Analysis of gut microbiota in patients with acute myocardial infarction by 16S rRNA sequencing

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Background: An increasing number of studies have shown that gut microbiota are associated with human cardiovascular disease, but the characteristics of intestinal flora in patients with acute myocardial infarction (AMI) are still unclear. In this study, we aimed to investigate the difference of intestinal microflora between patients with AMI and healthy people, and to find the effect of percutaneous coronary intervention (PCI) on intestinal microflora.

Methods: A total of 60 stool samples and 60 peripheral blood samples were collected from 20 previously diagnosed AMI patients and 20 healthy people serving as controls. Gut microbiota communities were analyzed via 16 ribosomal RNA-sequencing (16S rRNA). Gut microbiota-derived metabolites, trimethylamine N-oxide (TMAO) and short-chain fatty acids (SCFA), in the blood were detected using stable isotope dilution high-performance liquid chromatography with on line electrospray ionization tandem mass spectrometry (LC/MS/MS).

Results: The results showed that a distinct pattern of gut microbiota was observed in AMI patients compared to healthy controls. AMI patients had lower microbiological richness but no significant change in diversity. *Bacteroidetes* and *Verrucomicobia* showed an upward trend, whereas *Proteobacteria* showed a downward trend in AMI patients. During a longitudinal study to compare the changes in bacteria before and after treatment, we found routine cardiac admission therapy 1 week after PCI surgery had no effect on the microbial community structure in patients. There were significantly higher levels of plasma TMAO in AMI patients' microbiota than that in the control group. Contrarily, there was no obvious change in SCFA.

Conclusions: The gut microbiota of patients with AMI differs from that of normal people, and the metabolic products of microflora are more abundant in the plasma of AMI than control cases. Microflora may act on the cardiovascular system through metabolites, and regulation of the microfloral structure may be used in the future treatment of cardiovascular diseases.

Keywords: Acute myocardial infarction (AMI); gut microbiota; 16S rRNA

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Introduction

Acute myocardial infarction (AMI) is caused by myocardial necrosis due to an acute or persistent reduction in the coronary artery blood supply and an insufficient oxygen supply (1). AMI is often accompanied by malignant arrhythmia, cardiogenic shock, and even sudden death (2). With the aging population, stresses of modern life, changes in eating habits, and influence of social and psychological factors, cardiovascular disease-related deaths, such as coronary heart disease and AMI, have become the primary cause of human mortality and pose a serious threat to human health. Percutaneous coronary intervention (PCI) can help to quickly open the responsible blood vessels, mitigate ischemia, and save lives; therefore, early PCI is invaluable for improving the overall survival rate of AMI patients.

In recent years, the close relationships between the microbiome and human diseases have been demonstrated, and studies have confirmed that gut microbiota are associated with extraintestinal diseases, such as liver-, brain-, and immunity-related conditions (3-5). The digestive tract is directly connected to the outside environment and contains up to 100 trillion microorganisms, mainly in the colon (6). The number of genes associated with gut microbes is thought to exceed that of the host by approximately 150-fold (7-9). Trimethylamine (TMA) and short-chain fatty acids (SCFA) are both produced by gut microbiota and can be absorbed into the blood through the intestinal mucosa. TMA is then converted into trimethylamine N-oxide (TMAO) in the liver (10,11). SCFA, including acetate, propionate, and butyrate, are absorbed by intestinal epithelial cells, providing energy for the intestinal epithelium and entering the blood stream to participate in distal metabolic functions. Kimura *et al.* found that SCFAs and ketosomes directly regulate sympathetic nervous system (SNS) activity at the sympathetic ganglion level via the G protein-coupled receptor 41 (GPR41) (12). These derived products may have profound roles in host metabolism, immunity, and nutrition and can produce either beneficial or harmful metabolites in the human body as a result of changes in the environment and diet (13). The gut microbiota and gut metabolites have associated with hypertension, dyslipidemia, systemic inflammation, which are risk factors of AMI. Human study showed that hypertensive patients have lower alpha diversity of gut microbiota and higher LPS-producing microbiota (14). Animal study point a direct role for SCFA in blood pressure regulation (15).

Recently, the emerging study of gut microbiota with acute myocardial infarction have shown that gut microbiota has alteration in patients (16,17), but there are few studies about the changes of gut microbiota post-treatment. In this study, we have collected three group fecal sample from health group, AMI patients' group and post-treatment group. And we analyzed the community of microbiota of AMI patients and compared the effect of post-PCI treatment on gut microbiota for AMI patients. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5671/rc).

Highlight box

Key findings

- The results showed that a distinct difference of gut microbiota was observed in AMI patients compared to healthy controls.
- PCI treatment and routine drugs have no effect on gut microbiota in patients with AMI.

What is known and what is new?

- The disorder of gut microbiota structure was shown in cardiovascular patients.
- The expression of TMAO, a derivative of microbial metabolite, increased in peripheral blood of patients with AMI.

What is the implication, and what should change now?

- Attention should be paid to the treatment of gut microbiota in patients with AMI.

Methods

Patients and healthy controls

A total of 20 AMI patients and 20 healthy people aged 45–85 years were consecutively recruited from the Department of Cardiology, Yijishan Hospital of Wannan Medical College between December 2018 and May 2019. The AMI patients in this study were diagnosed with ST-segment elevation myocardial infarction according to electrocardiographic (ECG) examination and laboratory examination of myocardial enzymes. The eligible volunteers were selected according to the following conditions: (I) age 45–85 years old; (II) no digestive system-related diseases in the past 3 years; (III) no history of surgery; (IV) no history of smoking, alcoholism, and various metabolic diseases; (V) all participants were volunteers and signed their informed consent voluntarily. Hypertension as an independent factor should be controlled below 135/90 mmHg. All of the healthy controls were from the patients' families or close

relatives with similar age and no other diseases.

Collection and detection of fecal samples

We collected a total of 40 stool samples from the healthy control group and AMI patient group. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics board of Wannan Medical College (No. 2020371) and informed consent was taken from all the patients. In AMI patients, a week after PCI, stool was collected again as the post-treatment group (AMI-T). All fresh fecal samples from all participants were collected in container with liquid protectant from Hangzhou Guhe Information Technology Co. Ltd. (Hangzhou, China) and stored at 4 ℃ within 2 h of collection. Subsequently, samples were returned to the company for testing. The primers for polymerase chain reaction (PCR) amplification of the V4 region of the bacterial 16S ribosomal RNA (16SrRNA) gene were the forward primer 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and the reverse primer 806R (5'-GGACTACHVGGTWTCTAAT-3'). Sequencing was performed on the Illumina HiSeq4000 pair-end 2×150 bp platform (Illumina, San Diego, CA, USA) after quantification.

TMAO and SCFA measurement

Blood samples were collected from all participants, including the control group, within 12 hours after admission to the hospital before clinical treatment or after a week of clinical treatment. Within 60 min of collection, the plasma from the blood supernatant were separated after centrifugation at 3,000 rpm at 4 ℃ and stored at –80 ℃. TMAO and SCFA were detected by liquid chromatography/stable-isotope dilution/multiple-reaction monitoring/mass spectrometry (LC/MS/MS) (18) by Laboratory of School of Pharmacy, Wannan Medical College.

Date analysis

The data for each sample were separated from the original data, according to the barcode and primer sequences. After removing the barcode and primer sequences, the reads of each sample were spliced with a data analysis software (Vsearch v2.4.4) to get the original Tags data (Raw Tags). Concurrently, the quality of the sequence was controlled and filtered. The criteria for screening low quality sequences were as follows: the sequence was less than 150 bp, the average mass value was less than 20, the sequence contained unclear bases, and the single nucleotide repeat sequence contained >8 bp. The chimera sequence was removed and the final effective data were obtained. Operational taxonomic units (OTUs) analysis was performed using Vsearch v2.4.4, including de-repeating sequences, clustering, and de-chimerism (Rognes 2016). The differences in the UniFrac distance between the drawing box graph were compared using the *t*-test and the Monte Carlo test.

Statistical analysis

Results were expressed as mean ± SEM and differences between groups were determined by Student's *t*-test for continuous variables or χ^2 test for categorical variables using Graphpad Prism version 8.0 (Graphpad Software, La, Jolla, CA). A value of $P<0.05$ was considered statistically significant.

Results

Participants' baseline characteristics

The baseline characteristics of patients with AMI and healthy controls were investigated in detail within 24 h of admission and are shown in *Table 1*. To select a suitable healthy control group, patients' family members or healthy people living close to patients were recruited to the trial. The ranges and proportions of age, body mass index (BMI), sex, and diabetes mellitus in the AMI group were not significantly different from those in the control group. However, the rate of hypertension was significantly higher in the patient group than the control group.

Characteristics of fecal microbiota in AMI patients

To analyze the changes in gut microbiota in patients with AMI, we collected a total of 123,906.2±9,989.6 tags from control, 123,915.3±16,551.6 tags from AMI, and 125,706.7±14,864.7 tags from AMI-T groups by 16s rRNA sequencing, and we screened 1,554±155.2, 2,244±163.7, 2,241±154.6 OTUs, respectively (*Table 2*). According to the OTU clustering results, a Venn diagram was drawn for OTUs at 97% similarity by default, and the number of OTUs common to the different groups were compared. The types of OTUs shared between the different groups are shown in the Venn diagram (*Figure 1A*). There was no significant change in the alpha diversity of the flora in the

Table 1 Baseline characteristics of AMI patients and healthy controls

Variables	Healthy control (n=20)	AMI patients (n=20)	P value
Ages (years)	63.6±16.2	65.8±14.1	0.65
Sex (female)	11	8	–
BMI (kg/m^2)	21.67±2.4	22.81±2.5	0.15
Hypertension**	2	12	<0.01
History of smoking	0	2	–
Dyslipidemia	3.8±0.59	4.0±0.85	0.27
AST (U/L)**	21.1±7.26	143.5±135.34	<0.01
ALT (U/L)**	18.55±10.84	30.4±17.85	<0.01
BUN (mg/dL)**	3.88±1.79	6.6±2.81	<0.01
HDL-C (mg/dL)	1.5±0.93	1.3±0.32	0.29
LDL-C (mg/dL)	1.9±0.80	2.3±0.84	0.16
TG (mg/dL)	1.48±0.31	1.4±0.51	0.59

Data are presented as mean ± SEM or number (n=20). **P<0.01, AMI *vs*. healthy control group. AMI, acute myocardial infarction; BMI, body mass index; AST, aspartate aminotransaminase; ALT, alanine transaminase; BUN, blood urea nitrogen; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; SEM, standard error of the mean.

Table 2 16S rRNA sequence statistics

Groups	Fecal samples number	Tags (number of reads)	Clean-tags (number of reads)	OTUs
Control	20	123,906.2±9,989.6	115,192.5±12,038.0	1,554±155.2
AMI	20	123,915.3±16,551.6	113,519.1±17,839.0	2,244±163.7
AMI-T	20	125,706.7±14,864.7	116,514.4±16,996.9	2,241±154.6

The data were presented as mean ± standard deviation. Control, healthy control group; AMI-T, AMI patients treatment group. AMI, acute myocardial infarction; OTUs, operational taxonomic units.

AMI group, as shown by Shannon and Simpson indexes, but the richness of the flora significantly decreased, as shown by Chao1 and ACE (*Figure 1B-1E*, *Table 3*). Principal component analysis (PCA), based on weighted UniFrac distance of the overall community structure, did not show any separation (*Figure 2A*). In terms of the total number, *Figure 2B* illustrates the differences between gut microbiota in the healthy control and AMI groups at the species level. In addition, no significant difference was found between the phylum level and the genus level (*Figure 2C,2D*). At the phylum level, *Bacteroidetes*'s proportion (32.20%±5.9%) was slightly elevated in AMI patients, whereas *Firmicute*'s (50.27%±16.8%) and proteobacteria (13.02%±6.3%) had no clear change (*Figure 3A-3D*). At genus level, the proportion of *Bacteroides* and *Faecalibacterium* had no change (*Figure 3E,3F*). *Streptococcus* only accounted for 0.34%±0.05% of the fecal microbiota in the healthy group; the proportion increased 10 times in the AMI patient group, and there was no decrease after treatment (*Figure 3G*). On the contrary, *Lachnospira* showed a downward trend, from 5.5% to 1.4% (*Figure 3H*).

Species markers

An analysis of similarities (ANOSIM) non-parametric test showed that there were significant differences among groups with R=0.223 and a statistical probability of <0.05 (*Figure 4A*). To determine species differences between the groups, we used a linear discriminant analysis effect size (LEfSe) linear discriminant analysis (LDA)-based method. We used the logarithmic LDA score with significant difference set to 2 and found 5 genera, *Verrucomicrobia*, *Bifidobacterium*,

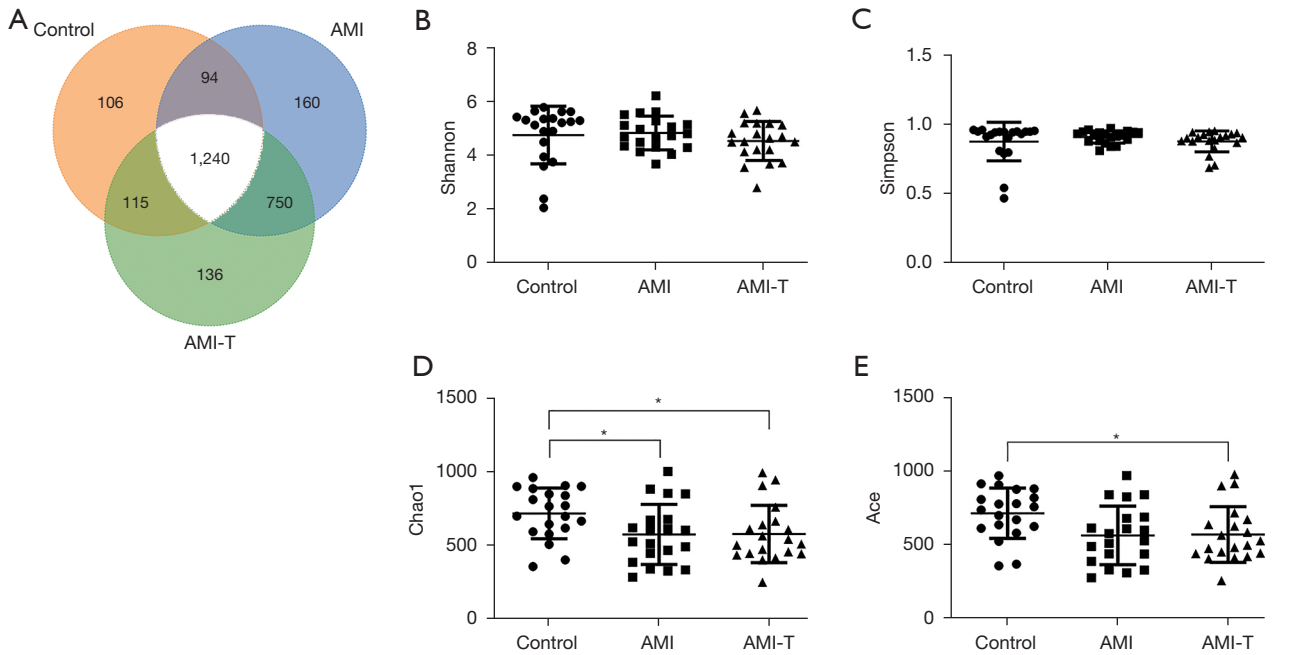

Figure 1 Characteristics of fecal microbiota and alpha diversity analysis. (A) Venn diagram of OTUs from control, AMI and AMI-T. The Scatter plot of Shannon index (B), Simpson index (C), Chao1 index (D) and Ace index (E) were showed to analyze microbiome diversity. *P<0.05; unpaired *t* test. AMI, acute myocardial infarction; OTUs, operational taxonomic units; AMI-T, AMI patients treatment group.

Table 3 The diversity index of gut microbiota

Groups	Shannon	Simpson	Chao1	Ace	Goods_coverage
Control	4.75±1.07	0.88±0.14	721.57±172.56	723.44±171.09	0.999
AMI	4.83±0.63	0.91±0.04	578.69±204.52*	572.28±199.27	0.999
AMI-T	4.53±0.72	0.89±0.08	581.88±195.16*	578.66±189.42*	0.999

The data shows the results of each index with mean ± standard deviation. *P<0.05 *vs*. control group. Control, healthy control group; AMI-T, AMI patients treatment group. AMI, acute myocardial infarction.

Bifidobateriates, *Dialister*, and *Collinsella*, that may be fecal bacteria markers for patients with AMI (*Figure 4B*). In addition, after routine drug treatment, we observed significant changes in gut microbiota species by comparing the AMI group with the AMI-T group.

Features of microbial metabolism

TMA and SCFA represent a major class of metabolites produced from microbial metabolism of dietary components, and TMA transforms into TMAO after entering the liver. We found that levels of TMAO were much higher in the plasma of AMI patients than in healthy people, and they decreased slightly after admission, but there was no statistical significance compared with levels of before treatment (*Figure 5A*). SCFA showed similar levels in plasma, and the difference was not statistically significant (*Figure 5B*). Using Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway classification, we found that the gut microbiota had a higher proportion of amino sugar, nucleotide sugar, fructose, and mannose metabolism compared to that of the healthy control group, whereas there was less microbial porphyrin and chlorophyll metabolism in AMI patients than the control group (*Figure 5C*).

Discussion

In this study, we used 16S rRNA technology to compare the

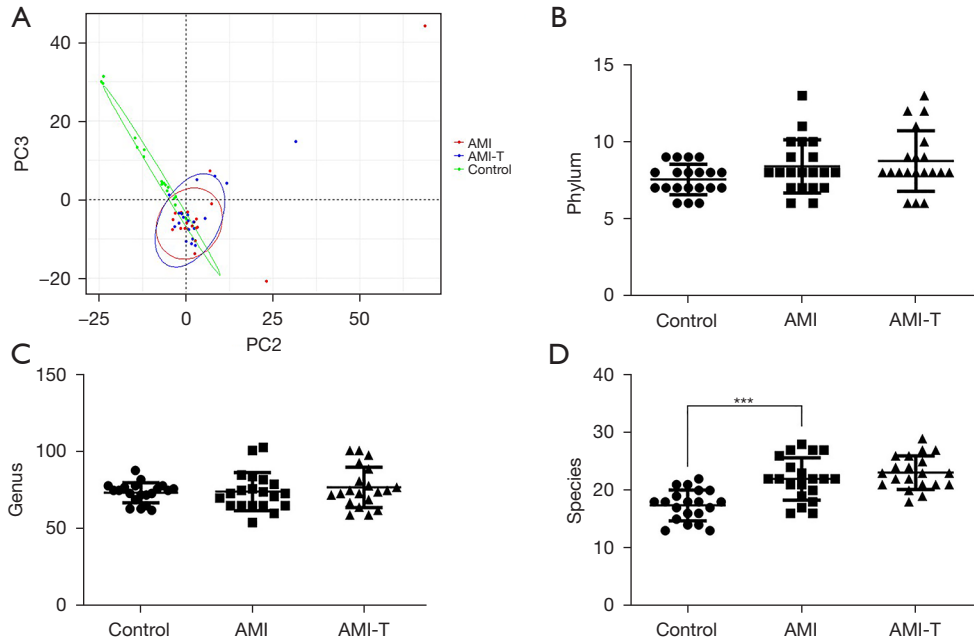

Figure 2 Comparison of the gut microbiota structures and Principal component analysis. (A) The beta diversity of the fecal microbiota was analyzed by PCA. (B-D) The taxonomic number of microbial communities at phylum, genus, species, respectively. ***P<0.001; unpaired *t* test. AMI, acute myocardial infarction; PCA, principal component analysis.

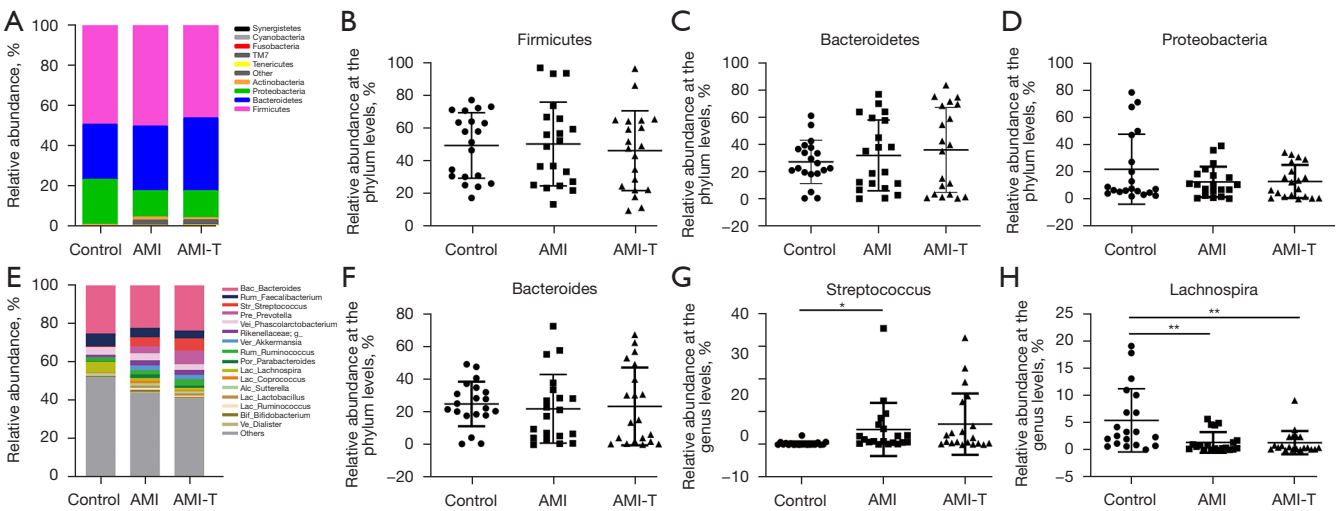

Figure 3 Abundance map of species composition between groups at phylum and genus levels. (A,E) Gut microbiota of total sample in phylum and genus classification of control group, AMI group and AMI-T group. (B-D) Relative abundance of *Firmicutes*, *Bacteroidetes*, and proteobacteria at phylum level. (F-H) Relative abundance of *Bacteroides*, *Streptococcus*, *Lachnospira*. *P<0.05; **P<0.01; unpaired *t* test. AMI, acute myocardial infarction.

differences in fecal microbiota between AMI patients and healthy controls. The results showed that the AMI group had a different pattern of intestinal microflora compared with healthy individuals. There was no significant difference in diversity but a significant difference in the richness of the intestinal microflora. After PCI and 1 week of medical

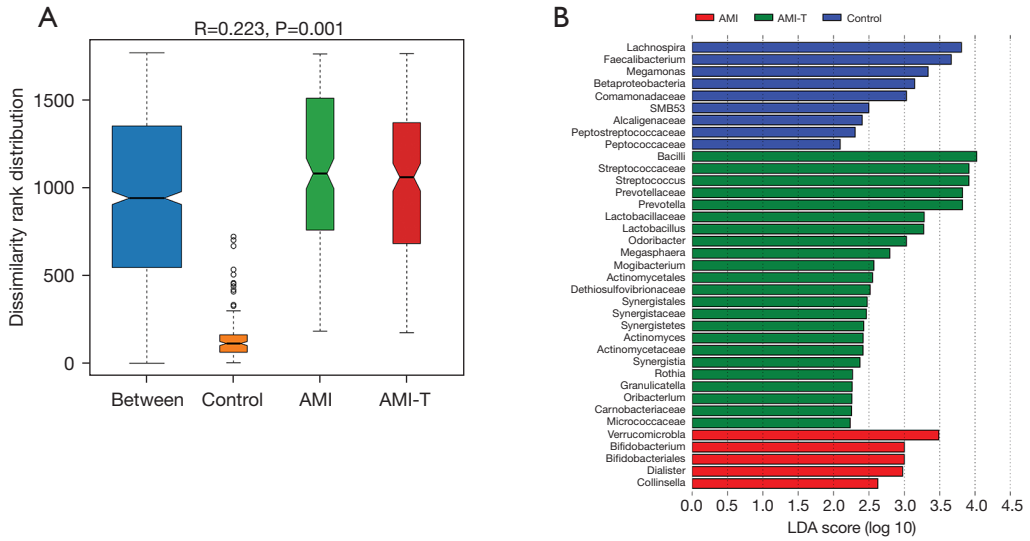

Figure 4 Analysis of species markers of fecal samples. (A) Nonparametric test Anosim analysis of three groups; (B) using LEfSe based LDA analysis method to compare and compare significant species between groups. AMI, acute myocardial infarction; LDA, linear discriminant analysis.

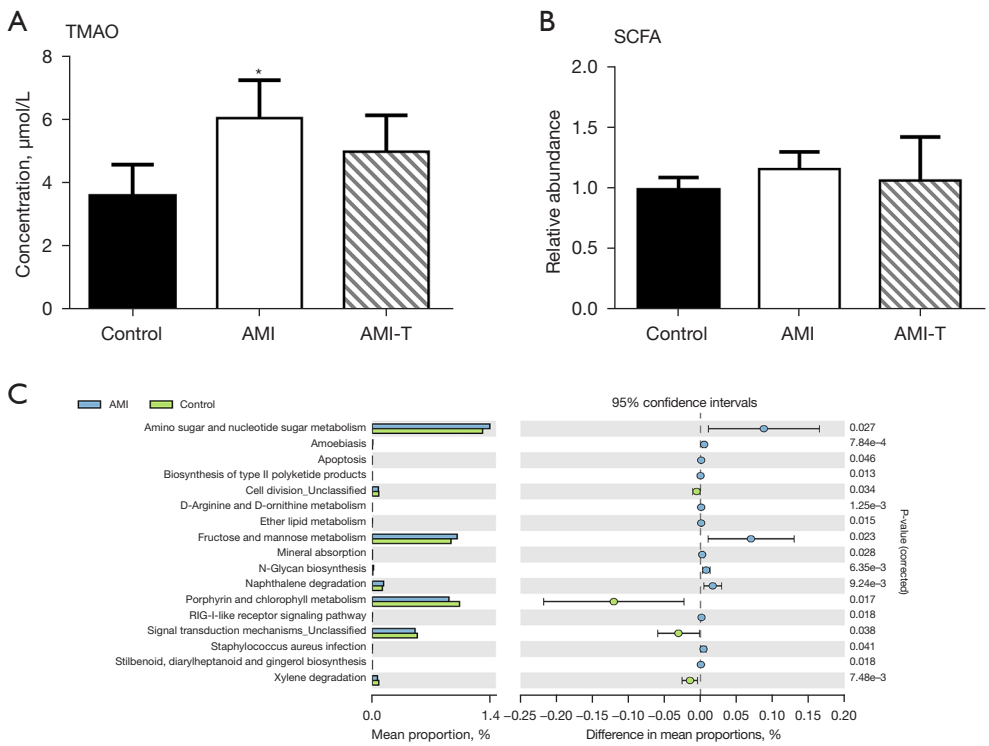

Figure 5 Analysis of bacterial metabolites and metabolic pathways. Detection of TMAO (A) and SCFA (B) in plasma from health control, AMI patients and after treatment. Analysis of the third layer classification difference of KEGG metabolic pathway (C). *P<0.05; unpaired *t* test. AMI, acute myocardial infarction; TMAO, trimethylamine N-oxide; SCFA, short chain fatty acids; KEGG, Kyoto Encyclopedia of Genes and Genomes.

treatment (Table S1), the gut microbiota of patients with AMI showed no significant difference compared with before treatment (*Figure 1B*). SCFA and TMAO are direct or indirect biological products of gut microbiota and can participate in the regulation of cardiovascular disease (19-21). In our research, plasma TMAO levels were higher in AMI patients than in the healthy individuals; however, this trend was not seen for SCFAs.

Gut microbiota of human contains a large number of obligative anaerobic and few aerobic bacteria and could be identified on species level by 16S rRNA sequencing, which is a mature sequencing technology with sequencing the V3-V4 hypervariable region of 16S rDNA gene (22). Some anaerobes were killed during collection, but the bacterial species can still be identified by the residual DNA sample. 16S rRNA sequencing of bacteria have been used in the study of human diseases. Cardiovascular disease accounts for more than 40% of deaths in China and is the leading cause of mortality (8). In recent years, studies have shown that, in addition to diabetes, hypertension and other factors, an imbalance of gut microbiota and their metabolites can affect blood lipid levels promoting the development of atherosclerosis and lead to cardiovascular disease (3,23). In this study, we found that *Firmicute* and *Bacteroidetes* were two major bacterial phyla in AMI patients with average relative abundance of 50.27% and 32.2%, respectively, which is similar to that found in a case-control study of coronary artery diseases (24). However, Liu's study showed that *Bacteroidetes* (59.6%) had a higher proportion than that of *Firmicute* (31.5%) (25). The reason for this significant difference results may be related to the Geographic factors, which is an important role for human microbiome diversity (26). *Bacteroidetes* and *Firmicutes* are two major groups of intestinal bacteria and their proportional structure can affect the intestinal microenvironment. We found that *Firmicutes* represented the largest microfloral component and *Bacteroidetes* the second largest. However, there is some controversy regarding the significance of the *Firmicutes/Bacteroidetes* (F/B) ratio (27), and there was no significant difference in the F/B ratio of AMI patients compared with controls in this study. In addition, two other groups showed significant differences between groups, *Proteobacteria* and *Verrucomicrobia*. The bacterial phylum *Verrucomicrobia* is now recognized as being ubiquitous and abundant in both soil and aquatic systems (28). Although *Verrucomicrobia* were significantly more abundant in AMI patients, the proportion was relatively small, and we speculated that the increase may have been due to environmental differences.

Metabolites of gut microbiota, including TMAO, SCFAs, Phenylacetyl glutamine (PAGln), are important mediator for gut microorganisms to participate in the regulation of human diseases. TMAO is the product of TMA by hepatoflavin dependent monooxygenase in the liver. Early clinical studies have shown that elevated levels of blood TMAO positively correlate with an increased risk of coronary heart disease (29). In animal study, exogenous effects caused the concentration of TMAO in the blood of mice to increase, but had no significant effect on cardiovascular disease (10). The reduction of TMAO level could enhance the stability of atherosclerotic plaque which is risk factor of AMI. At the same time, TMAO can increase the release of intracellular calcium ions (Ca^{2+}), enhance the reactivity of platelets, and increase the risk of thrombosis (30). The results of this study show that there is a significant higher concentration of TMAO in AMI patients' plasma than that of healthy humans. After PCI treatment, the concentrations of TMAO in patient plasmas showed a downward trend with no significant. The plasma concentration of TMAO in patients with AMI may be influenced by the disorder community of their gut microbiota. The results indicate that TMAO may be a biomarker in the development of AMI.

SCFA, including formic, acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids, are common metabolites of gut microbiota and are rapidly absorbed by the hindgut, and they not only store energy but also reduce osmotic pressure. SCFA play an important role in maintaining the normal function of the large intestine and the morphology and function of colonic epithelial cells. SCFA can be associated with GPR41 and directly regulate the sympathetic nervous system (12). In recent years, interest in the "microbiota-gut-brain axis" has emerged, in which gut microbiota metabolites play an important mediating role (5). Based on the study of gut microbiota in patients with cardiovascular disease, Yu *et al.* proposed a "gut-brain-heart" axis, through which SCFA may be involved in cardiovascular regulation (31). It is strange that the SCFA plasma levels of AMI patients in this study were not significantly higher than those of healthy individuals. More basic research is needed to ascertain the interactions and roles of SCFA in the cardiovascular system.

The present study had some limitations. First, in this study, to reduce the interference of other factors on gut microbiota, such as drinking or smoking, we chose the participants who had no history of drinking, smoking or diabetes. However, the daily diet was not considered,

which was an important confounding factor in affecting gut microbiota. Second, this study was just a small sample study and only 20 patients and 20 health control were recruited. The results of this experiment had certain limitations. Third, the study did not examine the expression of cytokines related to cardiovascular disease in peripheral and not assess the relationship of the gut microbiota and cytokine expression. In addition, analysis of changes in the gut microbiota should include changes in the number and species of microflora. However, the 16S rRNA assay used in this experiment was only able to detect differences in species, but cannot quantitatively analyze the proportion differences in the number of gut microbiota. In summary, our study found that the gut microbiota of AMI have a difference with that of health people and the microbial metabolites may have role in AMI development.

Conclusions

By comparing the gut microbiota of AMI patients and healthy people, it was found that the richness of gut microbiota of AMI patients was significantly reduced, but the content of metabolites, such as SCFA and TMAO, of gut microbiota in peripheral blood did not differ from that in the healthy group. Drug treatment and PCI treatment had no significant effect on the gut microbiota of patients with AMI. Changes to the structure of gut microbiota in AMI patients should be taken seriously. In the future, the treatment of patients with AMI after PCI should incorporate the regulation of gut microbiota.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5671/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5671/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5671/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics board of Wannan Medical College (No. 2020371) and informed consent was taken from all the patients.

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