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Research Article

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The composition of the gut microbiome in patients with sarcopenia

Sarkopenili hastalarda bağırsak mikrobiyomunun bileşimi

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Abstract

Objectives: The purpose of this study is to predict sarcopenia by analyzing the composition of the gut microbiota. **Methods:** We collected fecal samples for 16S rRNA sequencing, then we used the data results to analyze the diversity and composition of the gut microbiota and calculated the relationship between biochemical indexes and the microbiota.

Results: According to PCA and heatmap analysis, the characteristics of patients could be divided into two categories. Moreover, the P/B (*Prevotella/Bacteroides*) ratio of the sarcopenia group was higher than that of the control group in terms of relative abundance. A box plot based on the Chao1 and observed OTU values indicated both the relative abundance and diversity of the gut microbiota in sarcopenia patients were lower than those in the control group. After we applied binary logistic regression and ROC curve analysis to the data, we confirmed that three indexes (P/B value, *Coprococcus*, and *Lachnospiraceae*) could be used to predict sarcopenia.

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Conclusions: We can distinguish sarcopenia patients through the gut microbiota P/B index (over 1.7), the relative amount of *Coprococcus* (1.00–3.70%), and the relative amount of *Lachnospiraceae* (0.00–1.68%). Sarcopenia can be predicted with the help of the gut micro-community, which provides an improvement in methodology.

Keywords: bacteroides; diversity; elderly individuals; gutmicrobiome; interaction; prevotella.

Öz

Amaç: Bu çalışma, bağırsak mikrobiyotasını analiz ederek sarkopeniyi tahmini amaçlamaktadır.

Yöntem: 16S rRNA dizilimi için dışkı örnekleri topladık, ardından bağırsak mikrobiyotasının çeşitliliğini ve bileşimini analiz etmek için veri analizi yaparak biyokimyasal indeksler ile mikrobiyota arasındaki ilişkiyi hesapladık.

Sonuçlar ve tartışma: PCA ve ısı haritası analizine göre hastaların özellikleri 2 kategoriye ayrılabilir. Ayrıca sarkopeni grubunun P/B (Prevotella/Bacteroides) oranı oransal zenginliği açısından kontrol grubuna göre daha yüksekti. Chao1 ve gözlemlenen OTU değerlerine dayanan bir kutu grafiği, sarkopeni hastalarında bağırsak mikrobiyotasının hem oransal zenginliğinin hem de çeşitliliğinin kontrol grubundakilerden daha düşük olduğunu gösterdi. Verilere binary lojistik regresyon ve ROC eğrisi analizi uyguladıktan sonra sarkopeniyi öngörmek için üç indeksin (P/B değeri, Coprococcus ve Lachnospiraceae) kullanılabileceğini doğruladık.

Sonuç: Sarkopeni hastalarını bağırsak mikrobiyota P/B indeksi (1,7'nin üzerinde), oransal Coprococcus miktarı (% 1.00–3.70) ve oransal Lachnospiraceae miktarı (%0.00–1.68) aracılığıyla ayırt edebiliriz. Sarkopeni, metodolojide bir gelişme sağlayan bağırsak mikro topluluğu yardımıyla tahmin edilebilir.

Anahtar Kelimeler: Bağırsak mikrobiyomu; Prevotella; Bakterioidler; Çeşitlilik; Etkileşim; Yaşlı bireyler.

Introduction

With the development of society, the aging of the population is becoming more prominent. Nowadays, sarcopenia has become an important public health problem. According to the European Working Group on Sarcopenia in Older People (EWGSOP), the definition of muscle loss is based on muscle mass loss and functional disorder. Muscular reduction (referred to as sarcopenia) is a progressive, generalized syndrome of skeletal muscle mass associated with loss of skeletal muscle mass, decreased muscle strength and function, which increases the risk of falls and fractures in older adults. They are all caused by proteincatabolic or anabolic resistance [1].

One of the main causes of physical decline in the elderly is sarcopenia, which also leads to increased hospitalization and mortality rates among the elderly, increases the economic burden, and has a serious impact on the quality of life of the elderly. Due to the aging population, the development of sarcopenia has led to a significant increase in the demand for family medical care, which has also increased the burden on society. The high prevalence of sarcopenia in elderly people in a report aimed at elderly people over 80 years old in Beijing indicated that the incidence of sarcopenia was 45.7% in 2004. However, there are still some problems in the detection, diagnosis, and evaluation of sarcopenia.

The human gut microbiome can interface with muscle metabolism [2]. A new method to assess sarcopenia can be determined by analyzing the gut microbiota composition. In some cases, several chronic diseases such as obesity [2, 3] and inflammatory diseases [4-7] are associated with changes in gut microbiota. Compared with young people, the intestinal microflora of the elderly has greater individual differences, and changes in the intestinal microflora will have a great impact on the health of the elderly, which is worthy of research. The exact cause of primary sarcopenia is not known clearly, but studies have found that elderly patients with sarcopenia often have symptoms of malnutrition. Therefore, one of the main ways to prevent and treat sarcopenia is to provide adequate nutrition [8]. On the other hand, there is evidence of a strong link between gut microbiota, nutrition, and diet. However, the effects of dietary patterns, dietary composition, and nutrient composition on the intestinal microbiota of patients with sarcopenia have not been found. In addition,

some studies have shown that physical exercise can prevent and treat sarcopenia, but there is still a lack of research on the effect of exercise mode and intensity [9, 10]. The aims of this study are to investigate potential biomarkers of sarcopenia and to find potential methods that can decrease the risk of sarcopenia by changing the composition of the gut microbiome. Thus, a dietary pattern with a beneficial effect on the gut microbiome of elderly individuals with sarcopenia was developed and evaluated.

Materials and methods

Investigation of patients with sarcopenia and fecal sample collection

Patients in the hospital from September 2018 to November 2019, who met the criteria, were enrolled in the observation group (88) and the control group (104), respectively. The inclusion process is shown in Supplementary Figure 1. The observation group included the criteria according to the European Working Group on Sarcopenia in Older People 2 (EWGSOP2)'s criteria as follows: 1) Low muscle strength <27 kg, 2) low appendicular muscle mass <20 kg, 3) low physical performance measured with Short Physical Performance Battery eight points [11, 12].

All the patients did not receive antibiotics in the previous 6 months before sample collection and signed the informed consent forms. A total of 10 g of a fresh fecal sample from each person was collected and dissolved in 90 mL of sterile PBS (0.1 mol/L, pH 4.7). The samples were then stirred evenly and filtered three times using autoclaved double gauze to collect the filtrate (bacterial suspension) for inoculation. Before the study began, this specific study was reviewed and approved by the Research Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University; the reference number is 2018-576.

DNA isolation, PCR, and 16S rRNA gene analysis

DNA from different samples was extracted using the method we described previously [13, 14]. Based on the method described above and with simple modifications, we used the MicroElute Genomic DNA Kit (D3096-01, Omega, Inc., USA) to extract DNA from different samples. The total DNA was eluted in 50 μL of elution buffer by a modified version of the procedure described by the manufacturer (QIAGEN). The samples will be kept at -80 °C until PCR amplification (LC-Bio Technology Co., Ltd., Hang Zhou, Zhejiang Province, China).

16S rRNA gene sequences (V3-V4 regions) of bacteria were amplified from the whole genome of stool samples by a primer pair (319F 5'-ACTCCTACGGGAGGCAGCAG-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3') according to a previous method with a tiny modification [14].

The products of PCR were normalized by an AxyPrepTM Mag PCR Normalizer (Axygen Biosciences, Union City, CA, USA), which allowed skipping the quantification step regardless of the PCR volume submitted for sequencing. The amplicon pools were prepared for sequencing with AMPure XT beads (Beckman Coulter Genomics,

Danvers, MA, USA), and the size and quantity of the amplicon library were assessed on a LabChip GX (Perkin Elmer, Waltham, MA, USA) and with a Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. A PhiX Control library (V3) (Illumina) was combined with the amplicon library (expected at 30%). The library was clustered to a density of approximately 570 K/mm². The libraries were sequenced on a 300PE MiSeq. One library was sequenced with both protocols using the standard Illumina sequencing primers, eliminating the need for a third (or fourth) index reads.

16S rRNA gene sequences were processed and modified in previously described methods [14].

Statistics

All experimental parameters were recorded for each individual in the two groups. All data were expressed as mean \pm SD SPSS ver. 17.0 software was used to conduct multiple comparisons of variance analysis and Duncan's test. A value of p<0.05 was considered significant. For data analysis, the binary logistic regression method was introduced to calculate the probabilities of sarcopenia with three indexes, the P/B value, the relative amount of Coprococcus, and the relative amount of Lachnospiraceae. The precision and accuracy of our experimental indexes were determined by the ROC curve method. The 16S sequence data generated in the study were submitted to the NCBI Sequence Read Archive under accession number PRJNA724939.

Results

Data were available from 88 patients with sarcopenia and 104 persons in the control group. Supplementary Figure 1 summarizes the reasons for excluding volunteers. Some patients were treated with antibiotics or did not develop sarcopenia. The two groups were similar in age and sex and no significant difference was found in DBIL/TBIL, CRP, and GLU (Table 1). The A/G ratio of sarcopenia patients was lower than that of the control group, AST/ALT ratio of both patients and control group was greater than 1, and the value of sarcopenia patients was higher than that of control group (Table 1), indicating that all of the older adults surveyed had certain liver function injury, and sarcopenia patients were more serious.

Sequencing of the amplicon libraries resulted in a total of 3,964,204 reads assigned to the 192 samples. The average read length (± standard deviation) of reads before processing was 20,646.9 bp \pm 10,840.0 bp.

Based on OTU abundance, the Alpha diversity (microbiota diversity in every sample) was analyzed by the Chao1 index (Figure 1A) and observed OTU value (Figure 1B). As shown in Figure 1, the diversity of the control group (161.3 \pm 48.5) was higher than that of the muscle disease group (136.7 \pm 49.6) (p=0.00586). In the PCA (PC1 43.16%, PC2 25.82%), the composition of the gut microbiota

Table 1: Baseline characteristics and biochemical indexes screening results of patients with sarcopenia and control.

Variable		Patients (n=88)	Control (n=104)	p- Value
Female	n (%)	53 (47)	52 (54)	
Age, years	Mean (range)	77 (65–95)	70 (65–84)	0.06
BMI, kg/m ²	Mean (SD)	22.87 (3.17)	23.52 (30.39)	0.39
A/G	Mean (SD)	1.55 (0.32)	1.64 (0.60)	0.24
AST/ALT	Mean (SD)	1.45 (0.83)	1.35 (0.62)	0.38
DBIL/TBIL	Mean (SD)	0.40 (0.11)	0.40 (0.15)	0.86
CRP, mg/L	Mean (SD)	13.39 (22.92)	15.84 (22.94)	0.52
GLU, mmol/ L	Mean (SD)	5.23 (1.64)	5.36 (1.48)	0.59

n% presents the percentage of female in the group. The digital in the brackets is female number. BMI, body mass index: A/G, albumin/ globulin; AST/ALT, aspartate aminotransferase/alanine aminotransferase; DBIL/TBIL, direct bilirubin/total bilirubin; CRP, C-reactive protein; GLU, fasting blood-glucose.

of healthy people was to some extent distinguished from that of sarcopenia patients (Figure 2A). The samples of the control group were concentrated and covered by the sarcopenia group. After hierarchical clustering (HCL) analysis (Figure 2B), it was found that the distribution of the intestinal microbiome in patients with sarcopenia in PCA analysis was significantly divided into two categories. One group had a high content of Prevotella, while other bacteria represented a low proportion. It was surprising that in another patient group, Bacteroides and Ruminococcaceae had the highest abundance. Other microbiome constituents also exhibited scattered, irregular, and high relative abundances, such as Lachnospiraceae and Clostridiales.

At the phylum level (Figure 1C), when comparing the gut microbiota composition, Proteobacteria accounted for a lower proportion in sarcopenia patients than that in controls, while Firmicutes and Bacteroidetes were enriched in the patient group than in the control group. At the genus level (Figure 1D), the relative proportion of Prevotella in the control group was relatively small, at only 21.5%, and the relative proportion of *Bacteroides* was 18.0%. In contrast, the *Prevotella* proportion was 26.9%, and the proportion of Bacteroides was 14.6% in the sarcopenia group. In comparison, the P/B of the control group was 1.197, the P/B of the sarcopenia group was 1.842, and the P/B of the sarcopenia group was 1.538 times higher than that of the control group.

According to Spearman's analysis, we found that the genus Coprococcus had a positive relationship with sarcopenia and vice versa for the family Lachnospiraceae (Figure 3A). According to Figure 3B, we also found that the relative amount of Coprococcus was significantly higher in the sarcopenia group than in the control group and that

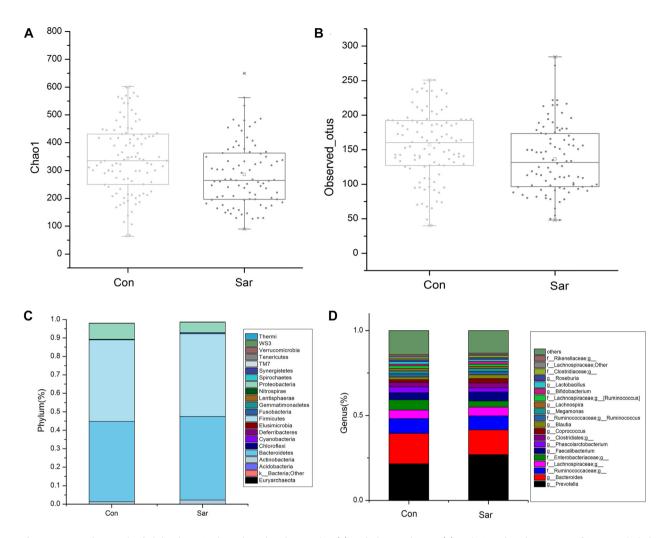


Figure 1: Box plot graph of alpha diversity based on the Chao1 value (A) and observed OTUs (B). Relative abundance map of intestinal phyla (C) and genera (D) in the control group and sarcopenia group.

the relative amount of Lachnospiraceae was significantly different between the two groups. In contrast to those in the control group (Figure 3D), the interactions among the different gut microbiota genera were stronger in the group of sarcopenia (Figure 3E), and most of the interactions were positive. Prevotella showed an inhibitory effect on other bacteria, especially against Bacteroides, Blautia, and Lachnospiraceae. In addition, there was a strong promoting effect between many of the gut microbiota genera. Lachnospira had strong positive interactions with Roseburia, g-unclassified (o-Clostridiales), g-unclassified (f-Lachnospiraceae), and Blautia. G-unclassified (f-Clostridiaceae) had a strong positive interaction with Coprococcus and Blautia. Coprococcus had a strong interaction with g-unclassified (o-Clostridiales) and g-unclassified (f-Ruminococcaceae). Ruminococcus had a strong interaction with g-unclassified (f-Ruminococcaceae) and g-unclassified (o-Clostridiales). There was a strong mutual promotion between the

g-unclassified (o-Clostridiales), *g-unclassified* (f-Lachnospiraceae), and *Blautia* genera. Based on the previous relationship of the gut microbiota genera, using the binary logistic regression method, we obtained the probability of sarcopenia in an equation as follows (Eq. 1). Then, we applied the probability into the ROC curve method to obtain an area of 0.61, which means that our model can predict sarcopenia (Figure 2C).

Sarcopenia =
$$0.244$$
 Lachnospiraceae
+ 12.538 Coprococcus + 0.002 P/B
- 0.512 (1)

Some patients in this study also underwent other blood testing, including that for albumin/globulin (A/G), AST/ALT, direct bilirubin (DBIL)/total bilirubin (TBIL), C-reactive protein (CRP), and fasting blood glucose(GLU) (Figure 3C). These indicators were correlated with the

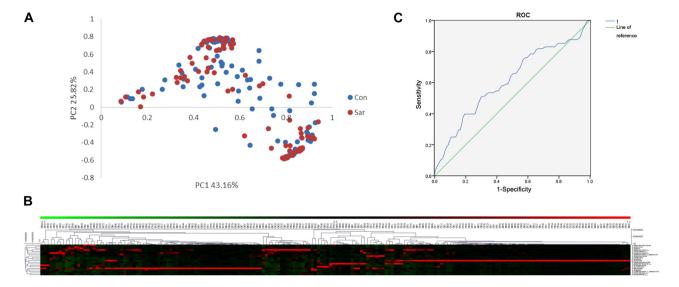


Figure 2: PCA map of the intestinal microbiome in the control group and sarcopenia group (A). Heatmap of gut microbiota abundance. "CF" means control female group; "CM" means control male group; "SF" means sarcopenia female group; "SM" means sarcopenia male group (B). ROC curves of the sarcopenia group for three indexes (C) (AUC=0.671, 95% confidence interval, p-values<0.01).

patient's gut microbiome by Spearman analysis, and a correlation heat map was obtained. From Figure 3C, we found that the A/G index had no strong interactions with the genera. However, the AST/ALT index had a strong negative interaction with g-unclassified (f-Lachnospiraceae). The DBIL/TBIL index had strong negative interactions with Prevotella and Megamonas and had strong positive interactions with g-unclassified (f-Lachnospiraceae) and g-Ruminococcus (f-Lachnospiraceae). CRP had strong negative interactions with Bacteroides and Megamonas. GLU had strong positive interactions with Coprococcus, Lactobacillus and g-unclassified (f-Clostridiaceae).

Discussion

In this study, the gut microbiomes from sarcopenia patients and control groups were distinguished. Moreover, the microbiota composition among sarcopenia patients was altered dramatically and could be divided into two categories. The first subgroup was defined by the high content of Prevotella, and the other group exhibited high content of Bacteroides. The Bacteroides-driven gut is primarily found in individuals with high protein and animal fat intake, while the Prevotella-driven gut is predominantly found in individuals who consume carbohydrate- and fiber-rich diets [15-17]. Some researchers have demonstrated that sarcopenia diseases were led by abnormal absorption of protein and insufficient protein intake, both of which were related to the two subgroups in our research [18, 19]. While patients can be divided into two categories based on their

microbiota composition, by analyzing the common characteristics, we can primarily utilize our PCA model and differentiate sarcopenia to find suitable treatments.

Animal-based diets increase the abundance of biletolerant microorganisms (Alistipes, Bilophila and Bacteroides) and reduce the level of Firmicutes bacteria that metabolize dietary plant polysaccharides [17]. In addition, both diet and exercise increased the percentage of Bacteroidetes and reduced the percentage of Firmicutes by 5.2%. In this study, we also found that the sarcopenia group usually contained more Firmicutes and than the control group, which is similar to previous studies. The relative proportion of Prevotella and Bacteroidetes (P/B) is considered to be an effective biomarker for diet and lifestyle and can also be considered a biomarker for a distinction between control and sarcopenia individuals [20]. In this study, the P/B of the control group was 1.197, the P/B of the sarcopenia group was 1.842, and the P/B of the sarcopenia group was 1.538 times that of the control group. All of the results indicated that the sarcopenia group was more likely to prefer the Mediterranean-style diet than the control group. This result may be due to a lack of high amounts of protein and animal fat in the diets of people with sarcopenia. Several recent studies have linked adherence to a Mediterranean-style diet to an overrepresentation of Prevotella [21, 22]. Prevotella is usually associated with plant-based diets. Those patients containing a high amount of Prevotella indicated that they did not consume enough protein or amino acids, which was also observed in our study. However, until now, there have been no reports about the P/B ratio and sarcopenia.

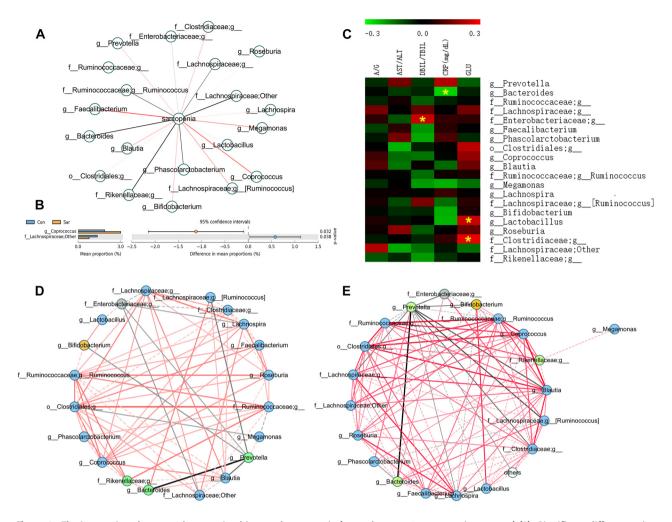


Figure 3: The interactions between the gut microbiota and sarcopenia (control group=0, sarcopenia group=1) (A). Significant differences in genera between the control group and the sarcopenia group (B). In Figure 3A, the red line indicates a positive correlation, the black line indicates a negative correlation, the solid line indicates the confidence level (double measurement) of a 0.01 significant correlation, and the broken line indicates the confidence level (double measurement) of a 0.05 significant correlation. The darker the color is, the greater the absolute value of the correlation coefficient. Heat map of Spearman interactions between blood indexes and the intestinal microbiome of sarcopenia patients. The color block marked with * indicates a significant correlation (C). Spearman interactions among gut microbiota constituents in the sarcopenia group (E).

Moreover, with the help of spearman analysis, several significant interactions were observed. In the sarcopenia group, there are more significant interactions between the genera than in the control group. This approach can be a useful tool to distinguish between sarcopenia and non-patient groups, but the reasons for this difference need to be further explored. Regarding diversity, the species richness of the sarcopenia group was significantly lower than that of the control group. Control subjects exhibited a higher intestinal microbial diversity, more stable microbiome structure and more stable intestinal environment compared with those of the patients with sarcopenia.

In our study, we tried to establish a connection between biochemical indicators and microbiota. The three indexes AST/ALT, DBIL/TBIL and CRP, had a strong relationship with some gut bacteria. The three indicators, albumin/globulin (A/G), ALT/AST, and direct bilirubin (DBIL)/total bilirubin (TBIL), were associated with liver function, which may be directly connected with sarcopenia and the microbiota. Furthermore, CRP is a nonspecific inflammatory marker and may be a risk factor for cardiovascular disease and sarcopenia is also associated with body inflammation, which is consistent with other studies [23–25]. Recent studies have shown that patients with

elevated basal CRP levels have an increased risk of diabetes [26, 27]. Patients suffering from sarcopenia experienced changes in blood composition that are closely related to the composition of the gut microbiota, which supports our model that gut microbiota composition could predicts sarcopenia.

Therefore, through a series of indicators, such as the gut microbiota P/B index (over 1.7), Coprococcus (1.00-3.70%) and the relative amount of Lachnospiraceae (0.00–1.68%), the control group and the sarcopenia group could be distinguished to some extent. The relative abundance and diversity of the sarcopenia microbiota were lower than that of the control group. According to the results of this experiment, sarcopenia can be predicted by intestinal microecological distribution. Since dietary patterns are closely related to the composition of gut microbiota, dietary intervention can be guided by the characteristics of the intestinal microbiome found in our results.

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Author contributions: Yunmei Yang and Yue Wu contributed to the design, organization, and performance of the whole experiment. Yanting Xia, Ji Yang, Shunmei Huang, Shiyu Liu, and Xuan Zhu contributed to the statistics and prediction. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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Ethical approval: The study was reviewed and approved by the Research Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University; the reference number is 2018-576.

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