Microbiome and Cardiovascular Disease

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Abstract

Atherosclerotic cardiovascular disease (ASCVD) is a prime example of a systems disease. In the initial phase, apolipoprotein B-containing cholesterol-rich lipoproteins deposit excess cholesterol in macrophage-like cells that subsequently develop into foam cells. A multitude of systemic as well as

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environmental factors are involved in further progression of atherosclerotic plaque formation. In recent years, both oral and gut microbiota have been proposed to play an important role in the process at different stages. Particularly bacteria from the oral cavity may easily reach the circulation and cause low-grade inflammation, a recognized risk factor for ASCVD. Gut-derived microbiota on the other hand can influence host metabolism on various levels. Next to translocation across the intestinal wall, these prokaryotes produce a great number of specific metabolites such as trimethylamine and short-chain fatty acids but can also metabolize endogenously formed bile acids and convert these into metabolites that may influence signal transduction pathways. In this overview, we critically discuss the novel developments in this rapidly emerging research field.

Keywords

ASCVD · Atherosclerosis · Bacteriophage · Bile acids · SCFA · TMAO

1 Introduction

Atherosclerotic cardiovascular diseases (ASCVD), including coronary heart disease and stroke, are still the main causes of death in the Western world. In the last decades, extensive research programs have attempted to unravel molecular mechanisms underlying these debilitating diseases. Substantial progress has been made particularly in identification of the causal role of low-density lipoprotein (Ference et al. 2017) and triglyceride-rich remnants (Nordestgaard 2016). Although the focal point in research is often directed to cholesterol-containing lipoproteins, the major predictive risk factor for ASCVD is age (Pencina et al. 2019). The vascular aging process that underlies ASCVD risk is a complex process in which lipoproteins and blood pressure interact with the vessel wall and a number of environmental factors exert a secondary influence. One of these factors, which has gained substantial interest in recent years, is the microbiome defined as all microorganisms that colonize the body. Although the vast majority of microbiome studies concentrate on the gut, the oral microbiome may play a significant role in the context of ASCVD development. In this overview, we will critically review recent literature that focuses on the role of the microbiome in ASCVD.

ASCVD is a prime example of a systems disease. The obligate substrate is deposition of cholesterol in the vessel wall. Once depot formation is initiated in the form of foam cells, a host of additional factors such as inflammatory processes further determine disease progression through a plethora of actions. Note that inflammatory processes may also control lipid levels in the blood, thereby exerting control on multiple steps in development of atherosclerosis. Until recently, most of the information about the importance of inflammation in ASCVD came from studies in animal models. The results of the CANTOS trial, which showed a beneficial effect on ASCVD events through antibody-mediated inhibition of interleukin-1β, have added critical insight in the role of inflammation in ASCVD development in

humans (Ridker et al. 2017). Inflammation is an extremely complex process by itself, and it is therefore not surprising that novel inflammation-modulating factors pop up continuously as potential ASCVD influencers. Yet, the onset of the disease requires lipid disposition mediated primarily by apolipoprotein B (apoB)-containing lipoproteins. The dominant role of these lipoproteins was nicely exemplified by Mendelian randomization studies that make use of natural mutations in the genes encoding lipoproteins (reviewed in Holmes et al. 2017). This enabled studies into the effect of variations in circulating cholesterol carriers on ASCVD risk (Ference et al. 2017). In addition, LDL-C lowering trials using statins with or without ezetimibe or PCSK9 inhibitors have provided critical insight in the role of cholesterol carriers and ASCVD risk (Wright and Murphy 2016). Interestingly, there is linear relation between LDL-C levels and cardiovascular events in which zero disease risk was associated with circulating LDL-C levels of around 1 mM (Wright and Murphy 2016). This suggests that at this level, the risk of cardiovascular events is close to zero and no disposition of lipid in the vessel wall will occur. In the general population, however, the concentration of apoB-containing lipoproteins is much higher than 1 mM, and some degree of atherosclerotic plaques development is almost inevitable. Indeed in the 1960s of the last century, studies in young soldiers that died in the Korean and Vietnam wars demonstrated early signs of atherosclerotic plaque formation as early as 25 years of age in about 5% of the soldiers (Virmani et al. 1987). Fortunately, certainly at ages lower than 60 years, most atherosclerotic plaques are not symptomatic. A majority of current research initiatives therefore focuses on processes that induce vulnerability of the plaque to rupture, one of the main causes for acute coronary syndromes.

In short, lipid deposition in the vessel wall and complex inflammatory processes are critical for development of ASCVD. In addition, aging and environmental factors such as smoking and diet play an important role herein. More recently, both oral and gut microbiome have entered the spotlights. Before considering the role of the microbiome in ASCVD development in detail, we will first give an update on recent oral and gut microbiome literature and focus on host-microbiome interactions relevant for progression of ASCVD.

2 Gut and Oral Microbiome Communities: Potential Drivers of ASCVD?

The gut and oral microbiome are the first and second, respectively, largest and most complex communities of microorganisms in the human body and comprise bacteria, fungi, viruses, archaea, and protozoa. It is critical to point out that the vast majority of publications on the role of the gut and oral microbiome in human health and disease are heavily biased toward bacterial members of this community. The upcoming awareness of the critical role of other members of the microbiome to composition and function of the community – and thereby contribution to human metabolism – is likely to change this bias in the coming decade. We will first elaborate on the bacterial component of the (gut and oral) microbiome and their

postulated role in ASCVD development. Mechanistically, both the gut and oral microbiome are currently considered to affect human metabolism and ASCVD by interaction with the host immune system (gut and oral) and by conversion of dietary components into hormone-like signals or biologically active metabolites (gut) (Fig. 1).

The gut microbiome (estimated number of species >1,000, 1.5–2 kg per person) is a critical component of digestion, maintenance of gut barrier function, and immunomodulation. The predominant bacterial phyla are *Bacteroidetes* and *Firmicutes*, with *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* being less abundant (Shetty et al. 2017). Methanogens are dominant among the archaea (Paterson et al. 2017). The vast majority of gut bacteria are shared among individuals at higher taxonomic levels (phylum). However, interindividual variation at lower taxonomic levels (species, strain) is very high. Alterations at this level, in particular reduced number and diversity of bacterial genes, have been associated with metabolic diseases including ASCVD (reviewed in Aron-Wisnewsky and Clément 2016; Liu et al. 2019; Tilg et al. 2019).

ASCVD risk factors have been reported to associate with the gut microbiome; for example, *Clostridiales* and *Clostridium* spp. correlate negatively with C-reactive protein, an inflammatory marker (Karlsson et al. 2012). The gut microbiome of ASCVD patients has been associated with decreased abundance of gut commensals such as *Bacteroidetes* (incl. *Bacteroides* and *Prevotella*) compared to healthy controls (Emoto et al. 2016). A metagenome-wide association study (Jie et al. 2017) in fecal samples of ASCVD patients and healthy controls reproduced findings on the reduced abundance of *Bacteroidetes* in ASCVD patients and further reported that these patients had reduced abundance of presumably beneficial short-chain fatty acid-producing bacteria such as *Roseburia intestinalis* and *Faecalibacterium prausnitzii*. Conversely, the microbiome of ASCVD patients was enriched in species belonging to the *Enterobacteriaceae* family, which are oftentimes associated with gut microbiome dysbiosis and (metabolic) disease development. Interestingly, the relative abundance of bacteria typical for the oral cavity, in particular *Streptococcus* spp., was also higher in the gut microbiome of patients with ACVD compared to healthy controls.

With over 700 bacterial species, the oral cavity comprises the second largest and most diverse microbiome community after the gut in the human body. These species are located in a plethora of very complex niches including the hard surfaces of the teeth as well as the soft mucus linings of the mouth. The mouth is the major entrance point to the human body. Bacteria from the oral cavity, in particular those associated with oral infectious diseases and adapted to thrive in an inflammatory environment (e.g., caries (tooth decay) and periodontitis (gum disease)), have been associated with "off-site" effects on systemic diseases such as ASCVD (Hajishengallis 2015).

Although the number of studies that have associated differences in gut and oral microbiome composition with ASCVD is plentiful, it is important to point out that data on a causal role for the gut microbiome in ASCVD development in humans is more difficult to find. The strongest evidence for a causal role of the gut or oral microbiome in progression of ASCVD has been derived from animal studies,

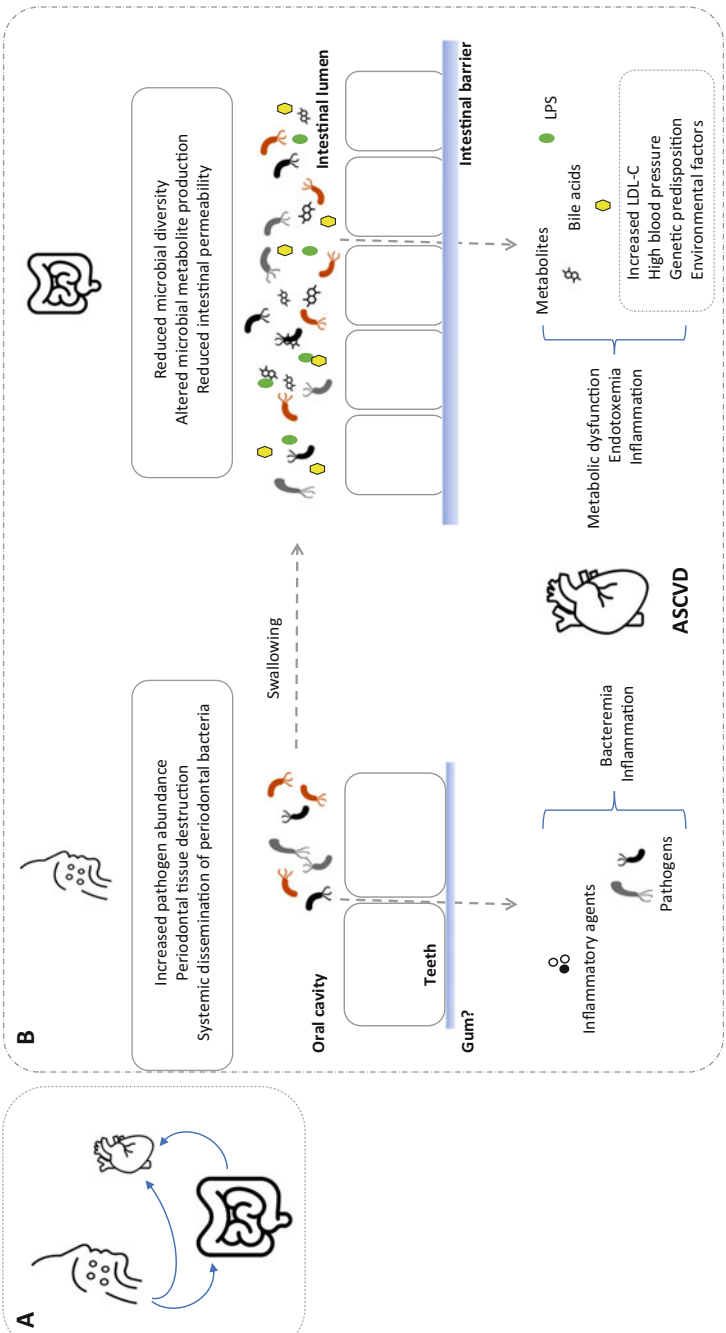

Fig. 1 Oral and gut microbiome have been implicated in the development of ASCVD (**a**). Increased pathogen abundance in the oral cavity, such as during periodontal disease, might reach the circulation and contribute to low-grade inflammation, a recognized risk factor for ASCVD. In addition, continuous swallowing of bacteria from the oral cavity has been suggested to alter gut microbiome composition, thereby contributing to ASCVD development (**b**). Please see text for details

Table 1 Overview of bacterial challenge studies carried out in germ-free mouse models

Mouse model	Diet + intervention(s)	Findings	Reference
Apoe−/− (gnotobiotic)	Chow, *Roseburia intestinalis* administration	Lesions and inflammation ↓in *R. intestinalis* vs control	Kasahara et al. (2018)
Apoe−/− (GF and CONV-R)	Chow, HFD, choline supplementation (both diets)	Chow: lesions and CH ↓ in CONV-R vs GF HFD: lesions and CH = in CONV-R vs GF Choline suppl: TMAO ↑, lesions = in CONV-R vs GF	Jonsson et al. (2018)
Apoe−/− (GF and CONV-R)	Chow	Lesions, LPS, and inflammation ↑ in CONV-R vs GF LDL-C ↓ in CONV-R vs GF	Kasahara et al. (2017)
Apoe−/− (GF by abx)	Chow without/with choline	Lesions, CH, and foam cells ↓ in choline-abx vs choline-no abx	Wang et al. (2011)
Apoe−/− (GF and CONV-R)	Chow without/with cholesterol	Chow without CH: lesions ↓ in CONV-R vs GF Chow with CH: lesions = in CONV-R vs GF	Stepankova et al. (2010)
Apoe−/− (GF and CONV-R)	HFD	Lesions = in CONV-R vs GF	Wright et al. (2000)

in particular mouse models (Table 1). Germ-free (sterile) apolipoprotein E-null (ApoE −/−) mice were shown to have increased atherosclerotic plaques compared to conventionally raised counterparts when fed a chow diet (Stepankova et al. 2010). These data were confirmed by Lindskog et al. but only with respect to the chow diet (Jonsson et al. 2018). Conversely, when germ-free ApoE −/− mice were fed a high-fat/high-cholesterol diet, the absence of microbiota increased atherogenesis, but the extent was still reduced compared to conventionally raised mice (Jonsson et al. 2018). However, there is some controversy around these data because Kasahara et al. reported decreased atherosclerosis in germ-free ApoE −/− mice on a chow diet (Kasahara et al. 2017). Since chow diets are not well characterized and composition differs from batch to batch, the apparent discrepancy may well be caused by subtle changes in the diet used in the different studies.

Convincing data for a causal role of the microbiota in ASCVD development came from fecal microbiota transplantation (FMT) studies showing that atherosclerosis was induced when FMT was carried out with feces derived from mice with proven atherosclerosis (Gregory et al. 2015). The question arises which factor is responsible for the atherosclerosis aggravating effect induced by the microbiome. Activation of inflammatory signaling pathways by the gut microbiome, or components thereof, has received a lot of attention in the past decade. This is exemplified by observations that transplantation of a pro-inflammatory microbiome into atherosclerosis-prone LDLR−/− mice accelerated phenotype development compared to LDLR−/− mice receiving a control microbiome (Brandsma et al. 2019). Translocation of lipopolysaccharide (LPS)

across the intestinal wall into the blood seems a good mechanistic candidate. LPS is the major molecular component of the outer membrane of gram-negative bacteria, the most abundant bacteria in the gut (Raetz and Whitfield 2008). The lipid A component of LPS is a pathogen-associated molecular pattern, which activates Toll-like receptor 4 (TLR4) (Aderem and Underhill 1999). High-fat diet has been shown to increase gut permeability in mice. This may enhance the translocation of LPS into the circulation thereby inducing metabolic endotoxemia (Cani et al. 2007). In line, germ-free mice were demonstrated to be resistant to high-fat diet-induced insulin resistance and obesity (Rabot et al. 2010). Whether these results in mice can be translated to humans remains to be established.

Identification of atherosclerotic plaque-associated bacteria is suggestive of a direct role in plaque progression (Koren et al. 2011; Jonsson et al. 2017). Interestingly, many of these bacteria were also localized in the oral microbiome of patients with atherosclerosis. Indeed, a close association between periodontitis and ASCVD risk has been reported in a number of studies (Hajishengallis 2015). Gingival bleeding caused by periodontitis offers oral bacteria an easy entry into the circulation where they can attach to the atherosclerotic plaque. Whether they stay alive when bound to the plaque is not clear. As far as we are aware, no live bacteria have been cultured from plaques obtained during surgery. Yet, upon entry into the blood, the orally derived bacteria may be capable of activating endothelial cells, possibly leading to expression and secretion of metalloproteinases that in turn may decrease plaque stability. The activity of periodontitis has been shown to be reflected in systemic inflammation. This is important given the role of inflammation in the pathogenesis of ASCVD. Plasma levels of the inflammatory marker C-reactive protein (CRP) have been correlated with periodontitis status (Noack et al. 2005; Yoshii et al. 2009) as well as the pro-inflammatory cytokine IL-6 (Loos et al. 2005). These immune modulators may be either produced locally in the oral environment and subsequently secreted into the circulation or arise as a result of low-grade, short-lived bacteremia (Torres De Heens et al. 2010). Given the high prevalence of periodontitis in the adult population, treatment of this disease may be an important modality to reduce the incidence of ASCVD (Lobo et al. 2019).

Summarizing these studies, it seems fair to conclude that activation of an inflammatory pathway is a reasonable way via which bacteria may promote progression of ASCVD. Whether the prokaryotes that enter the circulation via the oral cavity or gut play a role in affecting plaque stability has not yet been shown. In a study in mice, Jonsson et al. (2017) did not find differences in bacterial content between stable and labile plaques. However, activation of the inflammatory component of ASCVD may not be the only way by means of which the microbiota exert influence.

2.1 Other Microbiome Community Members

Although *bacteria* and *archaea* indeed account for >99% of microbiome mass (Shkoporov and Hill 2019), it should be realized, however, that both the

oral and gut microbiome contain vast numbers of viruses, fungi, and – in most humans – protozoans. Although many of these less abundant community members have been linked to human disease (Hoffmann et al. 2013; Huseyin et al. 2017; Paterson et al. 2017; Laforest-Lapointe and Arrieta 2018), surprisingly little is known about trans-kingdom community-level interactions and the consequences thereof for human health. In order to deepen our understanding about the complexity of host-microbial interactions, it will be critical to address such interactions in the relevant ecosystem (e.g., the gut or oral cavity).

2.1.1 Viruses and Bacteriophages

Bacteriophages (phages), viruses of bacteria, are of particular interest because of their proven role in shaping microbial communities in many ecosystems (Fernández et al. 2018; Warwick-Dugdale et al. 2019). Furthermore, phages are abundantly present in the gut (estimated 1:1 ratio with bacteria), either as free phage or integrated into the bacterial genome as prophage (Reyes et al. 2012; Walk et al. 2016; Carding et al. 2017).

The many studies that have described a strong association of specific bacterial strains with ASCVD generally characterize the phylogenetic core, dynamics, and stability of the bacterial ecosystem by high-throughput 16S ribosomal RNA gene sequencing-based approaches. This precludes identification of integrated bacteriophage DNA in the bacterial DNA (see for recent reviews (Shetty et al. 2017; Hornung et al. 2018; Falony et al. 2019)). Increasingly accessible and affordable shotgun and long-read nanopore sequencing approaches together with rapidly emerging computational tools to unravel novel phage genomes are now rapidly solving part of the challenges phage researchers have faced in the past. These include the fact that the majority of gut bacteria (phage hosts) are strict anaerobes and thereby extremely difficult to culture. This has, until recent years, limited researchers to microscopic characterization of phages. The current collection of known gut phages therefore is a vast underrepresentation of the gut phageome. Interestingly, a healthy gut status in humans has been shown to mainly comprise integrated phages (Reyes et al. 2010; Minot et al. 2011), whereas cases of intestinal bowel disease have been associated with higher levels of free phages (Norman et al. 2015; Duerkop et al. 2018). Prophage integration has been shown to affect bacterial fitness and metabolic function in the gut (Duerkop et al. 2012; Hsu et al. 2019; Oh et al. 2019). Moreover, evidence for a direct role of phages in activation of the mammalian immune system, a critical element of ASCVD development, has recently been put forward (Gogokhia et al. 2019; Sweere et al. 2019). In line with long-standing observations that bacteriophages are able to pass the intestinal wall to enter the bloodstream, at least in experimental settings (Van Belleghem et al. 2019), these results support the urgency to carefully look into these viral members of the microbiome community in the gut and beyond. Implications for phages as modulators of (immune)metabolism are yet to be confirmed, but we predict this will be highly relevant for studies addressing the role of the microbiome in human ASCVD development.

3 Microbiome-Derived Metabolites

Gut bacteria are also considered to modulate human metabolism by production of small molecules including conversion of dietary components into hormone-like signals or biologically active metabolites (Fig. 2). It has been estimated that about 10% of the small molecules in the circulation are derived from the gut microbiome (Holmes et al. 2012). This estimate can very well be an underestimation because

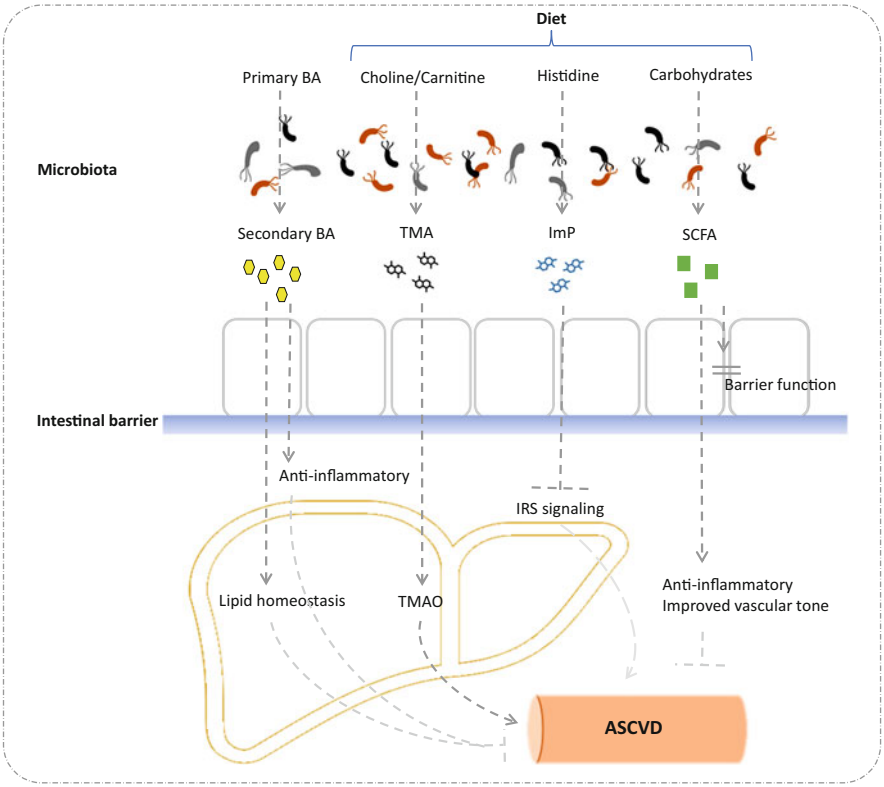

Fig. 2 Gut microbial metabolites have been implicated in ASCVD development. Primary bile acids are produced by the liver after which a small percentage is converted to secondary bile acids by colonic bacteria. Although evidence from human trials addressing if secondary bile acids might prevent ASCVD development is still warranted, secondary read outs associated with ASCVD (e.g., inflammation and LDL-C levels) have been reported to be reduced by bacteria capable of converting primary to secondary bile acids. TMA is produced by the microbiota from choline or carnitine precursors. In the liver, TMA is converted to TMAO which has been extensively linked to ASCVD development. ImP is a microbial metabolite of histidine. ImP directly inhibits insulin receptor-mediated signaling thereby leading to insulin resistance, a significant risk factor for the development of ASCVD. Whether ImP indeed affects ASCVD development is yet to be established. SCFA is a microbial fermentation product of complex carbohydrates. Many health benefits have been addressed to SCFA and include improved gut barrier function and reduced inflammation. Please see text for details

despite the major advances in development of metabolomics in the last decade, most circulating metabolites whether endogenous or microbial have not yet been identified.

3.1 TMAO

A wonderful example of how a bacterial metabolite can be identified to exert effect on ASCVD comes from the studies of the Hazen group in Cleveland Clinic (Zhu et al. 2017; Koeth et al. 2019; Wu et al. 2014). They demonstrated that nutrients such as choline and carnitine can be converted to trimethylamine (TMA) by gut bacteria that express TMA lyases. The gas TMA subsequently diffuses from the intestine into the circulation and is converted to trimethylamine N-oxide (TMAO) in the liver by the hepatic enzyme flavine monooxygenase 3 (FMO3) (Brown and Hazen 2015). TMAO activates atherosclerosis in mouse models, and TMAO plasma levels have been shown to correlate with incidence of cardiovascular disease in humans in a number of studies (Zhu et al. 2016, 2017). Several other studies, however, failed to show this correlation (Heianza et al. 2017; Kaysen et al. 2015; Mueller et al. 2015; Aldana-Hernández et al. 2019). Zhu et al. showed that TMAO may exert its action via influencing blood platelet hyperresponsiveness and thrombosis, which provides a mechanistic link between TMAO and cardiovascular risk (Zhu et al. 2016, 2017). Recently, Chen et al. showed that TMAO may induce ER stress (Chen et al. 2019). Unravelling the metabolic pathways involved in TMAO metabolism provides a beautiful example how the interaction between microbial activity and host metabolism can be elucidated. By developing specific inhibitors of TMA lyases, the Hazen group (Koh et al. 2018) may have produced the tools to treat patients at risk for ACSVD due to increased TMAO (Wang et al. 2015). In the original paper in which the Hazen group introduced the TMAO pathway, additional ASCVD-associated peaks in the MS spectra were observed (Wang et al. 2011). Characterization of these putative metabolites has not yet been published, but it seems justified to suggest that there is more to come.

3.2 Imidazole-Propionate

Metabolites produced by microbial metabolism of aromatic amino acids are good candidates. Recently the histidine derivative imidazole-propionate (ImP) has been linked to insulin resistance in humans. By detailed analysis of portal blood obtained from obese diabetic patients compared to “healthy” (nondiabetic) obese controls, the group was able to single out ImP as one of the compounds strongly increased in portal blood from the diabetics (Koh et al. 2018). The group of Backhed subsequently characterized the molecular mechanism of action in great detail (Koh et al. 2018). Extensive mechanistic studies in mice revealed that ImP impairs insulin signaling via p38 protein kinase. Identification of ImP is very recent, and its

putative role in ASCVD has not been investigated yet. Since ASCVD is an almost inevitable comorbidity of diabetes, an aggravating effect of IMP on ASCVD may be expected to be published in the near future.

3.3 Short-Chain Fatty Acids

The short-chain fatty acids (SCFAs) butyrate, propionate, and acetate are produced from colonic fermentation of complex fibers by the gut microbiota. SCFAs are the main product of the digestive actions of the gut microbiota making them interesting candidates in the quest for microbial-derived metabolites influencing ASCVD. Lower levels of SCFA or SCFA-producing bacteria have been correlated with arterial stiffness, high blood pressure, and related end-organ damage (Pluznick 2013; Kim et al. 2018; Menni et al. 2018). Despite the abundant literature on diverse aspects of SCFA, metabolism insight in their impact on ASCVD in humans is limited to association studies. However, interesting effects have been observed in studies in animal models. A case in point is the recent study of Kasahara et al. in *Nature Microbiology* (Kasahara et al. 2018) that focused on the ameliorating action of butyrate on atherosclerotic plaque progression in ApoE −/− mice. Germ-free ApoE−/− mice were first colonized with a mixture of eight low butyrate-producing bacterial strains. Subsequently, mice were inoculated with the high butyrate-producing strain *Roseburia intestinalis* as well. This led to a significant reduction in atherosclerotic plaque size (compared to controls). Interestingly, butyrate, whether added to diet or produced by the bacteria, had no effect on plasma cholesterol or TMAO levels. The observed beneficial effect on plaque progression seemed mainly due to a tightening of gut barrier function which potentially reduces translocation of LPS in this animal model. This local effect of butyrate makes sense because this particular SCFA is almost completely metabolized by the colonic enterocytes. Besides this putative effect on gut barrier function, which clearly requires confirmation, SCFA have been implicated in modulation of inflammatory processes (Ohira et al. 2017). The G protein-coupled receptors GPR41 and GPR43 serve as SCFA receptors and have been shown to elicit intracellular signal transduction cascades mediated by mitogen-activated protein kinases (MAPKs) and protein kinase C (PKC) (den Besten et al. 2013). In addition, butyrate has been shown to inhibit histone deacetylases, thereby altering the acetylation state of histones and other proteins which may induce epigenetic changes in gene transcription (Vinolo et al. 2011). A direct effect of SCFAs on expression of COX1 and 2 and hence possibly on eicosanoid production, important regulators of inflammatory processes, has also been proposed (Nurmi et al. 2005; Al-Lahham et al. 2010).

Most studies aiming at increasing insight into the molecular mechanism via which SCFA exert influence on inflammatory processes derive from in vitro experiments with cultured cells or tissues (Vinolo et al. 2011). Butyrate and propionate have been shown to affect neutrophil function by increasing apoptosis via a caspase-dependent pathway (Aoyama et al. 2010). A problem

with most in vitro studies is that supraphysiological concentrations are used to show the effects making translation to the human situation difficult. Furthermore, acetate, butyrate, and propionate sometimes exhibit contrasting effects (Cavaglieri et al. 2003) leading to controversy on their modes of action. It can, however, not be excluded that the SCFA concentration required to elicit an anti-inflammatory response varies with the type of inflammation (Al-Lahham et al. 2010).

Confirmation of the anti-inflammatory properties that are often associated with SCFA comes from animal experiments. In ApoE knockout mice, feeding with butyrate reduced atherosclerotic lesions and lowered macrophage migration accompanied by a decrease in pro-inflammatory cytokines (Aguilar et al. 2014). In mice treated intraperitoneally with acetate, inflammatory processes after kidney injuries were decreased leading to attenuation of the detrimental effects of inflammation on renal function (Andrade-Oliveira et al. 2015). Conversely, after systemic administration of supraphysiological doses of SCFA, renal tissue inflammation was increased due to dysregulation of T-cell response (Park et al. 2016). In another study in mice, SCFA receptors GPR41 and GPR43 were found to be required for an inflammatory response to bacterial infection and thus a protective pro-inflammatory response (Kim et al. 2013). In rodent models of colitis, oral acetate administration was shown to be protective (Masui et al. 2013).

As far as we are aware, no outcome trials have been carried out focusing on the effect of SCFA on ASCVD. However, a few human studies looked at the effect of SCFA on inflammatory aspects. A recent study investigated the effect of colonic infusions of SCFA, in concentrations found in the gut, on fasting levels of cytokines in overweight and obese subjects. The pro-inflammatory cytokine IL-1β decreased with a high acetate (60%) containing SCFA mixture compared to placebo and was significantly lower compared to a SCFA mixture containing high propionate (35%). Postprandial IL-1β levels as well as other pro-inflammatory cytokines including TNF-α, IL-6, and IL-8 did not change in the obese subjects neither in the fasting nor in the postprandial period (Canfora et al. 2017). In the study by van der Beek et al. (2016), a tendency for lower fasting plasma TNF-α concentrations was found after distal colonic acetate infusion with a 100 mmol/L yet not with a 180 mmol/L, as well as after proximal colonic acetate infusion.

3.4 Other Microbiome-Produced Metabolites Associated with ASCVD

A number of studies have appeared recently that aimed to identify bacteria as well as metabolites associated with different stages of cardiovascular disease (Wang et al. 2019; Würtz et al. 2015; Kurilshikov et al. 2019; Liu et al. 2019). Using a metagenomics approach, Kurilshikov et al. could link metabolic pathways encoded in the various bacteria to ASCVD risk but assessed risk directly in only one of the studied cohorts by measuring carotid IMT. In the other cohorts, a metabolic risk score was calculated from 33 established ASCVD biomarkers. An advantage of the metagenomics approach is that functional relations between

ASCVD risk and microbial pathway can be identified. This is important because many bacterial strains share metabolic pathways. ASCVD risk is associated strongly with pathways involved in amino acid metabolism (Newgard 2017). Metabolomics was investigated in this study using the NMR-based Nightingale platform which focuses mainly on lipoproteins, and the expected relations between ApoB-containing lipoproteins and ASCVD risk were observed. Using a multi-omics approach, in which state-of-the-art metabolomics was combined with 16S rRNA sequencing, a number of metabolic pathways and co-abundant bacterial groups were identified to associate with ASCVD severity (Liu et al. 2019). Although ASCVD severity was determined using coronary angiography, which enables accurate diagnosis of the extent of plaque formation, this limits the number patients that can be studied. By grouping bacteria by co-abundance, functional properties of these groups could be predicted linking ASCVD risk to taurine, sphingolipid, ceramide, and benzene metabolism. Identification of xenobiotics links environmental variables directly to microbiota and host metabolism which could lead in future studies to identification of molecular mechanisms.

4 Bile Acids

The most important endogenous molecules that undergo microbial modifications are the family of bile acids (BA).

4.1 Bile Acid Metabolism

These molecules are produced exclusively by the liver via two pathways that start separately but fuse after four steps to share most of the subsequent steps in the parts that produce the primary bile acid chenodeoxycholic acid (Kuipers et al. 2014). The so-called classic bile acid synthesis pathway starts with the conversion of cholesterol into 7-alpha-cholesterol catalyzed by the enzyme 7-alpha-hydroxylase and produce either cholic acid or chenodeoxycholic acid. In humans, this has been postulated to be the major pathway, but this hypothesis requires experimental validation. After synthesis is completed via a complex pathway consisting of enzymatic steps in the cellular cytosol as well as mitochondria, the molecules are conjugated in peroxisomes with either glycine or taurine (Russell 2003; Chiang and Ferrell 2019). In humans the ratio glycine/taurine is mostly around 3; rodents predominantly conjugate with taurine (Kuipers et al. 2014). Another substantial difference between rodents and humans is the fact that rodents convert the hydrophobic chenodeoxycholic acid into the very hydrophilic muricholic acids. This completely alters BA function and precludes direct translation of rodent data to humans (Kuipers et al. 2014).

In mice and man, BA are stored in the gallbladder and are expelled into the duodenum primarily after initiating intake of food (Behar 2013). The consensus is that sensors in the small intestine register arrival of fat and protein and activate

gallbladder contraction through release of cholecystokinin, although also in the absence of food regular small contraction of the gallbladder must occur to maintain BA concentrations observed in the circulation (Sips et al. 2018). BA arriving in the terminal ileum are extremely efficiently absorbed via the sodium-dependent bile acid transporter (ASBT or SLC10A2) (Hagenbuch and Dawson 2004). Bile acids are highly toxic for bacteria which is probably an important reason why the small intestine is sparsely colonized relative to the colon. Depending on small intestinal motility and the bile salt hydrolase activity of the microbiota colonizing the small intestine, a small amount of bile acids enters the colon and is metabolized into a myriad of so-called secondary or more recently tertiary bile acids. The degree of metabolism strongly depends on the colonic microbiota composition of a given subject (Ridlon et al. 2006).

Secondary bile acids are hydrophobic and consequently highly toxic for bacteria; apparently bacteria that are able to dehydroxylate bile acids have evolved to create a toxic environment for their neighbors. Because of the fact that the secondary bile acids are hydrophobic, they can passively diffuse across the colonocyte cell membranes and enter the bloodstream. Whether this process is purely diffusion or whether transporters are also involved is not known. In humans, it is estimated that about 5% of the bile acid pool is not reabsorbed and is excreted via the feces. The variability may in part be caused by changes in absorptive capacity which might directly influence the risk on ASCVD. Note that although only 5% of the bile acids escapes the enterohepatic circulation, bile acid excretion is the major pathway for cholesterol export from the body, apart from neutral sterol excretion. Aging correlates negatively with bile acid synthesis (Einarsson et al. 1985); thus also cholesterol excretion via this route decreases with age pointing to a possible causal relation between BA excretion and ASCVD. The plasma concentration of taurocholate has been found to negatively correlate with longevity (Cheng et al. 2015). This could be due to increased absorptive capacity possibly increasing with age and also accounting for the decrease in synthesis, but this still has to be addressed experimentally. One report has described a negative correlation between bile acid synthesis rates and ASCVD events (Charach et al. 2018). Though highly interesting this study requires confirmation.

4.2 Regulation by Bile Acids

Besides the direct role of BA in cholesterol metabolism, they control diverse metabolic pathways via membrane and nuclear hormone receptors signaling. Particularly G-protein linked receptor TGR5 and the farnesoid X receptor (FXR) are important in this respect. Both receptors show a great preference for the more hydrophobic bile acids; hence microbial metabolism plays a major role in regulating BA control of metabolism.

The role of FXR in controlling progress of ASCVD is ambiguous. Hanniman et al. reported increased atherosclerosis development in FXR/ApoE double KO mice (Hanniman et al. 2005), whereas two other studies reported that loss of FXR

in low-density lipoprotein receptor −/− (LDLR) mice and ApoE−/− mice reduced atherosclerotic lesion size (Guo et al. 2006; Zhang et al. 2006). Although differences in gut microbiota composition and sex of the mouse models used may play a role, the exact nature of these discrepancies is unclear. In contrast, FXR stimulation with the FXR agonists PX20606 and WAY-362450 did prevent atherosclerotic plaque formation in ApoE KO, LDLR −/−, or CETP transgene LDLR −/− models (Hartman et al. 2009; Hambruch et al. 2012). Additionally, FXR stimulation modulates inflammatory responses, thereby reducing pro-inflammatory cytokine production. The first FDA-approved FXR agonist obeticholic acid (OCA) is currently tested in human trials (Neuschwander-Tetri et al. 2015). Unexpectedly, OCA induced an increase in LDL cholesterol and a concomitant decrease in HDL-C (Nevens et al. 2016). The underlying mechanism is not clear, and the effect on ASCVD has not yet been assessed; but the induced phenotype makes OCA a less attractive option to treat atherosclerosis in humans.

The other important BA receptor, TGR5, has a high affinity for secondary BA in particular lithocholate (Klindt et al. 2015). TGR5 stimulation activates thyroid hormone deiodinase 2 which converts inactive thyroxine (T4) into active 3,5,3′-triiodothyronine 12 (T3) and stimulates energy expenditure (Watanabe et al. 2006). Interestingly, TGR5 activation has immunosuppressive effects. TGR5 has been shown to reduce cytokine expression via inhibition of nuclear translocation of NF-κB (Pols et al. 2011; Yoneno et al. 2013). Furthermore, TGR5 activation with INT-777 inhibits the inflammasome, a major driver of the inflammatory component of ASCVD progression (Hao et al. 2017). In addition to its effects on immune cells, TGR5 also effects metabolism in endothelial cells (Keitel et al. 2007) where it may control nitric oxide (NO) production, through phosphorylation of endothelial nitric oxide synthase (eNOS) (Kida et al. 2013). The immunomodulatory functions of TGR5 make this receptor an interesting target to treat atherosclerosis, and because of its high affinity for lithocholic acid and deoxycholic acid, it may explain beneficial effects of the gut microbiota in ASCVD. So far, the effects of specific TGR5 agonists have only been studied in animal models; hence it is not clear whether the results can be translated to humans.

5 Summary and Future Perspectives

The etiology of ASCVD starts simple with disposition of lipids in the vessel wall but develops into an extremely complex myriad of aggravating and inhibiting factors when it progresses. Because so many factors are involved, it seems justified to assume that ASCVD develops in a unique way in any single patient. Up to now treatment of ASCVD mainly focuses on inhibiting the initiating factor, disposition of lipid in the vessel wall. Although perhaps successful in inhibiting progress of the disease, it does not induce regression of the plaques to a significant extent. Attempts to induce plaque regression have been very unsuccessful so far. Enhancing cholesterol efflux through increasing plasma HDL concentration has not worked

probably by a lack of understanding of the molecular mechanism of cholesterol efflux in vivo.

The question arises whether influencing the composition of the microbiome can help. As discussed in this review, the bacterial component of the microbiome can influence the process of ASCVD development at many different stages. Particularly, the oral microbiome can easily invade a patient suffering from a very common periodontitis. This causes a systemic inflammatory response potentially aggravating atherosclerotic plaque progression.

Bacteria can initiate production of harmful molecules such as TMA or ImP that are likely to contribute to ASCVD development. Since many bacterial species are capable of TMA or ImP production, it is challenging to develop strategies to, e.g., eradicate these bacteria. Antibiotics treatment has been shown to reduce TMAO production in humans (Craciun and Balskus 2012) However, it is critical to point out that there are many objections to using antibiotics as means to intervene in microbiome-mediated cues to ASCVD development. These include significant consequences of antibiotic use for the gut microbial community, risk to develop antibiotic resistance, and the fact that antibiotics use has been associated with increased progression of ASCVD (Heianza et al. 2019).

Early initiatives aiming to specifically reduce production of TMA (instead of the bacterium) have shown promising results in lowering TMAO levels and ASCVD risk, at least in mice. Inhibition of the activity of TMA lyase, which hydrolyses choline to TMA, using the choline analog DMB reduced atherosclerosis burden in ApoE−/− mice fed a choline-rich diet (Wang et al. 2015). More recently, it was shown that strategies aiming to inhibit phospholipase D, a bacterial enzyme that frees choline from phosphatidylcholine lipids, might be an interesting novel target to reduce choline-derived production of TMA (Chittim et al. 2019). More upstream in the cascade of microbial-metabolite production, it might be beneficial to develop strategies that aim to reduce intake of precursors of the presumably harmful metabolites. Although it is too early to tell if reduction of choline intake or alternative dietary strategies to reduce TMAO production will prevent ASCVD development (Washburn et al. 2019), these initiatives might provide feasible and economic solutions for reduction of these and other (e.g., ImP from histidine) microbiome-derived atherogenic metabolites. Important in this context is that humans usually have very low coherence to dietary interventions. In addition, high interindividual differences in response to dietary interventions (Walker et al. 2011; Cotillard et al. 2013; Kovatcheva-Datchary et al. 2015) make diet a challenging intervention to alter the microbiome and (markers of) ASCVD development.

The microbial modification of primary into secondary bile acids is in part facilitated by the bacterial enzyme bile salt hydrolase (BSH). BSH activity has been postulated to alter cholesterol accumulation, inflammation, and atherosclerosis development (Tremaroli and Bäckhed 2012), and BSH activity is present in a very wide range of bacteria (Joyce et al. 2014). A modified *E. coli* strain carrying the BSH gene was shown to enhance expression of genes involved in cholesterol efflux, immune homeostasis, and energy metabolism in mice (Joyce et al. 2014).

In line, a dedicated intervention study using BSH-active *Lactobacillus reuteri* in hypercholesterolemic humans showed that this probiotic effectively lowered LDL-C compared to placebo-treated subjects (Jones et al. 2012). Of interest in this context is that many probiotic strains are characterized by BSH activity (Begley et al. 2006). Whether or not the BSH activity underlies the beneficial effects of probiotic strain administration on parameters of ASCVD risk/health remains to be determined. Nevertheless, many probiotic strains have been associated with ASCVD health. *Bifidobacteria* and *Lactobacillus plantarum* have been associated with lowering of cholesterol (Tahri et al. 1996). Interestingly, *Lactobacillus plantarum* (Nguyen et al. 2007) and *Lactobacillus rhamnosus* (Qiu et al. 2018) were also reported to decrease TMAO and atherosclerosis development in mice prone to develop the disease. Likewise, in hypercholesterolemic humans, *Lactobacillus rhamnosus* was reported to reduce cholesterol levels (Costabile et al. 2017).

One can speculate that beneficial bacteria may produce molecules that halt or even induce regression of the process. As far as we know, studies to find these compounds have not been carried out. A good strategy may be to use modern machine learning methods to analyze the plasma of subjects with atherogenic plasma profile that do not show signs of ASCVD.

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