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GUT MICROBIOTA AND ATHEROSCLEROSIS : EMERGING QUESTIONS

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ABSTRACT

Possible link between infectious agents and cardiovascular health was originally proclaimed by R Virchow and I Metchnikoff. There is a growing body of experimental and clinical evidence suggesting that some microbial communities of the human body, in particular the gut microbiota, are involved in atherosclerosis development. It has been recently shown, that products of microbial degradation of phosphatydilcholine - choline, trimetylamine N-oxide (TMAO) and betaine can predict risk for cardiovascular disease. Moreover, L-carnitine, a trimethylamine plentiful in red meat, also increases TMAO levels through a similar microbiota-dependent mechanism and promotes atherosclerosis by inhibiting reverse cholesterol transport and cholesterol disposal with bile. On the other hand, atherosclerosis is reportedly associated with activation of multiple bacterial genes involved in the peptidoglycan metabolism in gut. Possible therapeutic strategies and emerging questions related to the role of gut microbiota in cardiovascular health are discussed in this review.

INTRODUCTION

Mutually beneficial relations between microbial communities populating the human body and the host were first comprehensively described by the Nobel Prize Winner for Medicine (1908) Elie Metchnikoff (1845-1916) who linked the regular consumption of lactic acid bacteria from fermented dairy products with increased longevity in the Bulgarian peasant population (1, 2). Moreover, Metchnikoff was the first scientist to appreciate the complexity of the relationship between microbial populations and the human body ranging from mutualism to commensalism, and parasitism to pathogenicity. In particular, he suggested that autointoxication with bacterial by-products from the large intestine is a probable cause of age-related health decline and some human diseases (3, 4). Interdisciplinary scientific developments of Metchnikov's ideas over the past 100 years have paved the way for the introduction of probiotics in modern therapeutics and preventive medicine and, most importantly, established some new scientific categories (5). It is now widely acknowledged that the variety of micriobial communities (referred to collectively as microbiota) populating different areas of the human body outnumber human eukaryotic cells within an individual (6, 7). Moreover, advances in gene-sequencing technology have allowed emerging the concept of the microbiome, a collective genome of bacteria populating different areas of the human body, along with the initiation of a search to link the microbiome with various aspects of human health (8, 9). Although

the role of infectious agents in the initiation, progression and outcomes of different human diseases (cardiovascular disease, inflammatory bowel disease, Alzheimer's disease, multiple sclerosis and some others) was suspected from the first half of the 19th century, the cumulative evidence for the participation of infectious agents in the pathogenesis of atherosclerosis has principally gained new factual ground over the last few years. Even though it remains very unlikely that atherosclerosis will ever be unconditionally considered as an infectious disease caused by a single pathogen, there are new facts revealing the contribution of bacterial communities to the initiation, development and outcomes of atherosclerosis.

INFECTIOUS AGENTS AND ATHEROSCLEROSIS

The modern scientific concept of atherosclerosis originates from Anitchkov's classic cholesterol feeding studies (10). It was further crystallized around the axis formed by the identification of plasma lipoproteins (11) and the groundbreaking discoveries of LDL-receptors and the LDL receptor-mediated pathway (12). The subsequent introduction of statins and their tremendous therapeutic benefits in cardiovascular patients provides the best possible clinical evidence validating the role of abnormal cholesterol homeostasis in the pathogenesis of atherosclerosis. Although the possible role of infectious agents in atherosclerosis has attracted the attention of many researchers over the last 100 years dating back to R. Virchow (13), the efficacy of

antibiotic and/or antiviral drug use in cardiovascular patients has not been proven. Nevertheless, the existing solid body of epidemiological evidence linking acute as well as chronic infections with inflammation and atherosclerotic plaque formation in cardiovascular patients cannot be ignored. Various infectious agents are reportedly involved in the pathogenesis of atherosclerosis. Among them are *Chlamydia pneumoniae, Porphyromonas gingivalis, Helicobacter pylori,* influenza A virus, hepatitis C virus, cytomegalovirus and human immunodeficiency virus (14). *C. pneumoniae* seems to be the most well studied infectious pathogen associated with risk of atherosclerosis which can be detected and isolated in viable form from atherosclerotic lesions of cardiovascular patients (15, 16). Multiple sero-epidemiological data revealing that patients with cardiovascular disease have higher titers of anti-*C.pneumoniae* antibodies, as well as numerous experimental results implicating *C. pneumoniae* in the initiation and/or exacerbation of endovascular atherosclerotic lesions have fuelled the interest of researchers into the pathogen for the last three decades (17). The link between *C. pneumoniae* and atherosclerosis becomes less certain when the results of clinical trials are analyzed. Antibiotic therapy used for secondary prevention of cardiovascular events has little or no effect on the occurrence of secondary cardiovascular events in seropositive patients with atherosclerosis (17, 18). Therefore the fulfillment of classic Koch postulates or molecular Koch postulates as formulated by S. Falkow (19) to prove a

causal relationship between any infective agent, including C*. pneumoniae,* and atherosclerosis remains largely unaccomplished. However, over the last 3 years a new body of experimental and clinical evidence has emerged which allows the establishment of some new connections between mechanisms of atherosclerosis and the microbial communities of the human body, in particular the gut microbiota, rather than a sole infectious agent.

DEFINING INTESTINAL MICROBIOTA

A dense resident microbial community in the gut, referred to as the commensal intestinal microbiota confers protection against gut colonization by pathogens and provides biochemical transformation of nutrients which would otherwise be poorly absorbed (20, 21). Although there is no clear definition of the characteristics of a normal 'healthy' gut microbiota in human subjects (22) it is well known that the gastrointestinal tract hosts more than 100 trillion bacteria whose collective genome (metagenome) outnumbers the size of the human genome by a factor of 150 (23). Most of the intestinal bacteria are poorly or non-cultivatable using *in vitro* systems, which complicates gut microbiome categorization and the identification of individual species (24). However, based on the predominant bacterial genera in fecal specimens, recent metagenomic analysis (25) has allowed 3 major clusters of gut bacteria (named enterotypes) to be distinguished in humans: enterotype 1 (rich in *Bacteroides*), enterotype 2 (rich in *Prevotella*) and most

frequently in human populations, enterotype 3 (rich in Ruminococcus). Despite some disagreements regarding enterotype definition and categorization (26), the concept of dominant intestinal species is essentially right and can be traced back to Metchnikoff's ideas. Similar microbiota types resembling human enterotypes 1 and 3 were recently reported in mouse strains using 16S rDNA pyrosequencing (27). Although human enterotypes are not driven by ethnicity, race, geographical location of the individuals or environmental factors as originally claimed (25), the ratio between predominant bacterial species in the gut may be influenced by diet, cultural habits and socioeconomic status (28). Variations in the microbiome composition may occur within 24 hours of diet change (29). Protein and animal fat promotes the *Bacteroides* genera, whereas a carbohydrate diet switches enterotype identity towards the prevalence of *Prevotella* species (29). Latest results indicate (26) that individual variations in human microbiome composition may fall between previously defined (25) discreet enterotype categories.

GUT MICROBIOTA AND ATHEROSCLEROSIS

Since increased adiposity is a known risk factor for atherosclerosis and cardiovascular disease, the notion of a possible link between the gut microbiota and atherosclerosis gained its initial scientific ground when the first results regarding a relationship between lipogenesis, obesity and gut

microbiota were published in 2004. It was found by Gordon JL and his collaborators (30) that the intestinal microbiota promotes absorption of monosaccharides from the gut lumen, with subsequent induction of *de novo* hepatic lipogenesis and increased body weight. The same researchers found later that the gut microbiota of obese mice as well as obese human volunteers has an increased capacity to harvest energy from the diet due to changes in the relative abundance of the two dominant bacterial groups, the Bacteroidetes and the Firmicutes (31). Indeed, *Bacteroides* and *Parabacteroides,* which are major intestinal genera, have remarkable saccharolytic activity and derive energy primarily from carbohydrates and protein fermentation conferring thereby a higher glucose bioavailability in the gut (32). It was recently confirmed that predominance of major bacterial species in the gut microbiome affects some basic metabolic parameters of the human body including plasma lipids. Also it was shown recently that individuals with a high Prevotella\Bacteroids ratio tend to have higher plasma cholesterol values, as compared to volunteers with a lower abundance of Prevotella in the gut microbiome (33). On the other hand, a reduced Firmicutes\Bacteroidetes ratio in the gut microbiota caused by switching volunteers to a vegetarian diet was shown to be accompanied by a reduction in major plasma lipids (triglycerides, total cholesterol, low-density lipoprotein cholesterol) as well as improved hemoglobin A1c and fasting and postprandial glucose levels (33). Plasma lipid profile is also affected by the

richness of the gut microbiome. Very recent results (34) suggest that individuals with a lower number of gut bacterial genes are characterized by enhanced dyslipidemia, overall adiposity and insulin resistance.

Crucial clinical evidence revealing the participation of the gut microbiota in the development of atherosclerosis came from a recently published studies led by Dr. Hazen SL. In their initial paper Wang and collaborators reported (35) the results of metabolomics analysis in large clinical cohort which revealed that three major metabolites of dietary lecithin (phosphatidylcholine) – choline, trimetylamine N-oxide (TMAO) and betaine can predict risk for cardiovascular disease. These metabolites were shown to upregulate pro-atherogenic macrophage scavenger receptors and foam cell formation in apoE -/- mice, whereas dietary supplementation of apoE-/- mice with choline and TMAO promoted atherosclerosis. Remarkably, antibiotic pretreatment completely abolished plasma levels of TMAO and prevented atherosclerotic changes in choline-fed apoE-/- mice, suggesting a pivotal role of intestinal microflora in the formation of pro-atherogenic phosphatidylcholine metabolites. The same authors reported (36) that TMAO levels are markedly increased in the plasma of healthy volunteers after phosphatidylcholine ingestion, whereas pre-treatment with antibiotics inhibits the TMAO induction. Remarkably, withdrawal of antibiotics restores the phosphatidylcholine-induced spike in the plasma TMAO concentration. Moreover elevated plasma levels of TMAO were associated with a statistically

significant increased risk of major adverse events including death in cardiovascular patients. The link between plasma TMAO, intestinal microbiota and atherosclerosis becomes even more substantiated in the light of another set of results recently published by Dr. Hazen and collaborators (37). It was reported that L-carnitine, a trimethylamine plentiful in red meat, also increases TMAO levels through a similar microbiota-dependent mechanism and promotes atherosclerosis by inhibiting reverse cholesterol transport and cholesterol disposal with bile. Similarly, carnitine-induced levels of TMAO were abolished in antibiotic-treated volunteers and were restored upon antibiotic withdrawal. Notably in the same study, volunteers with a *Prevotella*–dominated enterotype demonstrated higher plasma TMAO levels than subjects with *Bacteroides* dominance, suggesting a possible role for the *Prevotella* genus in the formation of TMAO. However the link between atherosclerosis and intestinal microbiota seem to include some other players. As recently reported, symptomatic atherosclerosis is associated with activation of multiple bacterial genes involved in the peptidoglycan metabolism, whereas healthy volunteers had a clear upregulation of pathways responsible for bacterial synthesis of anti-inflammatory molecules, such as butyrate (38).

TMAO PATHWAY

Trimethylamine-N-oxide (TMAO) is a low molecular weight organic substance abundantly present in seawater fish as an osmolyte (39). It can be formed in the human liver (40) from its metabolic precursor trimethylamine (TMA) with the participation of the family of hepatic enzymes originally designated as flavin-containing monooxygenases (FMOs). TMA originates in the intestine with an involvement of the gut microflora from two principal dietary sources - either phosphatidylcholine/choline or L-carnitine (36, 40). This exclusively bacterial mechanism is maintained in the gut by a variety of intestinal microorganisms, in particular *Clostridia*, *Desulfovibrio, Shigella, Proteus* (41, 42). As recently reported, the FMO family is represented by 5 members (FMO1-FMO5) with relatively high amino acid sequence identity and conservation between mouse and human (43). FMO3 is the most enzymatically active family member, highly expressed in liver and capable of converting TMA to TMAO (43, 44). In transgenic mice hepatic FMO3 overexpression contributes to circulating TMAO levels, whereas FMO3 specific antisense nucleotides cause reciprocal effects, suggesting that FMO3 is the rate-limiting enzyme for TMAO formation. Besides distinct sexual dimorphism, FMO3 expression exhibits clear regulation by FXR due to presence in the FMO3 promoter FXRE sites (43). Collectively, these results suggest that hepatic expression of FMO3 might be controlled by FXR nuclear receptors which provide an important insight into mechanisms of pharmacological regulation of TMAO pathway.

TMAO: PENDING QUESTIONS

Despite its appealing sequel the intestinal microbiota-TMAO concept as formulated by Hazen SL and his collaborators (35, 36, 37, 43, 44) remains largely unopposed by other research projects. Most urgent question to be answered during next few years relates to the diagnostic and prognostic significance of TMAO levels in cardiovascular patients. There is a distinct but poorly understood association among plasma choline, betaine and TMAO levels in cardiovascular patients (44). Relevance of TMAO levels to the duration and severity of cardiovascular disease, gender and ethnic variations need to be also investigated. Larger epidemiological evidence required to set apart red meat consumption as an exclusive dietary factor promoting TMAO pathway and cardiovascular disease. Moreover, analytical procedure for TMAO measurement needs to be developed and introduced in the laboratory medicine. Strikingly, the molecular mechanism underlying the TMAO effects in atherosclerosis development and bacterial species involved remains elusive. Furthermore, there is some controversy regarding potential role of TMAO in cardiovascular disease. Fish consumption is known to upregulate TMAO levels in human body and to reduce an incidence of cardiovascular disease (45). These facts do not support the recent statements about pro-atherogenic effects of TMAO (35, 36). In addition, there is a solid clinical evidence suggesting that carnitine supplementation improves outcomes of cardiovascular disease (46), whereas results reported by Hazen SL and

collaborators (36) identify L-carnitine as a principal metabolic predecessor of TMA in human intestine.

ANTIBIOTICS, PROBIOTICS AND NUTRACEUTICALS

As shown in experimental and clinical conditions, diet-induced spikes in plasma TMAO can be completely abolished by antibiotic treatment which may reduce TMAO levels to undetectable levels (36). However treatment with antibiotics affects multiple quantitative characteristics of gut microbiota, its diversity and metabolic status (47) and showed no significant effect on outcomes of atherosclerosis (48). Therefore, even if TMAO pathway becomes an unquestionable target for treatment protocol of cardiovascular disease, treatment with antibiotic will remain a very problematic option.

As it was suggested by Metchnikoff a century ago, production of colonic bacterial toxins can be ameliorated by probiotics (49). Indeed, it was reported recently, that colonic TMA production and the further formation of TMAO can be blocked by antagonizing the colonic TMA producers with recently discovered methanogens, including *Methanomassiliicoccus luminyensis* (50). Other probiotic strains affecting TMAO pathway need to be identified. However, the establishment of new interactions in the intestinal ecology is a task of enormous challenge. Other strategies may arise from recently published results revealing molecular and dietary regulation of TMAO pathway.

First of all, hepatic expression of FMO3, a rate limiting enzyme of TMAO pathway is recently shown to be under control of FXR nuclear receptors. Therefore known FXR antagonists as well as nutritional interventions affecting FXR targets can be used to control the TMAO pathway.

Secondly, it is recently shown that some nutraceuticals affect the diversity and functional status of intestinal microbiota. In particular, cocoa-derived polyphenols, flavanols and procyanidins may have an impact on human disorders linked to intestinal microbiota (51). Regular consumption of cocoa-containing drinks promotes intestinal representation of Lactobacilli and Bifidobacteria reducing at the same time Clostridia species in the human gut (52). Reduction of Clostridium, Bacteroides and Staphylococcus species in feces was also observed in rats fed with cocoa-rich diet (53). It was hypothesized recently that interactions between dietary polyphenols and intestinal microbiota are multifaceted. While cocoa-derived compounds have clear effect on microbiota spectrum, certain intestinal bacteria promote bioavailability of cocoa polyphenols (54). Other food ingredients with known ability to modulate diversity of human microbiome may find proper application in the prevention and treatment of human disorders linked with intestinal microbiota.

CONCLUSION

Almost half of cardiovascular events develop in patients with no identifiable risk factors (18). Therefore, search for new modalities affecting initiation, progression and outcomes of cardiovascular disease remains a strategic goal of modern medicine. Recent developments allowed establishing a link between human microbiota and various diseases (cardiovascular disease, neurological abnormalities, inflammatory bowel disease, allergies). Moreover, a relationship between intestinal microbiota and atherosclerosis as well as a keystone role of phosphatidylcholine-carnitine-TMAO pathway has been declared. However, atherosclerosis is a polygenic disorder with multiple initiation mechanisms and variety of clinical manifestations. Therefore it is very unlikely that a single diagnostic or prognostic criterion of atherosclerosis will be ever identified. It is also implausible that there is a sole pathway mediating effects of gut microbiota on human health and cardiovascular disease. Further research is needed to support newly published results, answer remaining questions about role of intestinal microbiota in cardiovascular disease and develop effective interventional strategies.

DISCLOSURE STATEMENT

No conflict of interests involved.

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