

# Influence of TNF- $\alpha$ Inhibitors on Gut Microbiota and Immune Modulation in Treating Ankylosing Spondylitis: Insights into Therapeutic Mechanisms and Clinical Implications

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**Objective:** This study aims to evaluate the impact of tumor necrosis factor (TNF) inhibitors on the gut microbiota in patients with ankylosing spondylitis (AS) and investigate the potential therapeutic benefits of microbial modulation. Given the role of gut microbiota in immune regulation and its association with autoimmune conditions like AS, this research seeks to identify microbial targets that could enhance treatment outcomes.

**Methods:** Patients with AS undergoing TNF inhibitor therapy and healthy controls were recruited for this study. Gut microbiota samples were collected and analyzed using 16S rRNA gene sequencing. Assessed key parameters included  $\alpha$ -diversity and the relative abundance of dominant phyla, such as Firmicutes, Proteobacteria, Bacteroidota, Actinobacteriota, and Fusobacteriota.

**Results:** Tumor necrosis factor (TNF) inhibitor therapy was found to enhance the  $\alpha$ -diversity of the gut microbiota in patients with AS. The dominant phyla identified included Firmicutes, Proteobacteria, Bacteroidota, Actinobacteriota, and Fusobacteriota. Comparative analysis showed that patients with AS had elevated levels of Proteobacteria and Pasteurellaceae, which were normalized following TNF inhibitor treatment. Functional predictive analysis suggested that pathways associated with Terpenoid backbone biosynthesis and photosynthesis were reduced in patients with AS, bringing them closer to the profiles observed in healthy controls.

**Conclusion:** TNF inhibitors may contribute to the treatment of AS by promoting beneficial microbes, reducing the prevalence of disease-associated microbes, and modulating microbial functions. These findings bring valuable insights into the mechanisms of how TNF inhibitors act and highlight potential microbial targets for therapeutic interventions in AS.

**Keywords:** ankylosing spondylitis, gut microbiota, inflammation, immune system dysregulation, microbial diversity, TNF inhibitors

## Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory joint disease that presents a significant health challenge, primarily affecting the spine and pelvic regions. The condition can lead to severe consequences, including spinal joint fusion, abnormal curvature, chronic pain, and limited mobility, highlighting the urgent need for effective and long-term treatment strategies. Ankylosing spondylitis (AS) often extends beyond the spine, impacting other joints such as the hips and shoulders, with key symptoms including spinal stiffness, severe deformities, and restricted movement, especially during the early stages of the disease.<sup>1,2</sup>

Globally, AS prevalence ranges from 0.1% to 1.8%, representing a significant healthcare burden.<sup>3</sup> In China alone, approximately 5.19 million individuals are affected by AS.<sup>4</sup> These concerning statistics highlight the critical need for

identifying treatments that are not only effective but also provide long-term stability in mitigating the severe impact of AS.

More than 90% of patients with AS carry the human leukocyte antigen HLA-B27, suggesting a possible link between AS and autoimmune or auto-inflammatory disorders.<sup>5,6</sup> Among the various therapeutic options, tumor necrosis factor (TNF) inhibitors have become crucial in the management of AS.<sup>7</sup> However, the exact effects of TNF inhibitors on the gut microbiota and the potential role of the gut microbiota as a target for TNF inhibitor therapy in AS are still not fully understood.

While recent studies have explored the relationship between gut microbiota and immune modulation in autoimmune diseases such as rheumatoid arthritis and Crohn's disease, the impact of TNF inhibitors on gut microbiota composition in AS has been largely unexplored.<sup>7,8</sup> Existing studies have predominantly focused on the anti-inflammatory effects of TNF inhibitors, but the complex interactions between these treatments and the gut microbiota—along with their potential immunomodulatory effects—have yet to be thoroughly investigated. This represents a significant gap in our understanding, as the gut microbiota is increasingly recognized as a critical modulator of immune function and inflammation. Exploring this gap is essential not only for optimizing TNF inhibitor therapies but also for identifying new therapeutic targets in AS.

The gut microbiota, which consists of trillions of microorganisms residing in the human intestine, plays an essential role in regulating physiological functions such as digestion, metabolism, and immune responses.<sup>8,9</sup> It facilitates the absorption of vital nutrients and the generation of essential metabolites, including vitamins and short-chain fatty acids, which have systemic effects on immune and metabolic health.<sup>10–12</sup> Importantly, gut microbiota also contributes to immune homeostasis, helping to maintain immune tolerance and protect the host from excessive inflammation and autoimmune diseases.<sup>13</sup>

In the context of AS, we hypothesize that TNF inhibitors, while effective at managing the disease's inflammatory symptoms, may also influence the gut microbiota, potentially altering immune responses and contributing to the disease's pathophysiology. Understanding how TNF inhibitors interact with the microbiota could provide new insights into their broader immunomodulatory effects and uncover potential mechanisms by which microbiota changes might influence clinical outcomes in AS. This investigation into the interplay between TNF inhibitors, immune modulation, and gut microbiota could reveal novel therapeutic strategies, particularly for personalized treatment approaches that account for individual variations in microbiota composition.

## Materials and Methods

### Participants

Participants for this study were recruited from Longyan First Hospital Affiliated with Fujian Medical University between April 2022 and February 2023. We systematically collected fecal samples from adults aged 21 to 65 years. The study cohort included 13 patients with AS undergoing subcutaneous TNF inhibitors treatment, which is a standard procedure in our hospital, and an equal number of healthy controls. The duration of TNF inhibitor treatment in the AS group ranged from 6 to 24 months, with a median of 12 months. To minimize confounding variables, we applied propensity score matching to balance treatment and control groups for key variables including age, sex, disease duration, baseline disease activity, and other clinical parameters. This matching approach enhances comparability between groups and reduced potential selection bias, thus enhancing the internal validity of the study. Exclusion criteria included the use of antibiotics in the two months prior to enrollment and pre-existing conditions such as diabetes, hypertension, diarrhea, or other inflammatory diseases. Key clinical parameters such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), interleukin-6 (IL-6), and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) scores were recorded at baseline and following TNF- $\alpha$  inhibitor treatment.<sup>14</sup> In addition, we adjusted for potential confounders such as concomitant medications, comorbidities, lifestyle factors (eg, smoking and alcohol use), and baseline gut microbiota composition during statistical analyses. Ethical approval for the microbiota analysis was granted by Longyan First Hospital Affiliated with Fujian Medical University (approval number: Ethical Review of Longyan First Hospital Affiliated with Fujian Medical University [2019] No. 45). All participants provided written informed consent, and the study adhered to all relevant ethical and regulatory guidelines. The participants were systematically categorized, as shown in [Table 1](#).

**Table 1** Demographic and Anthropometric Characteristics

	AS	AS.TNF	HC
Number	13	11	13
Age	41.46±12.67	41.63±10.31	43.30±13.24
TNF inhibitor Dosage and Frequency	50 mg/week	50 mg/week	Not Applicable
ESR (mm)	45.69±23.86	9.23±4.94	\
CRP (mg/l)	38.12±42.64	3.49±5.67	\
IL-6 (pg/mL)	7.57±4.60	5.03±3.79	\
BASDAI	4.83±0.39	1.93±0.85	\

**Notes:** All data were presented as the mean ± SEM. A statistical analysis of physiological and biochemical data was conducted using GraphPad Prism version 7.0.

**Abbreviations:** ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; IL-6, interleukin-6; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; AS.TNF, AS patients receiving TNF inhibitor therapy.

## Fecal Samples and DNA Extraction

Fecal samples were collected from participants and immediately frozen at  $-20^{\circ}\text{C}$ , then stored at  $-80^{\circ}\text{C}$  for no longer than 24 hours prior to DNA extraction. DNA was extracted from the fecal samples using the QIAamp DNA Stool Minikit (Qiagen, Hilden, Germany), following the manufacturer's guidelines. DNA quality and concentration were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

## Microbiota Analyses by 16S rRNA Gene Sequencing

To profile the gut microbiota, we amplified the 16S rRNA (V4 region), 18S rRNA, and ITS genes using specific primers (eg, 515F-806R for 16S, 528F-706R for 18S). PCR reactions were performed using Phusion<sup>®</sup> High-Fidelity PCR Master Mix (New England Biolabs), with  $0.2\ \mu\text{M}$  of forward and reverse primers, and approximately 10 ng of template DNA. The thermal cycling protocol included an initial denaturation step at  $98^{\circ}\text{C}$  for 1 minute, followed by 30 cycles of denaturation ( $98^{\circ}\text{C}$  for 10 seconds), annealing ( $50^{\circ}\text{C}$  for 30 seconds), and elongation ( $72^{\circ}\text{C}$  for 30 seconds), with a final extension at  $72^{\circ}\text{C}$  for 5 minutes.

The PCR products were visualized on a 2% agarose gel stained with SYBR Green and purified using a QIAquick Gel Extraction Kit (Qiagen). Sequencing libraries were prepared using the NEBNext<sup>®</sup> Ultra<sup>™</sup> II DNA Library Prep Kit (New England Biolabs) and indexed for sequencing. Libraries were quantified using the Qubit<sup>™</sup> dsDNA Assay Kit (Thermo Fisher Scientific) and their size distribution assessed using a Bioanalyzer (Agilent Technologies). Libraries were then pooled in equidensity ratios and sequenced on an Illumina platform.

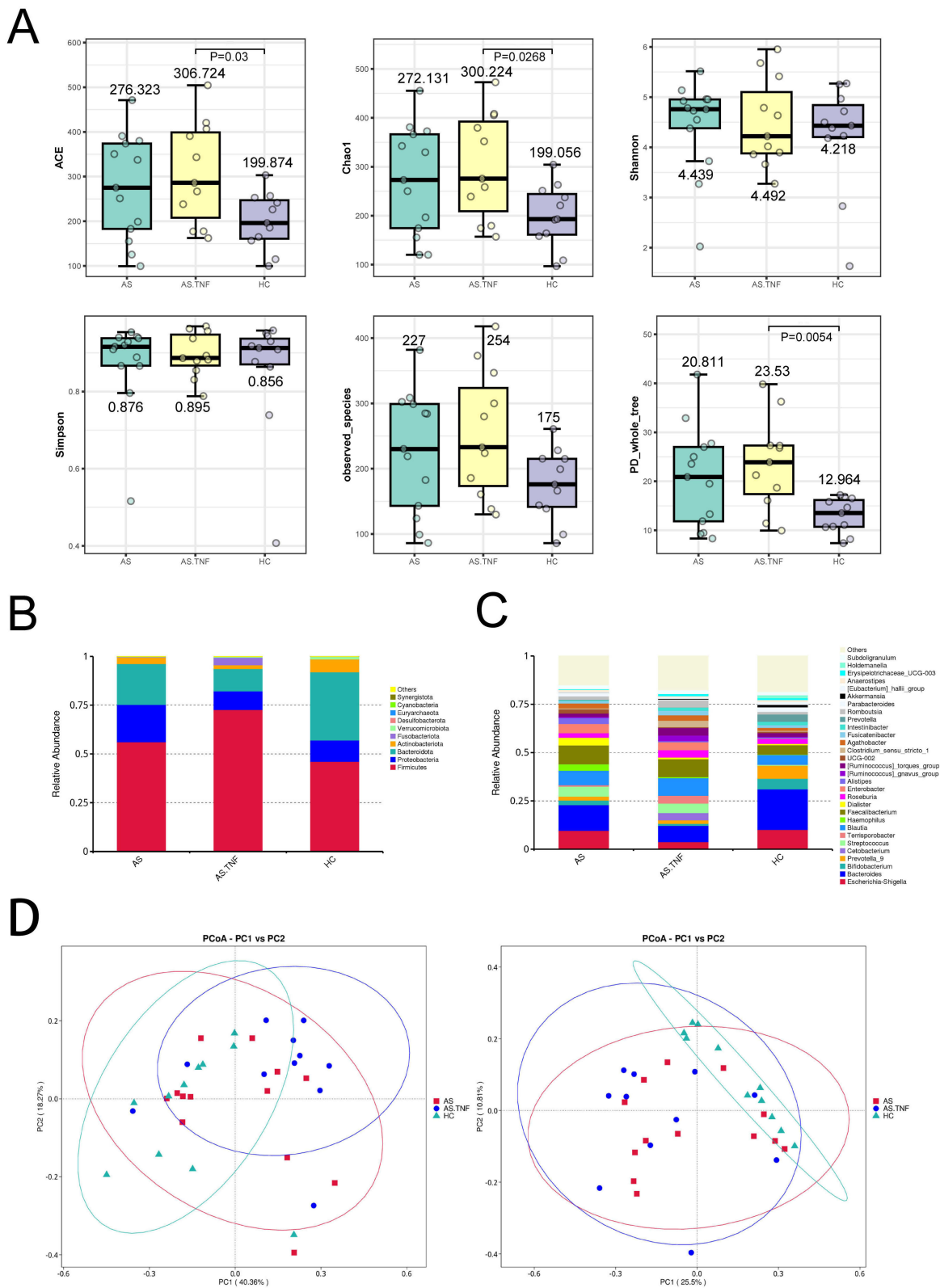
## Statistics

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## Results

### Effect of TNF Inhibitor on Gut Microbial Diversity and Composition

The  $\alpha$ -diversity analysis revealed that the observed species and diversity indices (ACE, Chao1, and Phylogenetic Diversity) were significantly lower in the HC group compared to the AS.TNF group ( $P < 0.05$ ) (Figure 1A). When comparing community structures among the groups, Synergistota, Cyanobacteria, Euryarchaeota, Desulfobacterota, Verrucomicrobiota, Fusobacteriota, Actinobacteriota, Bacteroidota, Proteobacteria, and Firmicutes were identified as the predominant phyla in all three groups (Figure 1B).

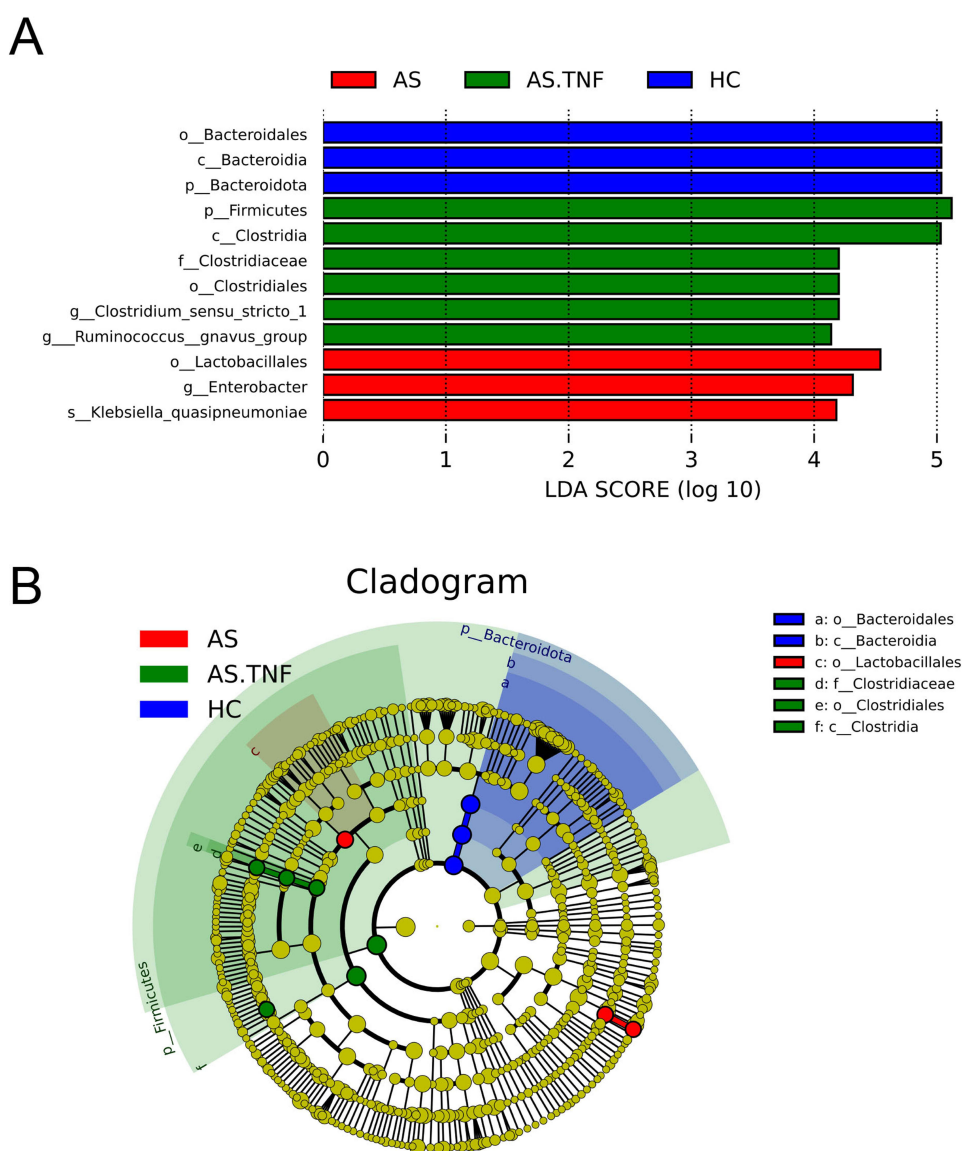


**Figure 1** The impact of TNF inhibitors on microbial diversity and composition is depicted as follows: **(A)**  $\alpha$ -Diversity was evaluated using indexes such as ACE, observed species, Chao1, Shannon, Simpson, and PD\_whole\_tree. **(B)** The TNF inhibitors' effect on the phylum-level taxonomic distribution of fecal microbiota was assessed. **(C)** Similarly, changes at the genus-level distribution were analyzed. **(D)** The PLS-DA plot revealed that while the AS and AS.TNF groups exhibited overlapping microbial profiles, the HC group showed a distinct trend of separation.

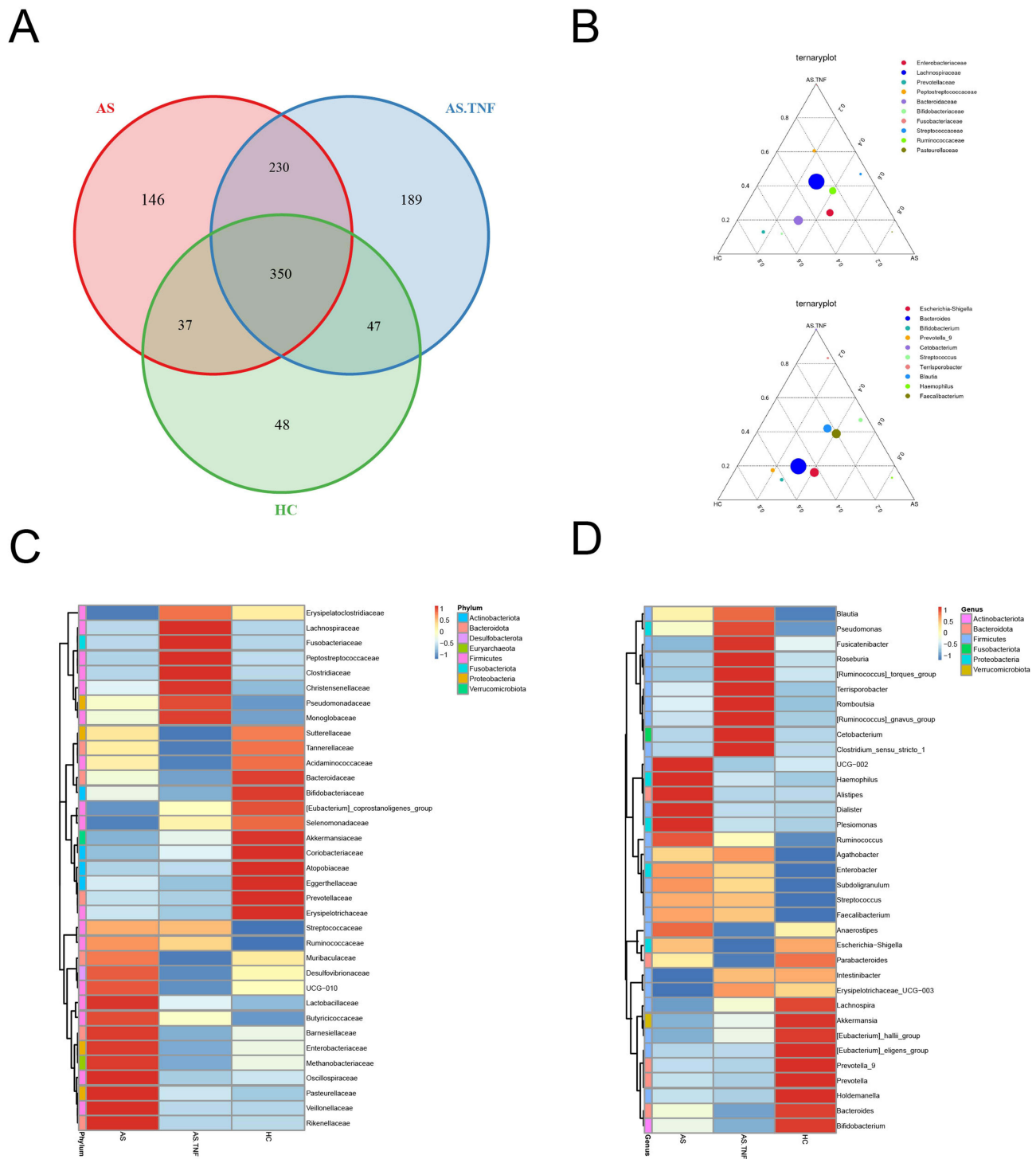
At the phylum level, Firmicutes, Proteobacteria, Bacteroidota, Actinobacteriota, and Fusobacteriota were the most prevalent. Proteobacteria, in particular, were significantly upregulated twofold in the AS group compared to the HC group. However, following TNF inhibitor treatment, the relative abundance of Proteobacteria in the AS.TNF group decreased, returning to levels comparable to the HC group.

At the family level, the predominant taxa included Enterobacteriaceae, Lachnospiraceae, Prevotellaceae, Peptostreptococcaceae, Bacteroidaceae, Bifidobacteriaceae, Fusobacteriaceae, Streptococcaceae, Ruminococcaceae, and Pasteurellaceae. Pasteurellaceae exhibited a sixfold increase in the AS group compared to the HC group, but decreased by 16-fold in the AS.TNF group post-treatment. PLS-DA analysis showed significant overlap between the AS and AS.TNF groups, with a clear separation of the HC group (Figure 1). Additionally, compared to the HC and AS.TNF groups, the AS group displayed a marked increase in the abundance of Lactobacillales, Enterobacter, and Klebsiella quasipneumoniae (Figure 2).

The AS, AS.TNF and HC groups comprised 763, 816, and 482 unique operational taxonomic units (OTUs), respectively. Specifically, the AS group contained 146 unique OTUs, the AS.TNF group had 189, and the HC group included 48 unique OTUs (Figure 3A). Ternary plot analysis revealed that the AS.TNF and HC groups were



**Figure 2** LDA Effect Size analysis is presented as follows: **(A)** Differential gut microbiota with an LDA score greater than 4 were identified between the HC, AS, and AS.TNF groups. **(B)** A cladogram illustrates the phylogenetic distribution of these differential gut microbiota across the HC and AS groups.



**Figure 3** Species abundance and diversity are illustrated as follows: **(A)** OTUs in the AS, AS.TNF, and HC groups. **(B)** Ternary plot results showing species distribution at both the phylum and genus levels. **(C)** Species abundance at the phylum level. **(D)** Species abundance at the genus level.

predominantly influenced by Bacteroides, Blautia, Bifidobacterium, and Prevotella, whereas Haemophilus and Pasteurellaceae were primarily associated with the AS group (Figure 3B).

The AS group showed significant upregulation of Muribaculaceae, Barnesiellaceae, and Rikenellaceae within the Bacteroidota phylum, but these microorganisms decreased in the AS.TNF group to levels similar to those in the HC group. Similar trends were observed for Desulfovibrionaceae (Desulfobacterota), UCG-010, Lactobacillaceae,

Butyricocccaceae, Oscillospiraceae, Veillonellaceae (Firmicutes), Pasteurellaceae and Enterobacteriaceae (Proteobacteria), and Methanobacteriaceae (Euryarchaeota) (Figure 3C).

Notable shifts were observed in Firmicutes and Proteobacteria phyla, where UCG-002 and Dialister (Firmicutes), as well as Haemophilus and Plesiomonas (Proteobacteria), exhibited higher relative abundances in the AS group. Both the AS.TNF and HC groups showed a decrease in these abundances (Figure 3D). In contrast, Intestinibacter and Erysipelotrichaceae UCG-003 (Firmicutes) showed lower relative abundances in the AS group but increased in both the HC and AS.TNF groups.

## Alteration of Microbial Function

Functional predictions based on microbial profiles revealed several key differences across the groups. At Level 1, major functions included Organismal Systems, Human Diseases, Cellular Processes, Environmental Information Processing, Genetic Information Processing, and Metabolism (Figure 4A). At Level 2, predominant functions included Cell Motility, Metabolism of Cofactors and Vitamins, Signal Transduction, Nucleotide Metabolism, Energy Metabolism, Amino Acid Metabolism, Translation, Replication and Repair, Membrane Transport, and Carbohydrate Metabolism (Figure 4B). At Level 3, significant functions involved Exosomes, Peptidases, Amino Acid-Related Enzymes, Pyrimidine Metabolism, Purine Metabolism, Transfer RNA Biogenesis, DNA Repair and Recombination Proteins, and Transporters (Figure 4C).

Notably, at Level 2, the AS group exhibited lower levels of bacterial functions related to Folding, Sorting and Degradation, Glycan Biosynthesis and Metabolism, Infectious Diseases, Lipid Metabolism, and Enzyme Families compared to the HC group. However, following TNF inhibitor treatment, these functionalities in the AS.TNF group increased, approaching levels observed in the HC group. Conversely, functionalities such as Genetic Information Processing, Carbohydrate Metabolism, Transcription, Xenobiotics Biodegradation and Metabolism, and Endocrine and Metabolic Diseases were higher in the AS group but decreased in the AS.TNF group to resemble the HC group. Key functionalities in the AS group, such as Amino Acid-Related Enzymes, Cysteine and Methionine Metabolism, Pyruvate Metabolism, Butanoate Metabolism, Glycolysis/Gluconeogenesis, Chromosome and Associated Proteins, were elevated. These functionalities decreased in the AS.TNF group, returning to a levels closer to those of the HC group.

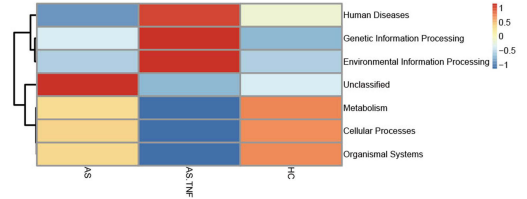
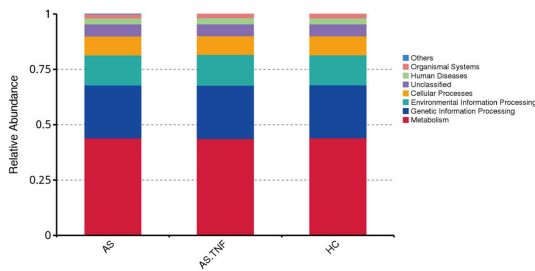
A comparative analysis of functional modules showed that the AS group had significant upregulation in Glycolysis/Gluconeogenesis, DNA Replication Proteins, Prokaryotic Defense System, Glucagon Signaling Pathway, and Vancomycin Resistance, among others ( $P < 0.05$ ) (Figure 5). Conversely, the AS group exhibited downregulation in modules related to Porphyrin and Chlorophyll Metabolism, Oxidative Phosphorylation, Sulfur Metabolism, and Photosynthesis ( $P < 0.05$ ).

The AS.TNF group showed significant upregulation in Messenger RNA Biogenesis, Glycerophospholipid Metabolism, Terpenoid Backbone Biosynthesis, and Photosynthesis Proteins ( $P < 0.05$ ), while it demonstrated downregulation in Amino Acid-Related Enzymes, Energy Metabolism, Lipid Biosynthesis Proteins, Biofilm Formation, and Vitamin B Metabolism ( $P < 0.05$ ) compared to the AS group (Figure 5A). Notably, Terpenoid Backbone Biosynthesis and Photosynthesis Proteins were also downregulated in both the AS.TNF and HC groups, suggesting a shared alteration independent of TNF inhibitor treatment.

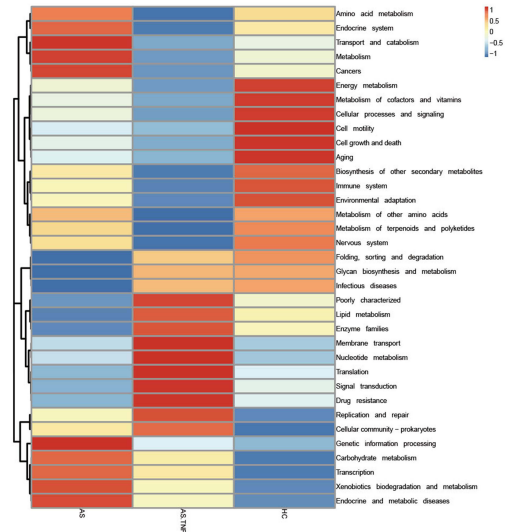
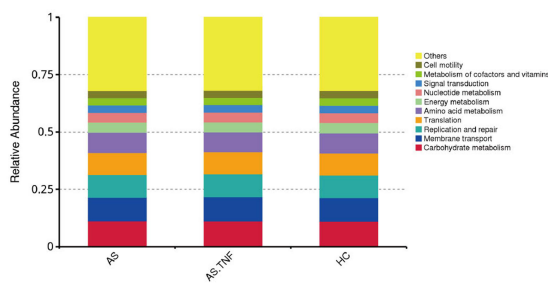
## Discussion

Ankylosing Spondylitis (AS) is a chronic inflammatory disease primarily affecting the spine and pelvic joints, characterized by immune system dysregulation, leading to inflammation primarily in the spine and pelvic regions.<sup>15</sup> This condition is believed to arise from an aberrant immune response, where the immune system erroneously identifies normal tissues as foreign, triggering an inflammatory reaction.<sup>2,16</sup> This inflammation predominantly affects tissues around the joints, especially in the spine and pelvic area. TNF is a critical player in the pathogenesis of AS, acting as a major inflammatory mediator.<sup>17</sup> TNF- $\alpha$ , a pro-inflammatory cytokine, is central to immune responses.<sup>18</sup> In patients with AS, excess TNF- $\alpha$  leads to an exaggerated inflammatory response and subsequent tissue damage.<sup>19</sup> TNF- $\alpha$  exerts its effects through interaction with two cell surface receptors: TNFR1 (TNF receptor 1) and TNFR2 (TNF receptor 2).<sup>20</sup> Activation of these receptors triggers inflammatory responses, including the release of additional pro-inflammatory cytokines, which

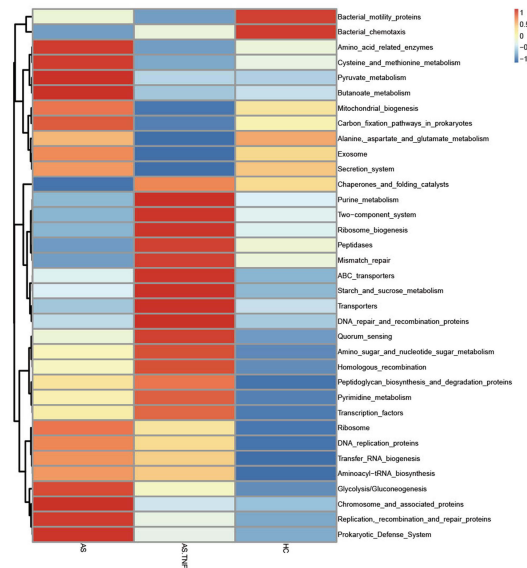
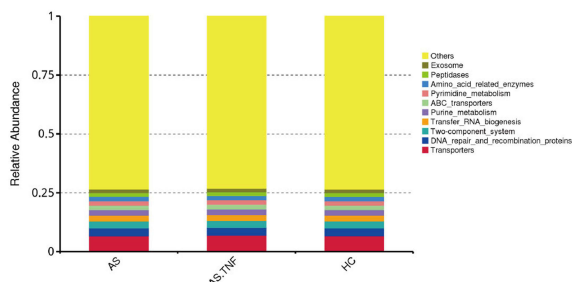
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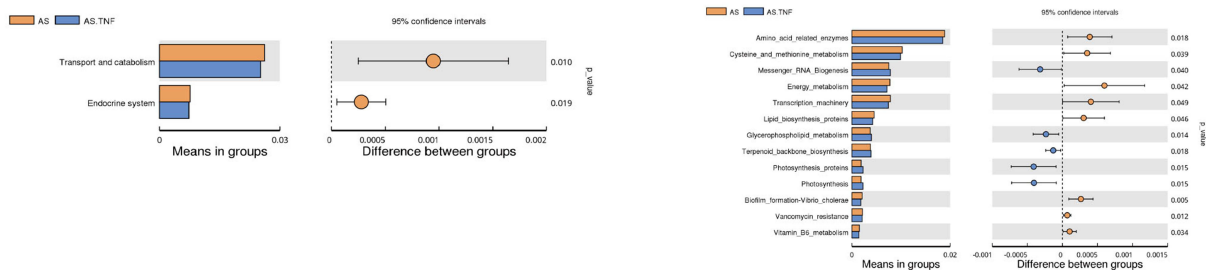
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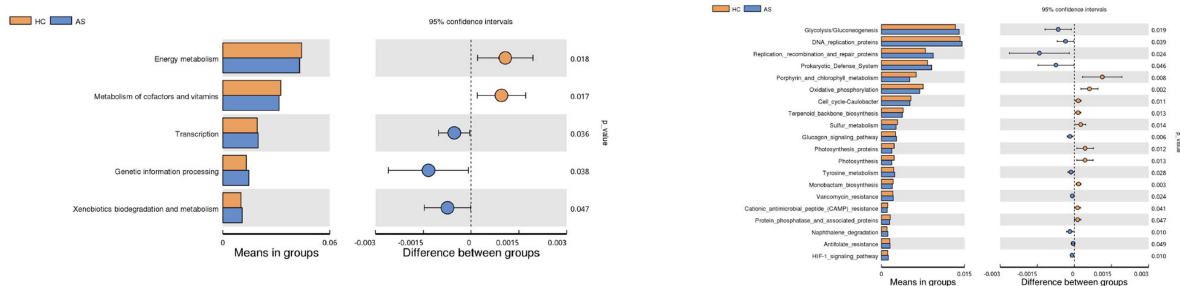
**Figure 4** The effect of TNF inhibitors on microbial function is assessed through correlation analysis of differential gut microbiota and differential metabolites among the AS, AS.TNF, and HC groups. This is depicted at different levels: (A) LV1, (B) LV2, and (C) LV3.



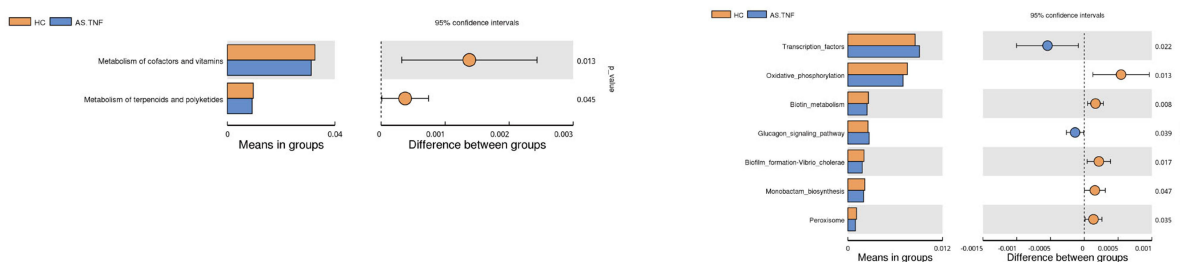
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**Figure 5** The effect of TNF inhibitors on microbial signaling pathways is illustrated by significant differences in microbial signaling pathways between the groups: **(A)** AS and AS.TNF, **(B)** AS and HC, and **(C)** HC and AS.TNF.

further exacerbate inflammation and contribute to joint and skeletal damage.<sup>21</sup> Consequently, TNF- $\alpha$  and its receptors are pivotal in the pathogenesis of AS.

Biologic agents targeting TNF- $\alpha$ , such as anti-TNF drugs, are commonly used in the treatment of AS.<sup>22</sup> These drugs work by inhibiting TNF- $\alpha$  activity, thereby reducing inflammation and alleviating symptoms. They are essential for controlling AS inflammation and slowing the progression of joint damage.<sup>23</sup> Despite their widespread use, the impact of TNF inhibitors on gut microbiota remains underexplored. Specifically, whether TNF inhibitors can exert therapeutic effects in AS by modulating the gut microbiota's composition and functionality is an area that warrants further investigation.

Despite the widespread use of TNF inhibitors in AS therapy, their impact on gut microbiota remains relatively underexplored. Gut microbiota is crucial for maintaining immune homeostasis, and its dysregulation is associated with various autoimmune and inflammatory diseases. Recent studies have started to investigate the potential relationship between TNF inhibitors and gut microbiota composition, particularly in the context of AS. Our study adds to this

emerging field by suggesting that TNF inhibitors may exert therapeutic effects in AS not only by directly reducing inflammation but also by modulating gut microbiota.

Our study provides new insights into the role of TNF inhibitors in modulating gut microbiota composition. We observed that TNF inhibitor treatment significantly increased microbial diversity in AS patients compared to untreated individuals. This aligns with recent studies suggesting that TNF inhibitors may restore a healthy microbial balance, which could contribute to their therapeutic effects.<sup>24,25</sup> Notably, our findings show that TNF inhibitors led to a reduction in the abundance of potentially pathogenic taxa, such as Proteobacteria and Pasteurellaceae, which have been implicated in inflammatory processes. These microbial changes resemble findings from studies on other immune-mediated diseases like Crohn's disease, indicating that TNF inhibitors may have a broader immunomodulatory effect on gut microbiota.<sup>10,26</sup>

Despite the promising results, several limitations must be considered. First, our study's cross-sectional design limits our ability to establish a causal relationship between TNF inhibitor use and alterations in gut microbiota composition. Longitudinal studies are needed to assess the temporal dynamics of these microbial shifts and their direct role in therapeutic outcomes. Additionally, while we observed significant changes in microbiota composition at the phylum and family levels, the functional implications of these changes remain unclear. Future research should explore the metabolic and immune-modulatory effects of these microbial alterations to better understand how TNF inhibitors affect gut health and contribute to disease management in AS.

Moreover, our reliance on 16S rRNA gene sequencing provides a broad overview of microbial composition but lacks the depth needed to fully explore microbial functionality. More advanced techniques, such as metagenomics, would offer a more comprehensive understanding of the microbial functions and their potential therapeutic implications.

## Conclusion

TNF inhibitors in AS patients show therapeutic potential by enhancing gut microbiota diversity and reducing dysbiosis. Our results indicate that TNF inhibitors may support beneficial microbial shifts and modulate metabolic pathways, potentially influencing host health beyond anti-inflammatory effects. These findings underscore the role of gut microbiota in AS treatment, suggesting avenues for more targeted and personalized therapeutic strategies.

## Abbreviations

AS, Ankylosing Spondylitis; TNF, Tumor Necrosis Factor; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; IL-6, interleukin-6; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; PLS-DA, Partial least squares discrimination analysis; LDA, Linear Discriminant Analysis; OUT, Operational Taxonomic Units; CAMP, Cationic Antimicrobial Peptide; TNFR1, Tumor Necrosis Factor Receptor 1; TNFR2, Tumor Necrosis Factor Receptor 2.

## Ethics Approval and Consent to Participate

This study was conducted with approval from the Ethics Committee of Longyan First Hospital Affiliated with Fujian Medical University (approval number: Ethical Review of Longyan First Hospital Affiliated with Fujian Medical University [2019] No. 45). This study was conducted in accordance with the declaration of Helsinki. Written informed consent was obtained from all participants.

## Data Sharing Statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

## Funding

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## Disclosure

The authors declare that they have no conflicts of interest in this work.

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