Influence of Gut Microbiota and Trimethylamine N-Oxide in Patients with Coronary Heart Disease

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Summary

In the current study, the gut microbiota of patients with and without coronary heart disease was compared and the relationship between gut microbiota distribution, intending to reveal the role of gut microbiota in the coronary atherosclerosis process, was investigated.

This study included 50 patients diagnosed with coronary heart disease (CHD) who received conventional coronary angiography or computed tomography angiography and 50 patients with CHD at Changshu No. 2 People’s Hospital, Suzhou, China, from May 2020 to January 2021. Trimethylamine N-oxide (TMAO) level was tested and feces were collected, the DNA of the gut microbiota was extracted, and the distribution by 16SrRNA gene sequencing was obtained from the two groups of patients.

Plasma TMAO concentrations were significantly higher in patients with CHD ($P < 0.001$). In the CHD group, 22 patients with multivessel disease had a higher level of TMAO compared with the 28 patients who had the single-vessel disease ($P < 0.001$). No difference in the gut microbiota diversity was noted between the two groups ($P < 0.001$). Patients with CHD had a significantly lower proportion of Bacteroidetes phyla and more proportion of Epsilonbacteraeota phyla. At the genus level, patients with CHD had an increased abundance of Enterococcus, whereas healthy controls had significantly higher levels of Streptococcus. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 analysis found that, in the KEGG ORTHOL-OGY, the level of choline trimethylamine-lyase gene expression correlated with TMAO production was higher in the fecal microbiome of the CHD group ($P < 0.05$).

Gut microbiota and its product were expected to become a diagnostic marker and a new target for preventing CHD.

Key words: 16S rRNA, TMAO, CutC

Many microflorae, called the second genome in our gut, exist. In recent years, gut microbiota has been one of the most studied fields. Eckburg, et al.1) performed a metagenomic analysis to determine that the gut microbial community consists of six phyla: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia. The primary dominants are the Firmicutes and Bacteroidetes phyla, accounting for >90% of the population. Accumulating evidence indicated that intestinal microbiota is associated with various diseases, including autoimmune disease,2) diabetes,3) and especially cardiovascular diseases.4) Coronary atherosclerotic heart disease (CHD), also called ischemic heart disease, refers to coronary artery stenosis or occlusion due to atherosclerotic lesions. This disease has been proven to be the primary cause of mortality. Unfortunately, neither drug intervention5) nor revascularization6) can effectively improve the prognosis of patients with CHD. The regulation of gut microbiota has brought light to the treatment of CHD. Both human and animal experiments have revealed that gut microflora dysbiosis can accelerate the onset of coronary atherosclerosis. Moreover, the gut microbiota converts the choline ingested by the host into trimethylamine (TMA), which is converted into trimethylamine N-oxide (TMAO) in the liver and released into the blood. TMAO affects a series of metabolites, including cholesterol metabolism, food choline, and TMAO supplements, which increase the expression of scavenger receptors (CD36 and SR-A1) on the surface of macrophages and promotes the formation of foam cells, thereby worsening atherosclerosis.7)
Methods
Participants and study design: The current study included 100 patients who underwent computed tomography angiography or conventional coronary angiography (CAG) at the hospital of the current study from May 2020 to January 2021. Patients were divided into the control (n = 50) and the CHD (n = 50) groups. Patients were diagnosed with CHD according to the Diagnostic and Therapeutic Guidelines for Coronary Atherosclerotic Heart Disease by the American Heart Association. This study was approved by the Ethical Committee of the Changshu No. 2 People’s Hospital, Suzhou, China. Informed consent was obtained from all the participants. Patients were excluded if they had taken antibiotics or other drugs that affect the gut flora within 3 months before the examination and if a history of gastrointestinal diseases, gastroenteritis, severe hepatic, renal dysfunction, tumor, severe infection, trauma, and connective tissue disease was noted. Each patient in the CHD group underwent coronary angiography with at least one coronary lesion >50%. Baseline demographic, clinical, and angiographic features were recorded for each patient.

Measurement of TMAO: Blood samples were collected via radial or femoral access before heparinization using vacutainer tubes containing ethylenediaminetetraacetic acid and stored in liquid nitrogen freezers at −80°C or colder until further analysis. Blood concentrations of TMAO were measured using an established stable isotope dilution high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. Both dilution high-performance liquid chromatography with TMAO were measured using an established stable isotope dilution high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. Both technicians and laboratory personnel were blinded to the case-control status of the samples.

DNA library construction and high-throughput sequencing: DNA was extracted from 0.5 g of patient fecal samples using the E.Z.N.A.® Stool DNA kit (D4015, Omega, Inc., Mountain Lakes, NJ, USA). The total DNA was stored at −80°C until measurement. The V4 region of the bacterial small-subunit (16S) rRNA gene was amplified with slightly modified versions of primers 515F (5’-GTGYCAGCMGCCGCGGTAA-3’) and 806R (5’-GG ACTACHVGGGTWTCTAAT-3’). The 5’ ends of the primers were tagged with specific barcodes per sample and sequencing universal primers. Polymerase chain reaction (PCR) amplification was performed in a volume of 25-μL reaction mixture containing 25 ng of template DNA, 12.5 μL PCR Premix, 2.5 μL of each primer, and PCR-grade water to adjust the volume. The PCR conditions to amplify the prokaryotic 16S fragments consisted of an initial denaturation at 98°C for 30 s; 32 cycles of denaturation at 98°C for 10 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s; and a final extension at 72°C for 10 minutes. The PCR products were purified by AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit (Invitrogen, Waltham, MA, USA). The amplicon pools were prepared for sequencing. The size and quantity of the amplicon library were assessed on Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. The libraries were sequenced on the NovaSeq PE250 platform.

Collection of basic information and prognosis: All patients’ basic information was recorded, including gender, age, body mass index, smoking history, previous medical history (hypertension, hyperlipidemia, and diabetes), biochemical outcome (LDL-c, albumin, creatine), TMAO levels, echocardiography (left atrial diameter of systolic, left ventricular ejection fraction), and coronary artery disease (degree of coronary artery stenosis, characteristic of the lesion, and so on).

Statistical analysis: Samples were sequenced on an Illumina NovaSeq platform. Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH. Chimeric sequences were filtered using Vsearch software (v2.3.4). After dereplication using DADA2, a feature table was obtained. Alpha diversity is applied in analyzing the complexity of species diversity for a sample through five indices, including Chao1, observed species, goods coverage, Shannon, and Simpson. The results are then illustrated using the principal coordinate analysis (PCoA). Differences in microbial abundance (at the phylum levels) were analyzed using the Mann-Whitney U test. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt®) was used to predict metagenome functional content from marker gene (16S rRNA) surveys. The data were analyzed statistically using STAMP.

Continuous data were expressed as mean ± standard deviation. Categorical variables are presented as frequencies and percentages. Comparisons between groups were performed using the Wilcoxon Mann-Whitney tests.0. The differences in categorical variables were assessed using the χ² test, whereas the Student’s t-test was applied for continuous variables. In addition, p < 0.05 was considered statistically significant. Statistical analysis was performed using the SPSS 22.0 software.

Results
Characteristics of the study population: The baseline characteristics of the study population are presented in Table 1. This study included 100 patients, divided into two groups. 50 CHD patients identified by coronary angiography constituted the CHD group, and the control group was composed of 50 patients with normal coronary angiography results. The incidence rates of conventional risk factors, e.g., hypertension, hyperlipidemia, and smoking) were also assessed, without significant differences between the two groups (P > 0.05; Table 1). Left atrial inner diameter was significantly increased in the patients in the CHD group (P = 0.036). The TMAO concentration was higher in the CHD group than that in the control group (P = 0.046).

Relationship between plasma TMAO levels and the number of diseased coronary arteries: The plasma TMAO concentrations were significantly higher in patients with CHD (3.31 [interquartile range (IQR): 2.80-3.80] μM versus 2.59 [IQR: 2.10-3.10] μM, 0.001; Figure 1A). CHD patients were divided into single- and multivessel diseases (≥2 vessels). The results showed that patients
with multivessel lesions had significantly higher TMAO levels than those with single-vessel lesions \( [2.91 \text{ IQR: } 2.57-3.43] \) μM versus \( 3.61 \text{ IQR: } 3.05-3.97 \) μM, \( P < 0.001; \) Figure 1B].

**Comparison of Differences in Gut microbiota Between CONTROL Group and Coronary Heart Disease Group:**

**Venn diagram** Operational taxonomic unit (OTU) analysis showed that 5,905 and 5,893 OTUs were obtained in the CHD and control groups, respectively. Moreover, 1,765 common OTUs were noted in the two groups (Figure 2A). **Principal coordinate analysis** Based on unweighted UniFrac and Bray-Curtis distance matrices of the 16S rRNA sequences, PCoA analysis was conducted to compare the composition of gut microbiota in the two groups by using the R software Vegan package. Sample contribution rates of the first PCoA (PC1) and second PCoA (PC2) were 12.65% and 6.06%, respectively, which reflected the microbial populations of the CHD patients away from that of the controls. The results showed no significant difference between the two groups \( (P = 0.067; \) Figure 2B).

**Alpha diversity** In this study, the Chao1, coverage, Simpson, and Shannon indices were used to evaluate the microbiota diversity. The results of the alpha diversity analysis showed that no significant differences exist between the two groups, including Chao1 \( [P = 0.84; \) Figure 3A], coverage \( [P = 0.54; \) Figure 3B], observed \( [P = 0.89; \) Figure 3C], Shannon \( [P = 0.64; \) Figure 3D], and Simpson \( [P = 0.62; \) Figure 3E] indices.

**The types and proportion of gut microbiota:** The relative abundance of the top 30 species with the highest abundance were calculated and the differences between the two groups at the phylum level were analyzed (Figure 4A). The top 4 abundance of reads were Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria. Phylum Firmicutes was found to have the highest abundance in the CHD group (50.0%), which was lower than that in the control group (54.05%, \( P = 0.82 \)). In addition, Bacteroidetes in CHD patients were also less abundant than that in the control group (13.01% versus 18.85%, \( P < 0.05 \)) while Epsilonbacteriaceota in the CHD group were more abundant (0.02% versus 0.01%, \( P < 0.05 \)). Phylum Proteobacteria and Actinobacteria was increased in CHD patients compared with those in the control group [22.22% versus 17.81%, \( P = 0.94 \); 12.58% versus 8.04%, \( P = 0.38 \) (Figure 4B)]. At the genus level. The top 5 abundance of reads: Escherichia-Shigella, Bacteroides, Bifidobacterium, Faecalibacterium, and Streptococcus. The abundance of Escherichia-Shigella and Bifidobacterium in the CHD group were more than that in the control group (13.17% versus 11.70%, \( P = 0.46 \); 7.23% versus 5.65%, \( P = 0.32 \)) while Bacteroides, Faecalibacterium, and Streptococcus were less (8.13% versus 11.71%, \( P = 0.06 \); 7.43% versus 8.42%, \( P = 0.51 \); and 4.82% versus 6.62%, \( P = 0.04 \); Figure 4C). In addition, 567 genera were detected of which 36 species had significant differences. In the top 30, a significant difference in Enterococcus was noted which was more abundant in CHD (2.13% versus 0.99%, \( P < 0.05 \)). However, Streptococcus in the CHD group was less abundant (4.82% versus 6.62%, \( P = 0.04 \); Figure 4D).

**Predictive function analysis:** PICRUSt based on closed-reference OTU was used to predict the abundances of functional categories. In the KEGG ORTHOLOGY, the level of choline trimethylamine-lyase (cutC) gene expression correlated with TMAO production was much higher in the fecal microbiome of the CHD group \( (P < 0.05; \) Figure 5).
Gut microbiota consists of trillions of bacteria and encodes more than 100 times as many genes as the human genome, which replace many of the functions of the host, consequently influencing the host’s health. The composition and function of gut microbiota, recognized as gut microflora, have been identified as a contributing factor in the development of cardiovascular disease. Meanwhile, metabolomics and animal model studies showed that TMAO, an intestinal microbiota-dependent metabolite formed from dietary trimethylamine-containing nutrients, is linked to the procession of coronary atherosclerosis. Currently, the composition of the gut microbiota is commonly quantified using DNA-based methods, e.g., next-generation sequencing of 16S ribosomal RNA genes or whole-genome shotgun sequencing. The current study adopted the former technology.

In this study, the TMAO levels of 100 patients undergoing CAG were tested and divided into the CHD (n = 50) and control (n = 50) groups. The TMAO concentration was higher in the CHD group. Some scholars also found the same results and recommended that plasma TMAO is a biomarker for CHD incidence. The concentration of TMAO and atherosclerotic coronary burden stratified based on the number of diseased coronary vessels was then examined. The study further divided all CHD patients into single-vessel disease (SVD, n = 22) and multivessel disease (MVD, n = 28). The median plasma TMAO levels in MVD were significantly higher in SVD, which suggested that TMAO levels are associated with an increased coronary atherosclerotic burden. The results of the current study are similar to Waleed’s study results in 2020. In addition, a study conducted by Li, et al. revealed that the TMAO level in acute coronary syndromes was an independent predictor of both short-term (30 days and 6 months) and long-term (7 years) major adverse cardiac events. However, in a large cohort of 2,529 CKD patients, subjects with higher levels of TMAO were independently associated with an increased risk of cardiovascular events. The results highlighted the participation of TMAO in CHD development and advised that TMAO tests were available for CHD patients to improve management and prognosis.
In the current study, the results showed no significant difference in microbial abundance among the two groups by alpha and beta biodiversity analysis, which was in line with the founding of Wan, et al.\textsuperscript{15} However, a randomized trial,\textsuperscript{16} which enrolled 169 CHD patients and 166 people without CHD, revealed that the CHD group was characterized by a lower number of OTUs compared with the control. This article was based on the Poland population, which may be the reason for different findings. The types and proportions of gut microbiota at the phylum level were then compared. The four major phyla (i.e., Firmicutes, Bacteroides, Actinobacteria, and Proteobacteria) account for $\geq 99\%$ of human gut microbiota.\textsuperscript{17} The same conclusion was reached as expected. In recent years, the importance of gut microbiota has increased due to growing evidence of their involvement in human pathophysiol-
Figure 4. A, B: The gut microbiota distribution of the top 30 at phylum level in CHD patients and normal controls. Each color represents one specie (A). Distribution of Bacteroidetes and Epsilonbacteraeota abundance in two groups (B). C, D: The gut microbiota distribution of the top 30 at the genus level in CHD patients and controls. Each color represents one specie (C). Distribution of differential genera abundance in two groups (D).

Figure 5. PICRUSt analysis in the KEGG ORTHOLOGY. Functional predictions for the fecal microbiome of the CHD group and the control group. Redline was an over-represented cutC gene in the gut microbiome of the CHD group.

Most cardiologists believe that this phylum and its producers play a substantial effect in the pathogenetic CHD process. Bacteroidetes, Firmicutes, and Firmicutes/Bacteroidetes ratio are regarded as cardiovascular risk...
markers because bacteria belonging to them have high functional redundancy. Previous studies have shown a decrease in the Bacteroidetes phylum and an increase in the Firmicutes phylum in coronary artery disease. This study found a reduction in the abundance of Bacteroidetes in the CHD group, similar to the study by Jie, in which stool samples were collected from 218 patients with coronary artery disease and 187 healthy controls. Although Firmicutes abundance did not differ between study groups, the results of the current study confirmed a higher relative abundance of Firmicutes in the control group contrary to previously mentioned findings provided by Sawicka-Smirnowska. Additionally, a significant decrease in Epsilonibacterota was also observed in CHD. Based on the results of the current study, gut microbiota was also suggested as a CHD diagnostic marker.

The CHD patients in the current study were diagnosed with high plasma TMAO levels and a lower relative abundance of Bacteroidetes. These findings agreed with those obtained by Falony, et al., suggesting that TMA production function is widespread in Firmicutes, whereas it appears to be absent in Bacteroidetes. Considering the primary role of microorganisms in TMA generation, gut microbiota’s composition and community structure play an important effect in circulating TMAO levels. This is further supported by Romano who screened a collection of 79 sequenced human intestinal isolates, including the major phyla found in the human gut, and identified nine strains capable of producing TMA from choline. Additionally, the difference at the genus level was also observed. Escherichia-Shigella, a human host-specific pathogen that infects intestinal epithelial cells, was the highest concentration in the CHD group. It is the causative agent of dysentery which is one of the inflammatory bowel diseases (IBD). The study by Czubkowski showed an increased risk of cardiovascular complications in IBD relapses. One of the pathogenetic pathways was that gut flora invades the defective intestinal mucosa, and microbial products are released from the inflamed mucosa into the circulation through a leaky intestinal mucosal barrier and lay a role in atherosclerotic plaque activation. For patients with CHD, intestinal management is indicated. Among the common genus, a significant difference was noted in the Enterococcus and Streptococcus. Streptococcus was more abundant in the control group than in the CHD group, the results are consistent with the study by Singh. Romano also confirmed that Streptococcus is not capable of TMAO production, which is partly due to the low TAMO level in the control group. Enterococcus was also found to be increased in the gut of CHD patients, but the role of Enterococcus in CHD has not yet been reported. Caesar has revealed that microbiota-derived bioactive compounds can signal to distant organs, contributing to the development of cardiovascular disease states. Based on the results of the current study, the gut microbiota is suggested to also be a CHD diagnostic marker.

The present study suggested that the plasma TMAO level and abundance of some bacteria differed between CHD patients and healthy controls while the diversity of gut microbiota did not. TMAO, as a gut microbiota product, may enhance atherosclerosis progression although the relationship between the changes in gut microbiota and increases in plasma TMAO were not established yet. A functional analysis using the data obtained from the KEGG showed that the cutC gene was markedly over-represented in the microbiota of CHD patients. Recent studies revealed that the TMA production level was dependent on cutC gene expression. Some intestinal microorganisms anaerobically convert choline to TMA by cutC. Many gut bacterial phyla, i.e., Firmicutes, Proteobacteria, and Actinobacteria, possess a choline degradation pathway (cutC), except for Bacteroidetes. The current study was consistent with the findings of Skye that cutC-expressing human commensal was sufficient to transmit TMA/TMAO generation and thrombosis potential.

Thus, the current study hypothesizes that increased TMAO levels may be related to the gene expression level of cutC in the microbiota of CHD patients. Gut microbiota and its product were expected to become a diagnostic marker and a new target for the primary prevention of CHD.

Specific foods and dietary patterns can influence the abundance of different types of bacteria. Bian, et al. found that consuming acesulfame K for 4 weeks perturbed the gut microbiota of CD-1 mice. Bacteroides were highly increased in acesulfame K-treated mice. Another study compared mice fed low concentrations of two commonly used emulsifiers and mice not fed with emulsifiers showed these changes after only 2 weeks. TMAO as a metabolite generated by the gut in response to choline, carnitine, and other rich nutrient consumption is also linked with diet. A study on obese subjects on a vegetarian diet for 8 weeks showed a 40% decrease in monitored plasma TMAO and returned to baseline levels after 4 weeks of unrestricted diet. In an international pooled analysis by Yang, including 16 population-based studies from the USA, Asia, and Europe, circulating TMAO was positively correlated with intakes of animal protein, saturated fat, monounsaturated fat, fish, shellfish, eggs, and red meat, and inversely associated with intakes of plant protein, carbohydrate, and nuts. Restrictive diets, containing some strict vegan diets, raw food, and low fermentable oligosaccharides, disaccharides, monosaccharides, and polyols diets are an effective strategy for treating CHD because dietary habits can affect the abundance of bacteria in both the gut and TMAO level.

Based on the above evidence, therapeutic targeting of the gut microbiome was suspected as a potential treatment or prevention strategy for cardiovascular diseases. Fecal microbiota transplantation is a method to directly change the recipient’s gut microbiota to normalize the composition and gain a therapeutic benefit, but it has not been proved to be successful in preventing the occurrence of CHD. Probiotics are nonpathogenic microorganisms acting via various means, including the production of organic acids and antimicrobial compounds, interaction with resident microbiota, and improving gut barrier integrity. Prebiotics are nondigestible food ingredients that selectively stimulate beneficial gut bacteria growth and activity; thus, benefiting host health. Symbiotics are a combination of synergistically acting probiotics and prebiotics that directly affect intestinal epithelium to regulate intestinal barrier function and correct intestinal microecological imbal-
Prebiotic, probiotic, and synbiotic supplementation has been shown to lower cholesterol levels; have antioxidant, antiplatelet, and anti-inflammatory properties; and improve blood lipid metabolism. These factors improve the prognosis of CHDs. Moreover, in the study by Farrokhan, synbiotic supplementation for 12 weeks among people with CHD had beneficial effects on serum hs-CRP, plasma NO, and MDA levels. Moreover, a recent study showed that gut microbiome composition can be influenced by commonly used drugs, including antibiotics, proton pump inhibitors (PPIs), metformin, laxatives, statins, antidepressants, and opioids. When performing microbiome studies, each patient was administered aspirin (100 mg/daily), clopidogrel (75 mg/daily), and statins for at least 3 days while individuals who had taken antibiotics, laxatives, antidepressants, and opioids within 3 months before the examination were excluded. However, PPI was used in 11 patients (six control patients and five CHD patients) in the current study for the prevention of nonsteroidal anti-inflammatory drug-induced gastroduodenopathy. A study from the Netherlands showed that PPIs were the drugs most associated with decreased diversity. In addition, 16 patients (nine control patients and seven CHD patients) were given metformin. Forslund, et al. found that changes in the gut microbiome thought to be driven by the underlying T2D were caused by the use of metformin. Thus, the results of the current study may be affected by the use of these drugs to some extent. This study had several limitations, mainly related to the single-center retrospective study. The baseline data was not completely matched, and the sample size was small. A study with a larger sample is needed to confirm the results of the current study.

Disclosure

Conflicts of interest: None.

Authors’ contributions: All authors contributed significantly to this study. Yanqi Liu and Guanqun Zheng designed the study. Xiaoqi Jin collected the data. Tao Fan and Zhixian Chen analyzed the data. Yanqi Liu, Guanqun Zheng, and Xiaodong Sheng wrote the paper. All authors reviewed and approved the manuscript.

References

with human gut commensals containing CutC is sufficient to transmit enhanced platelet reactivity and thrombosis potential. Circ Res 2018; 123: 1164-76.