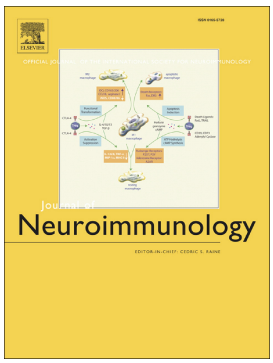


Dysbiosis of gut microbiota in patients with neuromyelitis optica spectrum disorders: A cross sectional study

Ziyan Shi, Yuhan Qiu, Jiancheng Wang, Yihuan Fang, Ying Zhang, Hongxi Chen, Qin Du, Zhengyang Zhao, Chao Yan, Mu Yang, Hongyu Zhou

PII: S0165-5728(19)30384-4

DOI: https://doi.org/10.1016/j.jneuroim.2019.577126

Reference: JNI 577126

To appear in: *Journal of Neuroimmunology*

Received date: 2 August 2019

Revised date: 8 December 2019

Accepted date: 9 December 2019

Please cite this article as: Z. Shi, Y. Qiu, J. Wang, et al., Dysbiosis of gut microbiota in patients with neuromyelitis optica spectrum disorders: A cross sectional study, *Journal of Neuroimmunology* (2019), https://doi.org/10.1016/j.jneuroim.2019.577126

Dysbiosis of gut microbiota in patients with neuromyelitis optica spectrum disorders: a cross sectional study

Ziyan Shi[1#], Yuhan Qiu[1#], Jiancheng Wang[1], Yihuan Fang[2], Ying Zhang[1], Hongxi Chen[1], Qin Du[1], Zhengyang Zhao[1], Chao Yan[1], Mu Yang[3] and Hongyu Zhou[1*]

[1]Department of Neurology, West China Hospital, Sichuan University, Chengdu, China

[2]Department of Biology, University of California, San Diego, La Jolla, California, United States of America

[3]Translational Center for Oncoimmunology, Sichuan Cancer Hospital and Research Institute, Sichuan Cancer Center, Chengdu, China

[#]Ziyan Shi and Yuhan Qiu contributed equally to this work.

*Correspondence to: Hongyu Zhou, PhD, MD (zhouhy@suc.edu.cn). Department of Neurology, West China Hospital, Sichuan University, #37 Guo Xuexiang, Chengdu 610041, China. Tel: + 86 28 85423550; Fax: + 86 28 85423550

Abstract

Background

Accumulating evidence points to an association of alternations in the gut microbiota with health and disease, including the development of neurological diseases. However, there are relatively scarce studies of the role of the gut microbiota in neuromyelitis optica spectrum disorders (NMOSD). Therefore, the aim of the present study was to evaluate the differences in the intestinal microbiota composition between patients with NMOSD and healthy control subjects.

Methods

This was a cross-sectional study. Stool samples were obtained from 20 patients with NMOSD and 20 healthy family members of the patients as controls (HC). The bacterial 16S rRNA gene amplification sequencing (V3-V4 region) was used to detect the composition and structure of the intestinal microbiota community in the two groups.

Results

The gut microbiota compositions clearly differed between the NMOSD and HC groups, although there was no significant difference in the overall microbial community structure. In detail, patients with NMOSD had an increased abundance of the pathogenic genera *Flavonifractor* (P = 0.004) and *Streptococcus* (P = 0.007) compared with the HC. In addition, several intestinal commensal bacteria were

detected at significantly lower abundance in the NMOSD patients compared to the controls, including *Faecalibacterium*, *Lachnospiracea_incertae_sedis*, *Prevotella*, *Blautia*, *Roseburia*, *Romboutsia*, *Coprococcus*, and *Fusicatenibacter* (all $P < 0.05$). ROC curve analysis suggested that the abundance of microbiota has predictive power to distinguish NMOSD from controls (area under the curve = 0.9275, 95% confidence interval: 0.849–1). Functional analysis further indicated that the gut microbiome of NMOSD patients is associated with three significantly downregulated metabolic pathways: "Photosynthesis" ($P < 0.001$), "Photosynthesis proteins" ($P < 0.001$), and "Thiamine metabolism" ($P = 0.007$). These differences remained significant even after correction for multiple comparisons (all $P_{FDR} < 0.05$).

Conclusion

Our results reveal the dysbiosis of intestinal bacteria and regarding metabolic abnormalities in patients with NMOSD. Further studies are warranted to elucidate the potential mechanism by which dysbiosis of microbiota contributes to the onset and progression of NMOSD.

Introduction

Neuromyelitis optica spectrum disorders (NMOSD) represent a group of autoimmune inflammatory demyelinating diseases of the central nervous system (CNS) characterized by acute myelitis, optic neuritis, and area postrema syndrome, which usually result in severe motor disability and visual impairment (Wingerchuk et al., 2015). The discovery of serum immunoglobulin G (IgG) antibodies against the astrocyte aquaporin-4 (AQP4) in most patients with NMOSD along with further evidence demonstrating the involvement of AQP4-IgG in the pathogenesis of NMOSD have contributed great insight into this disease (Lennon et al., 2004; Papadopoulos et al., 2012; Waters et al., 2012). Although numerous studies have demonstrated the pivotal roles of AQP4-IgG in NMOSD, the initial pathophysiological trigger leading to disease onset that results in antibody production remains unclear.

Previous studies have suggested that environmental factors play a key role in the pathogenesis of NMOSD (Eskandarieh et al., 2018). Emerging evidence indicates that disruption of the gut microbiota is associated with the risk and progression of neurological and autoimmune diseases (Tremlett et al., 2017a). Along these lines, an antibody response against gastrointestinal antigens has been observed in patients with demyelinating diseases of the CNS, especially for those with AQP4-seropositive myelitis (11). Interestingly, *Escherichia coli*-stimulated peripheral blood mononuclear cells from patients with neuromyelitis optica had elevated levels of interleukin (IL)-1β,

IL-6, and IL-17, released by CD4 (+) T helper cells, and these levels were positively related with the disability scores of the patients (Barros et al., 2013). Recently, two studies reported that gastrointestinal microbiota alternations were associated with the development of NMOSD (Cree et al., 2016; Gong et al., 2018), although the results were inconsistent. Cree et al. (2016) first reported that *Clostridium perfringens* was overabundant in NMOSD patients compared to controls, and shared a highly homologous sequence with the pathogenic epitope of $AQP4_{63-76}$. Subsequently, another study from a Chinese cohort demonstrated the overgrowth of *Streptococcus* in NMOSD patients (Gong et al., 2018). These somewhat inconsistent findings might be caused by the different genetic backgrounds and living environments of the patients; thus, more studies including populations from different regions are required to clarify the specific associations between the gastrointestinal microbiota and NMOSD. Toward this end, we performed a case-control study to evaluate the differences in the structure and composition of the intestinal microbiota between patients with NMOSD and healthy controls (HC).

Methods

Participants

According to the power and sample-size estimation for microbiome studies based on pairwise distances and permutational multivariate analysis of variance using distance matrices (PERMANOVA), 20 participants per group was an appropriate sample to obtain adequate statistical power for detecting variation in the community structure or

composition of gut microbiota between groups (Kelly et al., 2015). Specifically, five subjects per group offered statistical power of 52% with ω^2 of 0.036, 10 subjects per group provided 90% power with ω^2 of 0.036, 15 subjects per group provided 94% power with ω^2 of 0.014, and 20 subjects per group provided 96% power with ω^2 of 0.008 (Supplementary. **Fig. S1**). Therefore, 20 patients with NMOSD were consecutively enrolled in our study from West China Hospital, Sichuan University between November 2017 and December 2018. Each patient met the 2015 international diagnostic criteria for NMOSD and tested positive for serum AQP4-IgG using cell-based assays (Wingerchuk et al., 2015; Jarius et al., 2010). In addition, 20 paired healthy family members who had been living with the patients for at least 6 months and without NMOSD or other neurological disease were included as the HC group. This contributed to minimize any alternations in gut microbiota that could be caused by different dietary patterns. The exclusion criteria were active infection, gastrointestinal disorders, malignancy or other systemic diseases, or taking antibiotics. Notably, four patients were excluded from our study, including one patient was AQP4 antibodies negative, one had tuberculosis and received anti-TB treatment, one patient had hepatitis C and syphilis, and one took antibiotics during collecting stool.

Demographic information and clinical characteristics, including age, gender, body mass index, other autoantibodies, and treatments, were collected at enrollment. The study was approved by the Medical Ethics Committee of the West China Hospital, Sichuan University (No. 201828), and was performed in accordance with the ethical

standards of the Declaration of Helsinki. All participants provided informed consent prior to their inclusion in this study.

Sample collection and genomic DNA quality detection

A fresh stool sample was obtained from each participant immediately after defecation using a sterile stool collector and stored at −80°C until DNA extraction. The middle part of stool was taken to avoid contamination of urine and surrounding bacteria. The bacterial genomic DNA was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration and purity were detected on a Nanodrop 2000 spectrometer (Thermo Fisher Scientific, USA) and the integrity was confirmed by regular 0.8% agarose gel electrophoresis. The DNA quality requirements were a concentration ≥ 20 ng/μl, optical density ratio at 260 nm and 280 nm of 1.8–2.0, and total DNA of each sample ≥ 500 ng.

Polymerase chain reaction (PCR) amplification and sequencing of 16S rRNA

The bacterial genomic DNA was amplified with the 341 forward primer (5'-CCTACGGGNGGCWGCAG-3') and 805 reverse primer (5'-GACTACHVGGGTATCTAATCC-3') targeting the V3-V4 hypervariable region of the 16S rRNA gene. Each sample was independently amplified three times, and the PCR products from the same sample were pooled. The pooled PCR product was used as a template, and the index PCR was performed by using index primers for adding the Illumina index to the library. The amplification libraries were sequenced on an

Illumina MiSeq Benchtop Sequencer (Illumina, USA) for generating 2 × 250-bp pair-end reads, followed by bioinformatics analysis conducted at Genesky Biotechnologies Inc. (Shanghai, China).

Bioinformatics analysis

The raw reads were quality filtered to remove low-quality reads and merged by using FLASH (Magoc et al., 2011). The reads were filtered by Quantitative Insights Into Microbial Ecology (QIIME) quality filters using default settings for Illumina processing. After chimeras and singletons were detected and removed, operational taxonomic units (OTUs) were clustered on the basis of 97% similarity cutoff by UPARSE pipeline (Dong et al., 2017).

The microbial community richness and diversity were calculated by alpha-diversity indices, including observed species, Chao1, and ACE for microbial richness, and the Shannon index and Simpson index for microbial diversity. Beta-diversity was calculated to determine the difference in the shared microbiota between the NMOSD and HC groups, including principal coordinates analysis (PCoA) and ADONIS analysis that is based on PERMANOVA. Furthermore, the linear discriminant analysis (LDA) Effect Size (LEFSe) method was performed to identify species with significant differences in abundance between groups ($|LDA| > 2$ and $P < 0.05$) (Zhang et al., 2013). We further analyzed the microbiota at the community level to investigate differences in the microbial composition between the two groups and to identify taxa with significantly different abundance (relative abundance > 0.001 and P

< 0.05).

Functional predictions of the identified taxa with differential abundance based on the sequencing data were determined using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) and summarized as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Tankou et al., 2018).

Statistical analysis

Demographic and clinical data are presented as mean ± standard deviation or frequencies (number and percentages), which were analyzed using SPSS statistical software V25.0 (SPSS Inc., Chicago, IL, USA). The difference in the relative abundance of microbiota between patients with NMOSD and controls was evaluated by the Mann Whitney U test, and then subgroup analysis was conducted within the patient group according to no treatment and treatment with immunotherapy using GraphPad Prism 6.0 software. To evaluate the potential of microbial genera for distinguishing NMOSD patients from HC, we constructed the receiver-operating characteristic (ROC) curve using the R package pROC. The area under the ROC curve (AUC) value was estimated for each genus, and the candidate biomarkers with $P < 0.05$ were included in fitting logistic regression models to evaluate the accuracy in differentiating NMOSD from HC. The false discovery rate (FDR) was calculated for correction of multiple comparisons (Glickman et al., 2014; Li et al., 2019). A value of $P < 0.05$ was considered statistically significant in all analyses.

Results

A total of 40 stool samples were collected from the 20 patients with NMOSD and 20 healthy NMOSD-household controls. All samples were sequenced for 16S rRNA (V3-V4 region). The detailed demographic information and clinical characteristics of the participants are shown in Table 1. Among the patients with NMOSD, 13 received different immunotherapy treatments (eight patients treated with a corticosteroid and five patients treated with mycophenolate mofetil), whereas the other seven patients had not received any treatment.

Alpha diversity

The rarefaction curves of the samples indicated that the sequencing depth for 16S rRNA basically covered all of the species in the stool samples (**Fig. 1A**), and the species accumulation curve demonstrated that the collected samples were sufficient for the analysis (**Fig. 1B**). Comparison of alpha diversity was conducted to assess the overall difference in the microbial community structure between the NMOSD patients and HC, revealing no significant differences in microbial community richness (observed, Chao 1, and ACE indices) or diversity (Shannon index, Simpson index, and coverage) between patients with NMOSD and HC (**Fig. 1C**).

Beta diversity

The PCoA plot based on the Bray-Curtis distance showed that samples from the NMOSD and HC groups clustered separately (**Fig. 2A**). ADONIS confirmed that the

composition of microbiota in NMOSD patients significantly differed from that in the HC based on Bray-Curtis distance ($P = 1 \times 10^{-4}$, $R^2 = 0.070$; **Fig. 2B**). LDA was then performed to further identify the genera with significant differences between the two groups, revealing a total of 14 significant differences in taxonomic biomarkers at the genus level (LDA score > 3.0 and $P < 0.05$), including six enriched in the NMOSD group and eight enriched in the HC group (**Fig. 3**).

Community analysis

The results of community-level analysis for gut microbiota showed that Bacteroidetes, Firmicutes, and Proteobacteria were the most common bacteria at the phylum level in both the NMOSD and HC groups, accounting for more than 90% of the entire intestinal flora (**Fig. 4A**). In addition, *Bacteroides*, *Alistipes*, *Escherichia-Shigella*, *Faecherlibacterium*, and *Lachnospiracea_incertae_sedis* were the top five most abundant taxa at the genus level (**Fig. 4B**).

After excluding genera with relative abundance less than 0.001, we identified 10 genera with significantly different abundance between patients with NMOSD and HC. Overall, patients with NMOSD showed a significant increase in the relative abundance of *Flavonifractor* ($P = 0.004$) and *Streptococcus* ($P = 0.007$) compared with those of the controls. In contrast, several intestinal commensal bacteria showed remarkably lower abundance in the NMOSD group than in the HC group, including *Faecalibacterium*, *Lachnospiracea_incertae_sedis*, *Prevotella*, *Blautia*, *Roseburia*, *Romboutsia*, *Coprococcus*, and *Fusicatenibacter* (all $P < 0.05$; **Fig. 5A**), which was

consistent with the LDA results. However, there was no significant difference in the relative abundance of these genera between the NMSOD patients that received immunotherapy and the untreated patients ($P > 0.05$; **Fig. 5B**).

Given the difference in the sex ratio between the NMOSD and HC groups, we further compared the relative abundance of gut microbiota between females and males in the control group. However, we did not any significant find differences related to sex (Supplementary **Fig. S2**).

Potential of gut microbiota to differentiate NMOSD from HC

Based on the ROC curve, microbial genera that showed differential abundance between the patients and controls were then screened by univariate analysis, which identified *Faecalibacterium* as the most likely marker to distinguish between the groups, followed by *Lachnospiraceae_incertae_sedis, Blautia, Roseburia, Romboutsia,* and *Coprococcus* (**Table 2**). After combining the seven candidate markers by multivariate logistic regression analysis, the fitted ROC curve indicated that these candidate gut microbiota have potential to differentiate patients with NMOSD from HC (**Fig. 6**).

Functional prediction

A total of 20 gut microbe-related KEGG pathways were identified to be differentially enriched between the two groups, including eight enriched in the NMOSD group and 12 enriched in the HC group (**Fig. 7**). Among these pathways,

three metabolic pathways, including "Photosynthesis" ($P < 0.001$, $P_{FDR} = 0.007$), "Photosynthesis proteins" ($P < 0.001$, $P_{FDR} = 0.007$), and "Thiamine metabolism" ($P = 0.007$, $P_{FDR} = 0.041$), remained significantly reduced in patients with NMOSD after FDR correction. According to the KEGG pathway database, "Photosynthesis" and "Photosynthesis proteins" are classified as energy metabolism pathways, and "Thiamine metabolism" is involved in the metabolism of cofactors and vitamins, which is associated with thiamine pyrophosphokinase (TPK) deficiency disease.

Discussion

Using 16S rRNA sequencing, we identified clear dysbiosis of the gut microbiota in patients with NMOSD compared to their healthy family members that share a living environment, along with abnormal microbial-related gut metabolic pathways. The results of community analysis and beta diversity estimates showed remarkable differences in the bacterial composition between the two groups. In particular, compared with the HC group, two gut pathogenic genera showed increased abundance and seven commensal genera showed decreased abundance in NMOSD patients. We further demonstrated that these significantly different genera had potential as a combined biomarker for differentiating NMOSD from controls. In addition, functional prediction analysis revealed three significantly down-regulated gut metabolic pathways in NMOSD patients. Thus, our study provides evidence for alterations of intestinal microbiota contributing to the pathogenesis of NMOSD.

The interaction between gut microbiota and the immune system plays pivotal

roles in influencing the host immune response; thus, disturbance of the gut microbiota and dysfunction in immune tolerance are implicated in autoimmune diseases (Brown et al., 2019). In the past decade, the relationship between gut microbiota and autoimmune diseases has attracted extensive research attention, and numerous studies demonstrated that an imbalance in gut microbiota plays a role in the development of multiple autoimmune diseases such as systemic lupus erythematosus, primary Sjogren's syndrome, and rheumatoid arthritis (Maeda et al., 2016; Rosser et al., 2016; van der Meulen et al., 2019). In addition, recent studies revealed a relationship between gut microbiota with multiple sclerosis and NMOSD (Chen et al., 2016; Gong et al., 2018; Tankou et al., 2018; Tremlett et al., 2017b; Zeng et al., 2019).

In particular, we found that the relative abundance of the pathogenic genera *Flavonifractor* and *Streptococcus* was increased in patients with NMOSD. A previous study on NMSOD in a Chinese population also showed a relationship to *Streptococcus* (Gong et al., 2018). However, in contrast to our study, Cree et al., (2016) reported an overabundance of *Clostridium perfringens* in NMOSD patients, which might be explained by the genetic and environmental backgrounds of the Caucasian populations sampled, which were different from Asian populations. Notably, an increased abundance of *Streptococcus* was also observed to be associated with several other autoimmune diseases, including Crohn's disease, ulcerative colitis, and multiple sclerosis, suggesting a common dysbiosis among immune-mediated inflammatory diseases (Forbes et al., 2018). In addition, overrepresented *Streptococcus* was also reported to be associated with the reduction of short-chain

fatty acids (SCFAs) in NMOSD patients, thereby increasing the activity of CD4 (+) T cells to promote inflammatory reactions (Gong et al., 2018; Luu et al., 2019). Moreover, the increased abundance of *Streptococcus* could promote inflammation by increasing levels of the inflammatory cytokines tumor necrosis factor-alpha, IL-6, and interferon-gamma (Jiang et al., 2015).

This is the first report to highlight an increase in the abundance of *Flavonifractor* in the intestinal environment of patients with NMOSD, although the specific mechanism remains to be further clarified. *Flavonifractor* abundance has been reported to be positively correlated with the excretion of multiple pro-inflammatory cytokines such as C5a, IL-6, IL-8, IL-7, IL-1β, IL17A, and IL-21(He et al., 2016a; He et al., 2016b; Huang et al., 2019). Collectively, our results and previous studies indicate that excessive growth of *Streptococcus* and *Flavonifractor* might contribute to the pathogenesis of NMOSD by inducing inflammatory cells and cytokines.

Compounding these effects, our results indicated marked reduction in the abundance of many intestinal commensal bacteria in patients with NMOSD, including *Faecalibacterium*, *Lachnospiracea_incertae_sedis*, *Prevotella*, *Blautia*, *Roseburia*, *Romboutsia*, *Coprococcus*, and *Fusicatenibacter*. As the most abundant bacterium in the gut microbiota of healthy adults, *Faecalibacterium* contributes to suppressing the inflammation response by facilitating the production of SCFAs and the anti-inflammatory cytokine IL-10, and also helps to enhance intestinal barrier integrity to prevent pathogen colonization (Miquel et al., 2013; Sokol et al., 2008).

Moreover, a decrease in the abundance of *Faecalibacterium* has also been observed in patients with other autoimmune diseases such as Graves' disease, type 1 diabetes, systemic sclerosis, as well as multiple sclerosis (Bellocchi et al., 2018; Ishaq et al., 2018; Leiva-Gea et al., 2018; Swidsinski et al., 2017; Zeng et al., 2019). *Prevotella*, as another common human gut commensal bacterium, also showed significantly decreased abundance in patients with NMOSD, which was consistent with findings in patients with inflammatory diseases (Zeng et al., 2019). Recent studies have indicated that *Prevotella* could suppress Th17 inflammatory responses and increase the level of IL-10 secretion, along with inducing multiple cell types with inhibitory inflammatory activity (Mangalam et al., 2017; Marietta et al., 2016). *Roseburia* is a newly identified health-associated bacteria, and is considered to be a major butyrate and propionate producer (Lordan et al., 2019). An increased abundance of *Lachnospiracea_incertae_sedis* and *Coprococcus* was reported to be negatively associated with IL-17A expression levels (Huang et al., 2019), whereas *Fusicatenibacter* was found to be positively correlated with SCFAs production (Jin et al., 2019). Collectively, these results implied that an imbalance of gut microbiota, including a decrease in the gut commensal bacteria and increase in opportunistic pathogenic bacteria, might be involved in the development of NMOSD by regulating multiple inflammatory cytokines and anti-inflammatory metabolites. Thus, further detailed investigation is needed to determine whether these differentially abundant gut bacteria are risk factors of NMOSD or rather a consequence of the disease course.

Regardless of the causal relationship, the ROC curve analysis further indicated that

these differentially abundant microbiota, especially *Faecalibacterium*, could serve as disease biomarkers by significantly distinguishing patients with NMOSD from HC, although further studies are required for validation. According to previous reports, immunotherapies have potential to enrich the population of opportunistic pathogens while decreasing probiotic strains (Li et al., 2019); however, no significant difference was found between patients treated with or without immunotherapies in the present study. This conflicting result may be related to the small number of untreated patients in our sample; thus, more patients should be included for subgroup analysis in future studies.

Furthermore, functional comparisons between the two groups revealed downregulated metabolism pathways of energy and thiamine (vitamin B1) in the NMOSD patients in comparison to the controls. Thiamine deficiency has been reported to increase the Th1 and Th17 cell subpopulations (Ji et al., 2014). Zhang et al., (2019) further demonstrated that perturbed thiamine metabolism was related to a significant reduction of TPP, which plays important roles in the persistence of microbes through vitamin B metabolism. Notably, low vitamin B12 levels were previously found in the serum of patients with AQP4-IgG sera-positive NMOSD (Jarius et al., 2012), and decreased levels of vitamin D were also detected in NMOSD patients, which could be related to the decrease in photosynthesis proteins (Gao et al., 2019).

A previous study showed that alternations in gut microbiota caused disruption

of the tight junctions in epithelial cells, an important component of the intestinal barrier, leading to intestinal permeability changes that promoted the inflammatory response in the gut and neurological system via the gut-brain axis (Buscarinu et al., 2019). In addition, vitamin D deficiency was suggested to reduce calcium absorption and increase intestinal permeability (Ghareghani et al., 2018). Thus, gut microbiota dysfunction causes overall gut barrier breakdown, providing the opportunity for more endotoxins from the gut microbiota to enter the peripheral blood that will promote the development of neuroinflammation (Buscarinu et al., 2019; Ghareghani et al., 2018). Nevertheless, the detailed mechanisms by which changes in the gut microbiota affect the pathogenesis of NMOSD remain to be elucidated.

There are some limitations of our study worthy of mention. First, we included healthy family members of the patients as the control group so as to minimize the influence of different dietary patterns between the HC and NMOSD patients. Unfortunately, it was difficult to balance the sex ratio between groups, since the majority of the patients with NMOSD were females and half of the controls were their male spouses. Therefore, we further compared the composition of gut microbiota between females and males in the control group, and found no significant difference. Second, despite attempting to control for dietary variation with this design, detailed dietary information might be useful to understand the influence of food intake of each participant on the results. Furthermore, 13 patients treated with immunotherapies (mycophenolate mofetil and/or steroids) were included in this study, which might have affected the gut microbiota compositions, although we did not find a significant

difference between treated or untreated patients in the subgroup analysis. Finally, this was a cross-sectional study, and thus a longitudinal study with more samples is required to confirm our results.

In conclusion, this study revealed that dysbiosis of the gut microbiota was present in patients with NMOSD, including excessively abundant pathogenic bacteria and significantly decreased abundance of gut commensal bacteria, as well as abnormal metabolism-related pathways. These findings further point to a role of the gut microbiome in mediating the pathogenesis of autoimmune diseases such as NMOSD, although the mechanism by which gut microbiota alternations contribute to the onset and progression of NMOSD remain to be clarified.

Acknowledgments

We are grateful to Dr. Li Caihua, Dr. Weiming Xiong, and Dr. Yu Tao at Genesky Biotechnologies Inc. (Shanghai, China) and Dr. Junli Gong (School of Life Sciences and Biomedical Center, Sun Yat-Sen University, Guangzhou, China) for their support with the bioinformatics analysis for microbiology. We also appreciate all of the participants of this study for their support.

Declarations of interest

None

Funding

This work was funded by the National Key R&D Program of China (Grant Nos. 2017YFC0907704 and 2017YFC0907705), and the Department of Science and Technology of Sichuan Province (Grant No. 2018SZ0388).

Author Contributions

Dr. Hongyu Zhou and Ziyan Shi had full access to all of the data in this study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Ziyan Shi and Hongyu Zhou. Acquisition, analysis, or interpretation of data: All authors. Drafting of the manuscript: Ziyan Shi and Yuhan Qiu. Critical revision of the manuscript for important intellectual content: Ziyan Shi, Yuhan Qiu, and Hongyu Zhou. Statistical analysis: Ziyan Shi and Yuhan Qiu. Obtained funding: Hongyu Zhou. Administrative, technical, or material support: Hongyu Zhou. Study supervision: Hongyu Zhou.

References:

Banati, M., Csecsei, P., Koszegi, E. et al., 2013. Antibody response against gastrointestinal antigens in demyelinating diseases of the central nervous system. Eur J Neurol 20, 1492-1495.

Barros, P.O., Linhares, U.C., Teixeira, B. et al., 2013. High in vitro immune reactivity to Escherichia coli in neuromyelitis optica patients is correlated with both neurological disabilities and elevated plasma lipopolysaccharide levels. Hum Immunol 74, 1080-1087.

Bellocchi, C., Volkmann, E.R., 2018. Update on the Gastrointestinal Microbiome in Systemic Sclerosis. Curr Rheumatol Rep 20, 49.

Brown, E.M., Kenny, D.J., Xavier, R.J., 2019. Gut Microbiota Regulation of T Cells During Inflammation and Autoimmunity. Annu Rev Immunol 37, 599-624.

Buscarinu, M.C., Fornasiero, A., Romano, S. et al., 2019. The Contribution of Gut Barrier Changes to Multiple Sclerosis Pathophysiology. Front Immunol 10, 1916.

Chen, J., Chia, N., Kalari, K.R. et al., 2016. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. Sci Rep 6, 28484.

Cree, B.A., Spencer, C.M., Varrin-Doyer, M., Baranzini, S.E., Zamvil, S.S., 2016. Gut microbiome analysis in neuromyelitis optica reveals overabundance of Clostridium perfringens. Ann Neurol 80, 443-447.

Dong, L.N., Wang, J.P., Liu, P. et al., 2017. Faecal and mucosal microbiota in patients with functional gastrointestinal disorders: Correlation with toll-like receptor 2/toll-like receptor 4 expression. World J Gastroenterol 23, 6655-6673.

Eskandarieh, S., Nedjat, S., Abdollahpour, I. et al., 2018. Environmental risk factors in neuromyelitis optica spectrum disorder: a case-control study. Acta Neurol Belg 118, 277-287.

Forbes, J.D., Chen, C.Y., Knox, N.C. et al., 2018. A comparative study of the gut microbiota in immune-mediated inflammatory diseases-does a common dysbiosis exist? Microbiome 6, 221.

Gao, M., Yao, X., Ding, J. et al., 2019. Low levels of vitamin D and the relationship between vitamin D and Th2 axis-related cytokines in neuromyelitis optica spectrum disorders. J Clin Neurosci 61, 22-27.

Ghareghani, M., Reiter, R.J., Zibara, K., Farhadi, N., 2018. Latitude, Vitamin D, Melatonin, and Gut Microbiota Act in Concert to Initiate Multiple Sclerosis: A New Mechanistic Pathway. Front Immunol 9, 2484.

Glickman, M.E., Rao, S.R., Schultz, M.R., 2014. False discovery rate control is a recommended alternative to Bonferroni-type adjustments in health studies. J Clin Epidemiol 67, 850-857.

Gong, J., Qiu, W., Zeng, Q. et al., 2018. Lack of short-chain fatty acids and overgrowth of opportunistic pathogens define dysbiosis of neuromyelitis optica spectrum disorders: A Chinese pilot study. Mult Scler, 912916228.

He, Z., Shao, T., Li, H., Xie, Z., Wen, C., 2016a. Alterations of the gut microbiome in Chinese patients with systemic lupus erythematosus. Gut Pathog 8, 64.

He, Z., Shao, T., Li, H., Xie, Z., Wen, C., 2016b. Alterations of the gut microbiome in Chinese patients with systemic lupus erythematosus. Gut Pathog 8, 64.

Huang, S., Mao, J., Zhou, L., Xiong, X., Deng, Y., 2019. The imbalance of gut microbiota and its correlation with plasma inflammatory cytokines in pemphigus vulgaris patients. Scand J Immunol, e12799.

Ishaq, H.M., Mohammad, I.S., Shahzad, M. et al., 2018. Molecular Alteration Analysis of Human Gut Microbial Composition in Graves' disease Patients. Int J Biol Sci 14, 1558-1570.

Jarius, S., Paul, F., Ruprecht, K., Wildemann, B., 2012. Low vitamin B12 levels and gastric parietal cell antibodies in patients with aquaporin-4 antibody-positive neuromyelitis optica spectrum disorders. J Neurol 259, 2743-2745.

Jarius, S., Probst, C., Borowski, K. et al., 2010. Standardized method for the detection of antibodies to aquaporin-4 based on a highly sensitive immunofluorescence assay employing recombinant target antigen. J Neurol Sci 291, 52-56.

Ji, Z., Fan, Z., Zhang, Y. et al., 2014. Thiamine deficiency promotes T cell infiltration in experimental autoimmune encephalomyelitis: the involvement of CCL2. J Immunol 193, 2157-2167.

Jiang, W., Wu, N., Wang, X. et al., 2015. Dysbiosis gut microbiota associated with inflammation and impaired mucosal immune function in intestine of humans with non-alcoholic fatty liver disease. Sci Rep 5, 8096.

Jin, M., Kalainy, S., Baskota, N. et al., 2019. Faecal microbiota from patients with cirrhosis has a low capacity to ferment non-digestible carbohydrates into short-chain fatty acids. Liver Int.

Kelly, B.J., Gross, R., Bittinger, K. et al., 2015. Power and sample-size estimation for microbiome studies using pairwise distances and PERMANOVA. Bioinformatics 31, 2461-2468.

Leiva-Gea, I., Sanchez-Alcoholado, L., Martin-Tejedor, B. et al., 2018. Gut Microbiota Differs in Composition and Functionality Between Children With Type 1 Diabetes and MODY2 and Healthy Control Subjects: A Case-Control Study. Diabetes Care 41, 2385-2395.

Lennon, V.A., Wingerchuk, D.M., Kryzer, T.J. et al., 2004. A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. Lancet 364, 2106-2112.

Li, Y., Wang, H., Li, X. et al., 2019. Disordered intestinal microbes are associated with the activity of Systemic Lupus Erythematosus. Clin Sci (Lond).

Lordan, C., Thapa, D., Ross, R.P., Cotter, P.D., 2019. Potential for enriching next-generation health-promoting gut bacteria through prebiotics and other dietary components. Gut Microbes, 1-20.

Luu, M., Pautz, S., Kohl, V. et al., 2019. The short-chain fatty acid pentanoate suppresses autoimmunity by modulating the metabolic-epigenetic crosstalk in lymphocytes. Nat Commun 10, 760.

Maeda, Y., Kurakawa, T., Umemoto, E. et al., 2016. Dysbiosis Contributes to Arthritis Development via Activation of Autoreactive T Cells in the Intestine. Arthritis Rheumatol 68, 2646-2661.

Magoc, T., Salzberg, S.L., 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27, 2957-2963.

Mangalam, A., Shahi, S.K., Luckey, D. et al., 2017. Human Gut-Derived Commensal Bacteria Suppress CNS Inflammatory and Demyelinating Disease. Cell Rep 20, 1269-1277.

Marietta, E.V., Murray, J.A., Luckey, D.H. et al., 2016. Suppression of Inflammatory Arthritis by Human Gut-Derived Prevotella histicola in Humanized Mice. Arthritis Rheumatol 68, 2878-2888.

Miquel, S., Martin, R., Rossi, O. et al., 2013. Faecalibacterium prausnitzii and human intestinal health. Curr Opin Microbiol 16, 255-261.

Papadopoulos, M.C., Verkman, A.S., 2012. Aquaporin 4 and neuromyelitis optica. Lancet Neurol 11, 535-544.

Rosser, E.C., Mauri, C., 2016. A clinical update on the significance of the gut microbiota in systemic autoimmunity. J Autoimmun 74, 85-93.

Sokol, H., Pigneur, B., Watterlot, L. et al., 2008. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A 105, 16731-16736.

Swidsinski, A., Dorffel, Y., Loening-Baucke, V. et al., 2017. Reduced Mass and Diversity of the Colonic Microbiome in Patients with Multiple Sclerosis and Their Improvement with Ketogenic Diet. Front Microbiol 8, 1141.

Tankou, S.K., Regev, K., Healy, B.C. et al., 2018. A probiotic modulates the microbiome and immunity in multiple sclerosis. Ann Neurol 83, 1147-1161.

Tremlett, H., Bauer, K.C., Appel-Cresswell, S., Finlay, B.B., Waubant, E., 2017a. The gut microbiome

in human neurological disease: A review. Ann Neurol 81, 369-382.

Tremlett, H., Bauer, K.C., Appel-Cresswell, S., Finlay, B.B., Waubant, E., 2017b. The gut microbiome in human neurological disease: A review. Ann Neurol 81, 369-382.

van der Meulen, T.A., Harmsen, H., Vila, A.V. et al., 2019. Shared gut, but distinct oral microbiota composition in primary Sjogren's syndrome and systemic lupus erythematosus. J Autoimmun 97, 77-87.

Waters, P.J., McKeon, A., Leite, M.I. et al., 2012. Serologic diagnosis of NMO: a multicenter comparison of aquaporin-4-IgG assays. Neurology 78, 665-671, 669.

Wingerchuk, D.M., Banwell, B., Bennett, J.L. et al., 2015. International consensus diagnostic criteria for neuromyelitis optica spectrum disorders. Neurology 85, 177-189.

Zeng, Q., Junli, G., Liu, X. et al., 2019. Gut dysbiosis and lack of short chain fatty acids in a Chinese cohort of patients with multiple sclerosis. Neurochem Int 129, 104468.

Zhang, C., Li, S., Yang, L. et al., 2013. Structural modulation of gut microbiota in life-long calorie-restricted mice. Nat Commun 4, 2163.

Zhang, Q., Yin, X., Wang, H. et al., 2019. Fecal Metabolomics and Potential Biomarkers for Systemic Lupus Erythematosus. Front Immunol 10, 976.

Figure legends

Fig. 1 Alpha diversity analysis of gut microbiota diversity and richness between patients with NMOSD and HC. (**A**) Sample rarefaction curves and (**B**) species accumulation curve. (**C**) No significant differences in microbial community richness or diversity between patients with NMOSD and HC. NMOSD, neuromyelitis optica spectrum disorders; HC, healthy family controls.

Fig. 2 Beta diversity analysis of bacterial composition between the NMOSD and HC groups. (A) Principal coordinates analysis (PCoA) plot based on the Bray-Curtis distance showing that samples from the NMOSD patients and HC clustered separately. Each point in the figure represents a sample and the distance between any pair of points represents the similarity of samples. (**B)** ADONIS analysis based on Bray-Curtis distance showing that the compositions of gut microbiota in NMOSD patients significantly differed from those of HC. NMOSD, neuromyelitis optica spectrum disorders; HC, healthy family controls.

Fig. 3 Abundance of gut microbiota between the NMOSD and HC groups. LDA scores were calculated by LEFSe analysis to identify taxa with significantly different abundance between groups (taxa with an LDA score > 3 and $P < 0.05$). NMOSD, neuromyelitis optica spectrum disorders; HC, healthy family controls; LDA, linear discriminant analysis; LEFSe, linear discriminant analysis Effect Size.

Fig. 4 Community analysis for gut microbiota between the NMOSD and HC groups. Community structure of intestinal microorganisms between the two groups at the

phylum level (**A**) and at the genus level (**B**). NMOSD, neuromyelitis optica spectrum disorders; HC, healthy family controls.

Fig. 5 Differentially abundant gut microbiota between the NMOSD and HC groups. (**A**) Analysis of relative abundance obtained ten genera with significantly different abundance between the NMOSD and HC groups, including two enriched in the NMSOD patients and eight enriched in HC (relative abundance > 0.001 and $P < 0.05$). (B) Difference in the abundance of genera in treated or untreated NMOSD patients ($P > 0.05$). NMOSD, neuromyelitis optica spectrum disorders; HC, healthy family controls. $^{*}P < 0.05$, $^{**}P < 0.01$.

Fig. 6 ROC curves to evaluate the potential of different gut microbiota for differentiating NMOSD from HC. Genera with significantly different abundance between groups were screened, and six of them (AUC with $P < 0.05$) were finally fitted in a logistic regression model to evaluate the accuracy for NMOSD diagnosis. ROC, receiver operating characteristic; AUC, area under the curve; NMOSD, neuromyelitis optica spectrum disorders; HC, healthy family controls.

Fig. 7 Functional prediction analysis of the gut microbiota in the NMOSD and HC groups. A total of 20 significantly different KEGG pathways were identified, including eight enriched in the NMOSD group and 12 enriched in the HC group. Three decreased pathways in NMOSD patients, including "Photosynthesis", "Photosynthesis proteins", and "Thiamine metabolism", remained significantly different after correcting for multiple comparisons with FDR < 0.05. NMOSD,

neuromyelitis optica spectrum disorders; HC, healthy family controls; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#}P_{FDR} < 0.05$.

Table 1 Demographic and clinical characteristics of patients with NMOSD and controls

	NMOSD	HC[a]	P values
Stool samples, n	20	20	
Female, n (%)	18 (90%)	9(45%)	0.01
Age, years, mean ± SD	48.15±12.37	47.65±12.09	0.90
BMI, kg/m^2, mean ± SD	22.40±3.03	22.97±2.57	0.57
Age at onset, years, mean ± SD	43.4±13.96	NA	NA
Disease durations, years, mean ± SD	3.95±4.48	NA	NA
Serum AQP4-IgG, n (%)	20 (100%)	NA	NA
Treatments, n (%)	13 (65)	0 (0)	NA
Corticosteroids	8 (40)	0 (0)	NA
Mycophenolate mofetil	5 (25)	0 (0)	NA
Autoantibodies, n (%)	5 (20)	0 (0)	NA
SSA-Ab	3 (15)	0 (0)	NA
SSB-Ab	1 (5)	0 (0)	NA
TPO-Ab	1 (5)	0 (0)	NA

SD, standard deviation; BMI, body mass index; NMOSD, neuromyelitis optica spectrum disorders; [a]HC, healthy family controls, including nine husbands, three mothers, three daughters, three sisters, one father, and one son; Ab, antibody; NA, not applicable.

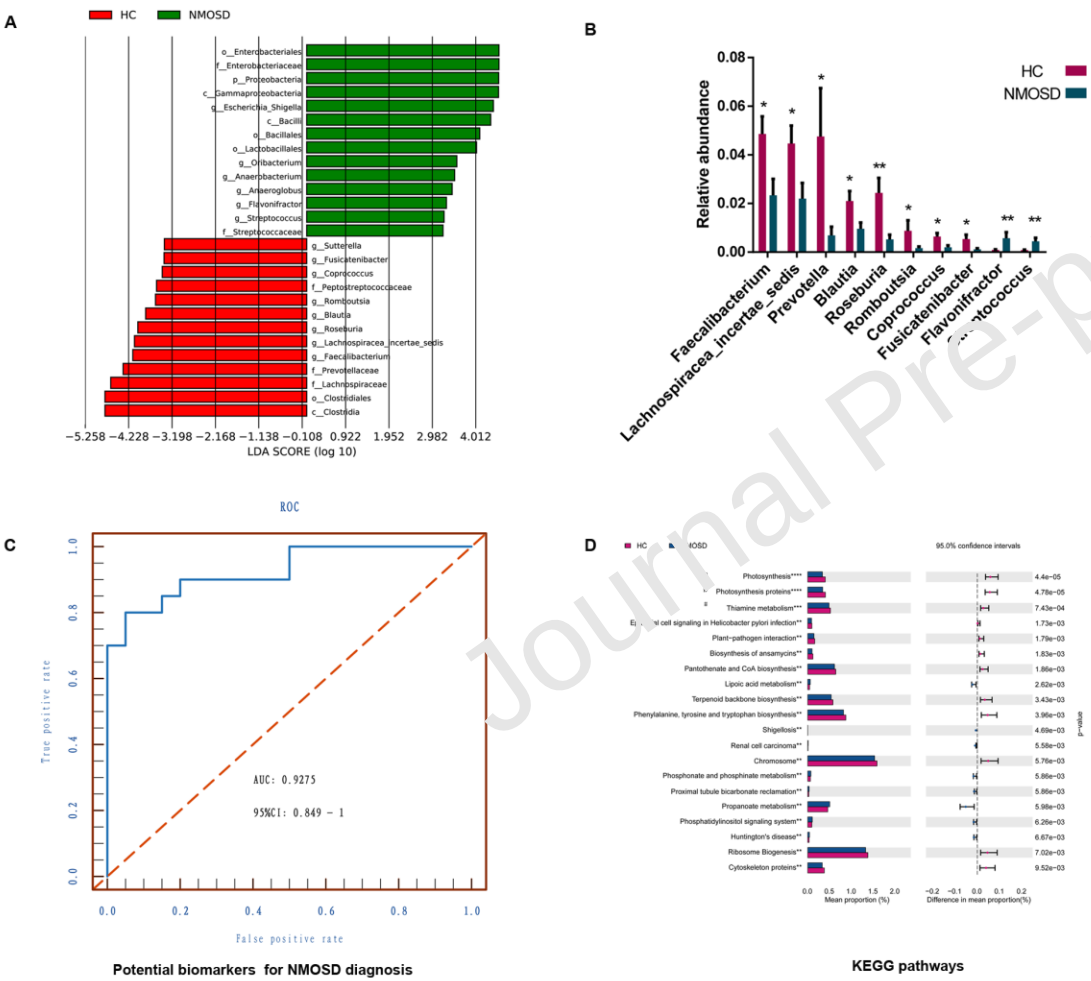
Table S1 ROC anaylsis for each differently abundant genera

	z value	specificity	sensitivity	accuracy	AUC	P-values
Romboutsia	-1.98	0.75	0.80	0.78	0.81	0.05*
Roseburia	-2.39	0.65	0.80	0.73	0.79	0.02*
Lachnospiracea_incertae_sedis	-2.11	0.90	0.50	0.70	0.73	0.04*
Blautia	-2.15	0.55	0.85	0.70	0.69	0.03*
Coprococcus	-2.19	0.80	0.75	0.78	0.76	0.03*
Faecalibacterium	-2.26	0.90	0.70	0.80	0.77	0.02*
Flavonifractor	2.02	0.70	0.80	0.75	0.75	0.04*
Fusicatenibacter	-1.71	0.90	0.55	0.73	0.72	0.09

Prevotella	-1.51	0.25	1.00	0.63	0.58	0.13
Streptococcus	1.60	0.75	0.65	0.70	0.71	0.11

AUC, area under the curve; * indicates P value < 0.05.

Different abundance of gut microbiota and metanolism between patients with NMOSD and healthy controls



Potential biomarkers for NMOSD diagnosis

KEGG pathways

1. significant different compositions of gut microbiota between NMOSD patients and healthy controls
2. different abundance of genera had potentials to distinguish between patients with NMOSD and healthy controls
3. three KEGG metabolic pathways, namely “Photosynthesis”, “Photosynthesis proteins”, and “Thiamine metabolism” pathways were downregulated in NMOSD patients.