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Normal gut microbiome in NMDA receptor encephalitis

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Abstract

Objective

To determine whether the gut microbiota shows overabundance of commensal bacteria species in patients with anti-NMDA receptor (NMDAR) encephalitis, similar to patients with MS or neuromyelitis optica where they potentially balance pro- and anti-inflammatory immune responses or participate in disease pathogenesis by molecular mimicry.

Methods

Intestinal microbiota was characterized in patients with NMDAR encephalitis (n = 23, mean age: 34 ± 12.7 years; 21 females) and age/sex/environment-matched healthy controls (n = 24, 40 ± 14.2 years; 22 females) using stool bacteria 16S rDNA sequencing and classification in operational taxonomic units (OTUs). Statistical analyses focused on intraindividual and interindividual bacterial diversity and identification of differentially abundant taxa.

Results

Patients with NMDAR encephalitis and controls had similar microbiome profiles of the gut microbiota regarding intraindividual bacterial diversity, OTU distribution, ratio between regional and local species diversity when testing all OTUs, and genera with a relative abundance greater than 0.5%. Similarly, the subgroup of NMDAR encephalitis patients with an ovarian teratoma (n = 3) showed no differences in microbiome variation compared with controls. Patients in the acute encephalitis stage (n = 8) showed significant differences in the numbers of *Clostridium XVIII*, *Clostridium IV*, *Oscillibacter*, *Prevotella*, and *Blautia*; however, significance was lost after correction for multiple testing.

Conclusion

Patients with NMDAR encephalitis and controls both had a normal gut microbiome. The lack of overabundance of certain bacterial species in patients suggests that microbiome changes are no major contributors to the pathogenesis, disease course, or prognosis in NMDAR encephalitis. Despite the small sample size and heterogeneous groups, findings indicate differences to other neuroimmunologic diseases.

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Glossary

AQP = aquaporin-4; **EAE** = experimental autoimmune encephalomyelitis; **GLM** = generalized linear model; **MDS** = multidimensional scaling; **NMDAR** = NMDA receptor; **NMOSD** = neuromyelitis optica spectrum disorder; **OTU** = operational taxonomic unit.

The role of the gut microbiota has been increasingly recognized in neurologic diseases. Overabundance of commensal bacteria species was seen in Guillain-Barré syndrome, Parkinson disease, MS, and neuromyelitis optica spectrum disorder (NMOSD).[1–8] Experimental data in germ-free mice demonstrated increased susceptibility to experimental autoimmune encephalomyelitis after selective bacterial colonization, indicating a role of the gut microbiota for balancing proinflammatory and anti-inflammatory immune responses.[9,10] Intestinal microbiota may even be a novel therapeutic target for extraintestinal inflammatory diseases as shown for possible beneficial effects in patients with MS from nutritional administration of a probiotic[11] and potentially from fecal microbial transplantation.[12] Recently, overabundance of the bacterium *Clostridium perfringens*, a commensal bacterium of the gut microbiota, was demonstrated in 16 patients with NMOSD, indicating a potential role in the disease pathogenesis.[5]

Similar to NMOSD, anti-NMDA receptor (NMDAR) encephalitis is an antibody-mediated disease of the CNS, and there are even cases of overlap between the 2 entities.[13] Autoantibodies against the NR1 subunit of the NMDAR are directly pathogenic and underlie the clinical disease spectrum ranging from amnesia, psychosis, and epileptic seizures to dyskinesias, coma, and vegetative dysfunction.[14,15] The potential role of the gut microbiome for the development of NMDAR encephalitis is particularly interesting as only few disease triggers are known, such as ovarian teratomas,[16] viral infections of the brain,[17,18] and potentially seasonal factors.[19] We therefore aimed to investigate the intestinal microbiome of patients with NMDAR encephalitis and healthy controls.

Methods

Standard protocol approvals, registrations, and patient consents

The study was approved by the Charité ethics committee (EA1/274/16) and Charité data protection (#0626/16/ST3). All study participants gave their written informed consent.

Study population

Twenty-eight patients with NMDAR encephalitis were recruited from the Charité Centre for Autoimmune Encephalitis during spring 2017. Five patients and 3 controls received antibiotic treatment within the last 6 weeks before stool collection (for cystitis, pyelonephritis, tonsillitis, sinusitis, or bacterial vaginosis) and were therefore excluded from the analyses. Of the remaining 23 patients with NMDAR encephalitis, 2 (9%) had acute encephalitis, 6 (26%) were in the recovery phase with persisting clinical deficits, and 15 patients (65%) had recovered. Three patients (13%) had an ovarian teratoma removed during clinical workup. Almost all patients (n = 21; 91%) were female; thus, sex was not considered a covariate. Demographic and clinical characteristics are shown in table e-1 (links.lww.com/NXI/A156).

All patients had received immunotherapy according to current guidelines. These included the following:

1. Steroids in 18 patients (78%), 1,000 mg IV methylprednisolone for 5 days, and mean 2.0 cycles;
2. IV immunoglobulins in 11 (48%), 2 g/kg body weight, and mean 1.9 cycles;
3. Plasmapheresis in 15 (65%) and mean 3.6 series of 5–10 sessions each;
4. Rituximab in 13 (57%), 1,000 mg per cycle every 6 months, and mean 2.7 cycles (3 patients ongoing);
5. Azathioprin in 3 (13%), 250 mg/d for 4 months, 75 mg/d for 1.5 years, and 100 mg/d for 6 months, respectively;
6. Cyclophosphamide in 2 (9%), 750 mg/kg once in 1 patient, and 5 times in the second;
7. Methotrexate in 1 (4%), 10–15 mg/wk over 3.5 years;
8. Bortezomib in 1 patient (4%) and 2 cycles of 4 subcutaneous injections of 1.3 mg/m^2.

Five patients (22%) still received immunosuppression at the time of stool collection. In the others, the mean immunotherapy-free interval was 4.4 years.

The control group consisted of 24 case-matched healthy individuals. Matching was prioritized for similar living environment, lifestyle, and dietary habits. Most controls were friends of the same sex and age or family members (mostly siblings) who shared the apartment. Controls had no history of autoimmune or gastrointestinal disease or cancer.

Study protocol and clinical assessment

Patients and controls collected fecal samples at home in standard stool collection tubes. The samples were shipped immediately (within 24 hours) at room temperature and were stored at −80°C until processing.

A standardized survey was completed by all patients and controls including demographic data (birth, sex, body size and weight, and size of city of residence [metropolis, city, center city, small town, and village]), information on smoking history (never smoked, current smoker, and the number of pack-years), general activity level (type and extent of physical activity),

defecation history (general character of feces using Bristol stool scales, incidence of constipation or diarrhea within the last 3 months [never, sometimes 25%, often 50%, very often 75%, and always 100%], number of average defecation per week, rectal tenesmus, and presence of hematochezia and abdominal pain), dietary habits (nutritional style [omnivore, ovo-lacto-vegetarian, pesco-vegetarian, vegan diet, and others] and regular intake of probiotics), preexisting illnesses (including fever in the last 7 days), regular medication (beginning of intake, cause of medication, dosage, and antibiotics within the last 6 months), current general state of health (very good, impaired, bad, very bad, and unacceptable), need for current antibiotic treatment, and travel history (longer than 4 weeks within the last 12 months).

DNA extraction, 16S rDNA sequencing, and quality control

DNA was extracted using the QIAcube and the QIAamp DNA stool kit (Qiagen) and a prior beat-beating step. Variable regions v1-v2 of the 16S rRNA gene were amplified using the primer pair 27F/338R. PCR products were normalized using the SequalPrep Normalization Plate Kit (Life Technologies) and sequenced on an Illumina MiSeq (2 × 300 bp).

Raw data were obtained with exact agreement of the index sequences, demultiplexed with a dual-indexing approach, and subjected to quality control. This consisted of trimming low-quality sequence ends,[20] combining forward and backward amplicon reads into a single sequence (VSEARCH), quality control based on estimated error (VSEARCH), and sequence quality (FastX toolkit). Reference and DeNovo-based identification of chimeric sequences (VSEARCH) took place,[21] as well as a filtering step to exclude nonbacterial (and thus unspecific or erroneous) sequence data (Simple non-Bayesian taxonomy classifier [SINTAX]). The clean sequences were classified in operational taxonomic units (OTUs) (VSEARCH), normalized to 10,000 sequences per sample, and taxonomically annotated (SINTAX).[22] Based on this information OTU, an operational definition used to classify groups of closely related individuals, and taxon abundance tables were created for subsequent analysis.

Statistical analysis

Statistical analysis was performed on alpha- (intraindividual) and beta- (inter-individual) diversity measures (Bray-Curtis dissimilarity and UniFrac distance). For differences in alpha-diversity, the Wilcoxon rank-sum test was used when value distribution deviated from normality, otherwise the 2-sample *t* test. Alpha-diversity indices contained the Shannon diversity index (accounts for both abundance and evenness of the species present) and the Chao1 index (reflecting species richness).

Unconstrained multidimensional scaling (MDS) plots of beta-diversity measures were generated using the cmdscale function in R.[23] MDS plots generally visualize the level of similarity of individual cases of a data set. To test for differences in beta-diversity, permutational multivariate analysis of variance was performed using the adonis function of the *vegan* software package with the option sqrt.dist = T when using abundance tables, but not when using UniFrac distances, and 10,000 permutations. Tests for differential abundance were performed using zero-truncated generalized linear models (GLMs) with negative binomial distribution on taxonomic groups present in at least 2 samples per group and 10 samples in total.

Data availability

Anonymized data will be shared by request from any qualified investigator.

Results

Study participants

Subject characteristics of the 23 patients with NMDAR encephalitis and 24 unaffected controls are given in table e-1 (links.lww.com/NXI/A156). Forty-three of 47 participants were female, and the mean age was 34 years in patients and 40 years in controls. There were no differences between groups regarding smoking, obesity, place of residence, defecation at the time of stool collection with general stool consistency and stool frequency per week, occurrence of hematochezia, occasional abdominal pain, nutritional intake, diet, travel history, and general medication, such as for hypertension, contraception, or hypothyroidism (table e-1). Five (22%) patients with NMDAR encephalitis with persisting symptoms still received immunosuppression or neuroleptic medication (rituximab [n = 3], cortisone [n = 1], bortezomib [n = 1], risperidone [n = 2], and lamotrigine [n = 1]), which could theoretically alter the gut microbiome. Two patients and 3 controls had probiotic intake.

Similar microbiome profiles in patients with NMDAR encephalitis and controls

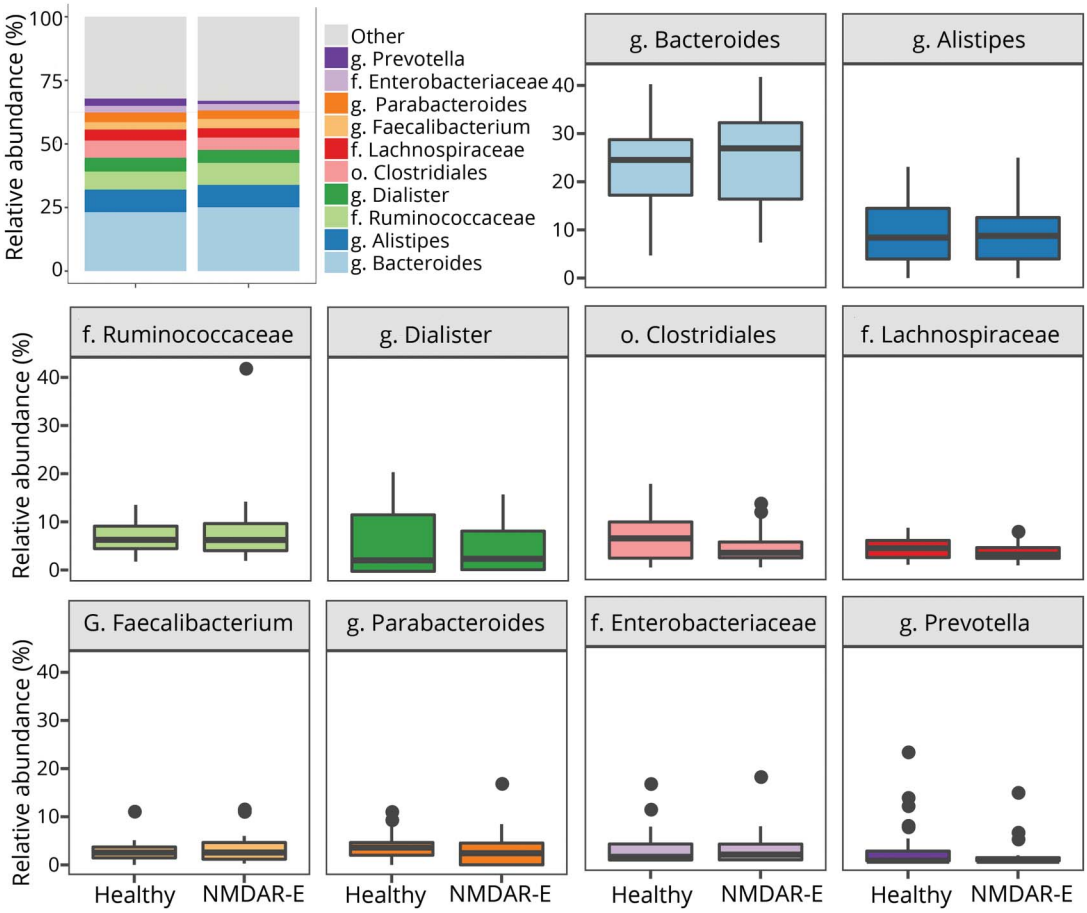
The average microbiome profile of patients with encephalitis and healthy controls did not reveal any differences between groups (figure 1). Regarding alpha-diversity (reflecting intraindividual bacterial diversity), no difference in species diversity within the gut microbiota was observed. The Shannon diversity index based on OTU distribution (groups of closely related individuals) did not reveal any significant difference between both groups. Similarly, the Chao1 index was not different (Mann-Whitney *U* test, figure 2).

Beta-diversity (interindividual dissimilarity) also did not differ significantly between patients and healthy controls. The lack of differences for genus and OTU is visualized using MDS plots (figure 3), i.e., showing the level of similarity of individual cases (adonis analysis: genera: $p > 0.3$; OTU: $p > 0.8$). All OTUs and genera with a mean abundance greater than 50 reads per sample (equivalent to 0.5% relative abundance) were tested in a GLM or hurdle model. None of the results were significantly different (all $q > 0.05$) after correction for multiple testing.

Alpha- and beta-diversity in patients with NMDAR encephalitis with a teratoma

Three of the 23 patients with NMDAR encephalitis had a teratoma, thus representing a subgroup with potentially different

Figure 1 Average gut flora profiles of patients with NMDAR encephalitis and healthy controls



Shown are all families and genera with a mean abundance of more than 2.5%. Remaining genera are summarized in the group "other."

immunologic mechanisms. Patients were compared with age- and sex-matched controls living in the same household. None of the 3 patients received immunosuppressive medication at the time of stool collection, and 2 had L-thyroxine for hypothyroidism. None of the controls required medication including antibiotics. All subjects were on omnivore diet, and the stool was inconspicuous.

No differences in microbiome variation were seen between patients with NMDAR encephalitis after teratoma removal and controls. Alpha-diversity was equal between both groups regarding Shannon diversity (genera/OTUs), the number of genera/OTUs, or the Chao1 estimator at the generic and OTU levels. Also, beta-diversity was not significantly different at the genus ($p = 0.62$) and OTU levels ($p = 0.32$, data not shown).

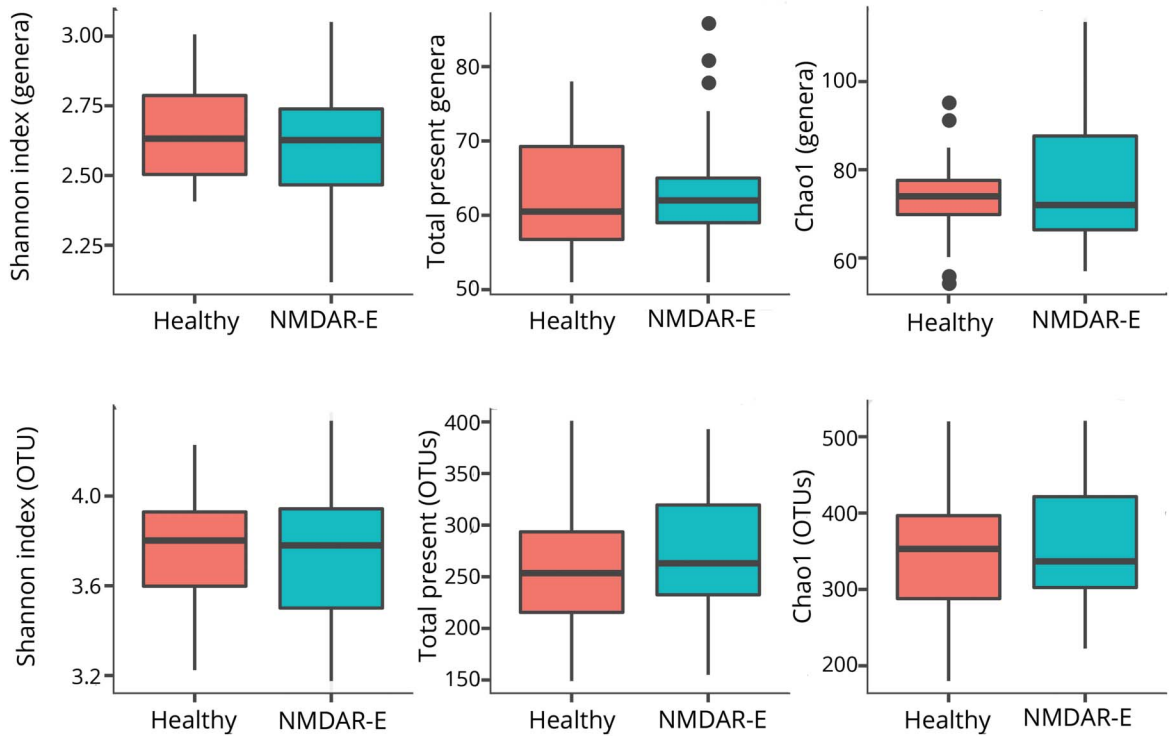
Alpha- and beta-diversity in patients with NMDAR encephalitis with acute illness

Eight of the 23 patients still had clinical symptoms of NMDAR encephalitis at the time of examination. Seven (88%) were female, and 5 (63%) received immunosuppressive therapy at the time of stool collection. These patients were compared with the 15 NMDAR encephalitis after disease recovery (14 [93%] females, 2 [13%]) on immunosuppressive therapy. All participants had normal stool habits and stool conditions at the time of examination. Comparing the intraindividual bacterial diversity between 2 patient groups showed no significant differences in the Shannon diversity index. However, significant differences in the number of genera were detected for *Clostridium XVIII*, *Clostridium IV*, *Oscillibacter*, *Prevotella*, and *Blautia* (figure 4), although significance was lost after Bonferroni correction (table e-2, links.lww.com/NXI/A156). Regarding beta-diversity, there were again no differences at the genus ($p = 0.082$) and OTU levels ($p = 0.15$).

Discussion

In this first study on the gut microbiome in patients with NMDAR encephalitis, data showed no overabundance of certain bacterial taxa compared with controls. Both patients and healthy participants had a "normal" microbiome consistent with previous reports of the healthy gut where gram-negative Bacteroides and gram-positive Firmicutes, including Clostridiales and Lactobacillaceae, dominate.[24,25] Similarly, no

Figure 2 Boxplots of the alpha-diversity indices

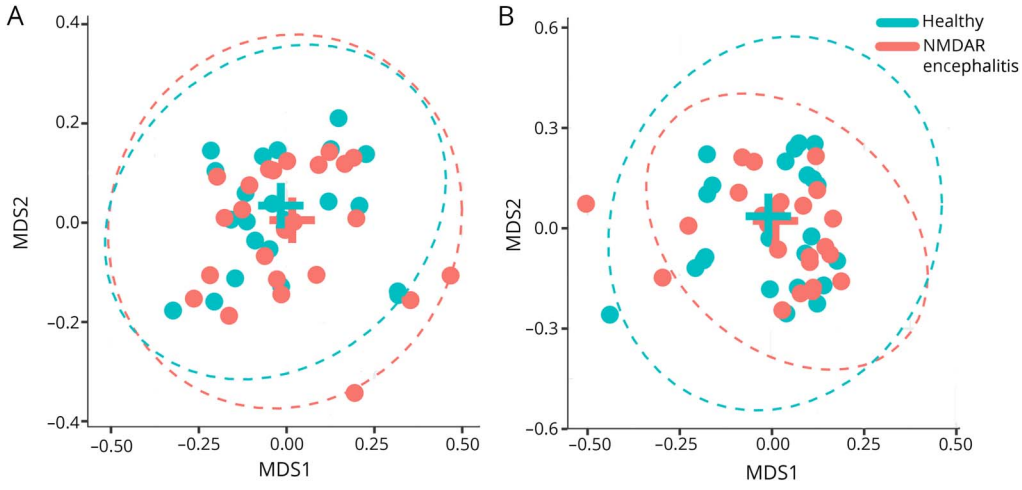


There were no significant differences in diversity (Shannon Index) and number or richness (Chao1) of gut microbiome genera and species within genera (OTUs) between patients with NMDAR encephalitis and healthy controls.

differences were seen in subgroups of patients with ovarian teratomas or in patients during the active disease phase.

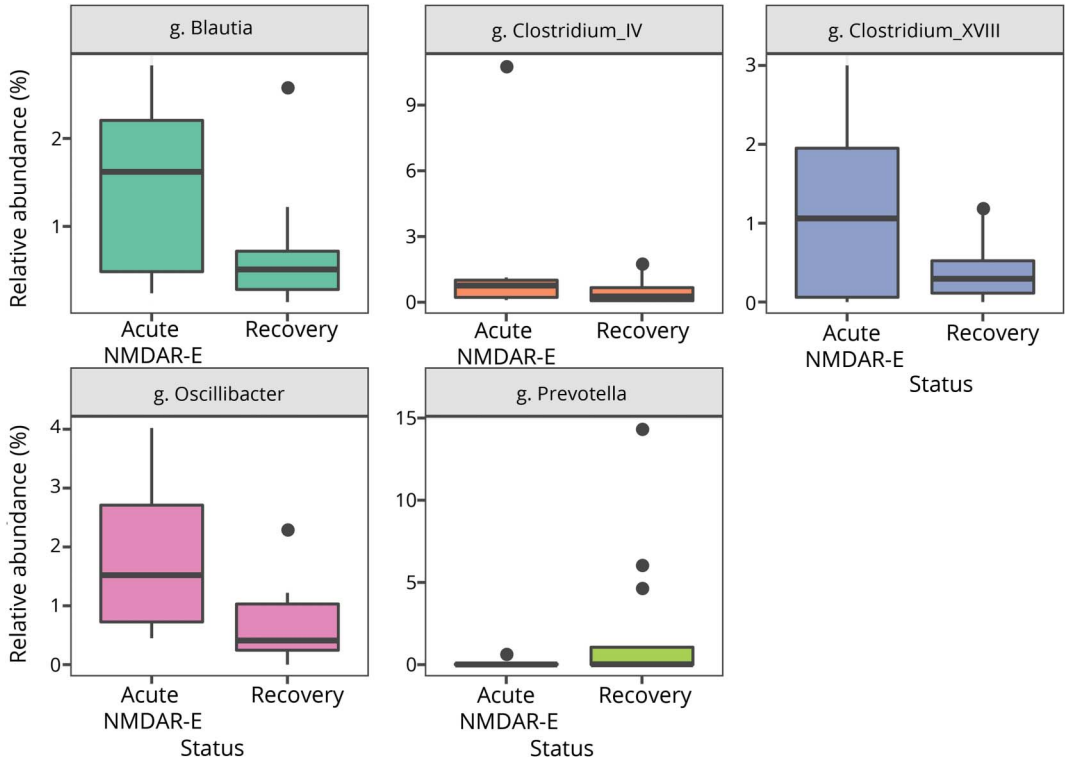
The study therefore provides no support for the hypothesis that microbiome changes are major contributors to pathogenesis, disease course, or prognosis in NMDAR encephalitis, although it is still possible that higher patient numbers could reveal differences. It is also possible that the microbiome is different only during the initial phase of the disease compared with remission. Indeed, our subgroup of

Figure 3 MDS plots of Bray-Curtis distances at the genera and OTU levels



Data show a high level of similarity regarding interindividual differences between patients with NMDAR encephalitis and healthy controls for genera (A) and OTU level (B). The ellipses represent the 99% CIs of the SDs within the groups around the mean values marked by the position of the group names.

Figure 4 Overabundance of bacterial species in active NMDAR encephalitis



Five genera showed significant difference in overall abundance in patients with acute NMDAR encephalitis compared with recovered patients. The differences were not present after correction for multiple testing.

patients with acute encephalitis had overabundance of 5 genera including *Clostridium XVIII* and *IV*, which, however, did not withstand statistical correction for multiple testing.

In the somewhat related neurologic disease NMOSD with autoantibodies targeting the water channel aquaporin-4 (AQP4), *C perfringens* was enriched in the gut microbiota of 16 patients vs 16 controls.[5] As T cells from NMOSD cross-react with a homologous sequence of a *C perfringens* adenosine triphosphate-binding cassette transporter,[26] the authors concluded that the microbiota might be involved in NMOSD pathogenesis.

It is therefore likely that different mechanisms initiate and drive disease in NMOSD and NMDAR encephalitis. For example, approximately one-third of female patients with NMDAR encephalitis have an ovarian teratoma, which contains neuronal elements expressing NMDAR and is thought to trigger the encephalitis.[16,27] In contrast, tumors are rare in patients with NMOSD. Another potential difference is the role of T cells for disease. Although T cells in patients with NMOSD proliferate in response to AQP4 protein,[26,28] no such role has yet been established for T cells from patients with NMDAR encephalitis. In fact, neuropathologic findings including the absence of relevant numbers of T cells in the brain suggested that NMDAR encephalitis is a predominantly humoral autoimmune disease.[27,29] Furthermore, molecular mimicry of NMOSD T cells between AQP4 and a *Clostridium* protein might contribute to NMOSD pathogenesis, whereas no cross-reaction of T cells and antibodies were so far observed in NMDAR encephalitis. The absence of detection of a consistent infectious agent makes an immune response by molecular mimicry unlikely.[14]

Differences in immunosuppression between groups had no effect on the composition of the gut microbiota, although conclusions about a clear effect would likely require higher patient numbers or stool samples from untreated patients with NMDAR encephalitis. These might be difficult to obtain as immunotherapy is usually started immediately after confirmation of the diagnosis with positive serum and CSF antibodies. In NMOSD, rituximab treatment did not account for changes in the microbiome.[5]

Despite the limitations of relatively small sample sizes, heterogeneity of the groups, and the majority of patients being in the recovery phase, the present work argues against a major influence of the gut microbiome in NMDAR encephalitis. It thereby reminds us of how little we still know about etiology and disease mechanisms in NMDAR encephalitis, in particular about the role of T cells and further immune cells. It is finally possible that microbiota-driven effects are still in place—not reflected by mere

overrepresentation of certain bacterial species, but rather by dysfunctional immune responses to numerically normal microbiota, potentially shaped by genetic, metabolic, or psychosocial factors.

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Disclosure

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Corinna Bang, PhD	Christian Albrechts University of Kiel, Germany	Author	Major role in the acquisition of data and drafted the manuscript for intellectual content
Malte C. Rühlemann, PhD	Christian Albrechts University of Kiel, Germany	Author	Major role in the acquisition of data and drafted the manuscript for intellectual content
Carsten Finke, MD	Charité – Universitätsmedizin Berlin, Germany	Author	Major role in the acquisition of data and revised the manuscript for intellectual content
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Andre Franke, MD	Christian Albrechts University of Kiel, Germany	Author	Major role in the acquisition of data and revised the manuscript for intellectual content
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