Materials and Methods

Research Participants and Sample Collection

A cohort consisting of 64 Chinese NMOSD patients (all with a relapsing-remitting form fulfilling the criteria of Wingerchuk[1], seropositive for AQP4-IgG, were consecutively recruited from Multiple Sclerosis Centre, the Department of Neurology, and 36 healthy controls (HC) was recruited from the Health Examination Center of the Third Affiliated Hospital, Sun Yat-sen University from November 2016 to May 2017. The flowchart of enrollment is shown in Figure S1. The participants in the patient group had different degrees of disability [Expanded Disability Status Scale (EDSS) score from 0 to 6]. Of the 84 NMOSD patients, 66 had been treated by immunosuppressants, including 27 treated by mycophenolate mofetil (mmf), 32 treated by azathioprine and 7 treated by cyclophosphamide, methotrexate or rituximab. The control group matched for body mass index (BMI), age and gender. Subjects consuming probiotics or antibiotics within one month before admission were excluded. The study was approved by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University and informed consent was obtained from all participants.

Fecal sample aliquots from the participants were frozen at -80°C immediately after collection. Written informed consent and questionnaires addressing previous and current diseases, lifestyles and medication were obtained from all subjects included (Table S1)

DNA Extraction, PCR amplification, and pyrosequencing

The bacterial DNA was extracted from fecal samples with a QIAamp DNA stool Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. The genomic DNA isolated from the clinical samples was amplified using barcoded primers that amplified the V3-V4 hypervariable region of the 16S rRNA gene (~500 bp long): primer forward: 5'-

ACTCCTACGGGAGGCAGCA-3', and reverse: 5'-GGACTACHVGGGTWTCTAAT-3'. The oligonucleotide sequence barcodes are fused to the forward primer. The PCR reaction mixture (20 μL volume) contained: 10 μL of 2×Phanta Max Master Mix (Vazyme Biotech Co., Ltd), 1 μL of each primer (10 μmol/L), 20 ng of the template DNA, and ddH_2O to make up the volume. The PCR thermal cycling scheme was set as follows: initial denaturation at 95℃ for 3 min, 30 cycles of denaturation at 95℃ for 15 s, annealing at 55℃ for 15 s, and extension at 72℃ for 30 s, followed by a final extension period at 72℃ for 5 min. PCR products were examined on a 2 % (w/v) agarose gel and further purified using E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek). Purified amplicons were pooled in equimolar amounts for library preparation. Construction of sequencing libraries and paired-end sequencing (2×250bp) was performed on an Illumina MiSeq platform at Biomarker Technologies Co, Ltd. (Beijing, China) according to standard protocols. The raw reads were deposited in the Sequence Read Archive database, and the accession numbers ranged from SRR6394750 to SRR6394849.

Sequence assembly and analysis

Custom Perl and Bash scripts were used to demultiplex the reads and assign barcoded reads to individual samples. Reads were kept only when the sequence included a perfect match to the barcode and the V3-V4 16S rRNA gene primers and were within the length expected for the V3-V4 variable region. The raw data were merged using FLASH[2]. Sequences were quality filtered using Trimmomatic[3], and Chimera sequences were removed using UCHIME algorithm[4].

The remaining effective sequences were binned into OTUs using USEARCH software with a cutoff of 97% identity[5]. For each OTU, reads with the highest frequencies were chosen as representative sequences. Taxonomic assignments for each OTU were made by extracting the best hits from the SILVA database[6] and then verified by using the Ribosomal Database Project

(RDP) SeqMatch tool (http://rdp.cme.msu.edu) and using BLAST against the Greengenes databases (http://greengenes.lbl.gov). Taxonomy was assigned based on hits with the highest percentage identities and coverage. When multiple hit fulfilled this criterion, classification was reassigned to a higher common taxonomy. QIIME pipeline[7] was used for (i) alpha diversity analysis, (ii) summarizing OTUs by different taxonomic levels, and (iii) PCoA analysis. Bar plots were created with the help of origin 8.0, and the box plots were created with the help of Graphpad Prism 6.0 software.

Determination of SCFAs

Fecal SCFAs were analyzed by Shenzhen Academy of Metrology & Quality inspection. Feces (~100 mg) was thawed and suspended in 1 mL of NaOH (0.5 mol/L), and homogenized (Ultra Turrax T 25, Sweden) for about 10 min, and then centrifuge at 13000g for 5 min. Filter the supernatant through a 0.22 μm filter before GC analyses. Acetate, propionate and butyrate standards were purchased from Sigma-Aldrich (Bornem, Belgium). All standards were of analytical quality (at least 99% purity). Chromatographic analysis was carried out using an Agilent 7890B GC system equipped with a flame ionization detector (FID) and an Agilent 7693 automatic liquid sampler (Agilent, USA). A fused-silica capillary column with a polyethylene glycol phase (HP-INNOWax, J&W Scientific, Agilent Technologies Inc., USA) of 30m×250 μm i.d.×0.25 μm was used. (HP-INNOWax, Agilent Technologies Inc., USA). Nitrogen was supplied as the carrier gas at a flow rate of 1 mL/min. The initial oven temperature was 80°C, maintained for 1.0 min, raised to 200°C at 25°C/min and held for 1.0 min, then increased to 250°C at 30°C/min, and finally held at 250°C for 2 min. The injected sample volume for GC analysis was 1 μL, and the run time for each analysis was 18.3 min. Data handling was carried out with a HP ChemStation Plus software (A.09.01).

Statistical Analyses

Statistical analysis was performed using unpaired two-tailed t test using GraphPad Prism 6.0 software, and the Wilcoxon rank sum test was used to compare difference between two groups. A value of $P<0.05$ was considered statistically significant in the compared groups. The results are expressed as median and range. PCoA was performed from binary_otu_gain distance in regard to every pairwise combination of all samples. The linear discriminant analysis (LDA) effect size (LEfSe) pipeline[8] (available on http://huttenhower.sph.harvard.edu/galaxy/) was used for biomarker detection. Firstly, taxa with significant differential abundances were detected by the nonparametric factorial Kruskal-Wallis (KW) rank sum test. Secondly, the (unpaired) Wilcoxon rank sum test was used to investigate the biological consistency among subclasses. LDA was used to evaluate the effect size of each differentially abundant trait, and a threshold of 4.0 was chosen for logarithmic LDA scores. Alpha values of 0.05 were used for the KW rank sum test, respectively. Besides, metastats[9] was also employed to identify differentially microbes that distinguish patients from healthy controls. A value of $P<0.05$ was considered statistically significant between two groups. The correlations between the abundance of microbial and fecal SCFAs levels were calculated by Pearson correlation coefficient and visualized by heatmap in R using the “corrplot” package [10].

References:

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