Alterations in the human gut microbiome in anti-N-methyl-D-aspartate receptor encephalitis

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Abstract

Objective: To explore the diversity and composition of the fecal microbiota in patients with anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis. Methods: We enrolled 10 patients in the acute stage with naïve treatment, seven patients with relapse, 13 patients without relapse in the remission phase, and 12 paired healthy controls. The fecal microbiota in different groups was compared by 16S ribosomal DNA (rDNA) gene pyrosequencing. Results: Prominent dysbiosis in the gut microbiome of patients with anti-NMDAR encephalitis was found. Our primary findings showed that the overall species richness (alpha diversity indexes) of the microbiota was higher in patients than in controls (P < 0.05). Distance-based community analysis revealed that the microbiota differed substantially within all subgroups of patients and controls (P < 0.05). The relative abundance of species heatmap showed a tendency toward depletion for some commensal genera, such as Prevotella_6, Bifidobacterium, Faecalibacterium, and other short-chain fatty acid (SCFA)-producing bacteria. Additionally, our results showed that all subgroups had a distinct bacterial species, with an increase in the genus Firmicutes in the acute phase group and the genera Streptococcus and Parabacteroides in patients with relapse. However, the genus Bacteroides was very abundant in patients without relapse. Although the findings regarding the Firmicutes/Bacteroidetes (F/B) ratios across the four comparison groups were not statistically significant, the F/B ratio gradually increased in patients from the acute phase group (0.87), to the disease remission group with relapse (1.06), to the group without relapse (1.28), to the healthy group (1.63). Interpretation: Patients with anti-NMDAR encephalitis exhibit a substantial alteration in fecal microbiota composition.

Introduction

Autoimmune-mediated encephalitis is a severe and devastating neurological disorder affecting approximately five to eight individuals per 100,000 persons.1 The most frequent form of autoimmune encephalitis is N-methyl-D-aspartate receptor (NMDAR) encephalitis,2 which is characterized by changes in behavior, psychosis, impairment of memory, seizures, stereotypical movements, autonomic dysfunction, or even coma.3 Immunotherapy (glucocorticoids, intravenous immune globulin, plasma exchange, or rituximab) is an effective method to treat this disease.4 However, relapses still occur in approximately 20-25% of patients.5,6 Recent studies have shown that tumors and viral infection are two potential triggers of autoimmune encephalitis; however, most autoimmune encephalitis occurs in patients with no apparent immunologic triggers, leading some investigators to postulate a genetic predisposition to these disorders. However, in recent studies, no specific association between HLA and anti-NMDAR encephalitis was found.7 Knowledge of the pathogenesis of anti-NMDAR encephalitis remains limited.

The gut microbiota, which is also referred to as the second brain, may affect brain activity through the gut-microbiota-brain axis under both physiological and pathological conditions.8 Dysbiosis in the gastrointestinal microbiota influences proinflammatory T-cell
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Subjects and Methods

Study subjects

Patients who were between 15 and 60 years of age and had a definitive diagnosis of anti-NMDAR encephalitis were recruited in this study and classified into subgroups according to the active (group AE1) or remission phase. Patients in the remission phase were further grouped into subgroups with relapse (group AE2) or without relapse (group AE3). We also enrolled healthy controls matched for age, sex, body mass index (BMI), eating habits, and geographical space. The detailed inclusion and exclusion criteria are shown in Figure 1.

Consecutive patients in the acute disease phase (group AE1) and healthy controls (group Con) were prospectively recruited from the inpatient department of the neurology center and health examination center of West China Hospital between June 2017 and December 2017. Participants who presented with acute psychiatric symptoms, seizures, or focal neurological signs were provided with a 50-mL sterile tube and disposable sterile forceps for fecal collection. These patients who met the inclusion criteria for anti-NMDAR encephalitis were recruited in our study:11 (1) rapid onset of less than 3 months and one or more of the following six major groups of symptoms: abnormal behavior or cognitive dysfunction, speech dysfunction, seizures, movement disorder, decreased level of consciousness, and autonomic dysfunction or central hypoventilation (N = 45); (2) positive results for anti-NMDAR antibodies in both cerebrospinal fluid (CSF) and serum (N = 19); (3) negative for antibodies for other autoimmune encephalitis diseases, such as a-amino-3-hydroxy-5-methyl-4-isoxazole receptors, contactin-associated protein-2 (N = 1), leucine-rich glioma-inactivated protein-1, gamma-aminobutyric acid receptors (N = 3), dipeptidyl-peptidase-like protein 6, IgLON5 or neurologic paraneoplastic antibodies (anti-Hu, anti-Ri, anti-Yo, anti-CV2, anti-Ma, anti-amphiphysin, anti-Tr, PCA-2, and anti-GAD); and (4) 18 years or older. In total, we acquired 10 subjects in the AE1 group and matched healthy control individuals with similar age, gender, weight, height, BMI, blood pressure, and constituent ratio of dietary nutritional factors. All these factors can affect the fecal microbiota.

Additionally, 100 patients definitively diagnosed with anti-NMDAR encephalitis based on an existing registry between January 2015 and May 2017 were included retrospectively and invited into our study. Relapse of encephalitis was defined as new onset or worsening of symptoms occurring after an initial improvement or stabilization of at least 2 months.4 In addition to the above inclusion criteria, patients in both the AE2 and AE3 groups also met the following criteria: (1) at least 6 months in the period of recovery from the last disease onset of encephalitis before sample collection and (2) no oral administration of glucocorticoids, antiepileptic, or antipsychotic drugs for at least 3 months. For the 25 cases with recurrence, two patients were in the active phase, and 16 unqualified specimens were excluded from this experiment. Ultimately, only seven patients in group AE2 and 12 patients in group AE3 were enrolled.

The exclusion criteria for all patients and healthy controls were as follows: (1) antibiotic or probiotic use within the past 3 months; (2) history of gastroenteritis within the past 3 months; (3) history of other autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus, type 1 diabetes, etc.); (4) history of bowel surgery; (5) pregnancy or lactation; and (6) history of neuropsychiatric disorders [depression, anxiety disorder, schizophrenia, multiple sclerosis (MS), etc.].

Clinical data collection

Demographic and clinical information for these patients was collected by an experienced neurologist from the patient review and/or medical records. To further control for diet, we requested all of the participants to complete a validated food frequency questionnaire (CHNS2015-FFQ). Then, we used nutritional database (http://db.foodmate.net/yingyangsu/) to provide information regarding 27 nutrients including carbohydrate, protein, fat, etc. Written informed consent was obtained from all patients or their guardians prior to enrollment in the study. This study was approved by the Research Ethics Committee of West China Hospital of Sichuan University.
Fecal sample collection and analysis of fecal microbiota

Fecal samples were collected in the morning when the patients had an empty stomach. All fecal samples were delivered to West China Hospital within 2 h and stored at $-80^\circ$C. Finally, the fecal samples were used for DNA extraction and analyses. After extraction of total DNA from subjects, we performed polymerase chain reaction amplification and pyrosequencing of the V4 region of the bacterial 16S rDNA genes and used these sequences for taxonomic assignments. Please refer the detailed methods in the Appendix S1.

Statistics

ANOVA was used to compare the mean values of dietary factors as well as age, height, weight, and BMI across the groups (AE1, AE2, AE3, and Con). Differences were considered significant if the F-test yielded a $p$ value of
Post hoc tests were used to assess pairwise comparisons for variables that differed among the groups. Alpha and beta diversity were investigated in QIIME. The significance of group differences was determined using the Mann–Whitney U test with R software (Version 2.15.3). Taxa with significant differential abundances were detected by the nonparametric factorial Kruskal–Wallis (KW) rank sum test, and the (unpaired) Wilcoxon rank sum test was used to investigate the biological consistency among subclasses. The P values were corrected for multiple testing using the Bonferroni correction.

**Results**

**Clinical characteristics**

Overall, we included 30 patients, among whom 10 patients were in the acute phase of encephalitis (group AE1) and 20 patients had at least a 6-month recovery period from the last symptom onset of encephalitis [seven patients had relapsed (group AE2), and 13 patients had not relapsed (group AE3) before sample collection], and 12 unrelated healthy individuals all living in Chengdu, Sichuan Province. Our study variable distributions, such as age, gender, weight, height, BMI, blood pressure, and constituent ratio of dietary factors, did not differ between both patients and controls (Table 1).

**Cluster assignment of 16S pyrosequencing reads and fecal community composition analysis**

Fecal samples from 30 anti-NMDAR encephalitis patients and 12 healthy individuals were sequenced and analyzed using the IonS5TMXL platform. The number of total raw reads obtained from all AE patients and healthy individuals was 2548416 and 966203, respectively. After chimeraism and filtration, we acquired a total of 2296174 and 906576 reads obtained from all AE patients and healthy individuals. When we calculated the estimators of Chao1 [(AE1 vs. AE2, P = 0.01), (AE2 vs. Con, P = 0.01), (AE3 vs. Con, P = 0.02), but the results also showed no differences between patients in the AE1, AE2, and AE3 groups (Figure 4A), which means a distant species phylogeny relationship between patients and controls. When we calculated the estimators of Chao1 [(AE1 vs. AE2, P = 0.08), (AE1 vs. Con, P = 0.02), (AE3 vs. Con, P = 0.05)] (Figure 4B) and ACE [(AE1 vs. Con, P = 0.02), (AE3 vs. Con, P = 0.04)] (Figure 4C), the results showed statistical significance, which suggest that anti-NMDAR encephalitis participants tended to have greater species richness. For the Coverage (Figure 4D), Shannon (Figure 4E), and Simpson (Figure 4F) indexes, there were no differences in both community diversity and species evenness between study subjects. All of the above mentioned data showed a trend toward a higher species richness in anti-NMDAR encephalitis patients than in healthy controls and the samples from patients with NMDAR encephalitis did not display significant differences in global community composition compared with samples from controls.

At the genus level, there was also a tendency for the feces of anti-NMDAR encephalitis patients to be enriched for some bacterial taxa, such as *Streptococcus*, *Escherichia-Shigella*, and *Bacteroides*, but the feces were depleted of some commensal genera, such as *Prevotella_6*, *Bifidobacterium*, and other short-chain fatty acid (SCFA)-producing bacteria (as shown in Figure 3B at the genus level).

In addition, we calculated the *Firmicutes-to-Bacteroidetes* (F/B) ratio in 30 patients and 12 healthy individuals. Although there was no statistically significant difference between AE and controls, nor differences between any pairs of the four subgroups, the outcome of the F/B ratio was lowest in patients in the acute phase (average AE1: 0.87) and gradually increased from patients in the remission phase with and without relapse [(average AE2: 1.06) and (average AE3: 1.28)] to the healthy group (average Con: 1.63) (Figure 3C).

**Quantification of fecal microbial diversity in anti-NMDAR encephalitis patients differs from that of healthy controls, particularly in patients with relapse**

To identify whether anti-NMDAR encephalitis was associated with a change in microbiota richness and complexity, we also compared significant differences in the p values for alpha diversity indexes among all subgroups of anti-NMDAR encephalitis patients and healthy participants (as shown in Figure 4A–F). The phylogenetic diversity (PD)-whole tree index showed that all subgroups of patients with anti-NMDAR encephalitis were significantly higher than those of the healthy group (AE1 vs. Con, P = 0.01), (AE2 vs. Con, P = 0.01), (AE3 vs. Con, P = 0.02), but the results also showed no differences between patients in the AE1, AE2, and AE3 groups (Figure 4A), which means a distant species phylogeny relationship between patients and controls. When we calculated the estimators of Chao1 [(AE1 vs. AE2, P = 0.08), (AE1 vs. Con, P = 0.02), (AE3 vs. Con, P = 0.05)] (Figure 4B) and ACE [(AE1 vs. Con, P = 0.02), (AE3 vs. Con, P = 0.04)] (Figure 4C), the results showed statistical significance, which suggest that anti-NMDAR encephalitis participants tended to have greater species richness. For the Coverage (Figure 4D), Shannon (Figure 4E), and Simpson (Figure 4F) indexes, there were no differences in both community diversity and species evenness between study subjects. All of the above mentioned data showed a trend toward a higher species richness in anti-NMDAR encephalitis patients than in healthy controls and the samples from patients with NMDAR encephalitis did not display significant differences in global community composition compared with samples from controls.
The modified Rankin Scale (mRS): The scale runs from 0 to 6, running from perfect health without symptoms to death. 0 - No symptoms; 1 - No disability. Unable to attend to own bodily needs without assistance, and unable to walk unassisted; 2 - Severe disability. Requires constant nursing care and attention, bedridden, incontinent; 3 - Moderate disability. Requires some help, but able to walk unassisted; 4 - Moderately severe disability. Unable to attend to own bodily needs without assistance, and unable to walk unassisted; 5 - Severe disability. Requires constant nursing care and attention, bedridden, incontinent; 6 - Dead.  

To evaluate the extent of similarity between the microbiota communities, beta diversity values were analyzed. We performed unweighted UniFrac-based principal coordinate analysis (PCoA). The plots of community structures of each fecal sample are shown in Figure 5. The PCoA results revealed that the overall microbial composition of patients with anti-NMDAR encephalitis deviated from that of healthy controls; the first two
components were 11.05% and 8.90% (Figure 5A). The boxplot of our study also revealed that the unweighted UniFrac-based PCoA index of microbiota differed substantially within all subgroups of the patients [(AE1 vs. AE2, \(P = 0.078\)), (AE1 vs. AE3, \(P = 0.06\)), (AE2 vs. AE3, \(P < 0.001\)), (AE1 vs. Con, \(P = 0\)), (AE2 vs. Con, \(P < 0.001\)), (AE3 vs. Con, \(P = 0\))] (Figure 5B).

The abundance levels of certain bacteria are crucial to anti-NMDAR encephalitis

A supervised comparison of the microbiota between subgroups of anti-NMDAR encephalitis patients and the healthy group was performed by utilizing LEfSe analysis (Figure 6), which is often used to identify the presence and effect size of region-specific OTUs among various groups. Linear discriminant analysis was used to evaluate the effect size of each differentially abundant trait, and a strict threshold of 4.0 was chosen for logarithmic LDA scores. We particularly focused on the differences in taxa at the genus level. The relative abundances of the genus *Fusobacterium* were more enriched in the AE1 group, whereas the representative abundances of the genera *Streptococcus* and *Parabacteroides* were outnumbered in the AE2 group. However, the genus *Bacteroides* was more abundant in patients in the AE3 group. No specific genera were found in the healthy control group, whereas the *Clostridia* taxa class and the order *Clostridiales* were much higher in the healthy control group.

Discussion

In recent years, antibody-mediated encephalitis has attracted growing interest. Whether genetic etiology or environmental inciting factors trigger the autoimmune process in anti-NMDAR encephalitis is still unclear,
Figure 3. Relative abundance composition of the fecal microbiome in different patient groups and the control group. (A) Variations in the relative top 10 abundances of fecal bacterial taxa at the phylum level; (B) variations in the heatmap of the top 35 abundances of fecal bacterial taxa at the genus level; (C) box plot representation of the relative abundance ratio of the phyla Firmicutes to Bacteroidetes between subgroups of anti-NMDAR encephalitis patient and healthy control profiles.

Figure 4. Box plot representation of gut microbiota richness and diversity distribution across the histogram of alpha diversity indexes. (A) PD-whole tree index to reflect the relationships of species within the community. The results suggest a distant species phylogeny relationship between different paired groups; (B) Chao1 estimator and (C) ACE estimator were used to identify community richness. The results suggest anti-NMDAR encephalitis participants tended to have greater species richness (D) Coverage was used reflecting sequencing depth, which suggest that good coverage was achieved in the tests for all groups. (E) Shannon and (F) Simpson indexes were used to identify community diversity for both diversity and evenness between subgroups of patients and healthy controls, which suggests that there were no differences in both community diversity and species evenness between study subjects (*P < 0.05).
Despite many studies. Thus, we performed a study to define the community structure of the fecal microbiota in patients with anti-NMDAR encephalitis using high-throughput 16S rDNA gene sequencing.

Disease phase-specific outcome analyses were conducted in our study. Although the healthy controls were enrolled with matched age, sex, BMI, eating habits, and geographical space, and we further used diet data to control for the microbiome findings, there was no global differences in the overall composition of the gut microbiota between patients and controls (see Figure 4) but rather on the level of microbiota abundance in our study (see Figure 6), which is an interesting phenomenon, since this clustering profile distincts from most other systematic (auto)immune diseases.13,14 In this study, fecal microbial diversity was unexpectedly somewhat greater in anti-

![Figure 5](image)

**Figure 5.** Differences in bacterial composition between anti-NMDAR encephalitis patients with various subgroups and controls. (A) The unweighted UniFrac principal coordinates analysis (PCoA) scatter plot based on the Wilcox rank sum test of patients’ fecal sample composition structure showed the trend that the two groups were well separated from healthy controls. The first two components were 11.05% and 8.90%. (B) The PCoA analysis box plot showed a significant difference within all the subgroups compared to the healthy group (AE1 vs. AE2, \( P = 0.0779 \)), (AE1 vs. AE3, \( P = 0.0616 \)), (AE2 vs. AE3, \( P < 0.001^{***} \)), (AE1 vs. Con, \( P < 0.0001^{****} \)), (AE2 vs. Con, \( P < 0.001^{***} \)), (AE3 vs. Con, \( P < 0.0001^{****} \)).

![Figure 6](image)

**Figure 6.** (A) LEfSe analysis was utilized to identify the “signature” taxa that are highlighted in the phylogenetic tree in cladogram format. (B) The LD scores showed at phylum, family, order, class, and genus level.
NMDAR encephalitis patients. Although a greater bacterial diversity could be potentially beneficial to human health, increased gut microbiome diversity and richness were also detected in some disease situations, for example depression and autism. The precise role of the microbiota in central nervous system diseases remains open to debate. The remarkable deviation in beta diversity in the microbiota composition structure of the AE2 group, which indicates a latent role of hard-to-correct fecal flora dysbiosis, might be associated with susceptibility to recurrence exacerbation and contribute to resistance to immunotherapy.

The fecal F/B ratio may influence host metabolism and inflammation since it might play a potential anti-inflammatory role. Considering that patients with autoimmune disease exhibit intestinal dysbiosis, we wanted to determine whether an altered F/B ratio might be associated with the fecal microbial composition in anti-NMDAR encephalitis patients. We observed the direction of the lowest F/B ratio in the acute disease stage, which is in stark contrast with an earlier study appearing in a significant increase in the F/B ratio associated with immune disorder and obesity-related diseases. However, lower F/B ratios were also observed in primary systemic sclerosis and systemic lupus erythematosus patients. Therefore, the precise consequences of a decreased F/B ratio for NMDAR encephalitis remain unclear. We conjectured that dietary differences between Western and Eastern countries and medications taken before the window of our investigation (such as prior usage of steroids and anti-epilepsy or antipsychotic drugs) may have also contributed to differences in bacterial composition. Overall, our findings supported a potential link between a lower F/B ratio and the acute phase of this autoimmune disease.

From the LEfSe analysis, the Con and AE3 groups had distinct species of the order Clostridiales and the genus Bacteroides, respectively. Clostridia play a crucial role in gut homeostasis and host defense mechanisms against exogenous infections. Evidence of many beneficial functions of Bacteroides strains suggests their intervention capabilities in both the lipopolysaccharide-induced immune response and gut microbiome shift, even having potential as therapeutic probiotics to prevent inflammatory disorders.

We also discovered that the phylum Fusobacteria and genus Fusobacterium, which are known to contribute to the disease activity of MS and are associated with future relapse after adjusting for age and drug exposure, were prevalent in the AE1 group. Fusobacterium species are deemed pathobionts given their invasive nature and ability to translocate into the blood and contribute to systemic disease states.

Our work characterized the prominent pathogenic abundance of the genus Streptococcus and enrichment of the genus Parabacteroides in the fecal samples from the AE2 group. It is possible to speculate that during Streptococcus infection, antistreptococcal or anti-neuronal antibodies are generated by mediating the destruction of healthy neurons (via the NMDAR) through a molecular mimicry mechanism. Some studies have reported that Parabacteroides have immunoregulatory functions. And, greater abundance of the genus Parabacteroides has been found in mice exposed to social stressors and related to abnormalities of depression symptoms and inflammatory cytokines in human. Therefore, we presumed that patients with recurrence enrich the genus Parabacteroides might correlate with behavior impairments.

To our knowledge, this is the first report on the analysis of fecal microbiota of anti-NMDAR encephalitis patients differing from healthy controls. Our investigation of the gut microbiota in patients with anti-NMDAR encephalitis provides initial insights into the association between the fecal microbiome and this antibody-mediated disease. An altered microbiome due to the presence of disease would contribute to future prognosis and recurrence in some individuals. Nonetheless, our study has certain limitations. First, we cannot simply assign a direct link between the gut microbiome and the etiology of anti-NMDAR encephalitis. The gut microbiota is merely one of the several component risk factors that make people more susceptible to this disease. Conversely, fecal microbiota dysbiosis is likely a presentation of the illness. Second, we used strict exclusion criteria to minimize the effects of confounders and eliminate the potential influence of gastrointestinal condition factors. Medications taken before the window of our investigation (such as prior usage of steroids and anti-epilepsy or antipsychotic drug) may also influence gut microbiota composition. Furthermore, our study cohort is relatively small due to the rarity of the cases, and longitudinal collection of samples will be needed to investigate clinical characteristic and phenotype associations with altered fecal microbiota, such as cognitive function, residual symptoms, severity of disease, and efficacy in immunotherapy. Finally, although phylotype profiles of the fecal microbial were produced using hypervariable tag sequencing of the V4 region of the 16S rDNA gene, the use of shotgun metagenomic, metatranscriptomic, and metabolomic analyses might further reveal subtle network interference in fecal microbial genes, their expression, and the presence of microbial metabolites, which would elucidate the mechanism of antibody-mediated encephalitis disease occurrence and supplement the phylogenic and taxonomic data. Therefore, future studies are needed to shed light on how...
the sophisticated “gut-brain-axis” participates in the promotion of anti-NMDAR encephalitis.

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Author Contributions

Xue Gong designed and conceptualized the study, analyzed the data, and drafted the manuscript for intellectual content. Xu Liu, Chu Chen, Jing Fang Lin, and Ai Qing Li performed statistical analysis and interpreted the data. Dong Zhou and Zhen Hong designed, conceptualized, and revised the study.

Conflict of Interest

None of the authors have any conflict of interest to disclose.

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**Supporting Information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Appendix S1.** Details for DNA extraction, sequence, and data analysis.