS in ApoE$^{-/-}$ mice fed a HFD (Chung et al., 2017).

Regulating the Gut Microbiota to Reduce TMAO Levels

Evidence from experimental and clinical studies has confirmed the role of the gut microbiota in TMAO metabolism, providing a theoretical basis for regulating the gut microbiota to control TMAO levels and prevent or treat AS (Wang et al., 2011; Tang and

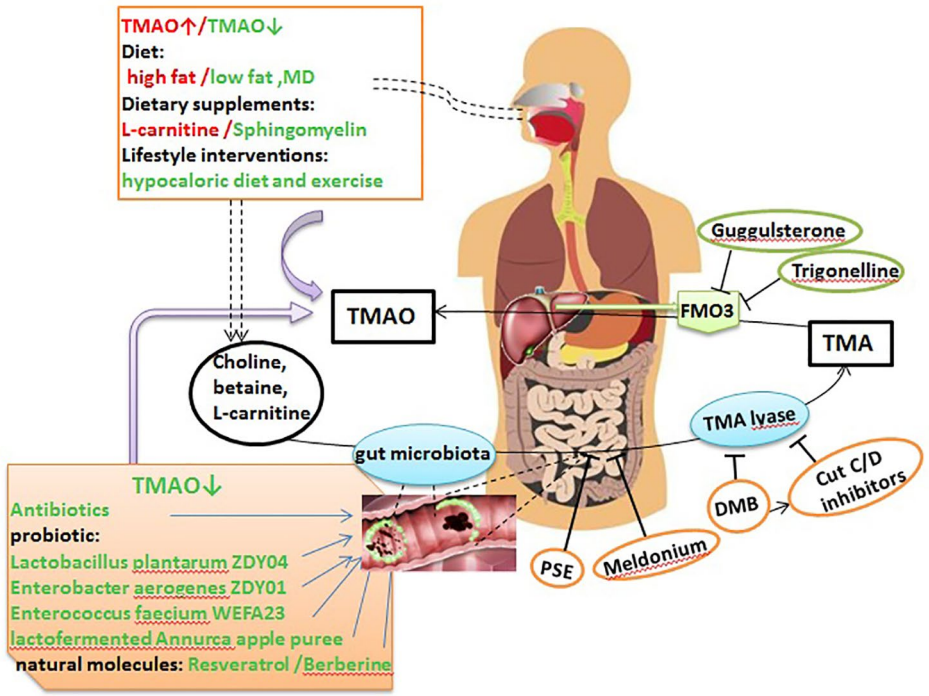

FIGURE 2 | TMAO potential as a therapeutic target in AS. Dietary choline, L-carnitine, betaine, and other choline-containing compounds are the major nutrient precursors of TMAO, and they are metabolized to TMA by the gut microbiota and various enzymes. Then, TMA can be absorbed in the intestines and delivered to the liver through the portal circulation, where it is converted to TMAO by FMO3. TMAO can be targeted as follows: *via* the diet, dietary supplements and lifestyle interventions can significantly affect TMAO levels; antibiotics, probiotics, probiotic functional products, and some natural molecules can markedly decrease TMA and TMAO levels by remodeling the gut microbiota; PSE, meldonium, DMB, and CutC/D inhibitors can suppress the generation of TMA; and trigonelline and guggulsterone can inhibit the conversion of TMA to TMAO by inhibiting FMO3. MD, Mediterranean diet; DMB, 3,3-dimethyl-1-butanol; PSE, plant sterol ester.

Hazen, 2014). The use of broad-spectrum antibiotics is the easiest method to alter the gut microbiota and thereby regulate TMAO levels. In healthy participants, plasma TMAO levels were increased in a time-dependent manner after phosphatidylcholine challenge; these levels were significantly reduced after administration of antibiotics, and then increased after discontinuation of antibiotic administration (Tang et al., 2013). In animal experiments, choline supplementation increased AS nearly 3-fold in male and female mice without antibiotics. In contrast, suppression of the gut microbiota with antibiotics completely inhibited this dietary choline-mediated enhancement in AS (Wang et al., 2011). Ageing also altered the abundance of the intestinal flora, and plasma TMAO levels increased accordingly. Inhibition of the gut microbiota by the addition of broad-spectrum antibiotics to drinking water for 3–4 weeks ameliorated age-related oxidative stress and arterial dysfunction in mice (Brunt et al., 2019). However, it should be noted that the chronic use of antibiotics may have adverse consequences, such as the emergence of antibiotic-resistant bacterial strains and inducing insulin resistance and obesity (Cho et al., 2012; Tang and Hazen, 2014). Thus, additional studies are needed to explore its safety.

The use of probiotics, probiotics and synbiotics, and probiotic functional products is a safer and potentially more effective way to alter the microbiota composition. In animal experiments, administration of the probiotic strains *Lactobacillus plantarum* ZDY04 (Qiu et al., 2018) and *Enterobacter aerogenes* ZDY01 (Qiu et al., 2017) markedly decreased choline-induced cecal TMA and serum TMAO levels by remodeling the gut microbiota in mice. Gut colonization with methanogenic archaea reduced plasma TMAO levels and attenuated the burden of AS, with decreased area and fat content in the atherosclerotic plaques, in Apo $E^{-/-}$ mice fed a TMA-supplemented or high choline diet (Ramezani et al., 2018). *Enterococcus faecium* WEFA23 improved the diversity of the gut microbiota in rats fed a HFD and decreased TMAO production and cholesterol levels (Huang et al., 2018). In human clinical trials, a study of 90 individuals with atherosclerotic CV disease risk factors suggested that Lactofermented Annurca apple puree was an effective functional food that could effectively control plasma TMAO and HDL-C levels (Tenore et al., 2019). Unfortunately, treatment with the multi-strain probiotic VSL#3 in non-obese males (Boutagy et al., 2015) and the probiotic strain *Lactobacillus casei* Shirota in patients with metabolic syndrome did not reduce the increased fasting plasma TMAO levels after a HFD (Tripolt et al., 2015). In general, probiotics that have been shown to effectively lower TMAO levels in human studies are relatively scarce.

Some natural molecules play a protective role against AS, primarily by remodeling the intestinal microbiota. Resveratrol (RSV), a natural plant antitoxin with probiotic activity, was found to attenuate TMAO-induced AS by lowering TMAO levels and increasing hepatic bile acid synthesis by remodeling the gut

microbiota (Chen et al., 2016). Berberine (BBR) has been shown to have antimicrobial effects; in BBR-treated male ApoE$^{-/-}$ mice fed a HFD, the abundances of *Firmicutes* and *Verrucomicrobia* were changed and the expression of hepatic FMO3 and serum TMAO levels were markedly reduced (Shi et al., 2018).

Inhibition of TMA Generation

Choline TMA-lyase (CutC/CutD) and carnitine oxygenase (CntA) are several enzymes that involved in converting dietary compounds into TMA. Rath et al. (Rath et al., 2017) examined the TMA-forming potential of microbial communities and found that *cutC* amplicons were related to various taxa, but that the sequences showed low nucleotide identities to reference sequences, whereas *cntA* amplicons showed high identities to reference sequences, principally sequences from *Escherichia coli*. This provided critical information for the development of particular treatment strategies that inhibit TMA producers. Wang et al. (2015) demonstrated for the first time that 3,3-dimethyl-1-butanol (DMB), a structural analogue of choline, could non-lethally inhibit TMA formation by inhibiting distinct microbial TMA lyases and thus prevent the development of atherosclerotic lesions in ApoE$^{-/-}$ mice. They further modified DMB and developed inhibitors targeting CutC and CutD, the major microbial TMA-generating enzyme pair, and when administered as a single oral dose, significantly decreased plasma TMAO concentrations for up to 3 days and rescued the diet-induced enhancements in platelet reactivity and thrombosis in animal models, without increasing bleeding risk or toxicity (Roberts et al., 2018). In addition, DMB significantly attenuated but did not completely eliminate pulmonary artery AS induced by an 8-week exposure to intermittent hypoxia and hypercapnia (IHC) in ApoE$^{-/-}$ mice and Ldlr$^{-/-}$ mice (Xue et al., 2017). Elevated circulating TMAO levels resulted in endothelial dysfunction in elderly rats, which was reversed by DMB treatment for 8 weeks (Li et al., 2017). Plant sterol esters (PSEs) were also shown to markedly dampen microbial production of TMA, attenuate cholesterol accumulation, and nearly abolish atherogenesis in ApoE$^{-/-}$ mice (Ryan et al., 2017). GBB is a pro-atherogenic intermediate in the conversion of L-carnitine to TMAO controlled by the gut microbiota (Koeth et al., 2014). Meldonium, an analogue of GBB, significantly decreased gut microbiota-dependent TMA/TMAO production from L-carnitine in Wistar rats (Kuka et al., 2014). These results indicate that targeting gut microbial TMA production and the use of non-microbicidal inhibitors are potential therapeutics for AS.

Inhibition of TMA–TMAO Conversion

FMO3 is a critical enzyme in the conversion of TMA to TMAO. It also plays an important role in modulating glucose and lipid homeostasis (Warrier et al., 2015; Shih et al., 2015; Shih et al., 2015), and knockdown or silence of hepatic FMO3 in different mouse strains reduced plasma TMAO levels, altered cholesterol and lipid metabolism, and decreased AS (Bennett et al., 2013; Warrier et al., 2015; Miao et al., 2015; Schugar and Brown, 2015). Furthermore, FMO3 suppression and overexpression were shown to directly impact systemic TMAO levels, platelet reactivity, and thrombosis rates in a murine model of $FeCl_3$-induced carotid artery injury (Zhu et al., 2018). A recent study showed that trigonelline, a compound from *Trigonella foenum-graecum*, inhibited the conversion of TMA to TMAO by inhibiting FMO3. In additon, culturing *Citrobacter freundii* in choline-enriched medium supplemented with trigonelline led to significant reductions in TMA and subsequent TMAO production. In an ex vivo study, TMAO production was reduced by a maximum of 85.3% in the presence of 300 μg/mL trigonelline (Anwar et al., 2018). Furthermore, activation of the nuclear receptor FXR induced the expression of FMO3 and the production of TMAO (Bennett et al., 2013). Recent evidence from *in vitro* and *in vivo* studies showed that guggulsterone, a FXR antagonist, lowered plasma TMA/TMAO levels (Gautam et al., 2018). However, inhibition of FMO3 expression led to a large accumulation of TMA, resulting in trimethylaminuria, which is better known as "fish odor syndrome," and seriously affected patient quality of life (Treacy et al., 1998; Messenger et al., 2013). In addition, since FMO3 plays a systemic role in catecholamine metabolism, inhibiting its function may not be innocuous (DiNicolantonio and McCarty M, 2019).

CONCLUSIONS

AS is a complex disease, which makes characterizing its underlying mechanisms difficult. However, increasing evidence suggests a correlation between TMAO levels and the risk of AS. TMAO might influence AS by activating immune and inflammatory responses, altering cholesterol metabolism, and promoting atherosclerotic thrombosis (**Figure 1**). In addition, elevated TMAO levels are related to an increased risk of incident MACE among patients presenting with AS; thus, the addition of TMAO levels could improve existing risk stratification tools. Finally, TMAO could be used as a novel approach for the prevention and treatment of AS. Current studies have demonstrated that inhibiting various steps of TMAO production can reduce TMAO levels and treat AS (**Figure 2**). However, it is worth noting that inhibition of TMAO may also have adverse effects. We hope that novel TMAO-targeting therapeutic strategies for AS will be established in the near future.

AUTHOR CONTRIBUTIONS

YX and MW designed the manuscript. SY wrote the manuscript. LL revised the manuscript. XinL and FY searched the literature. RZ, XP, JL, LT, and XiaL aided in the design of the illustrations. All authors approved the manuscript for publication.

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Fusobacterium nucleatum Activates Endoplasmic Reticulum Stress to Promote Crohn's Disease Development *via* the Upregulation of CARD3 Expression

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There is increasing evidence that members of the gut microbiota, especially *Fusobacterium nucleatum* (*F. nucleatum*), are associated with Crohn's disease (CD), but the specific mechanism by which *F. nucleatum* promotes CD development is unclear. Here, we first examined the abundance of *F. nucleatum* and its effects on CD disease activity and explored whether *F. nucleatum* aggravated intestinal inflammation and promoted intestinal mucosal barrier damage *in vitro* and *in vivo*. Our data showed that *F. nucleatum* was enriched in 41.21% of CD tissues from patients and was correlated with the clinical course, clinical activity, and refractory behavior of CD ($P < 0.05$). In addition, we found that *F. nucleatum* infection is involved in activating the endoplasmic reticulum stress (ERS) pathway during CD development to promote intestinal mucosal barrier destruction. Mechanistically, *F. nucleatum* targeted caspase activation and recruitment domain 3 (CARD3) to activate the ERS pathway and promote *F. nucleatum*-mediated mucosal barrier damage *in vivo* and *in vitro*. Thus, *F. nucleatum* coordinates a molecular network involving CARD3 and ERS to control the CD process. Measuring and targeting *F. nucleatum* and its associated pathways will provide valuable insight into the prevention and treatment of CD.

Keywords: *Fusobacterium nucleatum*, intestinal mucosal barrier, endoplasmic reticulum stress, Crohn's disease, gene regulation

INTRODUCTION

Crohn's disease (CD) is a chronic and disabling disease that can seriously affect quality of life. The natural history of CD can lead to intestinal damage, such as stenosis, fistula, or bowel resection (Malluta et al., 2019). Goals of therapy include resolution of symptoms and mucosal healing. However, many patients have suboptimal responses to currently available therapies. Therefore, understanding the mechanism of CD is critical to optimizing current treatment strategies.

In recent years, it has been determined that the gut microbiota plays a key role in CD (He et al., 2019). The intestinal flora and the intestinal immune system are always in homeostasis. Breaking this balance can trigger an excessive intestinal immune response and cause the damage to the intestinal mucosal barrier (Nishikawa et al., 2009; Allen-Vercoe and Jobin, 2014). *Fusobacterium nucleatum* (*F. nucleatum*) is a well-known pro-inflammatory bacterium that has been found in many patients with Crohn's disease (Kostic et al., 2013). Studies have shown that *F. nucleatum* is implicated in CD and that strains isolated from inflamed biopsy tissue from CD patients were significantly more invasive than strains that were isolated from healthy tissue from either CD patients or control patients (Cheung and Bellas, 2007; Han et al., 2010; Strauss et al., 2011). However, the effects and mechanisms of *F. nucleatum* on the CD disease process are not well-defined.

The intact intestinal mucosal barrier can prevent intestinal bacteria, toxins, and antigens from entering immune cells in the lamina propria (Actis et al., 2014). Mucosal healing has been considered the best therapeutic endpoint for CD patients because it is associated with sustained clinical remission followed by a lower incidence of hospitalization and surgery (Malluta et al., 2019). Previous studies have shown that in biofilms, *F. nucleatum* can penetrate the epithelial/basement membrane barrier and invade the collagen matrix after incubation if the bacterial biofilm is incubated in contact with cells in an organotypic cell culture model (Gursoy et al., 2010). *F. nucleatum* can invade into mucosa in patients with acute appendicitis and colorectal cancer (Yu et al., 2016). However, the damage to the intestinal mucosa by *F. nucleatum* has not yet been specifically clarified (Kumar et al., 2016).

Endoplasmic reticulum (ER), as a membrane-bound organelle, plays a crucial role in folding of secreted and membrane proteins (Huang et al., 2019). If the levels of the unfolded and misfolded proteins exceed the processing capacity of the ER, ER stress (ERS) occurs (Li et al., 2019). The ER chaperone protein BIP is a major regulatory factor of ER homeostasis and stress response (Li et al., 2016). Many factors can cause ER homeostasis to be disrupted, including bacterial infection (Ma et al., 2019). Studies have found that microbial infection can trigger ERS, and ERS-activating cells can regulate the expression and activation of ERS-related proapoptotic molecules, ultimately determining whether cells are adaptive or undergo apoptosis (Ma et al., 2019). This response allows pro-inflammatory molecules to be released during the chronic inflammation of the CD, leading to damage to the colon cells, and thereby impairing the integrity of the epithelial barrier. It has been found that the endogenous metabolite acrolein induces ERS, mediates epithelial cell death, leads to impaired intestinal epithelial barrier function and increased permeability, and causes the downregulation and/or redistribution of three representative tight junction proteins (i.e., zonula occludens-1, occludin, and claudin-1) that critically regulate epithelial paracellular permeability (Chen et al., 2017; Odenwald and Turner, 2017). This finding indicates that ERS is closely related to the integrity and function of the intestinal mucosal barrier. However, it is unclear whether *F. nucleatum* can induce intestinal mucosal damage by inducing ERS.

In this study, we investigated whether and how *F. nucleatum* affects the integrity of the epithelial barrier in patients with CD. We examined that the *F. nucleatum* abundance in colon tissue from patients with active CD was increased compared to that in tissues from healthy controls or patients with remitted CD. We then demonstrated that *F. nucleatum* plays a key role in mediating CD development by upregulating caspase activation and recruitment domain 3 (CARD3) and activating the ERS pathway.

MATERIALS AND METHODS

Collection of Clinical Samples

The patient materials used in this study were obtained from Wuhan University People's Hospital (Hubei, China). All participants provided informed consent, and the project was approved by the institutional review board (approval number: 2018K-C089). Inflamed intestinal biopsies were obtained from CD patients undergoing intestinal endoscopy. Normal tissue biopsies were obtained from healthy controls ranging in age from 15 to 65 years (to match the age of patients with CD) who underwent an endoscopy for colon cancer screening without a prior diagnosis of gastrointestinal illness. CD diagnosis was confirmed in conjunction with clinical and histological criteria. Clinical disease activity was assessed by the Harvey-Bradshaw activity index (HBI) and the colitis activity index. CD patients with an HBI of ≤4 were considered to be in remission, those with ≥5 to have active disease. Exclusion criteria included patients with previous inflammatory bowel disease (IBD) treatment, receiving antibiotics or probiotics in the last 12 weeks, receiving biologicals or immunosuppressants within the past 2 years, history of fecal microbiota transplant (FMT), age <15 years, presentation of other known chronic diseases, and pregnant or breastfeeding. Formalin-fixed, paraffin-embedded CD intestinal tissues were obtained from the pathology archives from June 2016 to June 2018. Clinicopathological data for each patient were obtained from hospital records.

Bacterial Strains and Cell Lines

The human normal epithelial cell line NCM460 and the FHC cell line ATCC were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. *F. nucleatum* was grown on tryptic soy containing 5% defibrinated sheep blood, under anaerobic conditions (10% H_2, 5% CO_2, and 85% N_2), with a 2-day anaerobic gas filling system at 37°C. *Escherichia coli* strain (Tiangen, China) was cultured in Luria-Bertani (LB) medium for 16 h at 37°C with shaking at 200–220 rpm. The *F. nucleatum* suspension was centrifuged at 2,500 × g for 5 min and was then resuspended in antibiotic-free DMEM before being used to infect normal epithelial cells. *E. coli*-infected cells were used as the control.

Mice

Five- to six-week-old male C57BL/6J CARD3 knockout (KO, $CARD3^{-/-}$) mice were kindly provided by Dr. Richard Flavell (Howard Hughes Medical Institute, Yale University, New Haven,

CT). CARD3 KO mice were backcrossed with C57BL/6 mice for at least six generations to yield CARD3 heterozygous mice. Then, the littermate offspring [card3 KO and wild-type (WT) mice] produced by inbreeding CARD3 heterozygous mice were used for further study. Five- to six-week-old male C57BL/6J WT (CARD3wt) mice were obtained from Nanjing Biomedical Research Institute of Nanjing University (NBRI). Mice were housed and bred in our specific pathogen-free animal facility (Yang et al., 2017). All animal procedures were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University, China.

Induction of Colitis

All mice were fed 2 mg/ml streptomycin for 3 days in their drinking water to ensure consistency of the conventional microbiota and promote *F. nucleatum* colonization. Mice were administered a daily dose of *F. nucleatum* cells [10^9 colony-forming units (CFUs)/ml] resuspended in phosphate-buffered solution (PBS) or PBS alone for 2 weeks. To induce colitis, 3% (wt/vol) dextran sulfate sodium (DSS) (wt. 36–50 kDa; MP Biomedicals) was added to the drinking water of the mice (Danese et al., 2007). Animal weight, water/food consumption, morbidity, stool consistency, and the presence of large amounts of blood in the feces or anus were measured or observed once daily. On the seventh day after induction with DSS, animals were quickly euthanized by inhalation of CO_2, the colon and cecum were quickly separated, the colon was photographed and used for length measurement, and feces and blood were gently removed with 4°C PBS. A small segment of the colon was fixed in paraformaldehyde for histological staining (H&E) and fluorescence *in situ* hybridization (FISH), and another portion of the tissue was immediately frozen in liquid nitrogen for PCR or Western blot (WB) analysis.

Inhibition of Endoplasmic Reticulum Stress With 4-Phenyl Butyric Acid

In the 4-phenyl butyric acid (4-PBA) treatment study, mice were injected intraperitoneally with 4-PBA every 3 days at a dose of 100 μg per mouse, and *F. nucleatum* (10^9 CFU/ml) resuspended in PBS were administered intragastrically 1 h after 4-PBA injection (Wu et al., 2018). And these mice were treated with DSS and continued for another week. Similarly, NCM460 cells were treated with 4-PBA and were then infected with *F. nucleatum* (MOI = 100) 1 h later.

RNA Extraction and Real-Time PCR

Total RNA was extracted using TRIpure Total RNA Extraction Reagent (ELK Biotechnology), and real-time PCR with three replicate wells per sample was performed on a StepOne™ Real-Time PCR machine (Life Technologies), using an EnTurbo™ SYBR Green PCR SuperMix kit (ELK Biotechnology, EQ001). Real-time quantitative PCR was performed in triplicate. The Ct values obtained from different samples were compared using the ΔΔCt method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal reference gene.

Fluorescence *in Situ* Hybridization

Microbial FISH was performed as described (Yu et al., 2016). Five-micrometer-thick sections were prepared and hybridized following the manufacturer's instructions (FOCOFISH, Guangzhou, China). The sequence of the "universal bacterial" probe (EUB338; Cy3 labeled) was 5′-GCT GCC TCC CGT AGG AGT-3′. The sequence of the *F. nucleatum*-targeted probe [FUS664; fluorescein isothiocyanate (FITC) labeled] was 5′-CTT GTA GTT CCG C(C/T) TAC CTC-3′. Slides were examined using a microscope (BX53F; Olympus, Tokyo, Japan). Five random 200× magnification fields per sample were evaluated by three observers blinded to the experimental protocol, and the average number of bacteria per field was calculated. We defined negative, low, or high abundance of *F. nucleatum* as those cases with <5, between 5 and 20, and >20 visualized FUS664 probes per field on average, respectively. Other bacteria were noted as positive with >5 bacteria per field with EUB 338 probe but negative with FUS664 probe.

High-Throughput Sequencing

DNA or RNA was sent to Adaptive Biotechnology (HuaDa, China) for sequencing. The DNA sequence data have been deposited in the NCBI Sequence Read Archive (SRA) database (accession number: PRJNA541040).

Cell Transfection

siRNA targeting the human CARD3 gene (siCARD3) and nontargeting siRNAs (control siRNAs) were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells were cultured and transfected with siRNAs according to the supplier's instructions. Lipid-based transfections were achieved with Lipofectamine 6000 (Beyotime, China) according to the manufacturer's protocol. Cells were incubated with the siRNA complex for 72 h, and protein was extracted for assessing transfection efficiency by WB.

Immunohistochemistry and Western Blotting

Immunohistochemistry (IHC) was performed using an UltraSensitive™ SP (mouse/rabbit) IHC kit (Maxib, Fuzhou, China) according to the manufacturer's instructions. For WB, primary antibodies against the following targets were used: CARD3 (CST), BIP (CST), XBP1 (Abeam), ZO-1 (Abeam), occludin (Abeam), and GAPDH (Bioworld).

Statistical Analyses

Statistical analysis was performed using GraphPad Prism software version 8.0. Data are expressed as the means ± SDs. Normally distributed data were analyzed by Student's *t* test. Differences among multiple groups were evaluated for significance using one-way ANOVA combined with Bonferroni's *post hoc* test. Statistical significance was defined as $P < 0.05$.

RESULTS

F. nucleatum Is Abundant in Crohn's Disease Tissues and Is Linked to Disease Severity

Previous studies have shown that the invasive potential of gut mucosa-derived *F. nucleatum* positively correlated with CD

status of the host (Zhou et al., 2016; Dinakaran et al., 2018). To verify the potential relationship between gut microbiota alterations and CD, we compared three inflamed tissues from patients with active CD (ACD) and three inflamed tissues from patients with remitted CD (RCD) by HiSeq 2500 sequencing. We also found that *F. nucleatum* was enriched in ACD tissues ($P < 0.05$; **Figures 1A, B**). We further examined the abundance of invasive *F. nucleatum* in 33 CD tissues (RCD = 11; ACD = 22) from patients and in 10 normal tissues using FISH. *F. nucleatum* was detected in a significantly higher percentage of CD tissues (41.21%) than control normal tissues (10%; $P < 0.05$; **Figure 1C**). Moreover, *F. nucleatum* was detected in a significantly higher percentage of ACD tissues (46.71%) than RCD tissues (13.52%; $P < 0.05$; **Figure 1C**). This result suggests that invasive *F. nucleatum* is present in CD tissues. We then evaluated the relationship between the abundance of *F. nucleatum* and clinicopathological features as shown in **Table 1**. The abundance of *F. nucleatum* was positively associated with the clinical course, clinical activity, and refractory behavior ($P < 0.05$). Thus, these data defined the potential value of the abundance of *F. nucleatum* in predicting CD activity.

F. nucleatum Destroys Epithelial Barrier Function *In Vitro* and *In Vivo*

We hypothesized that *F. nucleatum* infection may enhance the breakdown of intestinal epithelial barrier function in CD. To test this hypothesis, we incubated NCM460 cells (**Figure 2A**, **Supplementary Figures S1 A–D**) and FHC cells (**Figure 2B**, **Supplementary Figures S1 E–H**) with *F. nucleatum* (ATCC10953), DH5α, or PBS (Control, Con). Compared to DH5α and PBS treatment, *F. nucleatum* treatment downregulated the levels of ZO-1 and occludin in a time-dependent manner, suggesting that *F. nucleatum* may destroy epithelial barrier formation by interfering with tight junction protein expression at the protein level.

To investigate the roles of *F. nucleatum* in the development of colitis, we used a DSS-induced colitis model. Mice treated with *F. nucleatum* + DSS exhibited more severe colitis symptoms, including rapid weight loss (**Figure 2C**) and higher disease activity index (DAI) values (**Figure 2D**), than mice treated with DSS or *F. nucleatum* alone. The colon length was measured to determine the extent of colonic injury, and we found that the colons of *F. nucleatum* + DSS group mice were clearly shorter than those of DSS group mice (**Figures 2E, G**). Additionally, *F. nucleatum* enhanced epithelial damage, including mucosal erosion, crypt loss, and lymphocyte infiltration (**Figure 2F**). Consistent with these observations, histological assessment of the colons revealed a significantly higher histological score (HS) (**Figure 2H**) and more severe disease and disruption of mucosal structures in *F. nucleatum* + DSS-treated mice than in DSS-treated mice. *F. nucleatum*-treated mice exhibited a mild inflammatory phenotype (**Figures 2C–H**), suggesting that *F. nucleatum* may exacerbate the clinical and histological features of DSS-induced colitis. The high abundance of *F. nucleatum* was often accompanied by low levels of ZO-1 and MUC2 compared with those in normal tissues (PBS group) ($P < 0.05$; **Figure 2F**). In addition, the WB results showed lower levels of ZO-1 and occludin in colitis tissues from *F. nucleatum* + DSS-treated mice than in tissues from mice in the other groups (**Figure 2I**, **Supplementary Figures S1I, J**). Taken together, these results indicate that *F. nucleatum* possibly disrupt the mucosal barrier *in vivo* and *in vitro*.

F. nucleatum Damages the Mucosal Barrier *via* ER Signaling in Intestinal Epithelial Cells

To examine whether the ER signaling pathway could be activated by *F. nucleatum* infection, we measured the expression levels of BIP and XBP1 using WB. Compared to DH5α and PBS (Control, Con), *F. nucleatum* treatment upregulated BIP and XBP1 expression in NCM460 cells (**Figure 3A**, **Supplementary Figures S1 K–N**) and FHC cells (**Figure 3**, **Supplementary Figures S2 A–D**) in a time-dependent manner. Additionally, compared to DH5α and PBS treatment, *F. nucleatum* increased the mRNA levels of BIP (**Figure 3C**) and XBP1 (**Figure 3D**) in NCM460 cells ($P < 0.05$), suggesting that *F. nucleatum* may activate the ER pathway in intestinal epithelial cells.

We hypothesized that *F. nucleatum* destroys the intestinal mucosal barrier through the ER pathway. To test this hypothesis, we treated NCM460 cells with 4-PBA (10 μg/ml) or PBS 1 h before *F. nucleatum* (MOI = 100) treatment. Many investigations indicate that 4-PBA acts as a chemical chaperone that attenuates ERS in different cell types (Kolb et al., 2015). Our WB analysis showed that pretreatment with 4-PBA downregulated BIP and XBP1 expression, and blockade of the ER pathway by 4-PBA significantly attenuated the *F. nucleatum*-mediated decrease in ZO-1 and occludin ($P < 0.05$; **Figure 3E**, **Supplementary Figures S2 E–H**). These data indicate that *F. nucleatum* potentially damages the intestinal mucosal barrier *via* ER signaling in epithelial cells.

F. nucleatum Facilitates Intestinal Mucosal Barrier Destruction Through the ER Pathway in DSS-Induced Mice

To assess whether the ER pathway contributes to *F. nucleatum*-induced colitis, 4-PBA was administered to mice with DSS-induced colitis. Mice treated with *F. nucleatum* + DSS + 4-PBA exhibited a slower decline in body weight ($P < 0.05$; **Figure 4A**), a lower DAI ($P < 0.01$; **Figure 4B**), a significantly lower HS ($P < 0.01$; **Figure 4E**), and milder colitis (**Figure 4D**) than mice treated with *F. nucleatum* + DSS. DSS-induced or *F. nucleatum* + DSS-induced colon shortening was mitigated in mice administered 4-PBA ($P < 0.01$; **Figures 4C, F**). Moreover, WB showed that inflamed tissues from *F. nucleatum* + DSS + 4-PBA-treated mice exhibited lower levels of BIP and XBP1 and higher levels of ZO-1 and occludin than tissues from *F. nucleatum* + DSS-treated mice (**Figure 4G**, **Supplementary Figures S2 I–L**). Collectively, these data suggest that *F. nucleatum* possibly destroy the intestinal mucosal barrier at least in part *via* the ER pathway in mice.

F. nucleatum Upregulates CARD3 Expression

CARD3 is known for its role in inflammation (Irving et al., 2014). Previous studies have found that genetic loss of CARD3 is

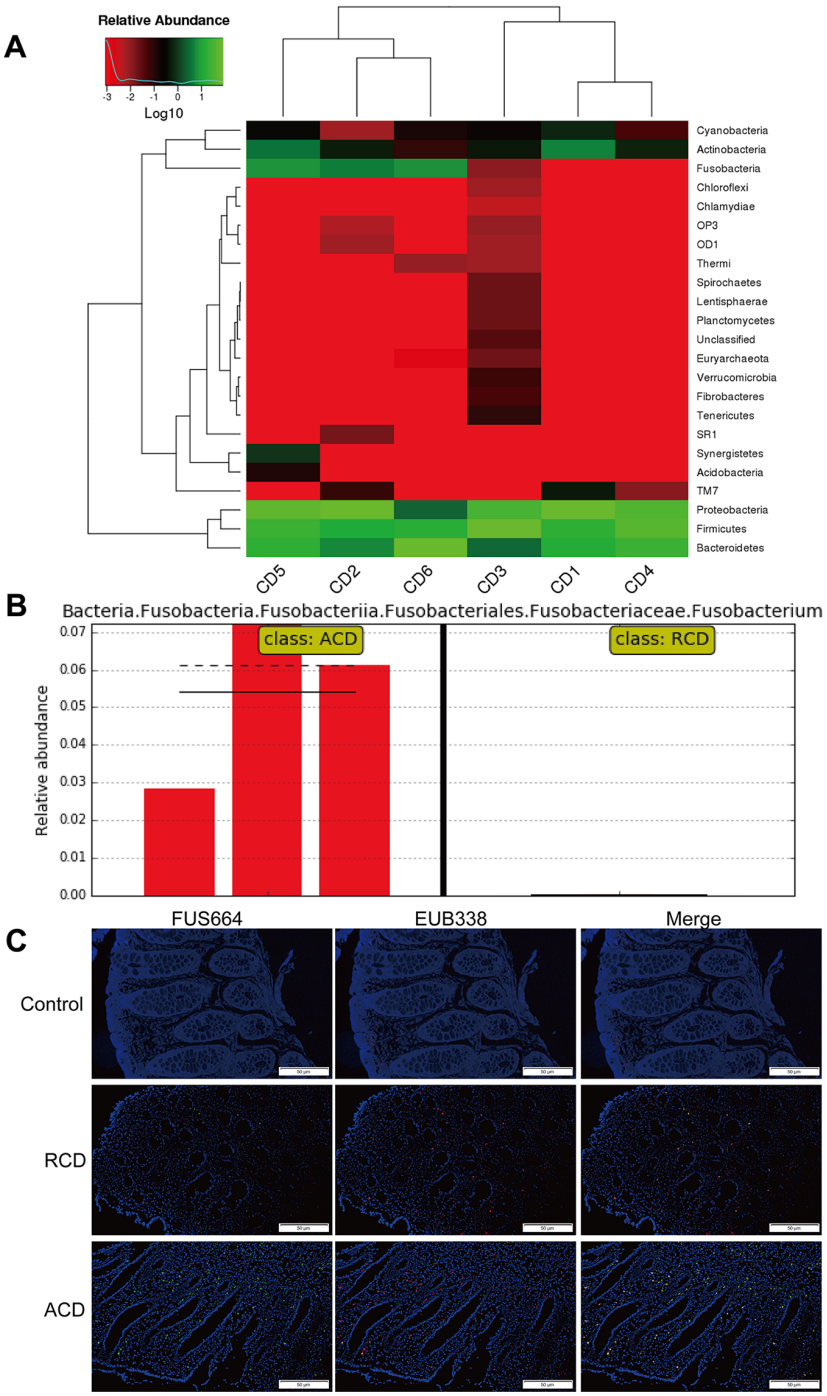

FIGURE 1 | *Fusobacterium nucleatum* is associated with Crohn's disease (CD) activity. **(A)** Hierarchically clustered heat map representing bacterial taxa (genus level) in six CD tissues from patients [active CD (ACD) = 3, remitted CD (RCD) = 3] by 16S rDNA sequencing. The relative percentages of bacteria are indicated by varying color intensities. Species with an abundance of less than 0.5% in all samples were classified as "Others." ACD: CD2, CD5, CD6; RCD: CD1, CD3, CD4. **(B)** The LEfSe algorithm was used to identify *Fusobacterium* in ACD and RCD tissues from patients. **(C)** Representative fluorescence *in situ* hybridization (FISH) images to assess the *F. nucleatum* abundance in ACD (n = 22), RCD (n = 11), and healthy tissues (n = 10). EUB338 (red) is a Cy3-conjugated universal bacterial oligonucleotide probe; FUS664 (green) is a fluorescein isothiocyanate (FITC)-conjugated *F. nucleatum*-specific oligonucleotide probe. Magnification, 200×. The sequence of the FITC-labeled Fn-targeted probe, FUS664, was: 5′- CTT GTA GTT CCG C(C/T) TAC CTC -3′.

TABLE 1 | Clinicopathologic characteristics in *Fusobacterium. nucleatum*-negative vs. *F. nucleatum*-positive CD.

Characteristics	*F. nucleatum*-negative (n = 15)	*F. nucleatum*-positive (n = 18)	*P* value[a]
Gender			
Male	6	12	0.126
Female	9	6	
Age			
≤16	0	1	0.505
≤40	11	11	
>40	4	7	
Clinical course			
Moderate	7	13	0.035*
Severe	0	2	
Remission	8	3	
Location			
L1	5	7	0.731
L2	4	3	
L3	6	7	
L4	0	1	
Behavior			
B1	7	5	0.495
B2	5	7	
B3	3	6	
Perianal disease			
Yes	0	4	0.405
No	0	14	
Surgery			
Yes	3	11	0.017*
No	12	7	

[a]*Chi-square test, *P < 0.05.*
CD, Crohn's disease.

protective against colitis through decreased epithelial cell apoptosis and consequent enhancement of intestinal epithelial barrier function (Yu et al., 2015). We hypothesized that CARD3 may be a target of *F. nucleatum* in disease development. To verify this hypothesis, we treated NCM460 cells with *F. nucleatum* (ATCC10953), DH5α, or PBS (Control, Con). Compared to DH5α and PBS treatment, *F. nucleatum* treatment increased the levels of CARD3 in a time-dependent manner (**Figure 5A**), suggesting that *F. nucleatum* may regulate CARD3 expression at the protein level. We next used FISH to visualize the amount of *F. nucleatum*, as well as the expression of CARD3, in inflamed tissues from CD patients. A high abundance of *F. nucleatum* in inflamed colon tissues was often accompanied by a high level of CARD3 expression ($P < 0.05$; **Figures 5B, C**). These data support the hypothesis that CARD3 is probably a downstream target of *F. nucleatum*.

F. nucleatum Modulates the ER Pathway *Via* Card3 Upregulation *In Vitro* and *In Vivo*

The increase in CARD3 expression in *F. nucleatum*-infected NCM460 cells led us to hypothesize that CARD3 may regulate *F. nucleatum*-mediated ER pathway activation. To test this hypothesis, we transfected NCM460 cells with CARD3-targeting siRNA (siCARD3) or nontargeting siRNAs (NC) (**Supplementary Figures S3 A, B**). Knockdown of CARD3 suppressed the *F. nucleatum*-induced increase in the protein levels of XBP1 and the *F. nucleatum*-induced decrease in the expression of ZO-1 expression ($P < 0.05$; **Figure 5D**, **Supplementary Figures S3 C, D**), highlighting the role of CARD3 in *F. nucleatum*-mediated ER pathway activation in NCM460 cells.

To further confirm our hypothesis that *F. nucleatum* promotes colitis progression through ER activation *via* the upregulation of CARD3 expression, we employed CARD3 knockout (KO, $CARD3^{-/-}$) mice. Both CARD3 WT ($CARD3^{wt}$) and $CARD3^{-/-}$ mice were initially administered *F. nucleatum* and were then subjected to DSS treatment. $CARD3^{-/-}$ mice exhibited slower body weight loss ($P < 0.01$; **Figure 6A**), a lower DAI ($P < 0.05$; **Figure 6B**), a significantly lower HS ($P < 0.05$; **Figure 6C, F**), a shorter colon length ($P < 0.05$; **Figures 6C–F**), and milder colitis (**Figure 6D**) than $CARD3^{wt}$ mice. After knockout of CARD3, treatment with 4-PBA or both, DSS-induced cecal edema, colon shortening and colitis were significantly suppressed ($P < 0.05$; **Figures 6C–F**). Within lesions, the levels of BIP and XBP1 were decreased, and the expression of ZO-1 was increased in $CARD3^{-/-}$ mice (**Figure 6G**, **Supplementary Figures S3 F–I**). Taken together, our data show that $CARD3^{-/-}$ mice are less susceptible to gut inflammation and *F. nucleatum* infection than $CARD3^{wt}$ mice, suggesting a role for CARD3 in the regulation of *F. nucleatum*-induced intestinal mucosal barrier destruction.

DISCUSSION

Recent studies have shown that *F. nucleatum* in the gut microbiota is involved in the development of colitis. Infection with *F. nucleatum* is often observed in patients with CD (Allen-Vercoe et al., 2011; Kaakoush et al., 2012; Purcell et al., 2018). Similarly, our high-throughput sequencing has demonstrated an increased abundance of *F. nucleatum* in colon tissues from CD patients. *F. nucleatum* infiltrated the epithelium and mucosa and is associated with the clinical activity and severity of colitis (Swidsinski et al., 2011; Strauss et al., 2011). We confirmed that *F. nucleatum* invades CD tissue and is associated with the degree of clinical activity and refractory behavior of CD. In addition, in DSS-induced colitis models, we used oral administration of *F. nucleatum* before the induction of acute colitis with DSS to initially assess the precise role of *F. nucleatum* in IBD. Through a combination of biological methods, *in vivo* models, and clinical studies, we demonstrated that *F. nucleatum* was enriched in CD tissues from patients and exacerbated colonic inflammation during CD development.

It has been confirmed that *F. nucleatum* contributes to the carcinogenesis of colorectal cancer by inducing inflammation and inhibiting host immunity (Feng et al., 2019; Wu et al., 2019). There is evidence that *F. nucleatum* induces the production of microbial peptides that upregulate the expression of proinflammatory cytokines and tumor necrosis factor and disrupt homeostasis and host defense barriers (Krisanaprakornkit et al., 2000; Repass et al., 2018). An *F. nucleatum* strain isolated from IBD patients showed upregulation of monocyte chemoattractant protein

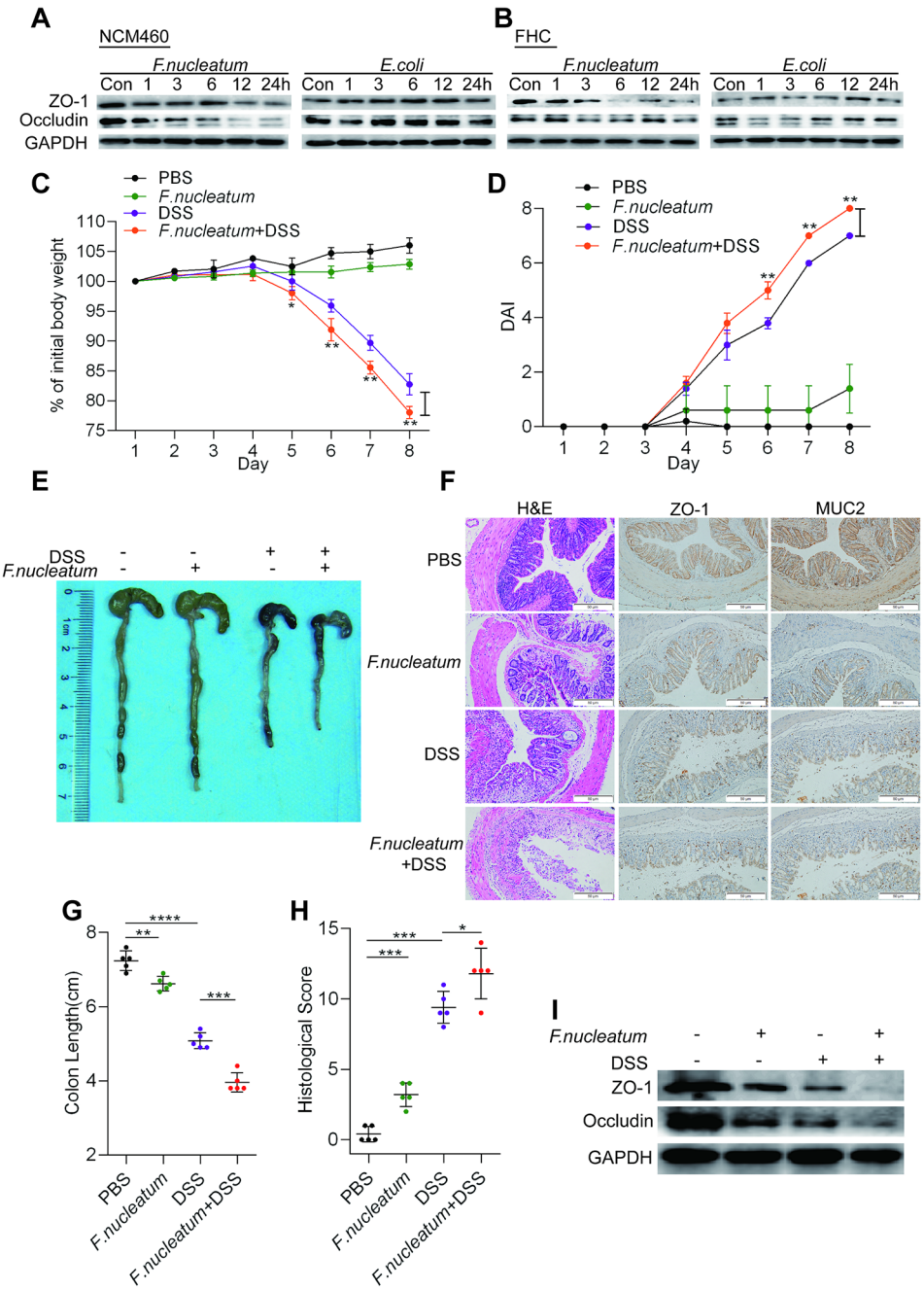

FIGURE 2 | *Fusobacterium nucleatum* destroys epithelial barrier function *in vitro* and *in vivo*. **(A, B)** Western blotting was performed to measure the expression of ZO-1 and occludin in NCM460 cells **(A)** and FHC cells **(B)** cocultured with *F. nucleatum*, *Escherichia coli*, or phosphate-buffered solution (PBS) (Control, Con). **(C, D)** Mice (n = 5 per group) were administered *F. nucleatum* or PBS for 2 weeks and treated with 3% dextran sulfate sodium (DSS) for 7 days. Colitis induction was evaluated by body weight loss **(C)** and the disease activity index (DAI) **(D)**. (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; one-way ANOVA combined with Bonferroni's *post hoc* test; the error bars indicate the SDs). **(E–G)** Representative colon morphology and length in the mice are shown in panel **(E)** and quantified in panel **(G)**. Representative images of histological analyses are shown in panel **(F)** and quantified in panel **(H)**. Representative images of MUC2 and ZO-1 expression are shown in panel **(F)** (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; unpaired Student's *t* test; the error bars indicate the SDs; 200× magnification). **(I)** Western blotting was performed to measure ZO-1 and occludin expression in mouse tissues.

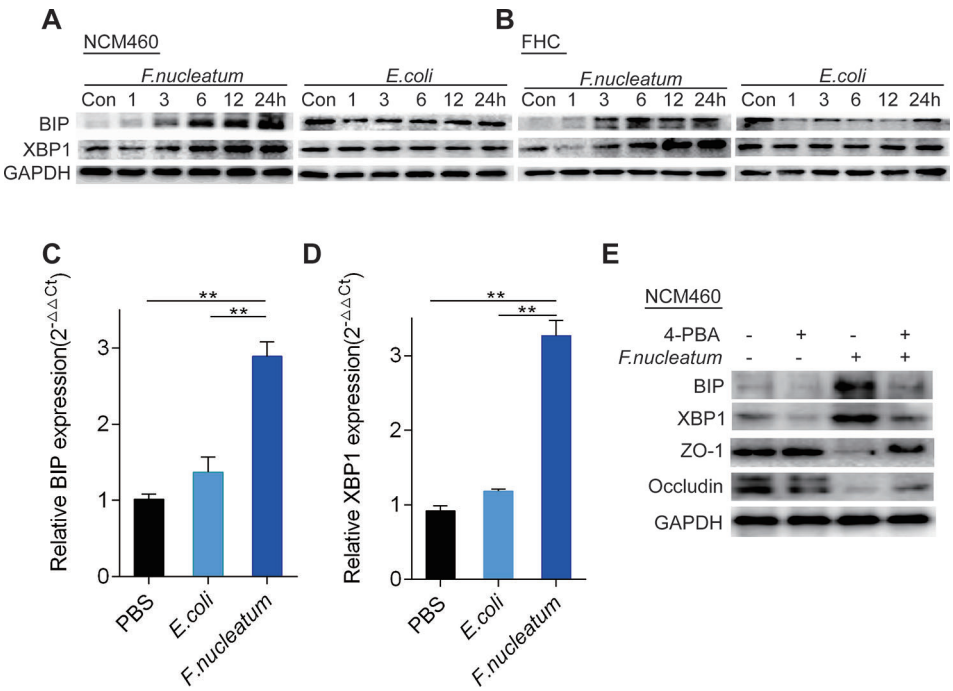

FIGURE 3 | *Fusobacterium nucleatum* activates the endoplasmic reticulum (ER) pathway and damages mucosal barrier-associated proteins *via* ER signaling in intestinal epithelial cell lines. **(A, B)** Western blotting was performed to measure BIP and XBP1 expression in NCM460 cells **(A)** and FHC cells **(B)** cocultured with *F. nucleatum*, *Escherichia coli*, or phosphate-buffered solution (PBS) (Control, Con). **(C, D)** The mRNA expression of BIP **(C)** and XBP1 **(D)** was measured in NCM460 cells cocultured with *F. nucleatum, E. coli*, or PBS (*$P < 0.05$, **$P < 0.01$; unpaired Student's t test; the error bars indicate the SDs). **(E)** Western blotting was performed to measure BIP, XBP1, ZO-1, and occludin expression in NCM460 cells cocultured with *F. nucleatum*, 4-phenyl butyric acid (4-PBA), or both.

(MCP)-1 and tumor necrosis factor (TNF)-α in an experimental model (McCoy et al., 2013). However, it is unclear how *F. nucleatum* mediates intestinal inflammation during the development of CD. We examined whether *F. nucleatum* induced the downregulation of membrane-associated proteins (ZO-1 and occludin), which are markers of intestinal mucosal barrier function. We found that *F. nucleatum* infection was negatively correlated with the expression of intestinal mucosal barrier-associated proteins (ZO-1 and occludin) in human epithelial cell lines and human colon tissues and significantly downregulated the expression of intestinal mucosal barrier proteins in a mouse model of DSS-induced colitis. *F. nucleatum* infection can disrupt intestinal mucosal function and has the potential contribution to induce associated colitis.

Next, we investigated the mechanism by which *F. nucleatum* causes intestinal mucosal barrier damage. Studies have shown that when ERS occurs, cells initiate a caspase12-dependent apoptosis program, which triggers the destruction of the intestinal mucosal barrier (Ge et al., 2019). We found that *F. nucleatum* infection significantly increased the expression of ERS-related proteins (BIP and XBP1), which are key regulators of ERS, *in vivo and in vitro* but that by inhibiting ERS with 4-PBA, the intestinal mucosal barrier function and the severity of colitis were significantly mitigated. Therefore, we concluded that *F. nucleatum* may induce intestinal mucosal damage partly by activating the ERS pathway to mediate CD, and colitis may be relieved under the treatment of 4-PBA. The majority of investigations suggest that 4-PBA acts as a chemical chaperone that attenuates ERS in different cell types (Kolb et al., 2015). But whether 4-PBA can act *via* different routes not just as an ERS inhibitor remains to be verified.

In addition, we investigated the mechanism by which *F. nucleatum* mediates the activation of the ERS pathway. Studies have reported that microbes can induce a CARD-dependent inflammatory response in epithelial cells (McCoy et al., 2013; Wang et al., 2013; Li et al., 2015). Previous studies have found that genetic loss of CARD3 is protective against colitis through decreased epithelial cell apoptosis and consequent enhancement of intestinal epithelial barrier function (Tigno-Aranjuez et al., 2014; Yu et al., 2015). Our previous report has found that the expression of NOD2 was upregulated in intestinal epithelial cells infected with *F. nucleatum* (Chen et al., 2019). Thus, we choose CARD3 as the downstream molecule of *F. nucleatum*. In this study, we found that CARD3 expression in CD patients was higher than that in healthy controls and that the abundance of *F. nucleatum* was positively correlated with the expression of CARD3. Some studies have shown that CARD3 is a nucleotide oligomerization domain (NOD)-independent nodal point of gut inflammation (Panda and Gekara, 2018). Decreased expression of NOD1/2 and interaction between NOD1/2 and CARD3 can decrease the severity of ERS (Zhang et al., 2016). It is clear that NOD1/2 and CARD3 are important mediators of ERS-induced

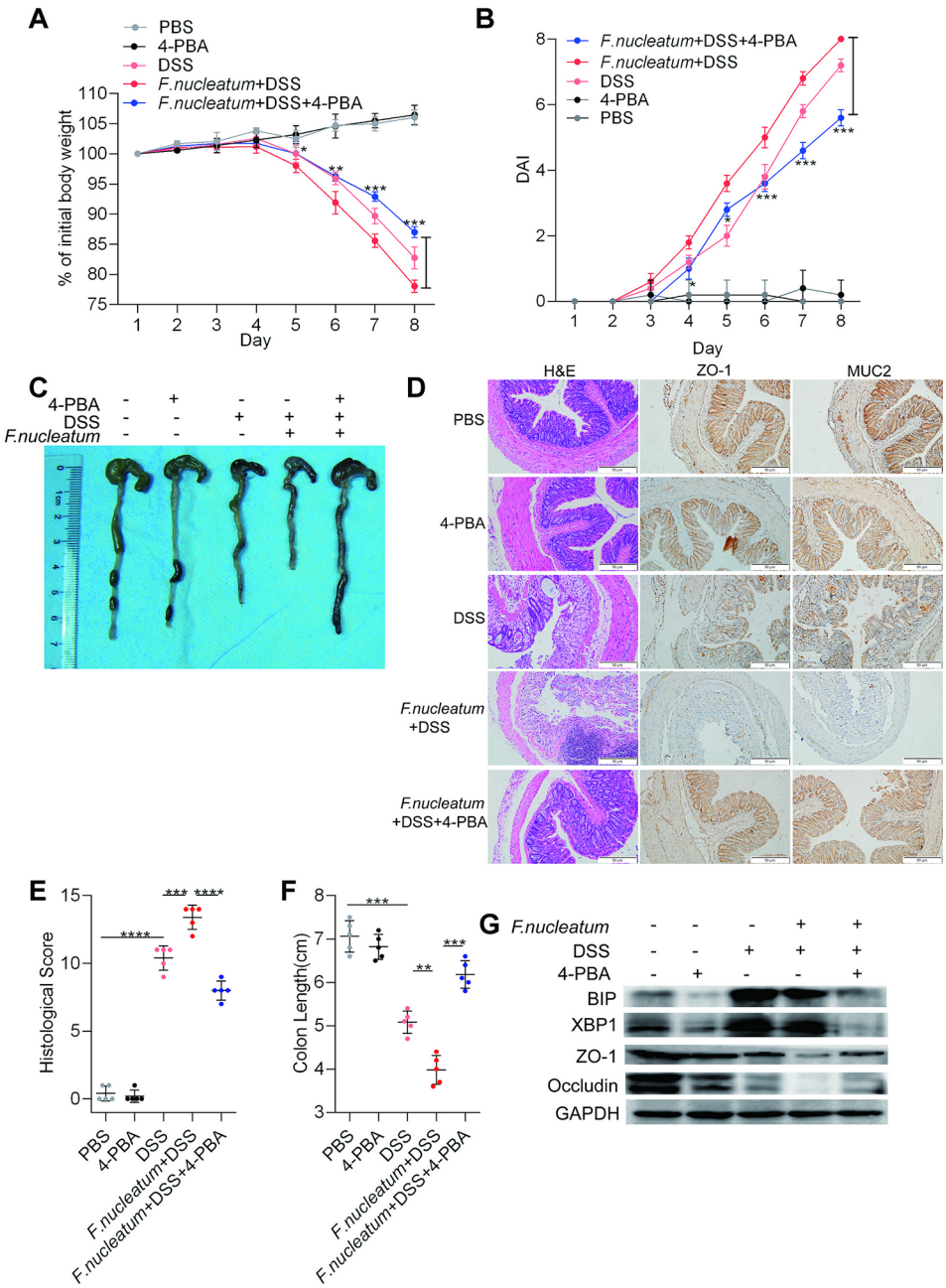

FIGURE 4 | *Fusobacterium nucleatum* facilitates intestinal mucosal barrier destruction through the endoplasmic reticulum (ER) pathway in dextran sulfate sodium (DSS)-induced mice. **(A–F)** Mice (n = 5 per group) were given 4-phenyl butyric acid (4-PBA) (100 μg per mouse every 3 days) and treated with *F. nucleatum* or phosphate-buffered solution (PBS) for 2 weeks. Then, these mice were administered DSS along with continued 4-PBA treatment for an additional 7 days. Colitis induction was evaluated by body weight loss **(A)** and the disease activity index (DAI) **(B)**. (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; one-way ANOVA combined with Bonferroni's *post hoc* test; the error bars indicate the SDs). Representative colon morphology and length in the mice are shown in panel **(C)** and quantified in panel **(F)**. Representative images of histological analyses are shown in panel **(D)** and quantified in panel **(E)** (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$; unpaired Student's *t* test; the error bars indicate the SDs; 200× magnification). **(G)** Western blotting was performed to measure the levels of BIP, XBP1, ZO-1, and occludin in colon tissues from mice.

inflammation in mouse and human cells (Keestra-Gounder et al., 2016). In the present study, we demonstrated that knockdown or knockout of the CARD3 gene can alleviate the extent of *F. nucleatum*-associated colitis and mitigate ERS. We demonstrated for the first time that *F. nucleatum* activates ERS and promotes CD development by upregulating CARD3 expression. However, we did not explore the mechanism by which *F. nucleatum* regulates CARD3, and whether other ERS or CARD3

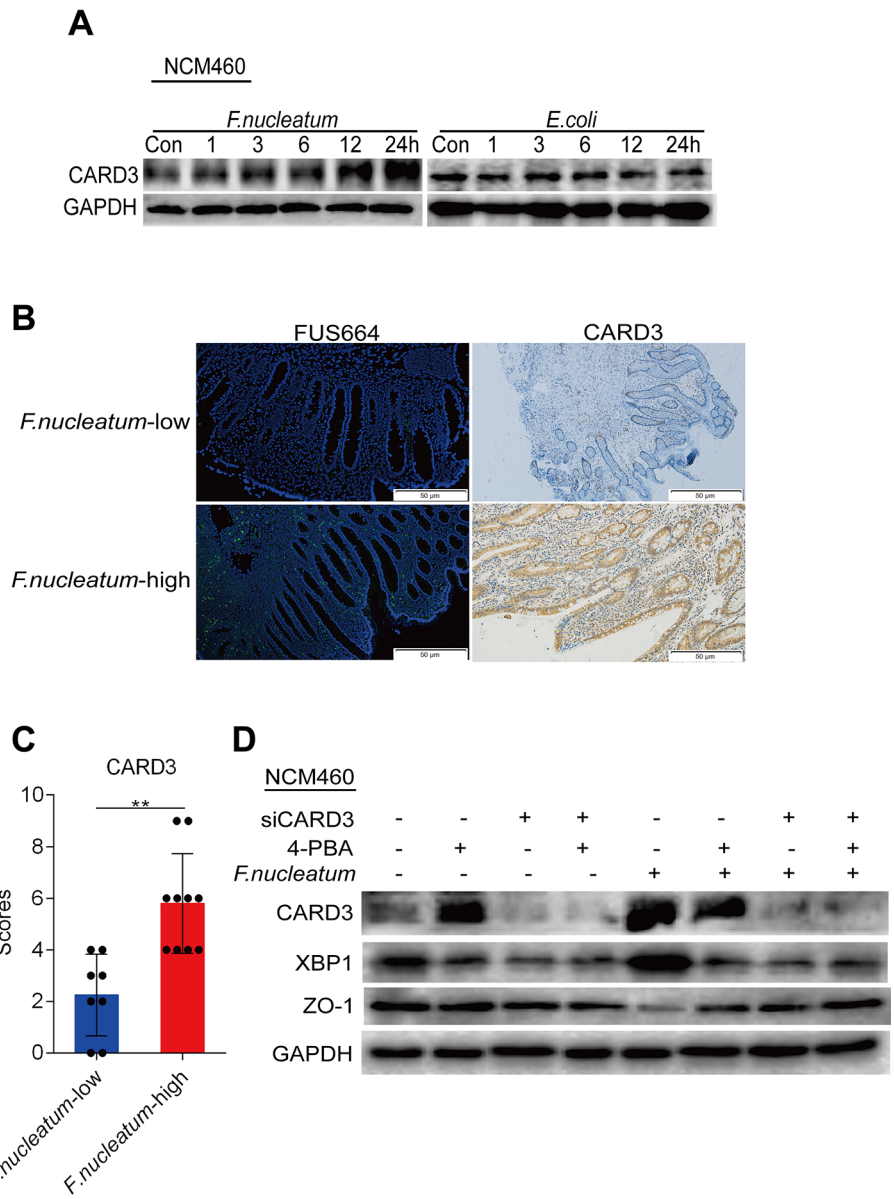

FIGURE 5 | *Fusobacterium nucleatum* is associated with caspase activation and recruitment domain 3 (CARD3) expression and activates the endoplasmic reticulum (ER) pathway *via* CARD3 in NCM460 cells. **(A)** Western blotting was performed to measure CARD3 protein expression over time in NCM460 cells cocultured with *F. nucleatum* or *Escherichia coli* over time. **(B, C)** Representative images showing that the abundance of invasive *F. nucleatum* in CD tissues is associated with high expression of CARD3 (**$P < 0.01$; unpaired Student's *t* test; the error bars indicate the SDs; 200× magnification). **(D)** Western blotting was performed to measure the protein levels of CARD3, XBP1, and ZO-1 in NCM460 cells transfected with nontargeting siRNAs (NC), 4-phenyl butyric acid (4-PBA), or siCARD3 and infected with *F. nucleatum*.

inhibitors can rescue the CD phenotype induced by *F. nucleatum* + DSS remains to be verified.

From a clinical perspective, since the abundance of *F. nucleatum* is related to the risk of CD activity, measuring the *F. nucleatum* abundance may be an effective method for predicting disease activity in patients. In addition, our data raise an important clinical question: Should CD patients with a high abundance of *F. nucleatum* be treated with conventional treatments against *F. nucleatum* and/or with ERS or CARD3 inhibitors? Our findings support this view. Therefore, it is important to determine the abundance and related pathways of *F. nucleatum* and to differentially manage patients with different abundances of *F. nucleatum*.

Overall, our results demonstrate that *F. nucleatum* promotes the development of CD by regulating the molecular mechanisms involving CARD3. Moreover, the clinical information that we

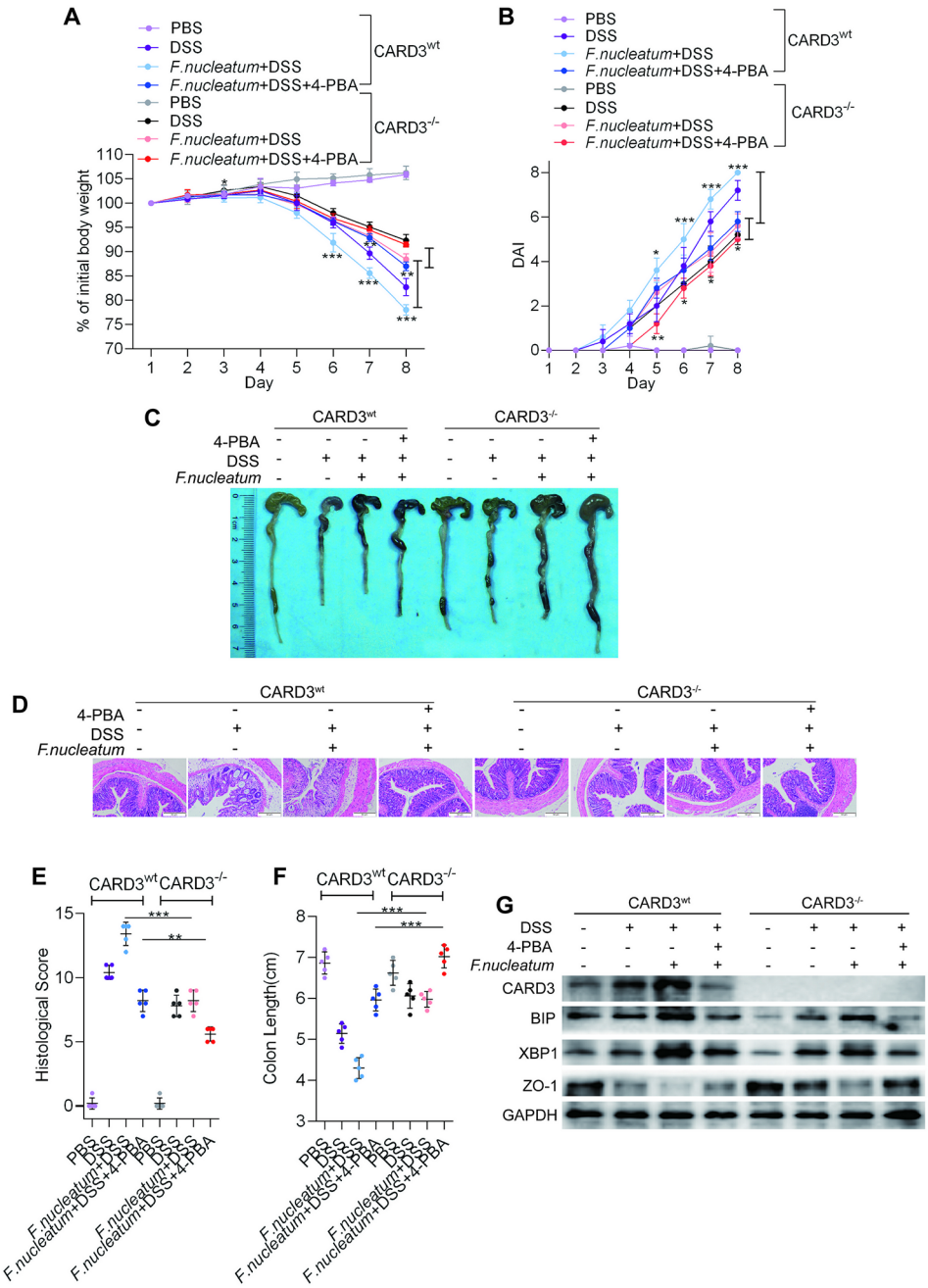

FIGURE 6 | *Fusobacterium nucleatum* activates the endoplasmic reticulum (ER) pathway through the upregulation of caspase activation and recruitment domain 3 (CARD3) in dextran sulfate sodium (DSS)-induced mice. **(A–F)** CARD3^{wt} mice and CARD3$^{-/-}$ mice (n = 5 per group) were administered intraperitoneal injections of 4-phenyl butyric acid (4-PBA) and treated with *F. nucleatum* or phosphate-buffered solution (PBS) for 2 weeks. Then, these mice were administered DSS along with continued 4-PBA treatment for an additional 7 days. Colitis induction was evaluated by body weight loss **(A)** and the disease activity index (DAI) **(B)**. (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; one-way ANOVA combined with Bonferroni's *post hoc* test; the error bars indicate the SDs). Representative colon morphology and length in the mice are shown in panel **(C)** and quantified in panel **(F)**. Representative images of histological analyses are shown in panel **(D)** and quantified in panel **(E)** (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$; unpaired Student's *t* test; the error bars indicate the SDs; 200× magnification). **(G)** Western blotting was performed to measure the levels of CARD3, BIP, XBP1, and ZO-1 in colon tissues from mice.

collected from CD patients also indicated that *F. nucleatum* and CARD3 are risk factors for a high degree of disease activity in CD patients. Our research provides new evidence demonstrating the pro-inflammatory effects of *F. nucleatum* in CD and offers new approaches to the assessment of microbial populations and genetic alterations for the treatment and prevention of CD.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI Sequence Read Archive (SRA) using the accession number PRJNA541040.

ETHICS STATEMENT

All participants provided written informed consent, and the project was approved by the Institutional Review Board (Approval number: 2018K-C089). The animal study was reviewed and approved by the Institutional Review Board of Renmin Hospital of Wuhan University, China.

AUTHOR CONTRIBUTIONS

Study conception and design: WD, PC, YYC; Specimen provision: NZ; Acquisition of clinical data: PC, YYC, YC; Data analysis and interpretation and statistical analysis: PC, YYC, YC; Animal experiments: PC, YYC, YC, WHS; Manuscript drafting: PC, YYC, WGD.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00106/full#supplementary-material

SUPPLEMENTARY FIGURE S1 | *F. nucleatum* destroys epithelial barrier function *in vitro* and *in vivo* **(A–H)** The protein expression of ZO-1 and Occludin in NCM460 cells **(A–D)** and FHC cells **(E–H)** cocultured with *F. nucleatum*, *E. coli* or PBS (Control, Con) were quantified. **(I, J)** The protein expression of ZO-1 and Occludin expression in mouse tissues were quantified. **(K–N)** The protein expression of BIP and XBP1 in NCM460 cells cocultured with *F. nucleatum*, E. coli or PBS (Control, Con) were quantified. Data are expressed as mean ± SD for three independent experiments. Statistical significance is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

SUPPLEMENTARY FIGURE S2 | *F. nucleatum* activates the ER pathway and damages mucosal barrier-associated proteins via ER signaling *in vitro* and *in vivo* **(A–D)** The protein expression of BIP and XBP1 in FHC cells cocultured with *F. nucleatum*, *E. coli* or PBS (Control, Con) were quantified. **(E–H)** The protein expression of BIP, XBP1, ZO-1 and Occludin in NCM460 cells cocultured with *F. nucleatum*, 4-PBA or both were quantified. **(I–L)** The protein expression of BIP, XBP1, ZO-1 and Occludin in colon tissues from mice were quantified. Data are expressed as mean ± SD for three independent experiments. Statistical significance is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

SUPPLEMENTARY FIGURE S3 | *F. nucleatum* activates the ER pathway through the upregulation of CARD3 *in vitro* and *in vivo* **(A–B)** The protein expression of CARD3 in NCM460 cells cocultured with PBS (Control, Con), NC, si001, si002 or si003 were detected by immunoblot and quantified. **(C–E)** The protein expression of CARD3, XBP1 and ZO-1 in NCM460 cells transfected with NC, 4-PBA or siCARD3 and infected with *F. nucleatum* were quantified. **(F–I)** The protein expression of CARD3, BIP, XBP1 and ZO-1 in colon tissues from mice were quantified. Data are expressed as mean ± SD for three independent experiments. Statistical significance is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Hypertension Is Associated With Intestinal Microbiota Dysbiosis and Inflammation in a Brazilian Population

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Hypertension is a major global health challenge, as it represents the main risk factor for stroke and cardiovascular disease. It is a multifactorial clinical condition characterized by high and sustained levels of blood pressure, likely resulting from a complex interplay of endogenous and environmental factors. The gut microbiota has been strongly supposed to be involved but its role in hypertension is still poorly understood. In an attempt to fill this gap, here we characterized the microbial composition of fecal samples from 48 hypertensive and 32 normotensive Brazilian individuals by next-generation sequencing of the 16S rRNA gene. In addition, the cytokine production of peripheral blood samples was investigated to build an immunological profile of these individuals. We identified a dysbiosis of the intestinal microbiota in hypertensive subjects, featured by reduced biodiversity and distinct bacterial signatures compared with the normotensive counterpart. Along with a reduction in Bacteroidetes members, hypertensive individuals were indeed mainly characterized by increased proportions of *Lactobacillus* and *Akkermansia* while decreased relative abundances of well-known butyrate-producing commensals, including *Roseburia* and *Faecalibacterium* within the *Lachnospiraceae* and *Ruminococcaceae* families. We also observed an inflamed immune profile in hypertensive individuals with an increase in TNF/IFN-γ ratio, and in TNF and IL-6 production when compared to normotensive ones. Our work provides the first evidence of association of hypertension with altered gut microbiota and inflammation in a Brazilian population. While lending support to the existence of potential microbial signatures of hypertension, likely to be robust to age and geography, our findings point to largely neglected bacteria as potential contributors to intestinal homeostasis loss and emphasize the high vulnerability of hypertensive individuals to inflammation-related disorders.

Keywords: gut microbiota, dysbiosis, hypertension, inflammation, immune profile

INTRODUCTION

Hypertension is a major global health challenge, as it represents the main risk factor for stroke and cardiovascular disease, the number one cause of death worldwide, as well as for kidney disorders (Hillege et al., 2000; Mills et al., 2016; de Oliveira et al., 2017). In Brazil, high blood pressure (HBP) has reached 30% of the adult population, 5% in children and adolescents, and 50% in elderly people (Picon et al., 2012; Ministério da Saúde, 2013). The identification of the causes of hypertension is still challenging but it is widely accepted that its etiology is multifactorial, involving an intricate set of endogenous and environmental factors contributing to its onset and progression. To date, HBP values have indeed been associated with over 50 genetic loci and metabolic pathways (such as those involved in the renin–angiotensin–aldosterone system) (Levy et al., 2009, 2017; Newton-Cheh et al., 2009; International Consortium for Blood Pressure Genome-Wide Association Studies et al., 2011; Kato et al., 2011; Ganesh S. K. et al., 2013; Tragante et al., 2014; Menni et al., 2015; Xu et al., 2015; Liu et al., 2016), as well as with lifestyle habits, such as dietary salt intake, alcohol consumption and lack of physical activity (Fuchs et al., 2001; Karppanen and Mervaala, 2006).

The human gut microbiota, i.e., the large array of microbes inhabiting our gastrointestinal tract, plays key roles for our physiology, by producing a wide and diverse pool of bioactive small molecules, including short-chain fatty acids (SCFAs, mainly acetate, propionate, and butyrate), which strongly impact on metabolic homeostasis, and regulate immune and nervous system function (Turroni et al., 2018). Emerging evidence suggests a role for the gut microbiota in various disorders, at both enteric and systemic level, including cardiovascular disease (Gregory et al., 2015; Lynch and Pedersen, 2016; Li et al., 2017; Yan et al., 2017; Tang et al., 2019). Specifically, several studies in hypertensive rat models have directly or indirectly emphasized the relevance of the gut microbiota in the regulation of blood pressure (Khalesi et al., 2014; Gómez-Guzmán et al., 2015; Mell et al., 2015; Moghadamrad et al., 2015; Yang et al., 2015; Durgan et al., 2016). Only more recently, a few papers have explored the intestinal microbial alterations underlying hypertension, especially in cohorts of Chinese and United States adult individuals, providing a range of phylogenetic and functional signatures, and advancing a possible causal role of the gut microbiota dysbiosis in contributing to the pathogenesis of hypertension (Li et al., 2017, 2019; Yan et al., 2017; Dan et al., 2019; Sun et al., 2019).

In an attempt to further extend this knowledge, including other geographic locations and expanding the age range, here we characterized the fecal microbiota from 48 hypertensive and 32 normotensive Brazilian adult individuals, by next-generation sequencing of the 16S rRNA gene. In addition, the cytokine production of peripheral blood samples was investigated. While corroborating previous evidence on the decrease of health-promoting SCFA producers in hypertension, our findings unveil minority microbiota components potentially linked to HBP, along with an overall inflamed profile.

MATERIALS AND METHODS

Study Population

Study population consisted of 80 volunteers aged >25 years, all resident in Governador Valadares, an urban municipality from the state of Minas Gerais (Southeast Brazil) and an endemic area for schistosomiasis. The subjects were participants of an institution called Casa Unimed, where they regularly went (twice a week) for exercise programs, lectures, community groups, music education, measurement of blood pressure and blood glucose tests, aimed at promoting health and preventing disease. Health conditions were checked according to standard clinical investigations and standard hematological and biochemical parameters. All subjects enrolled in this study were non-institutionalized and living in their own household.

The individuals were stratified into a control normotensive group and a hypertensive group based on the reported HBP (higher than 140 mmHg for systolic and 90 mmHg for diastolic) (Sociedade Brasileira de Cardiologia/Sociedade Brasileira de Hipertensão/Sociedade Brasileira de Nefrologia, 2010) taken previously at the time of diagnosis, and use of antihypertensive medication including diuretic drugs, such as hydrochlorothiazide and furosemide, angiotensin II receptor antagonists, such as losartan and valsartan, as well as adrenergic receptors antagonists (beta blockers), such as propranolol and atenolol. Some of the individuals used a combination of diuretics and other classes of drugs. The criterion for including them in the study was "reported and treated hypertension for more than 10 years," which would characterize them as chronic patients with HBP. The control subjects were those with no report of HBP.

General exclusion criteria were: infections, acute or chronic inflammation, *Schistosoma mansoni* infection, autoimmune diseases, undernourishment, anemia, leucopoenia, mood disorders, neurodegenerative diseases, neoplasia, and use of hormones (steroids) and drugs in the previous 2 weeks (alcohol, antidepressants, immunosuppressants, anticoagulants, antibiotics). Written informed consent was obtained from each participant, prior to inclusion in our investigations. Fecal and blood samples were collected from consenting participants over a period of 2 weeks in February 2014. Blood samples were immediately processed for peripheral blood mononuclear cell (PBMC) separation as described below, and stored along with the fecal samples at −80°C until use. The fecal samples were used for both microbiota analysis and parasitological exams. *S. mansoni* infection was assessed by Kato–Katz parasitological method with quantitative *S. mansoni* egg counts, using two slides prepared from three stool samples. Twenty-four hour dietary recalls were conducted for each enrolled subject over 3 days. We used the standard method in nutritional science of sampling 2 week days and 1 weekend day in an attempt to fully account for dietary habits and fluctuations. Records were analyzed using TACO (Brazilian table of food composition) (Universidade Estadual De Campinas – Núcleo de Estudos e Pesquisas em Alimentação NEPA – UNICAMP, 2006), and the caloric contributions of the main macronutrient groups were calculated. This work was approved by the Ethical Committee of Universidade Federal de Minas Gerais (UFMG) as well as the National Research Ethics Committee (CONEP) of Brazil.

Microbial DNA Extraction From Feces

Total microbial DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) with a modified protocol (Yu and Morrison, 2004). Briefly, 250 mg of feces were suspended in 1 ml of lysis buffer (500 mM NaCl, 50 mM Tris–HCl pH 8, 50 mM EDTA, 4% SDS). Four 3-mm glass beads and 0.5 g of 0.1-mm zirconia beads (BioSpec Products, Bartlesville, OK, United States) were added, and samples were treated in FastPrep (MP Biomedicals, Irvine, CA, United States) at 5.5 movements/sec for 1 min, repeated three times. Samples were heated at 95°C for 15 min, then centrifuged for 5 min at 14,000 rpm to pellet stool particles. Two hundred and sixty microliters of 10 M ammonium acetate were added to the supernatant, followed by 5-min incubation in ice and centrifugation at 14,000 rpm for 10 min. One volume of isopropanol was added to each sample and incubated in ice for 30 min. Precipitated nucleic acids were collected by centrifugation for 15 min at 14,000 rpm and washed with 70% ethanol. Pellets were suspended in 100 μl of TE buffer and treated with 2 μl of DNase-free RNase (10 mg/ml) at 37°C for 15 min. Protein removal by proteinase K treatment and DNA purification with QIAamp Mini Spin columns were performed following the manufacturer's instructions. DNA concentration and quality were evaluated using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States).

16S rRNA Gene Sequencing and Data Processing

For each sample, the V3-V4 hypervariable region of the 16S rRNA gene was amplified using the 341F and 805R primers with added Illumina adapter overhang sequences as previously described (Turroni et al., 2017). The resulting amplicons of approximately 460 bp were cleaned up with Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, United States). Indexed libraries were prepared by limited-cycle PCR using Nextera technology and further cleaned up as described above. Final libraries were pooled at equimolar concentrations, denatured with 0.2 N NaOH and diluted to 8 pM with a 20% PhiX control. Sequencing was performed on Illumina MiSeq platform using a 2 × 300 bp paired end protocol according to the manufacturer's instructions (Illumina, San Diego, CA, United States).

Raw sequences were processed using a pipeline combining PANDAseq (Masella et al., 2012) and QIIME (Caporaso et al., 2010). Quality-filtered reads were binned into Operational Taxonomic Units (OTUs) at 97% similarity threshold using UCLUST (Edgar, 2010). Taxonomy was assigned using the RDP classifier against Greengenes database (May 2013 release). All singleton OTUs were discarded. Alpha rarefaction was performed using the Faith's phylogenetic diversity, observed OTUs and Shannon index metrics. Beta diversity was estimated by computing weighted and unweighted UniFrac distances, which were used as input for Principal Coordinates Analysis (PCoA). OTUs assigned to genera of interest whose species were unclassified, were subjected to BLAST analysis (Altschul et al., 1990). Sequencing reads were deposited in the MG-RAST database (project ID, mgp84730). All statistical analysis was performed in R 3.3.2. UniFrac distances were plotted by the vegan package, and data separation in the PCoA was tested using a permutation test with pseudo-F ratios (function Adonis in vegan). Bacterial groups with the largest contribution to the ordination space were found by using the function envfit in vegan on the genus relative abundance and the weighted UniFrac ordination. Linear Discriminant Analysis Effect Size (LEfSe) algorithm with LDA score threshold of 2 (on a log10 scale) was applied after agglomerating data to genus and species level (Segata et al., 2011). Significant differences in alpha diversity and taxon relative abundances between groups were assessed by Mann–Whitney *U* test. When appropriate, *p* values were corrected for multiple comparisons using the Benjamini–Hochberg method. A p value ≤ 0.05 was considered as statistically significant, whereas a p value between 0.05 and 0.1 was considered a tendency.

Cytokine Measurements

PBMC Obtainment

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation (400 × *g* for 45 min at 18°C) over Ficoll-Hypaque gradient cushion (LMS Litton Biometries, Kensington, MD, United States), as described by Gazzinelli et al. (1983). After separation, PBMCs were washed in RPMI 1640 and resuspended to 1×10^7 cells/ml. One hundred microliters of PBMC suspension, containing 1×10^6 cells, were incubated for 6 days at 37°C, in humidified incubator containing 5% CO_2, in 24-well flat bottom plates, in the presence of 800 μl of RPMI 1640 medium, supplemented with 5% AB Rh-positive heat-inactivated human serum (Sigma, St. Louis, MO, United States), containing 3% of antibiotic/antimycotic solution, from the stock mix containing 10,000 IU of penicillin, 10 mg of streptomycin, and 25 μg of amphotericin B per ml (Gibco-BRL, Grand Island, NY, United States). Afterward, the culture supernatants were collected and immediately frozen at −80°C, and kept stored until flow cytometric cytokine measurements.

Cytokine Beads Array

Quantitative cytokine analysis was performed using the Cytometric Bead Array method (Human Cytokine Flex Set, Becton Dickinson, Pharmingen, San Diego, CA, United States) for simultaneous measurement of IFN-γ, TNF-α, IL-17A, IL-6, IL-10, IL-4, and IL-2, as recommended by the manufacturer.

Twenty-five microliters of supernatant from PBMC cell cultures were incubated with a mixture of beads coated with capture antibodies specific for each cytokine, labeled with distinct red fluorescence intensities. After incubation and wash steps, the system was incubated with 17 μl of phycoerythrin (PE)-labeled anti-cytokine antibody. After washings and flow cytometric acquisition, the relative fluorescence intensity for each bead was determined, and the results expressed as pg/ml for each cytokine, according to the standard curves. A total of 400 events/cytokine-specific-bead were acquired on a FACSVerse^{rmTM} Bioanalyzer (Becton Dickinson). Data analysis was performed using the FCAP array software (Soft Flow, Inc., St. Louis Park, MN, United States).

Cytokine Data Analysis

In the present study, one of the strategies used was an innovative model to analyze the immune response, referred to as functional cytokine signature. This model was designed to convert quantitative cytokine measurements into a categorical analysis of low and high cytokine producers as previously proposed by Luiza-Silva et al. (2011). This approach is able to detect subtle differences not detectable by conventional statistical analysis. Briefly, this categorical approach first converts the continuous cytokine measurements expressed in pg/ml into categorical variables, referred to as "Low" or "High" cytokine levels, taking the global median value as a specific cut-off edge for each cytokine. The use of such a global median cut-off for each cytokine allows for multiple comparative analyses between groups using the same criteria. Following data categorization, gray-scale diagrams were assembled to compile the cytokine pattern (columns) for each volunteer (rows). Column statistics was run for each diagram to calculate the frequency of "High Cytokine Producers" for each subject group. Next, the frequencies of "High Cytokine Producers" were ordered in an ascendant manner to create the ascendant curves referred to as "cytokine signatures." From each cytokine signature curve, the attributes with frequencies greater than 50% were selected for profile analysis. Then, cytokine ratios were calculated to evaluate the immunological profile for each subject group. The non-parametric Mann–Whitney *U* test was used to assess significant differences. A p value < 0.05 was considered as statistically significant.

Another innovative strategy adopted was the inflammatory score, calculated according to previous studies (Duncan et al., 2003; Recasens et al., 2005; Biagi et al., 2010). Specifically, the inflammatory score was calculated based on three inflammatory cytokines, whose production was assessed as relevant in the HBP group according to the cytokine ratio (**Figure 3**) and signature analysis (**Figure 4**). To obtain such a score, the global median for each selected cytokine was calculated, and every value greater than the median gave 1 point to the subject. The inflammatory score ranged from 0 to 3. Scores 0 and 1 were categorized as "Not Inflamed" and scores 2 and 3 were categorized as "Inflamed." Finally, the frequency of inflammatory score category for each group was calculated.

RESULTS

Study Population

Eighty subjects (21 males and 59 females) aged 26–87 years (mean 64.5), were recruited for the present study. This population was stratified according to the presence of reported HBP (higher than 140 mmHg for systolic and 90 mmHg for diastolic) (Sociedade Brasileira de Cardiologia/Sociedade Brasileira de Hipertensão/Sociedade Brasileira de Nefrologia, 2010) and the chronic use of antihypertensive medication that kept their HBP under control. Forty-eight subjects had HBP (HBP group) while 32 had not (control). The demographic data of individuals are summarized in **Table 1**. No differences were found in age or gender distribution between the two groups.

Intestinal Microbiota Diversity Is Altered in Hypertensive Subjects

Fecal samples were collected from the 80 subjects recruited in Governador Valadares, and the V3-V4 region of the bacterial 16S rDNA was sequenced. A total of 3,790,002 high-quality reads (mean per subject, 47,375; range, 3,745–1,50,790) were obtained and analyzed. Reads were clustered into 24,575 OTUs at 97% identity. Rarefaction curves obtained with phylogenetic diversity and OTU count did not reach saturation while the Shannon index for biodiversity plateaued at a sequence depth of about 1000 read counts (**Supplementary Figure S1**). According to this index (a combined parameter of richness and evenness), alpha diversity values were lower in the HBP group compared to normotensive controls ($p = 0.04$, Mann–Whitney *U* test; **Figure 1A**). Furthermore, HBP and control groups significantly, even if only barely, segregated according to weighted UniFrac distances ($p = 0.04$, $R^2 = 0.02$, permutation test with pseudo-F ratios; **Figure 1B**). Specifically, the PCo1 axis, which accounted for 43% of the total variance in the dataset, was negatively associated with the presence of HBP ($p = 0.03$, Mann–Whitney *U* test).

Hypertensive Subjects Show a Gut Microbiota Dysbiosis

At phylum level, the HBP-related gut microbiota tended to show a contraction of Bacteroidetes (mean relative abundance, 4.96% vs. controls, 8.97%; $p = 0.03$, FDR-adjusted $p = 0.1$, Mann–Whitney *U* test), resulting in a significantly increased Firmicutes/Bacteroidetes ratio in hypertensive vs. normotensive subjects ($p = 0.03$) (**Supplementary Figure S2**).

In order to identify the bacterial genera responsible for the separation between the microbiota structure of HBP and control subjects, relative abundance vectors with a statistically significant contribution were identified and overlaid onto the ordination space based on weighted UniFrac distances of **Figure 1B** ($p < 0.05$, permutational correlation test). Interestingly, several microbial taxa were found to drive the clustering pattern, i.e., *Lactobacillus*, *Akkermansia*, *Bifidobacterium*, and unclassified *Enterobacteriaceae*, which were associated with HBP, and well-known butyrate producers of the *Lachnospiraceae* and *Ruminococcaceae* families, including *Roseburia*, *Coprococcus*, *Dorea*, and *Oscillospira*, which were associated with controls (**Figures 2A,B**). Most of these genera were also identified as discriminating according to LEfSe analysis (**Figure 2C**). These differences are likely to be attributable to the species *Roseburia faecis*, *Faecalibacterium prausnitzii*, *Parabacteroides distasonis*, and unclassified species belonging to the genera *Fusobacterium* and *Coprobacillus*, which were representative of normotensive controls, and *Lactobacillus salivarius*, *Bacteroides plebeius* and an unclassified species belonging to *Eggerthella*, which were characteristic of hypertensive individuals ($p \leq 0.05$, FDR-adjusted $p \leq 0.1$, Mann–Whitney *U* test) (**Supplementary Figure S3**). According to BLAST,

TABLE 1 | Demographic and health data of individuals from Casa Unimed in Governador Valadares, stratified by the presence of high blood pressure (HBP).

Data	All subjects	Control group	HBP group	*p*-value
N.	80	32	48	–
Male	21	7	14	0.6056*
Female	59	25	34	
Age (mean ± SD)	64.5 ± 15.3	63.3 ± 15.0	65.3 ± 15.5	0.5611**
Age range	26–87	28–86	26–87	
High Blood Pressure	48	0	48	–

*, *Fisher's exact test*; **, *Student's t test*.

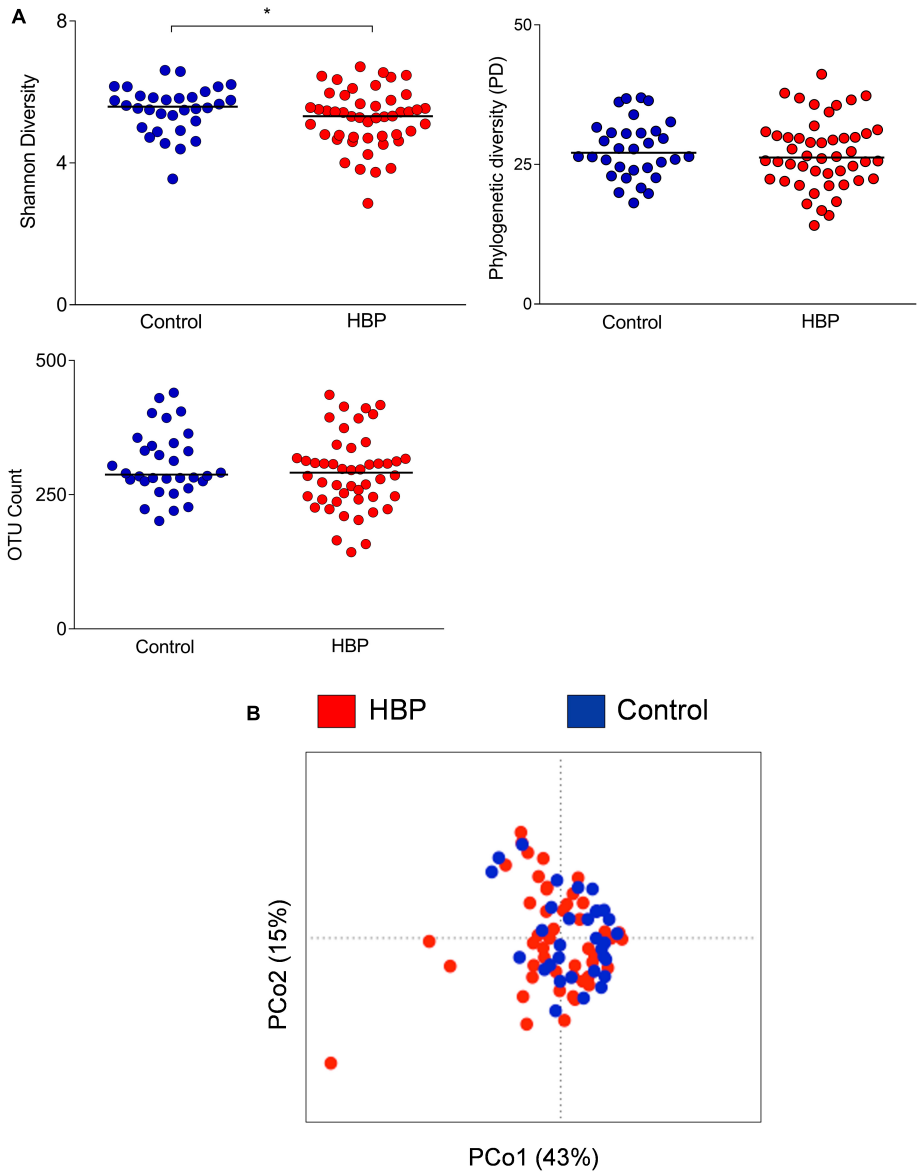

FIGURE 1 | Comparison of the gut microbiota structure between hypertensive (HBP) and normotensive (control) subjects. **(A)** Alpha-diversity values were calculated according to the following metrics: the Shannon index for biodiversity, phylogenetic diversity (PD) and OTU count. *, $p = 0.04$, Mann–Whitney *U* test. **(B)** Principal Coordinates Analysis of the weighted UniFrac distances for HBP and control subjects. The two components explain 43 and 15% of the variance, respectively. A significant separation was found between the two groups ($p = 0.04$, permutation test with pseudo-F ratios). Red, HBP subjects ($n = 48$); blue, controls ($n = 32$).

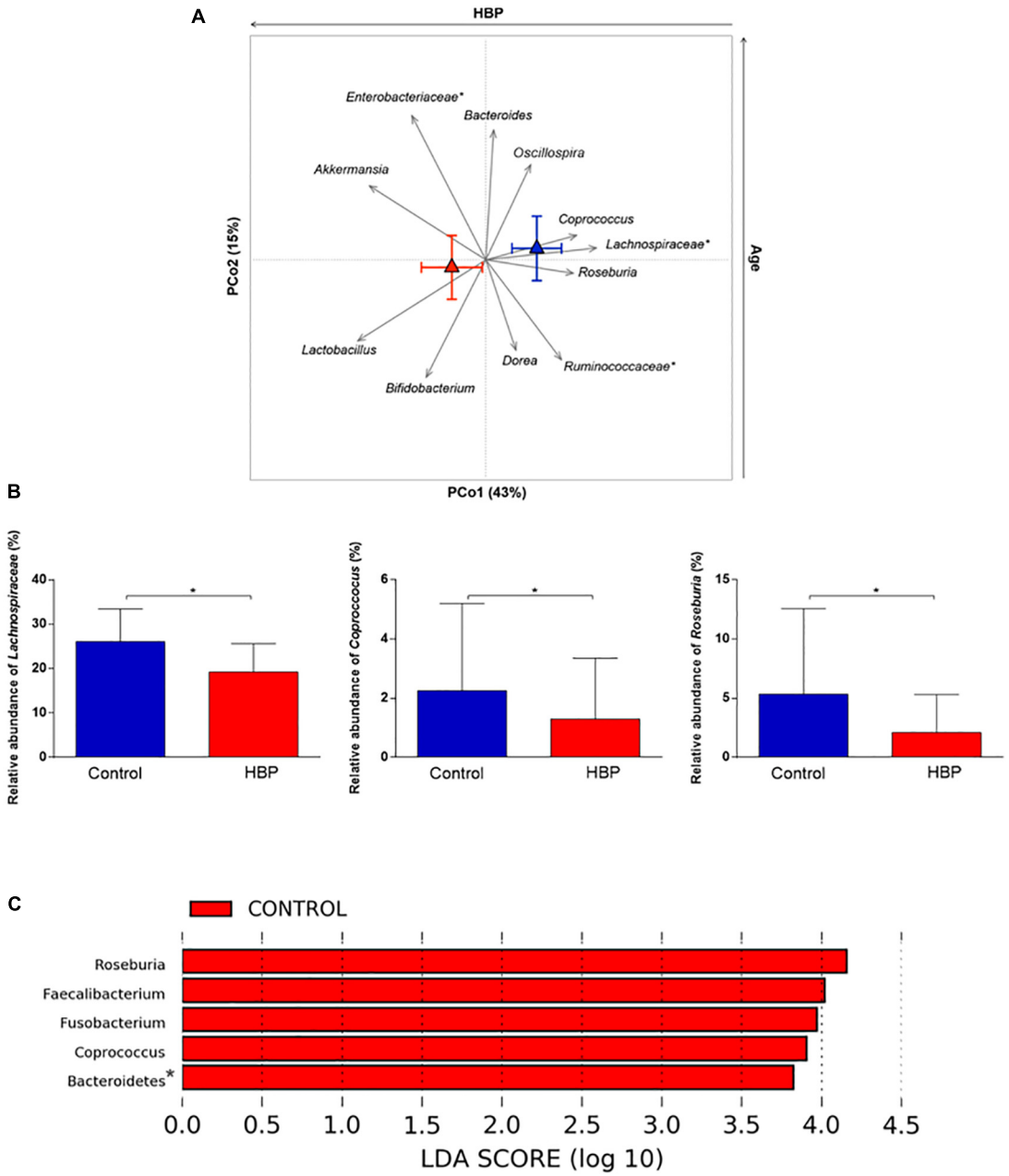

FIGURE 2 | Intestinal microbial components driving the separation between hypertensive (HBP) and normotensive (control) subjects. **(A)** Genus-level relative abundance vectors with statistically significant contribution to the ordination space ($p < 0.05$, permutational correlation test) were overlaid onto the PCoA plot of weighted UniFrac distances (see **Figure 1B**). Triangles, centroids for each group (red, HBP; blue, control) with indication of standard errors on each coordinate axis. *, unclassified OTU reported at higher taxonomic level. PCo1 was negatively associated with the presence of HBP while PCo2 correlated positively with age. **(B)** Bar plots showing the relative abundance of the *Lachnospiraceae* family and the genera *Coprococcus* and *Roseburia* in HBP and control subjects. *$p = 0.02$; $p = 0.04$; $p = 0.003$, respectively; Mann–Whitney *U* test. **(C)** Linear discriminant analysis (LDA) effect size (LEfSe) analysis. LDA scores indicate differentially represented genera between groups (the logarithmic threshold for discriminative features was set to 2.0). *, unclassified OTU reported at higher taxonomic level.

Eggerthella, *Fusobacterium* and *Coprobacillus* OTUs showed the highest percent identity with *Eggerthella lenta* (91%), *Fusobacterium (pseudo)periodonticum* (99%), and *Coprobacillus cateniformis* (95%), respectively. However, it should be stressed that, given the known resolution limits of 16S rRNA gene-based sequencing, these species-level results must be interpreted with extreme caution, at least until further information, through other approaches possibly based on full-length 16S rRNA gene sequencing or shotgun metagenomics, is available.

The Gut Microbiota Dysbiosis in Hypertensive Subjects Is Likely Independent of Age and Macronutrient Intake

Given the wide age range of volunteers, spanning young adults and elderly, we investigated the impact of age on the gut microbiota composition in our study cohort. According to weighted UniFrac distances, age was not associated with PCo1 (i.e., the axis along which HBP and control subjects clustered significantly), suggesting that the intestinal microbial dysbiosis in HBP may be independent of it (see **Supplementary Figure S4**). It was instead shown to correlate with the second axis of the PCoA plot, parallel to the relative abundance of several bacterial taxa (**Figure 2A**), confirming previous (HBP-independent) observations on the dynamics of the gut microbiota during aging (Odamaki et al., 2016; Biagi et al., 2017). In particular, higher age was found to be associated with higher proportions of *Oscillospira* and especially *Enterobacteriaceae*, already known to increase in the elderly in a sort of self-sustained pro-inflammatory loop. On the other hand, as expected, lower age was associated with increasing amounts of health-promoting bifidobacteria, along with SCFA producers of the gut microbiota, in particular *Dorea* and *Ruminococcaceae* members.

Furthermore, given the pivotal role of diet in shaping the gut microbiota (Zmora et al., 2019), we evaluated the dietary habits of volunteers through 3-day food frequency recalls. According to our data, no differences in the proportion of macronutrients between HBP and control individuals were observed (see **Supplementary Table S1**).

Hypertensive Subjects Show an Overall Inflammatory Cytokine Profile

In order to assess whether the hypertensive subjects also had a different immunological profile compared to controls, the cytometric bead array (CBA) method was used to evaluate the production of IL-6, IFN-γ, TNF-α, IL-17A, and IL-10. We excluded IL-4 and IL-2 from the analysis, because their measurements were too low (median = 0 for both cytokines). According to our findings, there were no differences in the cytokine concentration between control and HBP groups ($p > 0.05$, Mann–Whitney U test; **Figure 3A**). However, to fully explore the possible contribution of these cytokines to the immunological profile of our study groups, we calculated several cytokine ratios, commonly used in immunological analysis (Gómez et al., 2004; Coelho-dos-Reis et al., 2013; Tsurumi et al., 2016; Medeiros et al., 2018), by creating a measure of balance between cytokine profiles. Interestingly, an increased TNF-α/IFN-γ ratio was found in HBP compared to the control group ($p = 0.02$, Mann–Whitney U test; **Figure 3B**), suggesting an overall inflammatory profile for HBP subjects. No difference was detected for the other ratios (data not shown).

To further deepen the contribution of these cytokines, individuals were stratified in carriers of either low or high cytokine producers taking the global median of the cytokine index as the cut-off (**Figure 4**; please see the section "Materials and Methods" for the functional cytokine signature analysis), as previously reported (Luiza-Silva et al., 2011; Campi-Azevedo et al., 2012; Silveira-Nunes et al., 2017). When considering the frequency of high cytokine producers, a relevant production (i.e., more than 50% of individuals were high producers) was only found in the HBP group for TNF-α and IL-6 (**Figure 4B**).

Finally, to support the hypothesis of increased inflammation in HBP subjects, an inflammatory score, based on three inflammatory cytokines, whose production was assessed as relevant in the HBP group according to the cytokine ratio and signature analysis, i.e., IFN-γ, TNF-α, and IL-6 (please see section "Materials and Methods"), was calculated. Subjects were considered as inflamed for inflammatory scores of 2 and 3, and as non-inflamed for scores of 0 or 1. In this analysis (**Figure 5**), we observed that more than half (50% as a cut-off) of the individuals from the HBP group (54%) were categorized as inflamed by inflammatory score, a result we did not observe in the control group (47%).

DISCUSSION

Hypertension is a multifactorial clinical condition characterized by high and sustained levels of blood pressure, likely resulting from a complex interplay of endogenous and environmental factors, including the gut microbiota (Mell et al., 2015; Moghadamrad et al., 2015; Yang et al., 2015; Durgan et al., 2016; Galla et al., 2017; Oyama and Node, 2019). Epidemiological studies have shown that high levels of blood pressure are widely associated with increased risk of fatal and non-fatal cardiovascular events (Lewington et al., 2002; Williams et al., 2008). Although research in the field has grown considerably in recent years, the etiology of hypertension is poorly understood and there is still limited evidence on the role of the gut microbiota.

In an attempt to bridge these gaps, here we sequenced the bacterial 16S rRNA gene of fecal samples from a cohort of 48 hypertensive and 32 normotensive Brazilian individuals, and integrated the analysis with extensive immunological profiling. Consistent with recent findings in Chinese and United States cohorts (Li et al., 2017, 2019; Yan et al., 2017; Dan et al., 2019; Sun et al., 2019), our study highlighted a dysbiosis of the intestinal microbiota in hypertensive subjects, featured by reduced biodiversity and distinct bacterial signatures compared with the normotensive counterpart (see **Supplementary Table S2** for a review of available studies on gut microbiota and hypertension). Loss of microbial diversity is widely recognized as a hallmark of unhealthy microbiota and so far has been reported in a plethora of human disorders, including (but not limited to) gastrointestinal, immune and metabolic ones (Sonnenburg and Sonnenburg, 2014), thus likely to be part of a non-specific, shared response to the disease. With regard to the taxonomic structure, we found a higher Firmicutes/Bacteroidetes (F/B) ratio in the gut microbiota of hypertensive individuals compared to controls, resulting from a reduction in the relative proportion of Bacteroidetes. Interestingly, also Yang et al. (2015) reported an increased F/B ratio in a small group of hypertensive patients, as well as in rat models of hypertension. Although still debated, the F/B ratio has been widely used in the past as a marker of an obesogenic microbiota, with high values being generally

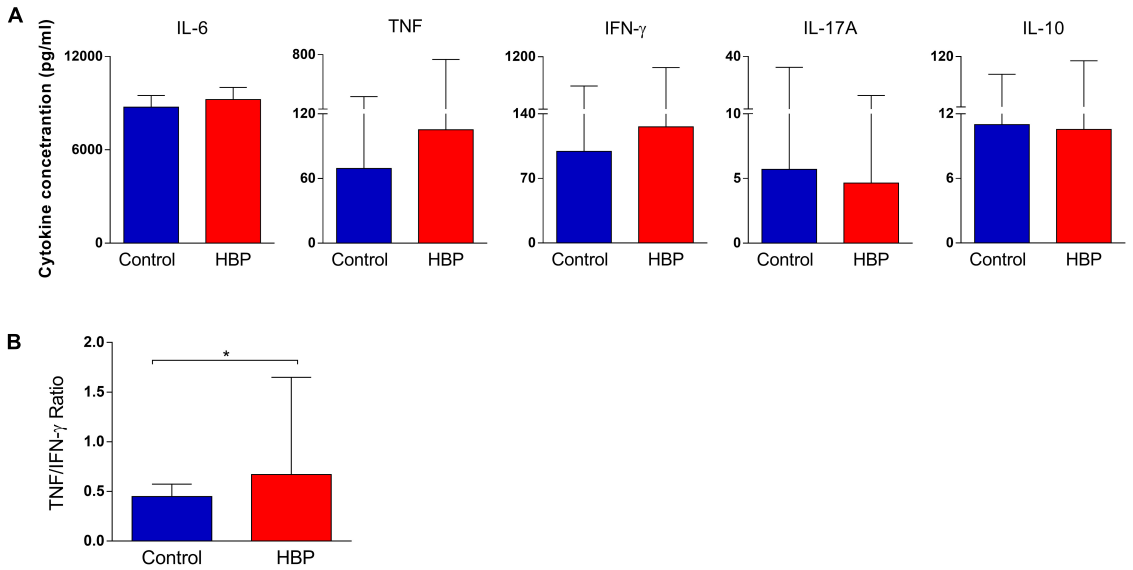

FIGURE 3 | Comparison of cytokine production in the supernatant of PBMCs between the high blood pressure group (HBP) and control group (Control). **(A)** The bars represent the median and interquartile of the cytokine concentration (pg/ml), as determined by flow cytometry, for each study group. **(B)** The bars represent the TNF/IFN-γ ratio. *, $p = 0.02$, Mann–Whitney *U* test.

associated with the consumption of high-fat low-fiber Western diets and inflammation (Turnbaugh et al., 2009; Everard and Cani, 2013; Trompette et al., 2014; Nakayama et al., 2017).

At the genus level, our results confirmed previous evidence on the relative abundance decrease of several SCFA (mainly butyrate) producers, generally recognized as beneficial components of the human gut microbiota, including members of the *Lachnospiraceae* and *Ruminococcaceae* families, such as *Roseburia* and *Faecalibacterium* (Li et al., 2017, 2019; Yan et al., 2017; Dan et al., 2019). Such alterations could therefore represent robust dysbiotic microbial signatures, regardless of geography and lifestyle. SCFAs are known to exert a key, multifactorial role in human physiology, not only locally but also at distant body sites, promoting the maintenance of metabolic and immunological homeostasis (Koh et al., 2016). In particular, they have been shown to regulate blood pressure via the sensory receptors Olfr78 (Olfactory receptor 78) and GPR41 (G protein-coupled receptor 41), acting in opposition to one another (Pluznick, 2014), and exert relaxant effects on resistance arteries (Mortensen et al., 1990), thus possibly improving microcirculation. Furthermore, SCFAs are known to have important anti-inflammatory effects (Vinolo et al., 2011; Vieira et al., 2012; Chang et al., 2014; Iraporda et al., 2015; Park et al., 2015; Yang and Zubcevic, 2017). Specifically, they are able to inhibit the activation of NF-κB in immune cells by binding to GPR43 and GPR41, thereby blocking inflammatory responses and suppressing the production of TNF-α and IL-6. Butyrate also suppresses inflammation by reducing IL-12 and increasing IL-10 expression (Säemann et al., 2000; Fukae et al., 2005; Tedelind et al., 2007), and it promotes transcription of the *Foxp3* gene, inducing the production of regulatory T cells (Furusawa et al., 2013). As thoroughly discussed by Million et al. (2018), butyrate could represent the best functional marker of the healthy mature anaerobic gut microbiota, and oxidative stress-sensitive butyrate-producing commensals could serve as new instrumental targets for the maintenance of a microbiota-immunity symbiotic loop and the prevention of a series of local and systemic dysbiosis-related disorders. It is also worth noting that *Roseburia*, one of the main butyrate-producing microbes that were found to be underrepresented in hypertension, also produces conjugated linoleic acid, which has been shown to have anti-inflammatory properties and potential blood pressure-lowering effects as well (McIntosh et al., 2009; Neyrinck et al., 2012; Van den Abbeele et al., 2013; Derrien and Veiga, 2017).

Interestingly, our study also led to the identification of other bacteria deserving further investigation for their possible role in hypertension, especially *Lactobacillus* (most likely *L. salivarius*), *Eggerthella* and possibly *B. plebeius*, which were found to be discriminating for hypertensive individuals. While the literature is consistent in reporting both *Eggerthella* and *B. plebeius* as potential pathogens for cardiovascular disease, with the former already found to occur at higher levels in patients with hypertension compared to controls (Yan et al., 2017) and the latter being associated with dyslipidemia (Liu et al., 2019), conflicting evidence is available for lactobacilli. Some clinical trials in fact support hypotensive effects for *Lactobacillus* species used as probiotics (Upadrasta and Madempudi, 2016), but this genus and/or certain species have also been found to be overabundant in some inflammatory disorders, including obesity (Million et al., 2012), coronary heart disease (Yamashita, 2017), and heart failure (Kamo et al., 2017). In particular, the species *L. salivarius* has recently been assigned to metagenomics linkage groups enriched in the gut microbiome of individuals with

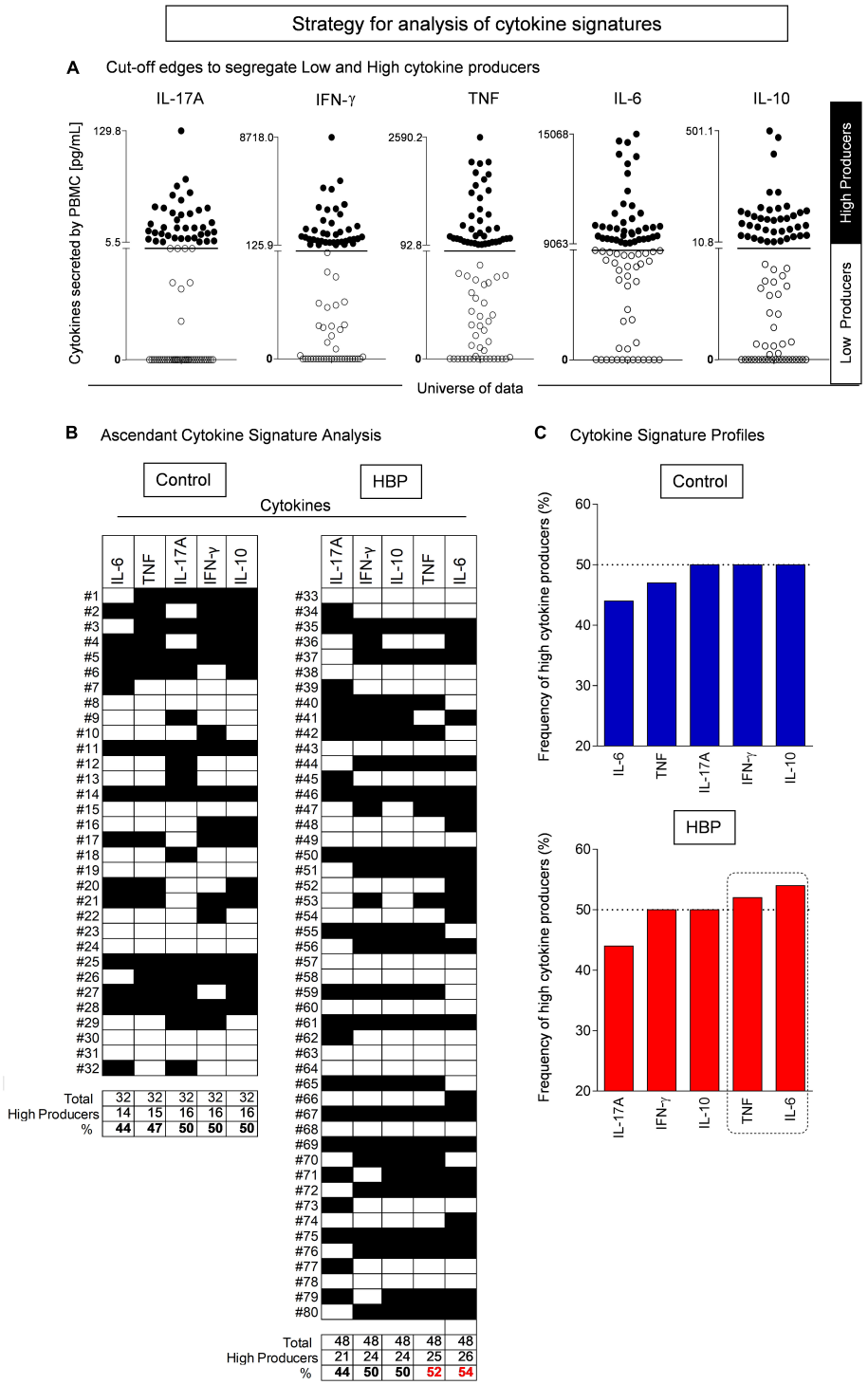

FIGURE 4 | Cytokine signature analysis in hypertensive (HBP) and normotensive (control) individuals. **(A)** Establishing cut-off edges to categorize the subjects as "Low" or "High" cytokine producers. The cytokine quantitative analysis was performed by flow cytometry using the Cytometric Bead Array as described in Methods. The cut-off line was established as the global median value calculated for each cytokine, taking the whole dataset, including all subjects. The use of such a global median cut-off for each cytokine subsidizes multiple comparative analyses between groups using the same criterion. The subjects were then categorized as "Low" (white box) or "High" (black box) cytokine producers. **(B)** Assembling the ascendant cytokine signatures. Diagrams were assembled to compile the cytokine pattern (columns) for each volunteer (lines). Column statistics was run for each diagram to calculate the frequency of "High Cytokine Producers" for each group of patients. **(C)** The cytokine signature profiles. The frequencies of "High Cytokine Producers" were ordered in an ascendant manner to create the ascendant patterns referred to as "cytokine signatures." From each cytokine signature curve, the attributes with frequencies higher than 50% (i.e., more than half of the group are high cytokine producers) were selected (dashed rectangle).

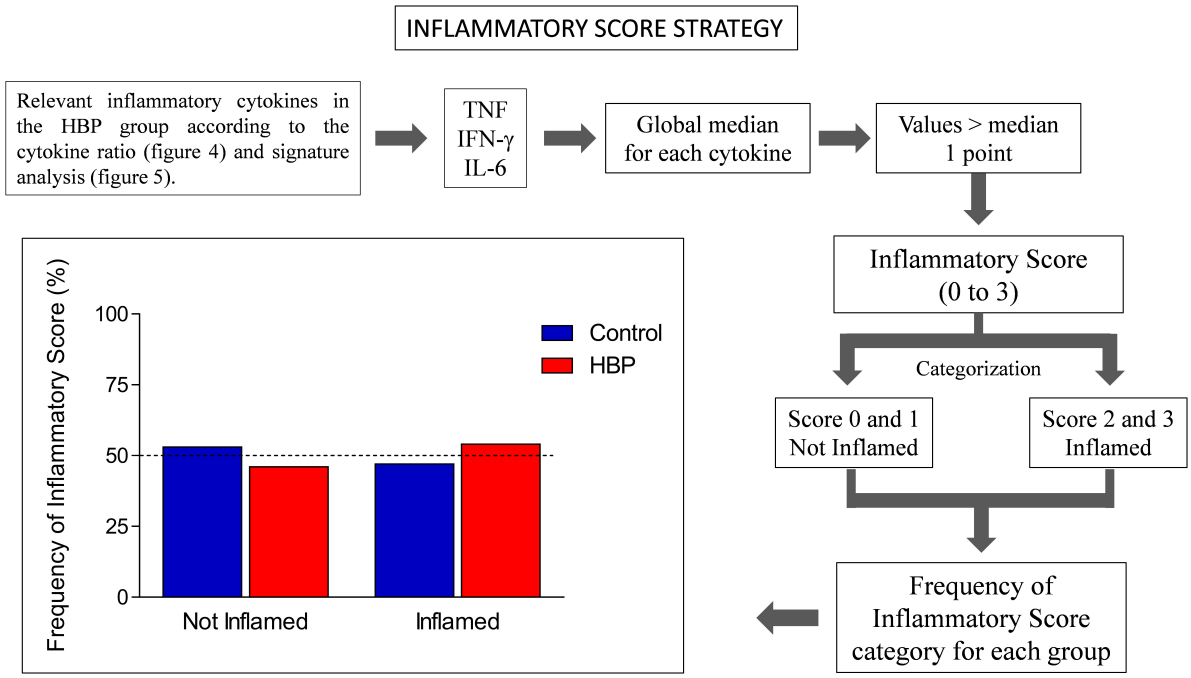

FIGURE 5 | Inflammatory score in hypertensive (HBP) and normotensive (control) subjects. The inflammatory score was calculated based on three inflammatory cytokines, whose production was assessed as relevant in the HBP group according to the cytokine ratio and signature analysis, i.e., IFN-γ, TNF, and IL-6. The global median for each selected cytokine was calculated, and every value greater than the median gave 1 point to the subject. The sum of the points for each subject defined its Inflammatory Score. The inflammatory score ranged from 0 to 3. Scores 0 and 1 were categorized as Not Inflamed and scores 2 and 3 were categorized as Inflamed. The frequency of inflammatory score category for each group was calculated. The bars represent the frequency of Inflamed (score 2–3) and Not Inflamed (score 0–1) subjects in control (blue) and HBP group (red).

atherosclerotic cardiovascular disease (Jie et al., 2017), thus opening fascinating perspectives on the possible use of this species as a non-invasive biomarker for blood pressure-related diseases. It is also worth noting that we found an association between hypertension and the mucin degrader *Akkermansia*. Although this microorganism is generally associated with improved metabolic profile (Everard et al., 2013), it has recently been reported to show greater abundance in hypertensive Chinese subjects (Dan et al., 2019) and exacerbate inflammation during infections by disturbing the mucus layer homeostasis (Ganesh B. P. et al., 2013). Recent evidence in human PBMCs and mice also indicates that *Akkermansia* promotes Th1 lymphocyte differentiation (Cekanaviciute et al., 2017). It is thus tempting to speculate that this microbiota member may play a role in hypertension, by sustaining and contributing to an overall pro-inflammatory environment.

Consistent with our hypothesis, we observed an inflamed immune profile in hypertensive individuals. Chronic inflammation is one of the biological mechanisms most commonly associated with hypertension (Blake et al., 2003; Cevenini et al., 2010; Chung et al., 2010; Singh and Newman, 2011; Youn et al., 2013; De Miguel et al., 2015). Bautista et al. (2005) demonstrated that the plasma levels of inflammatory cytokines, such as C-reactive protein, IL-6, and TNF-α, positively correlated with blood pressure in humans. In agreement with those findings, our results revealed an increase of type 1 cytokines, with an increased TNF/IFN-γ ratio, in hypertensive individuals when compared to normotensive ones, thus strengthening the hypothesis that inflammation plays an important role in hypertension. Specifically, according to our ascending profile analysis, TNF and IL-6 were the main cytokines responsible for the leukocyte immune profile of hypertensive individuals, and 50% of them had high inflammatory scores. The association of elevated levels of TNF-α with hypertension has been demonstrated through pharmacological and genetic approaches in experimental models, including angiotensin II-induced hypertension, lupus, metabolic syndrome and preeclampsia (Zinman et al., 1999; Ito et al., 2001; Guzik et al., 2007; Venegas-Pont et al., 2010; Ramseyer and Garvin, 2013). The IL-6 levels are also frequently elevated under hypertensive conditions. In particular, studies have shown that IL-6 is essential for the development of angiotensin II-induced hypertension and that the activation of the STAT3/JAK pathway by IL-6 plays a key role in the disease (Brands et al., 2010). In addition, human studies confirmed increased plasma levels of IL-6 in response to acute angiotensin II infusion, and also in hypertensive patients (Furuya et al., 2010; Chamarthi et al., 2011; De Miguel et al., 2015).

In summary, despite the small sample size, our work provides, to the best of our knowledge, the first evidence of an association of hypertension with altered gut microbiota and inflammation in

a Brazilian population. While lending support to the existence of microbial signatures of hypertension, possibly robust to age and geography, and stressing the need for species-level analysis in future microbiome-based studies, our findings point to bacteria widely neglected to date as potential contributors to the loss of intestinal homeostasis, and emphasize the high vulnerability of hypertensive individuals to inflammation-related disorders. Future studies in independent and much larger cohorts, also through other methods, including culture-dependent ones, are encouraged to assess alterations in the microbiota-host co-metabolic networks and their contribution to hypertension and related complications. This information will be instrumental to the design of rational nutritional interventions aimed at correcting the gut dysbiosis and mitigating dysregulated immune responses, for reduced incidence and severity of chronic diseases.

DATA AVAILABILITY STATEMENT

The 16S rRNA gene sequences generated for this study can be found in the MG-RAST database (project ID, mgp84730).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of Universidade Federal de Minas Gerais (UFMG) as well as the National Research Ethics Committee (CONEP) of Brazil. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AF and PB conceived and designed the study. LA and EC recruited volunteers, collected biologic samples, and applied the questionnaires. DD, AV, and GS-N performed the microbial DNA extraction from feces. GS-N and ST performed the 16S rRNA gene sequencing. GS-N, ST, and SR carried out the bioinformatics analysis and analyzed the microbiota data. TM and EC collected and analyzed the nutritional data. GS-N, ES, OM-F, RC-O, and AT-C performed and reviewed the cytokine measurements. GS-N and SR prepared **Figures 1**–**3** and **Supplementary Figure S1**. GS-N and ST wrote the main manuscript text. AF, OM-F, and RC-O revised and edited the draft. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00258/full#supplementary-material

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REVIEW
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Unraveling Host-Gut Microbiota Dialogue and Its Impact on Cholesterol Levels

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Disruption in cholesterol metabolism, particularly hypercholesterolemia, is a significant cause of atherosclerotic cardiovascular disease. Large interindividual variations in plasma cholesterol levels are traditionally related to genetic factors, and the remaining portion of their variance is accredited to environmental factors. In recent years, the essential role played by intestinal microbiota in human health and diseases has emerged. The gut microbiota is currently viewed as a fundamental regulator of host metabolism and of innate and adaptive immunity. Its bacterial composition but also the synthesis of multiple molecules resulting from bacterial metabolism vary according to diet, antibiotics, drugs used, and exposure to pollutants and infectious agents. Microbiota modifications induced by recent changes in the human environment thus seem to be a major factor in the current epidemic of metabolic/inflammatory diseases (diabetes mellitus, liver diseases, inflammatory bowel disease, obesity, and dyslipidemia). Epidemiological and preclinical studies report associations between bacterial communities and cholesterolemia. However, such an association remains poorly investigated and characterized. The objectives of this review are to present the current knowledge on and potential mechanisms underlying the host-microbiota dialogue for a better understanding of the contribution of microbial communities to the regulation of cholesterol homeostasis.

Keywords: gut microbiota, microbiome, gut metabolites, cholesterol, LDL-cholesterol, cholesterolemia, dyslipidemia and cardiovascular disease

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INTRODUCTION

Atherosclerosis is the underlying cause of the majority of cardiovascular disease (CVD) events, the complications of which can be fatal (myocardial infarction, sudden death, and ischemic cerebral accidents). According to WHO projections, exposure to multiple genetic and environmental risk factors and the growing number of dysmetabolic conditions (metabolic syndrome, type 2 diabetes mellitus, obesity, non-alcoholic fatty liver diseases) will contribute to making atherosclerotic cardiovascular disease (ACVD) a leading cause of death in the world by 2030 (Kaptoge et al., 2019). Among the etiological factors of this multifactorial pathology, circulating levels of total

cholesterol (TC) or LDL-Cholesterol (LDL-C) represent major risk factors for ACVD. Consistent evidence from numerous epidemiological, clinical, and genetic studies unequivocally establishes a causal role of LDL-C in ACVD (Ference et al., 2017). A major instigating event is the recognition of oxidized-LDL-C by immune cells due to molecular mimicry with foreign antigens, thus promoting chronic inflammatory and self-perpetuating responses (Wolf and Ley, 2019).

Genetics plays an important role in regulating the levels of TC and associated lipoproteins (van Dongen et al., 2013; Hoffmann et al., 2018), yet genetic variation may account for 20% of plasma cholesterol levels (Surakka et al., 2015; Hoffmann et al., 2018). Environmental factors such as the amount and composition of the diet (Mente et al., 2017) as well as dietary cholesterol intake (Griffin and Lichtenstein, 2013) are well-established contributors; however, the individual-level contribution of intestinal microbiota to cholesterol homeostasis and the relevant pathways through which microbiota may exert their actions need to be documented and characterized. The gut microbiota functions as an endocrine system, which communicates with distal organs through metabolic pathways (Cani and Knauf, 2016). Additionally, modifications in the gut microbial ecosystem induced by external factors may cause radical changes in the symbiotic relationship between the microbiota and the host, and thus contribute to the low-grade inflammation that is constitutive of metabolic diseases (Cani et al., 2007; Fändriks, 2017). As a consequence, these modifications may account for a substantial proportion of the variation of plasma lipids, including cholesterol levels (Fu et al., 2015). In this context, the objectives of this review are to explore pre-clinical and clinical evidence and mechanisms linking gut microbiota and host-cholesterol metabolism in conditions of normal or altered homeostasis.

CLASSICAL RISK FACTORS ASSOCIATED WITH CHOLESTEROLEMIA

Genome-wide association studies (GWAS) have identified multiple human genetic variants contributing to plasma LDL-C and TC concentrations (Willer et al., 2013; Surakka et al., 2015; Liu et al., 2017; Hoffmann et al., 2018). These latter studies identified 289 and 189 independent variants significantly associated with circulating levels of TC and LDL-C, respectively (Willer et al., 2013; Liu et al., 2017). These genetic polymorphisms collectively account for the phenotypic variance of nearly 20% of TC and LDL-C (Surakka et al., 2015; Hoffmann et al., 2018). Among them, only 1.7 to 2.5% of subjects with elevated LDL-C levels were carrying the known genetic variants identified from familial hypercholesterolemia (LDLR, APOB, and PCSK9) (Abul-Husn et al., 2016; Khera et al., 2016). The results of both twin and family studies estimated a heritability of 46-57% for TC and LDL-C (Yu et al., 2005; Goode et al., 2007; van Dongen et al., 2013). In this respect, we can estimate that environmental/lifestyle factors may account for not less than 50% and up to 80% of the complementary fluctuations of TC and LDL-C. Moreover, development of atherosclerosis and regulation of plasma TC and LDL-C levels are also closely linked to consumption of dietary fatty acids, dietary fibers, carbohydrates, and alcohol, as well as to obesity, tobacco use, and level of physical activity (Yusuf et al., 2004); most of these CV risk factors are correlated with significant changes in the gut microbial ecosystem (Cerdá et al., 2016; Costantini et al., 2017; Savin et al., 2018; Medina et al., 2019; Sarin et al., 2019). Among the best known, saturated-fatty acids, trans-fatty acids, and fibers are the nutritional factors that have the most significant impact on LDL-C (Fernandez, 2001; Riccioni et al., 2012).

Reduction in body weight in severely obese subjects has a modest influence on TC and LDL-C, with each kilogram lost associated with a decrease of ∼0.8 mg/dL in LDL-C. When weight reduction is even higher (e.g., bariatric surgery), the cholesterol-lowering effect is even more pronounced (Benetti et al., 2013). LDL-C can be reduced by regular physical activity (Leon and Sanchez, 2001), as suggested by new genetic variants interacting with physical activity and associated with cholesterol levels (Kilpeläinen et al., 2019). Additionally, other common causes of elevated LDL-C such as biliary obstruction, nephrotic syndrome, hypothyroidism, and pregnancy (Stone et al., 2014) have been connected to adverse effects on the gut microbiota composition (Ejtahed et al., 1969; Tsuji et al., 2018; Lv et al., 2019; Vieira-Silva et al., 2019).

THE NEW PLAYER: COMMENSAL GUT MICROBIOTA

In the last decade, research developments have positioned the commensal gut microbiota at the interface between living organisms and the environment and demonstrated its considerable influence on optimum metabolic functioning (Sekirov et al., 2010). One of the contributions of gut bacteria to host biology is the circulating pool of bacteria-derived metabolites (Nicholson et al., 2012), which can reach or exceed concentrations achieved by a typical drug dose (μM to mM). In many cases, these co-metabolites signal through specific receptors and impact multiple metabolic pathways and host biology. Nearly half of the circulating metabolites are believed to come from bacterial metabolism (Wikoff et al., 2009; Sridharan et al., 2014). In the symbiotic relationships established between resident microorganisms and the host, bacteria benefit from a stable environment (nutrients, temperature, pH, osmolarity, oxygen pressure), and the biological functions of the microbiota are increasingly seen as essential to health: maturation of the immune system, metabolic and nutritional functions, and protection against pathogens. A growing number of pathologies are associated with combined quantitative and qualitative dysbiotic changes in the intestinal microbiota composition and function: diabetes, obesity, cancer, inflammatory bowel disease, autoimmune and allergic diseases, autism spectrum disorders, anxiety, and depression (Lynch and Pedersen, 2016). The microbiota thus appears to be a critical player at the crossroads of physiology and multiple pathologies. It is also emerging as a

powerful transmission channel of environmental changes linked to diet and exposure to drugs, antibiotics, pollutants, and infectious agents. Microbiota modifications induced by recent changes in the human environment thus seem to be a determining factor in the current epidemic of chronic metabolic and inflammatory diseases.

More than 65 million years of mammalian-microbe co-evolution has led to an interdependence. The diversity of bacterial genes allows a wide variety of metabolic activities, such as energy extraction (5–10% of the daily energy requirements of the host) by digesting macromolecular complexes (polysaccharides, glycosaminoglycans, glycoproteins) that are not easily digestible by humans. Bacterial genes also allow the synthesis of vitamins (Yoshii et al., 2019), neurotransmitters (Onaolapo et al., 2020), and metabolites derived from tryptophan (Agus et al., 2018); they can also provide substrates that can feed critical metabolic pathways of the host (short-chain fatty acids) (Sun et al., 2017); they metabolize steroids such as cholesterol (Allayee and Hazen, 2015) or its derivatives, for instance, bile acids (Ridlon et al., 2014) and can thus influence the metabolism of lipids and cholesterol of the host. They can also contribute to or suppress the detoxification of xenobiotics and the biological activities of drugs (Koppel et al., 2017). In this ecosystem, eukaryotic and prokaryotic genes will constitute a reservoir of metabolic response that can be mobilized as a function of nutritional and xenobiotic intakes (Foster et al., 2017).

However, the complexity of intestinal microbial communities and their dialogue with the host's metabolic pathways make functional connections complicated to disentangle in these pathologies. The fundamental challenge now is to understand the causal dimension of these relationships.

OVERVIEW OF THE EPIDEMIOLOGY OF THE GUT MICROBIOTA-CHOLESTEROLEMIA RELATIONSHIP

Recent data from epidemiological studies report associations between phylum, bacterial taxa, and cholesterolemia (Koren et al., 2011; Karlsson et al., 2013; Le Chatelier et al., 2013; Fu et al., 2015) (**Table 1**). These data are based on microbial taxonomy derived from 16S rRNA gene sequencing (Karlsson et al., 2013; Fu et al., 2015) or whole-genome shotgun sequencing of microbial genes collectively present in feces (Karlsson et al., 2013; Le Chatelier et al., 2013), methods that reflect the current state of the art. However, these repertoires of genes or bacterial species do not make it possible to directly report microbial functions, which can vary considerably from one strain to another within the same species. Besides, the repertoire of genes identified at the bacterial DNA level does not necessarily reflect the repertoire of functions that can or will be expressed in the host. Nevertheless, cross-validation analysis on fecal taxonomy and on circulating lipid and lipoprotein levels from 893 individuals of the general Dutch population support a contribution of the microbiome to 1.5% of the variance in TC and 0.7% in LDL-C regardless of age, gender, and genetics, with the family of *Clostridiaceae/Lachnospiracease families* being specifically associated with LDL-C (Fu et al., 2015). Comparable results are found by whole-genome analysis approaches on the same cohort enlarged up to 1135 individuals (Zhernakova et al., 2016). Of note, this population is primarily composed of normolipidemic subjects displaying a mean TC and LDL-C of 1.97 ± 0.39g/L and 1.24 ± 0.36g/L, respectively. These convergent data indicate that circulating concentrations of TC and LDL-C are correlated with changes in microbiota composition, and a recent study conducted on the LifeLines-DEEP cohort (1293 subjects) supports this hypothesis. In this study, 92 plasma proteins associated with CV risk were quantified. Among them, the variance in the concentration of circulating LDL receptor is explained by microbial factors for 5%, while only 0.1% is explained by genetic factors (quantitative trait locus) (Zhernakova et al., 2018).

In patients with metabolic syndrome, interindividual variations in circulating TC and LDL-C are associated with microbial gene richness and diversity (Le Chatelier et al., 2013). The correction of diversity loss after nutritional intervention in dysmetabolic patients corrects hypercholesterolemia (Cotillard et al., 2013) and is associated with a higher abundance of *Akkermansia muciniphila* (Dao et al., 2016) (**Table 1**). When hypercholesterolemia coexists with obesity, hypertension, and glucose intolerance, it should be taken into account that multiple mechanisms can contribute to the

TABLE 1 | List of major clinical evidence.

Evidence	Cohort	Correlation Association	References
Epidemiological	268 healthy subjects (16S)	Enterotypes/ Cholesterol	(de Moraes et al., 2017)
	896 healthy subjects (16S)	Phylum/ Cholesterol	(Fu et al., 2015)
	1135 healthy subjects (MGS)	Taxa/ Cholesterol	(Zhernakova et al., 2016)
	Metabolic Syndrome (MGS)	Fecal microbial gene richness and diversity/Cholesterol	(Karlsson et al., 2013) (Le Chatelier et al., 2013)
	Dyslipidemic cohort	nd	nd
Dietary intervention	49 overweight/ obese adults (MGS)	Fecal microbial gene richness/ LDL-C	(Cotillard et al., 2013) (Dao et al., 2016)

nd, not done.

regulation of cholesterolemia, including pathways through which these pathologies are associated collectively (metabolic syndrome) or individually with dysbiosis (Ussar et al., 2015; Lim et al., 2017; Hoyles et al., 2018). Finally, a study in patients displaying clinical features of atherosclerosis in comparison with control subjects found associations between TC, LDL-C, and the oral abundance of some bacterial species (Koren et al., 2011).

Interestingly, studies performed in pigs, which have a metabolism and microbiome much closer to humans than rodent models, showed a significant contribution of the caecal microbiome of 5.6% to TC and of 2.8% to LDL-C (Huang et al., 2017). Additionally, most microbial taxa positively associated with TC and LDL-C belong to the pathogenic bacteria. These data are consistent with the known relationship between inflammation and serum cholesterol (Khovidhunkit et al., 2004), which needs to be further explored.

Although obtained in general populations, the influence of the gut microbiota on cholesterol levels would undoubtedly benefit from an investigation in a dyslipidemic cohort where reciprocal effects of hypercholesterolemia on microbiota functions may amplify dysbiosis and its consequences on host metabolism. Indeed, such correlative data do not establish a causal link. A disease may modify the gut microbiota, and conversely, the gut microbiota may trigger or aggravate a condition. Additionally, the bacterial species distribution is not homogeneous along the digestive tract, and fecal microbiota mostly reflects colonic species. Thus, feces analysis neglects the potential involvement of commensal species of the small intestine in dysbiosis, though this represents an essential site for the metabolism of cholesterol. Therefore, evaluation of the contribution of the microbiota to cholesterol levels is not optimal. In a recent study, albeit in a small cohort, the authors show that in hyperlipidemic patients, the higher prevalence of small intestinal bacterial overgrowth (SIBO) is positively associated with LDL-C levels (Kvit et al., 2019).

MODULATION OF THE MICROBIOTA AND ITS IMPACT ON CHOLESTEROLEMIA IN HUMANS

After birth primo-colonization of the digestive tract, the gut microbiota becomes richer and more diversified all through life as a result of environmental challenges such as those from nutritional status, cultural habits, and drug treatments (Rothschild et al., 2018). Thus, the effect of changes in diet composition, eating patterns, on cholesterolemia is likely related to the benefits of prebiotics (Beserra et al., 2015) or a Mediterranean (Filippis et al., 2016) or vegan (versus omnivorous) (Wu et al., 2016) diet, which correlate with variations in microbiota composition. Likewise, lipids are strongly modified at birth and at weaning, two periods associated with major changes in microbial composition (Nuriel-Ohayon et al., 2016) and bile acid (BA) metabolism (Jönsson et al., 1995), which can influence circulating lipid and lipoprotein concentrations (Joyce et al., 2014). When administered orally, antibiotics induce a reduction in circulating cholesterol, which is strongly associated with changes in the composition of microbiota-derived secondary BAs (Samuel and Whithe, 1961; Samuel et al., 1973; Miettinen, 1979).

GUT MICROBIOTA AND CHOLESTEROL TRAITS IN PRECLINICAL MODELS

Numerous studies that specifically evaluated the potential role of the microbiota in the regulation of cholesterol homeostasis have been conducted by using conventional approaches to eradicate the microbiota by either antibiotic therapy or an axenization procedure. These latter studies revealed that the absence of microbiota significantly influences cholesterolemia (**Table 2**). However, these associations are not all consistent in the normolipidemic context. Some teams show decreases in TC (Rabot et al., 2010) (Velagapudi et al., 2010; Joyce et al., 2014; Zhong et al., 2015), while others find no effect (Danielsson and Gustafsson, 1959; Sayin et al., 2013; Out et al., 2015; Caesar et al., 2016; Mistry et al., 2017; Zarrinpar et al., 2018) or even an increase (Danielsson and Gustafsson, 1959; Caesar et al., 2016). The potential underlying explanations of such variability involve (i) differences in microbiota composition between the animal facilities, (ii) normolipidemic wild type mice carry the majority of plasma cholesterol in HDL, (iii) reduced penetrance of the influence of the microbiota in a homeostatic context, as observed in the general human population (Fu et al., 2015). By contrast, in a commonly used mouse model of dyslipidemia (apolipoprotein-E and Ldl-r deficient mice), almost all studies show increased cholesterol levels in the absence of gut-microbiota (**Table 2**) (Wright et al., 2000; Stepankova et al., 2010; Chen et al., 2016; Kasahara et al., 2017; Lindskog Jonsson et al., 2018; Kiouptsi et al., 2019; Le Roy et al., 2019). The lipid-rich environment is associated with an impoverishment of gut microbiota diversity and richness (Martínez et al., 2013; Bo et al., 2017; Tran et al., 2019), increased intestinal barrier permeability, and endotoxemia (Netto Candido et al., 2018; Schoeler and Caesar, 2019; Wisniewski et al., 2019). Thus, in this inflammatory context, the influence of microbiota on cholesterolemia is revealed, as suggested by data obtained in the pig model (Huang et al., 2017). These findings are in agreement with data obtained in rodent models in which hypercholesterolemia associated with acute activation of innate immune receptors by endotoxin/lipopolysaccharide (LPS) is connected with an increase hepatic cholesterol synthesis and VLDL production and decreased VLDL and LDL clearance (also termed the lipemia of sepsis) (Harris et al., 2000). The underlying molecular mechanisms involve decreased nuclear receptor signaling of peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), farnesoid X receptor (FXR), and retinoid X receptor (RXR) (Khovidhunkit et al., 2004). It additionally involves inhibition of reverse cholesterol transport (RCT) at multiple points, including decreased hepatic production of apolipoprotein A1, cholesterol ester transfer protein (CETP),

TABLE 2 | List of major pre-clinical evidence.

Model	Diet	Phenotype in germ-free	References
Axenic Normolipidemic Rat	CD	Chol ↗	(Danielsson and Gustafsson, 1959)
	CD with 0.5% cholesterol	Chol →	(Danielsson and Gustafsson, 1959)
Axenic Normolipidemic Mice	HFD 0.03% cholesterol	Chol ↘	(Rabot et al., 2010)
	CD*	Chol ↘	(Velagapudi et al., 2010)
	CD	Chol →	(Sayin et al., 2013)
	WD with 0.2% cholesterol	Chol ↘	(Zhong et al., 2015)
	CD source of fat: lard	Chol ↗	(Caesar et al., 2016)
	CD source of fat: fish oil	Chol →	(Caesar et al., 2016)
	CD	Chol →	(Mistry et al., 2017)
Normolipidemic Mice (axenization by a mixture of antibiotics)	CD*	Chol ↘	(Joyce et al., 2014)
	CD	Chol →	(Out et al., 2015)
	CD	Chol →	(Zarrinpar et al., 2018)
	CD	Chol →	Personal observations
Axenic Dyslipidemic Mice	$Apoe^{-/-}$ 0.15% cholesterol diet	Chol →	Wright et al., JEM 2000
	$Apoe^{-/-}$ CD	Chol ↗	(Stepankova et al., 2010)
	$Apoe^{-/-}$ 2% cholesterol	Chol ↗	(Stepankova et al., 2010)
	$Apoe^{-/-}$ CD	Chol ↗	(Kasahara et al., 2017)
	$Apoe^{-/-}$ CD	Chol ↗	(Lindskog Jonsson et al., 2018)
	$Apoe^{-/-}$ WD	Chol →	(Lindskog Jonsson et al., 2018)
	$Ldlr^{-/-}$ CD	Chol ↗	(Kiouptsi et al., 2019)
	$Ldlr^{-/-}$ 0.2% cholesterol diet	Chol →	(Kiouptsi et al., 2019)
	$Apoe^{-/-}$ CD	Chol ↗	(Chen et al., 2016)
Dyslipidemic Mice (axenization by a mixture of antibiotics)	$Apoe^{-/-}$ 0.15% cholesterol diet	Chol ↗	(Chen et al., 2016)
	$Apoe^{-/-}$ CD	Chol ↗	(Le Roy et al., 2019)
	$Ldlr^{-/-}$ CD	Chol ↗	(Le Roy et al., 2019)
Dyslipidemic Mice FMT	$Apoe^{-/-}$ CD	Cholesterol levels transmitted**	(Le Roy et al., 2019)

*All studies involved mice of C57Bl6 genetic background, except for two studies that used Swiss Webster mice (indicated by *). CD, chow diet; HFD, high-fat diet; WD, western diet; FMT, fecal material transfer.*

***Phenotype in recipient mice.*

↗Increased; ↘decreased; →no changes.

ATP binding cassette transporters ABCG5 and ABCG8, and Cyp7a1. These findings are consistent with the association found of the lipopolysaccharide receptor Toll-like receptor 4 (TLR4) and NIMA-related kinase 7 (NEK7) polymorphisms with LDL-C in human (Zhu et al., 2015; Gomes Torres et al., 2019). NEK7 is a serine/threonine kinase required for NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome assembly. However, this relationship should be tempered as chronic TLR-signaling deficiency in $MyD88^{-/-}/Apoe^{-/-}$ (Björkbacka et al., 2004) (Michelsen et al., 2004), $TLR4^{-/-}/Ldl\text{-}r^{-/-}$, (Ferreira et al., 2015), $TLR2^{-/-}/Apoe^{-/-}$, and $TLR4^{-/-}/Apoe^{-/-}$ (Higashimori et al., 2011) mice is not associated with changes in cholesterol levels as compared to control mice. It should also be noted that the evidence for the role of microbiota in genetically modified mice is difficult to interpret, as numerous studies in the literature do not report the experimental conditions (production of experimental groups, use of littermates, housing conditions). Indeed, fecal microbiota is partially normalized by extended co-housing conditions, due to coprophagic and grooming behaviors, thus abrogating microbiota-genotype dependent phenotype.

Finally, using a standardized method, our recent work (Le Roy et al., 2019) demonstrates the microbiota-dependent transmissibility of a significant proportion of the cholesterol level (around 15-20%). Indeed, transplantation of the microbiota from hypercholesterolemic (without known genetic cause) human donors into recipient mice is sufficient to transfer the phenotype compared to the same experiment performed with normolipidemic donors. The more hypercholesterolemic phenotype is associated with "low hepatic cholesterol synthesis" and "high intestinal cholesterol absorption" traits in recipient mice. Several bacterial phylotypes affiliated with *Beta-proteobacteria phylum*, *Alistipes genus, and Barnesiella genus* were enriched in hypercholesterolemic mouse recipients. Similarly, *Alistipes* were recently associated with TC and LDL-C in HFD-fed hamsters treated with a chitin9-derived polysaccharide (chitosan) (Tong et al., 2019).

It is also of particular clinical interest to show the influence of the intestinal microbiota on the balance between absorption and cholesterol synthesis (Le Roy et al., 2019) since it has been observed in human cohorts that "high absorption" and "low synthesis" patterns are associated with higher LDL-C levels and are predictive of cardiovascular events (Matthan et al., 2009; Silbernagel et al., 2010; Weingärtner et al., 2011). Thus, inter-individual evaluation of microbiota diversity or dysbiosis opened up new opportunities for better therapeutic decision-making in ASCVD.

GUT BACTERIAL METABOLITES, THE NEW FRONTIER FOR DEFINING PATHOLOGICAL METABOLOTYPES

Primary Bile Acids

Bile acid (BA) biosynthesis is the predominant metabolic pathway for cholesterol catabolism in the human body. The

conversion of cholesterol to bile acids is a process performed by a set of hepatic enzymes necessary for the conversion of the steroid nucleus of cholesterol, the elimination of the side chain, amidation on the side chain with either glycine or taurine (GCA, GCDA, TCA, TCDCA) and eventually sulfonation or glucuronidation in the steroid backbone (Hofmann and Hagey, 2014). Another critical aspect of BA physiology is their circulation in the enterohepatic cycle, a finely tuned and orchestrated system, in which BAs synthesized in the liver are actively transported in the bile ducts, stored in the gallbladder, then secreted in the duodenum, absorbed again in the ileum, and recaptured by the liver *via* the portal circulation. Each stage of this enterohepatic cycle is influenced by diet, hormonal cross-regulation, and bacterial activities that maintain a functional and non-toxic supply of bile acids in circulation. Indeed, BAs have a pro-inflammatory and cytotoxic potential when they are not regulated, due to their detergent activity and destabilization of membranes, as shown in cholestatic liver diseases (**Figure 1**).

In the post-prandial period, primary BAs are released into the intestinal lumen. Due to their amphiphilic properties, primary BAs adsorb at an oil–water interface to form mixed micelles with other bile lipids (cholesterol, phosphatidylcholine), fat-soluble vitamins (such as vitamins A, D, E, and K) and lipolysis products (free fatty acids, 2-monoglycerides). In the absence of bile secretion, fat absorption is impaired (Hofmann, 1999; Dawson and Karpen, 2015). In the ileum, a highly efficient transporter system allows active reabsorption of conjugated-BAs redirected to the liver through mesenteric and hepatic portal veins (Dawson and Karpen, 2015). At each enterohepatic cycle (4-5 cycles per day), about 5% of the non-absorbed BAs are released into the colon, modified by bacteria, and then excreted. This represents around 600 mg per day, the loss of which is compensated for by an equivalent synthesis from hepatic cholesterol. The co-excretion of fecal sterols and BAs in a 2:1 ratio in humans therefore represents a significant pathway for regulating cholesterol homeostasis (Groen et al., 2014).

Secondary Bile Acids

Another complexity in BA metabolism is the modification of the BA structure by intestinal bacteria (Ridlon et al., 2014). BAs that are not reabsorbed encounter anaerobic resident bacteria in the colon (**Figure 1**). Microbial enzymes such as bile salt hydrolases (BSH) deconjugate conjugated -BAs, bacterial 7 α-dehydroxylases and 7 β-dehydroxylases convert CA and CDCA to deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. Bacterial 7β-isomerization of the 7α-hydroxyl group of CDCA forms ursodeoxycholic acid (UDCA). Sulfated and glucuronidated BAs formed during hepatic detoxification to facilitate their urinary and fecal excretion can be hydrolyzed of their ester linkage by microbial enzymes (Takikawa et al., 1983; Gérard, 2013), ultimately leading to the presence of a vast repertoire of secondary BAs. In the colon, unconjugated BAs produced by microbial metabolism can diffuse passively over the intestinal border and can eventually be captured by the liver through multispecific organic anion-transporting polypeptide (OATP) transporters that can vehicle unconjugated BAs

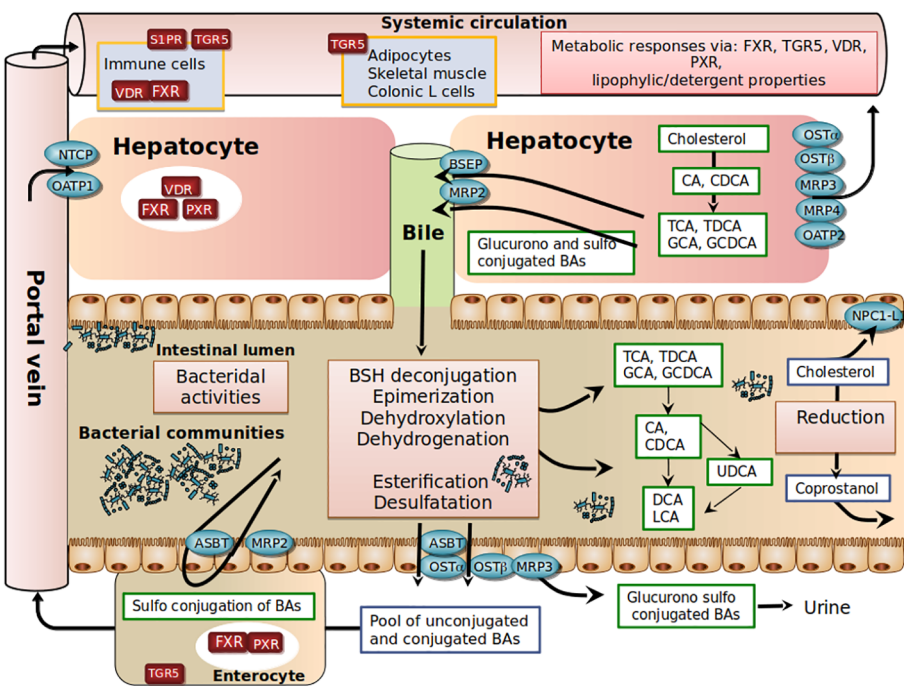

FIGURE 1 | Schematic view of host-gut microbial co-metabolism of bile acids and cholesterol in enterohepatic circulation.

(Unconj-BA) and sulfated BAs (Dawson and Karpen, 2015). In the liver, conjugated BAs are more efficiently recycled from portal blood at the hepatic basolateral membrane by the high-affinity sodium-dependent taurocholate cotransporting polypeptide (NTCP) than are unconjugated BAs (Angelin et al., 1982; Hofmann and Hagey, 2014; Eggink et al., 2018). The estimated hepatic fractional uptake of total BAs ranges from 50 to 90% depending on the bile acid structure (Angelin et al., 1982) and is reflected by differences in systemic blood concentration versus portal blood concentration (Eggink et al., 2018). During the completion of the enterohepatic cycle, unamidated BAs can be conjugated again in the liver, leading to the formation of their glycine or taurine conjugates (GDCA, GLCA, GUDCA, TDCA, TLCA, and TUDCA), while CDCA and LCA can be 6α-hydroxylated to form hyocholic acid (HCA) and hyodeoxycholic acid (HDCA), respectively (Bodin et al., 2005). UDCA represents about 4% of total fecal BAs, and a cholesterol-lowering effect has been reported in patients with primary biliary cirrhosis (Poupon et al., 1993) or hypercholesterolemia (Cabezas Gelabert, 2004). Other exclusively microbial activities, including esterification, oxidation, and desulfation, contribute to the high chemical diversity and changes in hydrophobicity (Ridlon et al., 2014; Devlin and Fischbach, 2015; Watanabe et al., 2017) and bile acid signaling activities (Thomas et al., 2008; de Boer et al., 2018) (**Figure 1**).

It should be noted that molecular species of BAs are involved not only in lipid metabolism but also in carbohydrate metabolism, energy homeostasis, and host immune responses through their agonistic or antagonistic activities on diverse receptors, the best characterized being the farnesoid X nuclear receptor (FXR) and the TGR5 membrane receptor (Thomas et al., 2008; de Boer et al., 2018); these aspects have been extensively reviewed elsewhere (Houten et al., 2006; Thomas et al., 2008; Lefebvre et al., 2009). Basically, FXR impacts cholesterolemia through the repression of CYP7A1, the rate-limiting enzyme that catabolizes conversion of cholesterol into BAs, resulting in decreased hepatic cholesterol content, followed by upregulation of the LDL-receptor expression and activity, which consequently reduces plasma LDL-C levels. This mechanism underlies the hypocholesterolemic effect of BAs sequestrants (Spinelli et al., 2016). Administration of obeticholic acid (FXR agonist) to chow-fed mice elevates liver LDL receptor expression by mRNA stabilization and reduces plasma LDL-C in mice (Singh et al., 2018). Of note, a recent study discovered a novel association of a variant in human NR1H4 gene (encoding the BA receptor FXR) with levels of TC and LDL-C (Deaton et al., 2018), thus highlighting the role of FXR in the regulation of plasma cholesterol levels in humans.

In the context of cholesterol reduction following antibiotic treatment, in humans (Jenkins et al., 2005), the prevailing hypothesis is that inhibition of the conversion of primary BAs to secondary BAs reduces their hydrophobicity, which results in poorer reabsorption by passive diffusion through the colonic epithelium. Similarly, this decrease in hydrophobicity of BAs is associated with a poorer micellization of cholesterol, which would, therefore, be less efficiently absorbed. These joint activities contribute to the outflow of BAs and cholesterol in the stool and therefore to a decreased sterol pool of the whole body. However, in humans, other molecular mechanisms must coexist to the extent that treatments with primary bile acids (CDCA) or secondary bile acid (LCA) only slightly alter the absorption of cholesterol and the serum concentrations of LDL-C (Wang et al., 2006). Other putative mechanisms qualitatively and quantitatively modulating the pool of bile acids may be at work; a pool of depleted BAs will be associated with the proliferation of pro-inflammatory microbes (Kakiyama et al., 2013) and intestinal barrier dysfunction (Kang et al., 2017), and a pool reconstituted after transfer of fecal material or liver transplantation will correct endotoxemia (Kang et al., 2017; Bajaj et al., 2018) and lipidemia (Kang et al., 2017).

Interestingly, the treatment of 51 naive type-2 diabetic patients with an antidiabetic (acarbose: a tetra-saccharide inhibiting hydrolysis of carbohydrates in the upper intestine and thus reducing glucose absorption) led to improvements of glycemia and cholesterolemia (Gu et al., 2017). These changes were correlated with variations in plasma BA profiles. The primary-BA/secondary-BA ratio and UDCA and T-DCA concentrations were negatively correlated with plasma cholesterol. Accordingly, metagenomics analysis confirmed a lower capacity for 7α/β dehydroxylation of BAs after acarbose treatment. The relative abundances of baiE (rate-limiting enzyme for 7α-dehydroxylation) and baiI (7β-dehydratase) were significantly decreased after acarbose treatment. Phylogenic analysis established a strong inverse correlation between *Lactobacillus rhamnosus* and plasma cholesterol and LDL-C levels. Interestingly, decreases in plasma cholesterol levels associated with acarbose treatment were associated with a decline in *allistipes* spp., in accordance with recent studies (Le Roy et al., 2019; Tong et al., 2019).

In conclusion, the mechanisms underlying bile acid-cholesterol-lowering relationships remain largely undefined. The specific roles of bile acids *in vivo* remain difficult to disentangle, due to the large number of compounds and biological properties involved, including detergent and bactericidal activities and FXR signaling potential (**Figure 1**). The picture is even more complex if we consider the newly involved receptors such as pregnane X receptor (PXR), vitamin D3 receptor (VDR), muscarinic acetylcholine receptors, and sphingosine 1-phosphate receptor (S1PR). In addition to qualitative parameters, it is also necessary to consider all the poorly known circadian and post-prandial quantitative variations of BAs that will need to be well-defined to better understand the impact of BAs on cholesterol homeostasis (Han et al., 2015).

Short-Chain Fatty Acids (SCFAs)

SCFAs are the main end-product produced by the bacterial fermentation of non-digestible dietary fibers in the caecum and proximal colon. Consumption of dietary fibers such as inulin, oat bran, and pectin is effectively associated with lower plasma cholesterol levels, with reductions in cholesterol level ranging from 0.5% to 2% per gram of intake (Ripsin et al., 1992). Fibers

reduce both TC and LDL-C (Anderson and Chen, 1979) through increased BA excretion and decreased hepatic synthesis of cholesterol (Vahouny et al., 1980; Jenkins et al., 2010). Other potential mechanisms are related to the microbiota-dependent formation of SCFAs (acetate, propionate, butyrate) that are produced and can be used as a macronutrient source of energy. Alternatively, SCFAs can act as hormone-like signaling, entering the portal circulation to ultimately bind to G-protein-coupled receptors (GPR) in numerous cells (Maslowski et al., 2009) and inhibit the histone deacetylase (HDAC), resulting in numerous epigenetic modifications in targeted cells (Riggs et al., 1977; Candido, 1978).

Several studies have described the role of SCFAs on immunity (Aguilar et al., 2014) and its protective effects against cardiovascular disease (Bazzano et al., 2003; Fernandez et al., 2019), yet few of them have individually examined the effects on cholesterol levels. *Ex vivo* and *in vivo* studies have shown that acetate and butyrate (but not propionate) are potential precursors of cholesterol synthesis that can be incorporated into the endogenous cholesterol synthesis pathway (Wolever et al., 1991; Demigné et al., 1995; den Besten et al., 2013). By contrast, earlier studies reported inhibition of cholesterol synthesis by propionate through decreased expression of the HMGCS and HMGCR genes (Bush and Milligan, 1971; Rodwell et al., 1976; Demigné et al., 1995), white more recent studies have not confirmed such effects (Zhao et al., 2017). Accordingly, in healthy subjects, the oral administration of propionate does not lower plasma cholesterol but does increase HDL and triglycerides levels (Venter et al., 1990). In vivo, supplementation of a cholesterol-rich diet with acetate in rats resulted in a lower increase of TC levels associated with lower hepatic HMGCS and increased conversion of cholesterol into BAs due to the upregulation of Cyp7a (Fushimi et al., 2006). A similar rise in Cyp7a was observed in Apoe-deficient mice fed with a cholesterol-rich diet supplemented with butyrate, with additional beneficial effects on the "reverse cholesterol transport" (RCT) (Du et al., 2019). The connection between SCFAs and BAs metabolism was further reported in Syrian hamsters (Zhao et al., 2017). The addition of acetate, propionate, or butyrate to a cholesterol-rich diet resulted in decreased CT levels and LDL-C/HDL-C ratio and to increased fecal excretion of BAs (LCA, DCA, CDCA, CA). Expression of the SREBP2, LDLR, and CYP7A1 genes was also involved (Zhao et al., 2017). The effects of SCFAs were more specifically addressed in mice deficient for FFAR2/GPR43, one of the SCFA receptors (Bjursell et al., 2011). High-fat fed GPR43-deficient mice displayed lower CT levels than control mice.

Collectively, the role of SCFAs in cholesterol levels is poorly defined. SCFAs may be used as precursors of cholesterol synthesis, but the overall hypocholesterolemic effect seems to be associated with the conversion of cholesterol into BAs. Translation studies into humans will be critical to move forward (**Figure 2**).

Trimethylamine-oxide (TMA-O)

The contributing role of intestinal microbiota to ACV diseases through the production of trimethylamine-N-oxide (TMA-O,

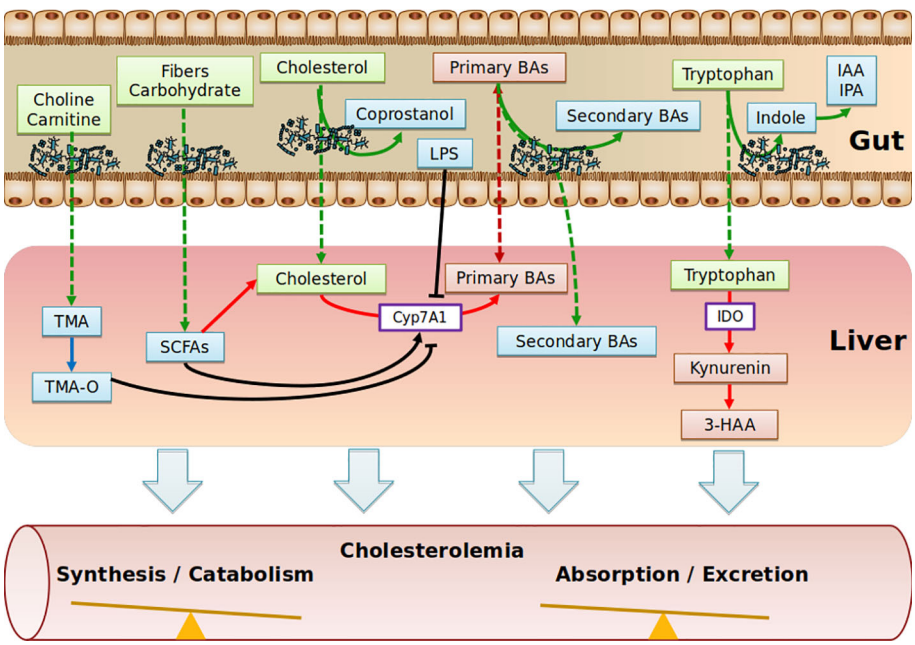

FIGURE 2 | Schematic depicting the range of putative pathways through which the gut metabolites impact on cholesterol metabolism. Diet nutriments (green boxes) are metabolized and transformed into microbial metabolites (blue boxes) by gut microbiota. Purple boxes represent enzymes. Red boxes represent host metabolites. TMA, Trimethylamine; TMA-O, Trimethylamine-Oxide; SCFAs, Short Chain Fatty Acids; BAs, Bile Acids; IAA, Indole-3-acetic acid; IPA, Indole-3-propionic acid; IDO, indoleamine2,3-dioxygenase; 3-HAA, 3-hydroxyanthranilic; LPS, lipopolysaccharides.

has been recently demonstrated (Randrianarisoa et al., 2016; Tang et al., 2017; Yang et al., 2019) and has been covered in recent reviews (Brown and Hazen, 2018; Yang et al., 2019). Essentially, trimethylamine-containing dietary nutrients (choline, phosphatidylcholine, γ-butyrobetaine, and carnitine) are metabolized by microbes, leading to the production of trimethylamine (TMA), which is rapidly converted by host hepatic flavin monooxygenase 3 (FMO3) into trimethylamine N-oxide (TMA-O). Interestingly, in dyslipidemic mouse models, TMA-O affects cholesterol homeostasis mainly by suppressing reverse cholesterol transport (RCT) (Koeth et al., 2013) and impacting the BA metabolic pathways at multiple levels (decreased expression of hepatic BAs synthetic enzymes (Cyp7a1 and Cyp27a1) and hepatic BA transporters (Oatp1, Oatp4, Mrp2, and Ntcp) (Koeth et al., 2013). Additionally, TMA-O appears to promote cholesterol uptake by macrophages by inducing scavenger receptors CD36 and SRA1, both of which are involved in the intracellular accumulation of modified lipoproteins (Wang et al., 2011). Likewise, under normal dietary conditions, TMA-O did not impact plasma cholesterol levels in mice deficient for FMO genes (Veeravalli et al., 2018). Identification of TMA-O receptors would be of particular interest to substantiate a potential association of TMA-O with cholesterol levels, albeit that no significant correlations between TMA-O and TC, LDL-C, even when excluding individuals taking cholesterol-lowering medications, have been observed (Li et al., 2017). This might explain why TMA-O has been demonstrated to be a prognostic marker for ACV diseases beyond traditional risk factors (Manor et al., 2018) (**Figure 2**).

Diet-Associated Tryptophan (Trp) Derivatives

Tryptophan is an essential amino acid that is degraded through the kynurenine pathway, leading to the generation of several biologically active compounds. Endogenous kynurenine metabolites contribute to the initiation of ACV disease. In human atherosclerotic plaques, Trp metabolites were found to be associated with unstable plaque phenotype (Taleb, 2019). Tryptophan is processed 95% by the kynurenine pathway (gut-microbiota independent) and 5% by the indole pathway (gut microbiota-dependent). Regarding the kynurenin pathway, supplementation of 3-hydroxyanthranilic (3-HAA), a tryptophan-derivative metabolite from the kynurenine pathway has anti-atherosclerotic effects, associated with lower plasma cholesterol levels in Ldl-r-deficient mice fed an HFD regime (Zhang et al., 2012) or a western diet (Berg et al., 2019). Correspondingly, indoleamine 2,3-dioxygenase (IDO) inhibition showed the exact opposite phenotype in Apoe-deficient mice fed an HFD (Polyzos et al., 2015; Liang et al., 2019), while no effect was reported in chow-diet-fed double-deficient mice for Apoe and IDO (Cole et al., 2015). IDO enzymes are involved in the catabolism of tryptophan, and the ratio of kynurenine to tryptophan (kyn/trp) can be used to reflect IDO activity. In clinical investigations, IDO activity has been reported to be positively correlated with a range of atherosclerosis risk factors in the female population, including LDL-C (Pertovaara et al., 2007). Concerning the indole pathway, tryptophan, indole-3-propionic acid, and indole-3-aldehyde were shown to be decreased in atherosclerotic patients, while kynurenine/tryptophan ratios were increased (Cason et al., 2018); still, no independent correlation with cholesterol has yet been reported. In conclusion, the scarcity of studies does not allow the indole pathway to be implicated in regulation of cholesterolemia (**Figure 2**).

CONVERSION OF CHOLESTEROL INTO COPROSTANOL

Cholesterol from the diet, bile, or intestinal cells is actively metabolized by intestinal bacteria, mainly in coprostanol (Gérard, 2013). Unlike cholesterol, coprostanol is very poorly absorbed by the intestine (Bhattacharyya, 1986). In a singular way, the rate of conversion of microbial cholesterol to coprostanol in the general human population appears to be multimodal, with an average of 65% of high converters (80% to 100% of luminal cholesterol is converted to coprostanol in the colon), 21% of intermediate converters and 14% of non-converters (Wilkins and Hackman, 1974; Midtvedt et al., 1990; Veiga et al., 2005; Benno et al., 2005). It was also demonstrated that this phenotypic characteristic was maintained in axenic rodents (without germs) colonized with a high-converter or non-converter human microbiota (Gérard et al., 2004). Finally, several clinical and preclinical studies support the hypothesis that the conversion of cholesterol in coprostanol could influence the bioavailability of cholesterol, leading to modulation of plasma cholesterol levels (Sekimoto et al., 1983; Li et al., 1995; Li et al., 1998). Larger studies are needed to validate this relationship. Notably, the disconnection between the major cholesterol uptake site (small intestine *via* the Niemann-Pick C1-Like 1 transporter (NPC1L1)) and the site of cholesterol conversion to coprostanol (colon) does not plead for a causal relationship. Nevertheless, normolipidemic subjects treated with Ezetimibe (NPC1L1 inhibitor) show residual absorption of cholesterol, which suggests as yet unidentified additional absorption mechanisms (Jakulj et al., 2016) (**Figures 1** and **2**).

IMPACT OF THE MICROBIOTA ON HYPOCHOLESTEROLEMIC DRUG EFFICACY

The gut microbiota has been shown to impact, negatively or positively, drug efficacy. This effect has been shown to result either from modifications in pharmacokinetic or pharmacodynamic properties or by synergistic/antagonistic effect of microbiota toward drugs. As a matter of fact, the impact of the microbiota on drugs is not restrained to oral drug intake, as studies have shown modifications in monoclonal antibody efficacy (Sivan et al., 2015; Doherty et al., 2018). The interaction of gut microbiota with drug efficacy/toxicity has recently been exhaustively reported upon (Spanogiannopoulos et al., 2016; Wilson and Nicholson, 2017; Clarke et al., 2019).

Direct links between the gut microbiota and hypocholesterolemic drugs are still thin. Statins, which are the leading pharmaceutical class

in hyperlipemia therapeutic care, are ineffective for almost 20% of patients treated and are sometimes even deleterious (Stroes et al., 2015). Several studies have explored and demonstrated that statins can directly influence the growth and virulence of bacterial pathogens and commensal bacteria as well as combating microbial infections, such as in sepsis and pneumonia (Hennessy et al., 2016; Zimmermann et al., 2019). The first statin, "Mevastatin," which is a metabolic product of Penicillium citrinum, was initially characterized for its antibiotic properties, and statins are now considered as adjuvant antibiotics that can impact antimicrobial resistance (Ko et al., 2017). Consequently, the role of statins deserves to be explored beyond their traditionally established indications in light of their antimicrobial potential as a regulator of gastrointestinal microbiota (Nolan et al., 2017). Only a few studies have shown the impact of the microbiota on statin efficacy. Still, these studies suggested that the microbiota participated in statin's effect (He et al., 2017) and was responsible for statin metabolization (Yoo et al., 2014) and that the microbiota from patients unresponsive to statin was different from that of responsive patients (Sun et al., 2018).

Additional studies are required in this context and should, therefore, also be conducted on other therapeutic classes of hypolipemic drugs. Understanding the impact of the microbiota on drug efficacy/toxicity should bring us closer to personalized medicine and should result in an improvement in therapeutic care.

CONCLUDING REMARKS

LDL-C is the primary target for the management of atherogenic dyslipidemia and the reduction of cardiovascular events. New actors such as the microbiota introduce more complexity into this multifactorial disease but allow new insight into pathogenicity and the development of new prevention and prophylaxis approaches. In addition to the usual pharmacological approaches (statins, ezetimibe, fibrates, resins, proprotein convertase subtilisin-kexin 9 (PCSK9) inhibitors), new biotherapies targeting the microbiota are possible. Indeed, the data in the literature support the notion that the microbiota has a causal contribution to the metabolism of lipoproteins and host cholesterol. The mechanisms of this reciprocal influence need to be clarified, and the advent of functional analyses of the microbiota, the development of new technologies allowing the culture of anaerobic microbes, and the advent of more and more better-performing technologies will make it possible to specify the dynamics of the relationship of the intestinal microbiota with cholesterol metabolism.

AUTHOR CONTRIBUTIONS

RV, PK, SB, MS, DR, MG, and PL originally conceived and wrote the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Calcific Aortic Valve Disease-Natural History and Future Therapeutic Strategies

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Calcific aortic valve disease (CAVD) is the most frequent heart valve disorder. It is characterized by an active remodeling process accompanied with valve mineralization, that results in a progressive aortic valve narrowing, significant restriction of the valvular area, and impairment of blood flow.The pathophysiology of CAVD is a multifaceted process, involving genetic factors, chronic inflammation, lipid deposition, and valve mineralization. Mineralization is strictly related to the inflammatory process in which both, innate, and adaptive immunity are involved. The underlying pathophysiological pathways that go from inflammation to calcification and, finally lead to severe stenosis, remain, however, incompletely understood. Histopathological studies are limited to patients with severe CAVD and no samples are available for longitudinal studies of disease progression. Therefore, alternative routes should be explored to investigate the pathogenesis and progression of CAVD.Recently, increasing evidence suggests that epigenetic markers such as non-coding RNAs are implicated in the landscape of phenotypical changes occurring in CAVD. Furthermore, the microbiome, an essential player in several diseases, including the cardiovascular ones, has recently been linked to the inflammation process occurring in CAVD. In the present review, we analyze and discuss the CAVD pathophysiology and future therapeutic strategies, focusing on the real and putative role of inflammation, calcification, and microbiome.

Keywords: severe aortic stenosis, calcific aortic valve, aortic valve replacement, TAVR, microbiome, immune system, inflammation

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INTRODUCTION

Calcific aortic valve disease (CAVD) is the most common valve disease worldwide (Nkomo et al., 2006). Epidemiological studies show that 2.8% of adults over 75 years old have some CAVD degree, and as many as 25% of adults over 65 years old have at least valvular sclerosis (Miller et al., 2011).

CAVD is a chronic process characterized by progressive fibrotic tissue remodeling and mineralization (Mathieu et al., 2015). Over the years, there is a disease continuum from sclerosis to chronic inflammation and finally leaflet calcification, culminating with severe stenosis. Human pathologic samples have shown that key features in the CAVD development include pathological concentrations of inflammatory cells and lipid species (Otto et al., 1994; O'Brien et al., 1996).

Several risk factors have been identified as relevant in the CAVD progression. Among others, male gender, high triglyceride levels, and smoking have been independently associated with early aortic valve replacement in the presence of CAVD. Levels of oxidative stress, higher in patients that are more exposed to certain risk factors than others, could be responsible for the starting of the sclerotic CAVD phase (O'Brien, 2006; Ferreira-Gonzalez et al., 2013).

Calcification is recognized as an active disease process driven by the native valvular interstitial cells (VICs) (Sun et al., 2013). These cells acquire an osteogenic and pro-calcific profile in response to different pathological stimuli, such as inflammatory mediators, endothelial damage, low-density lipoprotein (LDL) accumulation, reactive oxygen species (ROS), increase calcium/phosphate (Ca/Pi) levels, modified lipids, and cyclic stretch (Liao et al., 2008; Rajamannan et al., 2011; Rattazzi et al., 2014). The underlying process that ends with the ectopic mineralization of the aortic valve is still not entirely understood. Treatment options include operative valve replacement and percutaneous implantation of valve prosthesis (Lauten et al., 2013; Lauten et al., 2014; Daubert et al., 2016; Alushi et al., 2019; Wernly et al., 2019).

To date, there is no medical treatment available to prevent or reverse calcium deposition within the valve leaflets. Conventional cardiovascular drugs studied in clinical trials failed to influence the disease progression or reduce adverse outcomes, therefore additional research is required to understand the mechanisms of disease progression and, identify novel therapeutic targets (Cowell et al., 2005; Freeman and Otto, 2005; Rossebo et al., 2008; Chan et al., 2010).

CAVD BIOLOGY

Histological Structure of the Aortic Valve

The aortic heart valve consists of three leaflets that allow blood flow from the left ventricle to the aorta without regurgitation (Liao et al., 2008). Each leaflet has a trilaminar structure that is vital for the biomechanical properties of the aortic valve (Tseng and Grande-Allen, 2011; Lindman et al., 2016) as shown in **Figure 1**. Histologically, the leaflets are composed of three distinct layers: fibrosa, spongiosa, and ventricularis. Fibrosa and ventricularis are the external layers, facing the aorta and the left ventricle respectively. Fibrosa consists largely of collagen fibers with dispersed VICs, that are thought to be responsible for reinforcing the valvular structure (Dweck et al., 2012a).

The central layer, spongiosa, is rich in glycosaminoglycans (GAGs) and is responsible for absorbing some of the mechanical stress generated during the cardiac cycle.

The ventricularis is localized on the ventricular side of the leaflets and consists of collagen and elastin fibers (Chen and Simmons, 2011; Mathieu et al., 2015). The tissue composition of ventricularis provides more compliance and grants the

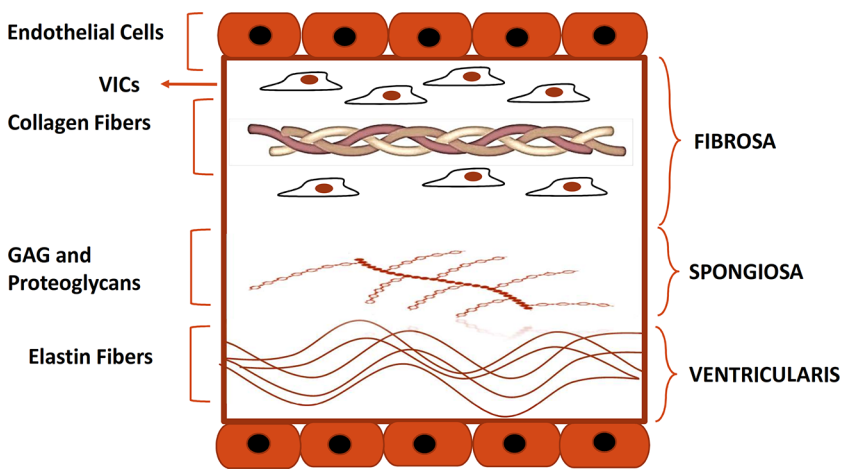

FIGURE 1 | Histological structure of the aortic valve. Depicted is the histological structure of the healthy aortic valve. Fibrosa, spongiosa, and, ventricularis are the three layers that make up the structure of a normal aortic valve. The Fibrosa layer is composed of type I and III collagen fibers and contains also VICs. Spongiosa and ventricularis layers are respectively composed of GAG and proteoglycans and elastin fibers. Endothelial cells form a monolayer on each side of the cusp. GAG, glycosaminoglycans; VIC, valve interstitial cell.

apposition of free edge leaflet regions, thus preventing the backward blood flow into the left ventricle during diastole (Lindman et al., 2016). The normal valve leaflet is avascular and free of infiltrating lymphocytes or monocytes.

Pathobiology of CAVD

The CAVD pathophysiology is schematically depicted in **Figure 2**. The process of progressive fibrocalcific remodeling of the aortic valve is multifactorial, involving genetic predisposition, endothelial shear stress, chronic inflammation, lipid deposition, and valve calcification (Freeman and Otto, 2005; Perrot et al., 2019; Thériault et al., 2019). In its early stages, CAVD resembles atherosclerosis. The existence of shared risk factors for the disease development and the correlation between the severity of CAVD and that of coronary calcification suggest a shared disease pathway, at least in the initial phases of both diseases.

Alike atherosclerosis, the triggering event in CAVD is endothelial damage resulting from increased mechanical stress and reduced shear stress. Physiologic fluid shear stress (FSS) contributes to valve homeostasis, whereas altered shear stress, on the contrary, stimulates the endothelial upregulation of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) (Sucosky et al., 2009; Sun et al., 2013). The endothelial damage following the altered shear stress allows the infiltration of lipids, especially LDL and lipoprotein (a) [Lp(a)], starting the recruitment of inflammatory cells into the leaflets. Later appears the formation of focal subendothelial plaque-like lesions constituted from LDL, Lp(a) and infiltrates of inflammatory cells which interact in processes that release ROS causing LDL oxidation. In time, the expansion of this inflammatory process triggers the release of factors that cause VICs to acquire an osteogenic profile, with the formation of microcalcifications initiating leaflet mineralization (Otto et al., 1994; O'Brien et al., 1996). The two disease processes differ in the later disease phases: while in atherosclerosis, smooth muscle cells are the active players in the chronic inflammation within the plaque, in CAVD fibroblasts are involved in the prominent mineralization process and only with a small number of smooth muscle cells is present.

The hallmark of the early CAVD stages is inflammation, characterized by the activation of the leaflet endothelium *via* enhanced expression of cell adhesion molecules (VCAM-1, ICAM-1) (Sun et al., 2013; de Sousa et al., 2017). Disease initiation involves the activation of VICs, recruitment of immune cells, and subsequent sclerosis of the valve leaflets owing to fibrosis and formation of calcific nodules. All these phenomena start and interest more the fibrosa layer within the valve. The first macroscopic change in the leaflets, seen as microcalcifications, or focal thickening with preserved valve function, is nominated aortic valve sclerosis, nonetheless, the initiating events likely occur much earlier. The activation degree of the immune system seems to be different on different examined valve areas. Interestingly, the thickening process and the formation of calcium nodules accompanied by neo-angiogenesis, are both localized near the aortic surface of the leaflets (Cote et al., 2013).

The final disease, namely calcific aortic stenosis, is characterized by large calcified noduli on the leaflet surface,

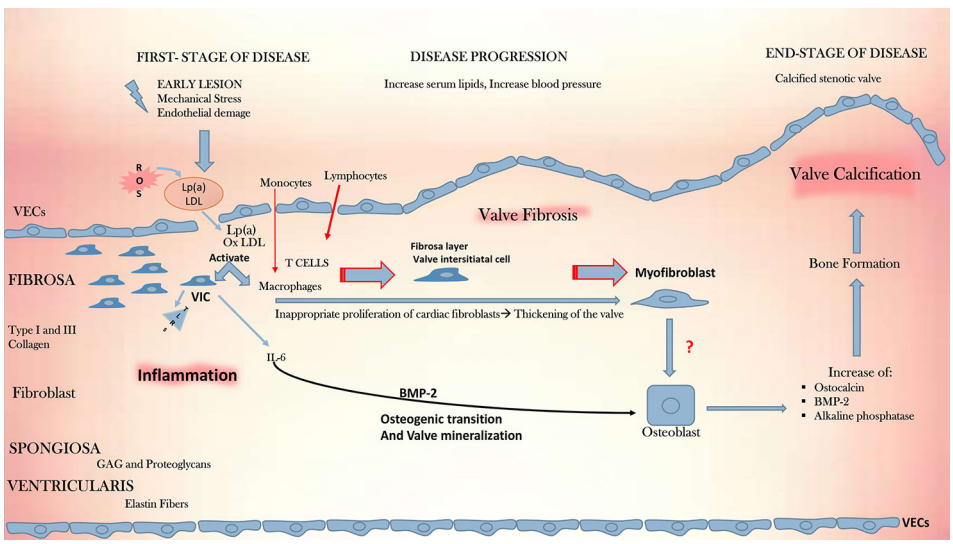

FIGURE 2 | CAVD development. Depicted is the pathophysiologic cascade leading to CAVD. Initial lesions, such as mechanical stress, endothelial damage and, production of ROS, initiate an ox-LDL-mediated inflammation in the endothelium and promote the activation of macrophages and quiescent VICs. These start to express TLRs and the cytokine pro -inflammatory IL-6, which plays a fundamental role in inflammation, together with macrophages and T cells. IL-6 mediates mineralization through the BMP-2 signal, driving the osteogenic transition and culminating with the aortic valve calcification. CAVD, calcific aortic valve disease; GAG, glycosaminoglycans; IL-6, interleukin 6; Lp(a), lipoprotein a; ox-LDL, oxidized low-density lipoprotein; ROS, reactive oxygen species; TLRs, toll-like receptors, VECs, valve endothelial cells; VICs, vascular interstitial cells, BMP-2, bone morphogenetic protein 2.

that protrude into the sinuses of Valsalva, hindering the leaflet mobility (Rajamannan et al., 2011). In this final phase the leaflets are infiltrated by immune cells and concomitantly angiogenesis occurs, along with deposition of lipids, proteoglycans, and cell debris (Chen and Simmons, 2011). Finally, the calcification of the valvular matrix leads to an increased stiffness and obstruction of the blood flow (**Figure 2**). With an orifice under 1 cm^2, versus 2.5–4.5 cm^2 observed in a normal valve, the stenotic valve generates a pressure gradient of over 40 mmHg, categorized as severe stenosis with an indication to valve replacement (Zigelman and Edelstein, 2009; Rutkovskiy et al., 2017).

Impact of Inflammation in CAVD Remodeling

Inflammation is the primary response of innate immunity and occurs after endothelial damage with its activation and lipid deposition. The innate immune response, with both its components, cellular and humoral responses are implicated in this process. As response to an injury induced by foreign organisms, dead cells or physical irritants, the innate immune system represents the first response to external or internal triggers and initiates the process of tissue regeneration (Hulin et al., 2018; De Almeida et al., 2019). Once the inflammation process has been triggered, it will proceed along a certain course of events until the inflammatory stimulus is eradicated and the healing mechanism can begin. However, if the inflammatory source cannot be eliminated, inflammation will progress, varying in intensity over time (Hakansson and Molin, 2011).

Histological samples of human CAVD valves are characterized by calcified areas rich in lymphocytes, macrophages, and osteoblast-like cells (Hulin et al., 2018). The inflammatory process can be acute or chronic (Pahwa and Jialal, 2019). Cote´ et al. showed that chronic inflammatory infiltrates, composed of the $CD45^+$ leukocytes, $CD68^+$ macrophages, and a few scattered $CD3^+$ T cells, were present near the calcified areas. Moreover, the chronic inflammatory infiltrates in the aortic valve were independently associated with several indices of remodeling, suggesting that inflammation may participate in mineralization and the fibrotic process (Cote et al., 2013). A significant association between the degree of aortic valve inflammation and the development of calcification has been previously reported (and recently confirmed) using 18F-fluorodeoxyglucose positron emission tomography. In these studies, a high degree of inflammation and calcification was documented in patients with severe CAVD, with the latter being the predominant pathogenic process (Marincheva-Savcheva et al., 2011; New and Aikawa, 2011; Dweck et al., 2012b). This could in part explain the failure of statin therapy in slowing the CAVD progression (Cowell et al., 2005; Rossebo et al., 2008).

Previously thought to be a passive disorder, CAVD is now recognized as an active process, whereby endothelial progenitor cells (EPCs) and inflammatory cells promote tissue remodeling (Yip and Simmons, 2011). In summary, mechanical shear stress and atherogenic factors activate VICs and initiate the recruitment of inflammatory cells. The following remodeling of extracellular matrix with leaflet stiffening and valvular dysfunction causes further mechanical stress that maintains the self-perpetuating cycle of endothelial dysfunction. As suggested by studies with molecular imaging, the continuous maintenance of this shear stress-inflammation cycle could result irreversibly to calcium deposition and finally to severe CAVD (New and Aikawa, 2011).

Role of Lipids

Over recent years, several studies have highlighted the central role of ox-LDL as an activator of inflammation, both in atherosclerosis and in CAVD (Cote et al., 2008). In atherosclerotic plaques, ox-LDL activates the inflammatory cells and the production of cytokines promoting tissue remodeling and disease progression (Hansson, 2005). Existing evidence of valve infiltration by ox-LDL in cases of CAVD, supports the concept that the development of valve calcification may be, at least in part, influenced by ox-LDL and, that an association exists between high plasma levels of ox-LDL and CAVD (Mohty et al., 2008). Although having some similarities with atherosclerosis, CAVD differs from it since the aortic valve has some inherent properties that differ from the vascular wall. Indeed, the cellular organization, as well as the hemodynamic stress imposed upon the aortic valve, vary from that of the arteries. Furthermore, the phases that characterize the pathological process occurring within the valve, from early inflammation stages to the calcification ones, are more numerous and qualitatively different from atherosclerosis, as shown in **Figure 2**. Most importantly, statins, although efficient in reducing adverse events in patients with atherosclerosis, are inefficient in CAVD (Cowell et al., 2005; Rossebo et al., 2008; Chan et al., 2010).

Role of the Immune Response in CAVD

The immune system, with innate and adaptive responses, plays a central role in the development of different chronic disorders, including atherosclerosis and CAVD. Evidence suggests that inflammation, as the primary response of the innate immunity, promotes the mineralization of VICs in response to several factors. Adaptive immunity, on the other hand, could play a role in instrumenting the immune response (Cote et al., 2013). In this regard, experimental studies have shown that the increased leukocyte density in mineralized aortic valves correlates with faster disease progression (Mathieu et al., 2015).

Innate Response in CAVD

The innate immune response in CAVD $|+|^{\}-^{-}$be initially triggered by several oxidized lipid species, through both the toll-like receptors (TLRs) and the nuclear factor-κ B (NF-κB) pathway. VICs express TLRs, known to play a key role in inflammation and initiation of antigen-specific adaptive immune responses. TLRs can be activated by several lipid species, especially ox-LDL, with the signal passing through the recruitment of specific adaptor molecules to the activation of the transcription factor NF-κB (Kawasaki and Kawai, 2014; Garcia-Rodriguez et al., 2018). In this way, TLRs signal inflammation through the NF-κB pathway and are vital for maintaining tissue homeostasis.

NF-Kappa B Pathway. The NF-κB plays an important role in signal integration by responding to mediators of endothelial

injury. VICs actively participate in the regulation of inflammation by producing a high level of cytokines, and the NF-κB pathway contributes to a stimulus-dependent and cell-type-specific manner. The NF-κB activation (canonical and non-canonical) pathway is mediated by different extracellular signals including angiotensin II, ox-LDL, CD40 ligand, advanced glycation end-products, and inflammatory cytokines. The canonic pathway is activated, among others, by tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β). Angiotensin II-induced ROS generated also on mineralized aortic valves, are involved in the activation of the NF-κB canonic pathway (Jamaluddin et al., 2007). The non-canonical pathway is activated, among others, by vasoactive peptides, ox-LDL, activated CD40 receptor, B cell-activating factor (BAFF), lymphotoxin β (LTβ) monocyte released cytokines, or advanced glycation end-products (Xiao et al., 2004; Morrison et al., 2005). In summary, activated NF-κB controls the initiation of vascular inflammation, through "leucocyte adherence" and "chemotaxis" and is a crucial signaling integrator of vascular injury.

Cytokines. One important target of the NF-kB pathway is IL-6. Highly expressed in valves with severe CAVD, IL-6 is a pleiotropic cytokine secreted by multiple vascular cell types, such as macrophages, lymphocytes, fibroblasts, endothelial cells, and smooth muscle cells. It mediates local vascular monocyte activation and protection from ROS-induced cellular stress *via* the downstream transcription effector signal transducer and activator of transcription 3 (STAT3) (Brasier, 2010; El Husseini et al., 2014; Rahat et al., 2016; Huang et al., 2019). By controlling the monocyte activation vial the IL-6 pathway, NF-κB mediates the systemic acute phase response and plays a central role in the initiation and maintaining of the vascular inflammation (Brasier, 2010; Perez et al., 2019).

IL-1β is another intriguing cytokine involved in the valve calcification process. Its levels are indeed increased in the stenotic aortic valve, making IL-1β subject to numerous research studies in the last decade (Isoda et al., 2010; Nadlonek et al., 2013; Lee and Choi, 2018). The IL-1β enhances the expression of matrix metalloproteinases (MMPs), a group of enzymes responsible for the degradation of extracellular matrix proteins. The MMPs exacerbate the process of valvular stenosis and activate the NF-κB pathway, leading to the production of several cytokines, such as IL-6, IL-8, and MCP-1 (monocyte chemoattractant protein-1) involved in both pathological processes, namely atherosclerosis and CAVD (Deshmane et al., 2009).

Finally, the IL-1 receptor agonist (IL-1Ra) plays a protective role in valvular disease, as its deficiency is closely associated with inflammatory cell infiltration and valve thickening (Isoda et al., 2010). Using *in vitro* and *in vivo* models, Zeng et al. elucidated the role of IL-37, an anti-inflammatory member of the IL-1 family. IL-37 attenuates the expression of bone morphogenetic protein 2 (BMP2) and alkaline phosphatase (ALP) enzyme, inhibiting the osteogenic process. In CAVD, the expression levels of IL-37 are very low, consequently, BMP2 is free to promote VICs calcification by ALP expression leading subsequently to aortic valvular thickening. Confirming the protective role of IL-37, *in vivo* experiments showed that mice expressing this human cytokine, displays significantly lower BMP-2 levels and a lower degree of aortic valve thickening (Zeng et al., 2017).

Adaptive Response in CAVD

The key role of lymphocytes in CAVD and the presence of T-lymphocyte clonal expansion in stenotic valves has been highlighted by various studies (Wallby et al., 2002; Mazzone et al., 2004; Mazur et al., 2018). In a study involving patients with severe CAVD, Wu et al. observed the presence of $CD8^{+}/CD28^{-}$ T cells near the mineralized nodules of the aortic valve and a higher prevalence of circulating $CD3^{+}$ T cells, namely the subset of $CD8^{+}$ and $CD57^{+}$ T cells expressing HLA-DR, in subjects with CAVD (Wu et al., 2007). $CD8^{+}/CD28^{-}$ T cells play a crucial role in CAVD, as demonstrated by the correlation between the degree of clonal expansion and the severity of valve calcification. Furthermore, these cells play a crucial role in both activation and differentiation of memory effector status among circulating T cells. Winchester et al., investigated the composition of the lymphocytic infiltration in patients with (bi-and tri-cuspid CAVD). They showed an aggregate of infiltrating lymphocytes containing $CD4^{+}$ and $CD8^{+}$ T cells, with a preponderance of CD4 T cells. The clones shared between blood and the valve were found in the memory-effector $CD8^{+}CD28^{-}$ T cell subset, demonstrating the trafficking of members of the same T cell clone between the peripheral circulation and the valve. Importantly, the proportion of activation of peripheral blood T-lymphocytes strongly correlated with the degree of valve calcification, indicating that the extent of these events is somehow related to CAVD severity (Winchester et al., 2011).

This data suggests that in patients with CAVD, an adaptive systemic immune response is occurring, coupled with the valvular lymphocytic infiltration, probably triggered by recognition of antigens expressed in the valve. The valvular events that lead to an immune response, such as intracellular pathogens, or self-antigens, remain unclear. One hypothesis is that in response to shear stress the VICs express stress-induced molecules which could be perceived as antigens and generate an immune response.

Mineralization

At some point during the CAVD progression, VICs start to produce a calcified matrix and enter an osteogenic phase. The mechanisms of this switch are still poorly understood. One hypothetical pathway involves IL-6 and the tumor necrosis factor ligand superfamily member 11 (also called receptor activator of NF-κB ligand—RANKL). IL-6 has been shown to induce the expression of RANKL in bone cells, which activates its cognate receptor RANK that finally activates VICs to produce extracellular matrix and might therefore promote matrix calcification (Wada et al., 2006). This could be a hypothetical pathway, which through IL-6-RANKL overexpression promotes the osteogenic reprogramming of VICs (Kaden et al., 2004). Another pathway involving the expression of BMP2 *via* inflammatory cytokines and oxidized lipid derivatives was shown to induce osteogenic reprogramming in several cell types including VICs (Shao et al., 2005).

Genetic Factors Implicated in CAVD

Numerous genetic factors are implicated in the CAVD pathogenesis (LaHaye et al., 2014). Two recent genome-wide association (GWAS) studies showed that a genetic variation in the *LPA* locus, mediated by levels of Lp(a), is involved in both atherosclerotic disease and CAVD and that several pathways are shared by both conditions. Lp(a) and non-high-density lipoprotein cholesterol as shared risk factors contributed to the frequent co-existence of these disorders (Thanassoulis et al., 2013; Helgadottir et al., 2018).

A recent transcriptome-wide association study (TWAS) identified *PALMD* (palmdelphin), a gene that promotes myoblast differentiation and muscle regeneration, as an additional key gene in CAVD. The study showed that lowered expression levels of *PALMD* mRNA in valve tissues are associated with risk alleles for CAVD and higher disease severity (Nie et al., 2017; Theriault et al., 2018). Recently, Theriault et al. identified some additional susceptibility genes in patients with CAVD. Using both techniques, GWAS and TWAS, *IL-6*, *ALPL* (alkaline phosphatase), and *NAV1* (neuron navigator 1) emerged as important contributors involved not only in pulse and blood pressure modulation—two important factors associated with blood flow turbulence, already identified as risk factors for CAVD development—but also in the valve mineralization process. Moreover, the *ALPL* gene codes for tissue-nonspecific alkaline phosphatase, a crucial enzyme involved in mineralization. This gene was present in calcified aortic valves contrary to non-calcified valves (Thériault et al., 2019).

All these findings taken together constitute a new step toward the further elucidation of the CAVD pathogenesis and may open new paths for the development of innovative therapeutic approaches.

Gender as a Risk Factor for CAVD

The role of gender as a biological variable, especially that of the male sex as a risk factor for cardiovascular diseases, including CAVD, has been long established (Carroll et al., 1992; Institute of Medicine Board on Population H and Public Health P, 2012). However, data regarding the significance of male sex in CAVD is lacking. Indeed, most studies addressing sex-specific differences in CAVD focus on conditions caused by CAVD, such as ventricular dysfunction, rather than CAVD itself (Dobson et al., 2016). It is mostly unclear whether the onset and progression of CAVD are inherently different in men and women, due to genetic and cellular-scale sex differences, or whether the initial disease process is common in both sexes but undergoes subsequent differentiation after (Porras et al., 2017). Aggarwal et al. reported that male gender correlates with an higher incidence of CAVD, as men displayed higher levels of calcification for the same degree of aortic stenosis when compared to women (Aggarwal et al., 2013). Another study showed significant differences between stenotic valves in males and females, where the latter were more prone to fibrosis. (Sharma and Eghbali, 2014) Evidence points towards genetic and epigenetic differences, specifically the differential production of miRNAs, being the origin of these sex-specific differences (Sharma and Eghbali, 2014).

Epigenetic Regulation of CAVD

The remarkable developments in the field of epigenetics could offer answers regarding the link between environmental and proatherogenic factors, induction and perpetuation of inflammation, and CAVD development. The four major epigenetic mechanisms, collectively known as "epigenome", include non-coding regulatory RNAs (ncRNA), DNA methylation, histone modifications, and chromatin remodeling (Murr, 2010). Studying the link between epigenome and CAVD could be clinically relevant since epigenetic markers could represent non-invasive biomarkers for monitoring CAVD initiation and progression and predicting prognosis.

ncRNAs, a group of single-stranded cellular RNAs made up of 18–25 nucleotides, represent a new area of interest in the field of human biology. These molecules, normally not coding for proteins, were previously regarded as "dark matter" since their function was largely unknown. Increasingly discovered to play a central role in the regulation of various molecular pathways and cell differentiation, ncRNAs are categorized in short, medium, or long (lncRNAs) based on their transcript size (Aryal et al., 2014). MicroRNAs (miRNAs) are the most well-studied ncRNAs, involved in cell development, proliferation, lipid metabolism, cancer metabolism, and angiogenesis (Palazzo and Lee, 2015). They regulate gene expression after transcription, by translational repression or transcript degradation. The role of miRNAs and especially lncRNAs in regulating atherosclerotic processes has been investigated in several studies suggesting that some miRNAs may be important inducers of proinflammatory pathways and others stimulators or inhibitors of calcification in the human aortic VICs (Gosev et al., 2017). Among others, miRNA-30b, miRNA-138, and miRNA-204 can suppress the differentiation of mesenchymal cells into osteoblasts whereas miRNA-29b and miRNA-214 promote calcification (Zhang et al., 2014; Wang et al., 2015; Fang et al., 2018; Lu et al., 2019; Zheng et al., 2019). miRNA-138 was indicated as a suppressor of osteoblastic differentiation of human aortic VICs by targeting FOXC1, representing, therefore, an inhibitor of VIC osteogenic differentiation during the development of CAVD (Lu et al., 2019). In 2017, the role of miRNA-29b as a promoter of valvular calcification, through the TGF-ß3/Smad3 and wnt3/ß-catenin pathways was reported (Fang et al., 2018) whereas in 2019, miRNA-214 was identified as a promoter of the human aortic VICs calcification by accelerating inflammatory chain reactions through MyD88/NF-κB signaling (Zheng et al., 2019).

Another epigenetic mark, the alteration of DNA methylation, could have a role in the osteogenic differentiation of aortic VICs by downregulating Notch/drosophila/homolog 1/translocation-associated (*NOTCH1*), a regulator of calcification-related gene networks in human valvular endothelium (Theodoris et al., 2015; Hadji et al., 2016). Markers of histone modification have also been reported to have a pro-inflammatory and osteogenic role in CAVD development. They participate in the shear-stress induced proinflammatory pathways *via* alteration of the silent information regulator-two (*SIRT)* gene expression (Roos et al., 2014).

In summary, increasing evidence supports the role of epigenetic factors as regulators of key processes underlying valvular tissue remodeling and participators in the landscape of phenotypical changes occurring in CAVD. Understanding the epigenetic mechanisms involved in the initiation and progression of CAVD will not only help to enlighten the pathology, but since these markers are potentially reversible, it could offer important targets for diagnostic and therapeutic interventions.

THE PUTATIVE ROLE OF THE MICROBIOME

Herein we hypothesize a putative role of the microbiome in the development and progression of CAVD, either *via* stimulation of immune response and valve calcification or *via* direct valvular damage caused by specific bacterial taxa. Different hints point to a role microbiome in the promotion of CAVD and its deterioration to severe calcified aortic stenosis. Some known risk factors for CAVD, such as age, male sex, hypertension, and diabetes mellitus, have a known relationship to the microbiome. In fact, patients with type II diabetes mellitus (T2DM), which is associated with the gut microbiota dysbiosis, are at higher risk for developing CAVD (Harsch and Konturek, 2018). Tissue histopathological studies demonstrated more calcification in tissue samples from patients with T2DM compared to non-diabetic patients (Mosch et al., 2017). Furthermore, the presence of metabolic syndrome is associated with faster disease progression and worse outcome in patients with aortic stenosis (Katz et al., 2009). Here we discuss in detail the involvement of oral and gut microbiome (GM) in pathophysiological processes that promote cardiovascular diseases, including CAVD.

Oral Microbiome

The oral microbiome represents, after the GM, the second-largest microbial community in humans and plays a fundamental role in maintaining the oral cavity homeostasis and preventing the development of several diseases (Jia et al., 2018; Deo and Deshmukh, 2019). The involvement of the oral microbiome in pathologies such as obesity, diabetes, cancer, and cardiovascular diseases is well documented (Gao et al., 2016; Eberhard et al., 2017; Long et al., 2017; Yang et al., 2019). Different studies suggest that oral pathologies with oral microbiome alteration, have a tight link with heart diseases; for example, the gingival ulceration in periodontitis causes bacteremia and could induce the formation of atherosclerotic plaques (DeStefano et al., 1993; Bartova et al., 2014). Increasing evidence suggests a close association between oral microbiome alteration and CAVD, demonstrating the presence of oral bacteria in the valvular tissue by using a polymerase chain reaction (PCR) (Nakano et al., 2006). Nomura et al., investigated the mechanisms through which *Streptococcus mutans* (*S. mutants*), the major pathogen responsible for dental caries, colonizes heart valves constituting a potential virulence factor for the development of infective endocarditis. They demonstrated that specific strains of *S. mutants* have selective virulence for infectious endocarditis (Nomura et al., 2014; Oliveira et al., 2015). There is an increasingly accepted hypothesis that bacterial endocarditis is one of the causes of aortic valve calcification, supported by *in vivo* studies showing larger calcifications in animals inoculated with endocarditis-related pathogens.(Cohen et al., 2004)

Gut Microbiome

The composition of the human microbiome is specific to each individual and the study of the GM is increasingly focusing on its role in the physiology of the host organism in during phases of health and disease (Blander et al., 2017). Changes in the composition of GM are involved in several diseases including atherosclerosis, hypertension, heart failure, chronic kidney disease, obesity, diabetes, inflammatory bowel disease, and colon cancer (Amedei and Barcelo-Coblijn, 2019). Growing evidence suggests that intestinal bacteria play an essential role in cardiovascular diseases. GM metabolites such as choline, betaine, and trimethylamine N-oxide (TMAO), are the subject of research studies investigating the correlation between the intestinal microbiome and the calcification of the aortic valve. Diets based on red meat, eggs, and dairy products are rich in choline, lecithin, and carnitine, constituting, therefore, a potential source of TMAO. Recent metabolomic studies indicate that fasting TMAO, choline, and phosphatidylcholine plasma levels are related to high risk of cardiovascular diseases. Furthermore, carnitine has been indicated as an independent predictor of major adverse cardiovascular events, with TMAO as the main driver behind the risk association (Velasquez et al., 2016; Canyelles et al., 2018). The idea that gut microbiota could contribute to CAVD is new. Very recently Kocyigit et al. reported a significant association between gut microbiota metabolites and CAVD (Kocyigit et al., 2017) whereas Liu et al. showed that patients with CAVD and those with coronary artery disease suffer from different gut microbial dysbiosis (Liu et al., 2019). The next section discusses the putative link between the GM and CAVD development.

The Role of the GM in Adaptive Immunity

Several studies indicate a very close connection between the GM and adaptive immunity. Bacterial metabolites function as a link between the commensal microbiome and the immune system, by affecting the shifting of the immune response between pro- and anti-inflammatory pathways. Hypothetically, the gap between the microbiome and immune system could be filled by short-chain fatty acids (SCFAs), generated by bacterial fermentation of dietary fiber in the intestinal lumen (Russo et al., 2016). SCFAs such as acetate, propionate, and butyrate act as important inductors of regulatory T (Treg) cell differentiation and reinforce the gut barrier function by expressing the transcription factor Foxp3 and consequently suppressing inflammatory responses in the intestine (Arpaia et al., 2013). A diet rich in SCFAs might antagonize several immunological defects, therefore GM-derived metabolites could offer a therapeutic advantage for patients suffering from various immunological conditions (Luu et al., 2019).

Significant links between fungal- and bacterial-induced cytokine responses and specific gut bacterial species were identified through the Human Functional Genomics Project. For instance, the production of interferon-γ (IFN-γ) and TNF-α is strongly associated with the GM (Schirmer et al., 2016). The fermentation of bacterial SCFAs acts through activation of G-protein coupled receptors by modulating the activity of intestinal epithelial cells and leukocyte life cycle. Furthermore, due to their direct effect on lymphocytes and by activating macrophages and dendric cells, SCFAs can induce a T-lymphocyte tolerogenic profile, acting as a link between the microbiome and the immune system (Correa-Oliveira et al., 2016).

In summary, the role of adaptive immunity in CAVD has been explored, but the valvular events that lead to an immune response, such as intracellular pathogens or self-antigens, remain largely undetermined. Alike atherosclerosis, one could hypothesize that the presence of a certain type of gut or mouth microbiome could lead to the presence of bacterial DNA inducing valvular specific antigens that could trigger an immune response. The putative interaction between the microbiome and immune response leading to CAVD development is schematized in **Figure 3**.

NOVEL POTENTIAL THERAPEUTIC STRATEGIES

Currently, no drug strategies can prevent or reverse CAVD and valve replacement remains the only treatment option. Patients with severe CAVD undergo valve replacement either with surgery or *via* percutaneous transcatheter procedures. Drugs already used to treat vascular complications might also improve CAVD outcomes, but the mechanisms of valve calcification differ from other vascular conditions (Hutcheson et al., 2014).

A randomized trial of intensive lipid-lowering therapy failed to stop ore reverse the progression of CAVD. However, the possibility that this specific pharmacological treatment induces a small reduction in disease progression or in the main clinical endpoints cannot be excluded (Cowell et al., 2005; Rossebo et al., 2008). Anti-inflammatory agents represent a promising therapeutic strategy that requires additional research in CAVD patients (Hurle et al., 2013). Due to the role of angiotensin II in CAVD development, inhibitors of angiotensin-converting enzyme (ACE-I) have also been explored. Notably, no significant difference in disease progression was observed in a retrospective cohort of more than 200 patients. The benefits of ACE-I are mostly related to their effect on left ventricular remodeling.

Human monoclonal antibodies have been used successfully in many conditions. In 2015 Lerman et al. found that denosumab, a human monoclonal antibody targeting the receptor activator of nuclear factor-κB ligand, may reduce valvular calcium deposition to basal levels (Lerman et al., 2015). Furthermore, denosumab reduced calcium deposition in the aorta, although the mechanisms by which it affected ectopic calcification, must be further examined. (Helas et al., 2009) Nonetheless, this field remains attractive and promising.

After GWAS and TWAS studies discovering several genes implicated in the CAVD pathogenesis, genetic therapy could also be a possible therapeutic development. A growing body of experimental evidence supports the role of epigenetic factors such as miRNAs and markers of DNA methylation, as regulators of key processes underlying valvular tissue remodeling and participators in the landscape of phenotypical changes

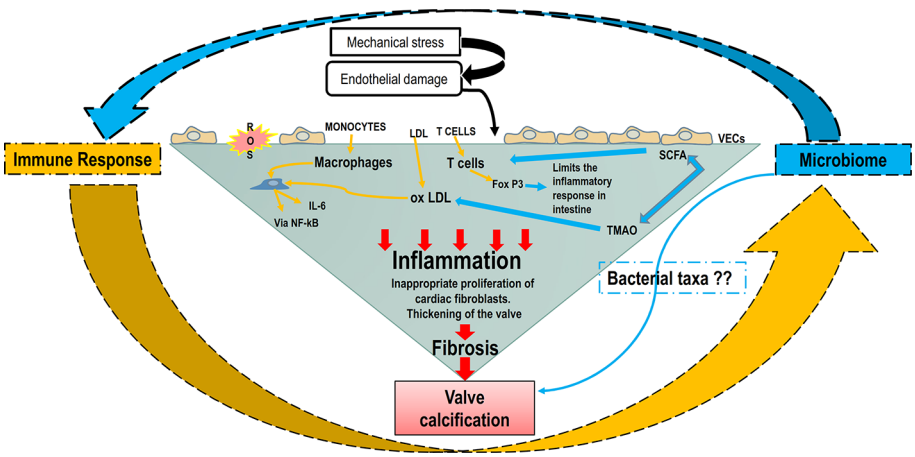

FIGURE 3 | The putative interaction between the microbiome and immune response in CAVD development. Increased shear stress, genetic factors, and oxygen reactive species cause the endothelial damage that allows ox-LDLs, immune and inflammatory cells to infiltrate the valve. Macrophages and ox-LDLs stimulate VICs inflammation through NF-κB/IL-6 signaling pathway. The microbiome and microbial metabolites can modulate the immune system through the intense effect of SFCAs on T cells' differentiation. Furthermore, the bacterial metabolite TMAO can induce ox-LDL and maintain the inflammatory loop. IL-6, interleukin 6; NF-κB, nuclear factor κ-light-chain enhancer of activated B cells; ox-LDL, oxidized low-density lipoprotein; SCFA, short-chain fatty acid; TMAO-Trimethylamine-N oxide; VECs, valve endothelial cells; VICs, vascular interstitial cells.

occurring in CAVD. Since these markers are potentially reversible, they could offer important targets for diagnostic and therapeutic interventions.

The recently introduced, tissue-engineered heart valves [tissue engineered heart valves (TEHVs)] may constitute a valid alternative to traditional strategies of valve replacement. In TEHVs, a decellularized scaffold is seeded with patient's cells subjected to an appropriate series of stimuli able to promote cellular activity. TEHVs could offer the advantages of biocompatibility and longevity, while preventing pathological tissue responses and regurgitation. Additionally, TEHVs could allow VICs to produce their own extracellular matrix, thus displaying the capacity for growth and remodeling, which is critical for pediatric patients. TEHVs can be produced from synthetic or natural materials (Berry et al., 2010).

CONCLUSIONS

Despite its high prevalence and associated mortality, there are no drugs to prevent or cure CAVD. Therefore, the development of new therapeutic strategies capable of counteracting the CAVD development is crucial.

Being a multifactorial disease, CAVD is characterized by a complex pathobiology that involves environmental and genetic factors, immune-molecular pathways, hemodynamic factors, and shear stress, all intertwined in the systemic district. Several of the many steps of disease progression have been clarified but some others, like the critical switch from inflammatory-fibrotic to osteogenic program, or the putative role of microbiota, remain still unclear. The quickly evolving field of epigenetic regulation of CAVD, involving especially miRNAs and lncRNAs, could offer novel potential biomarkers for the development of new diagnostic and therapeutic interventions. Furthermore, the microbiome could play a role in promoting and perpetuating CAVD by inducing the production of endogenous or exogenous antigens *via* the activation of inflammatory pathways. The elucidation of these conundrums will help identify new disease targets, host or microbial, and support the design of new potential therapeutic strategies to prevent or reverse the effects of CAVD.

AUTHOR CONTRIBUTIONS

BA, LC, AL, and AA were involved in the study concept and design. BA, LC, and MC drafted the manuscript. AL, UL, HG, and AA revised the manuscript for important intellectual contents. All authors had access to all the data, including figures, and approved the manuscript for final submission.

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