The human gut microbiota in people with amyotrophic lateral sclerosis

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Abstract
Objective: To characterize the gut microbiota in people with amyotrophic lateral sclerosis (ALS) relative to controls and to test the hypothesis that butyrate-producing bacteria are less abundant in the gastrointestinal tracts of people with ALS (PALS).

Methods: We conducted a case–control study at Massachusetts General Hospital to compare the gut microbiota in people with ALS to that in controls. Metagenomic shotgun sequencing was performed on DNA extracted from stool samples of 66 people with ALS (PALS), 61 healthy controls (HC), and 12 neurodegenerative controls (NDC). Taxonomic metagenomic profiles were analyzed for shifts in the microbial community structure between the comparator groups using per-feature univariate and multivariate association tests.

Results: The relative abundance of the dominant butyrate-producing bacteria Eubacterium rectale and Roseburia intestinalis was significantly lower in ALS patients compared to HC. Adjustment for age, sex, and constipation did not materially change the results. The total abundance of 8 dominant species capable of producing butyrate was also significantly lower in ALS compared to HC (p < 0.001).

Conclusions: The levels of several butyrate-producing bacteria, which are important for gut integrity and regulation of inflammation, were lower in people with ALS compared to controls. These findings lend support to the inference that the gut microbiota could be a risk factor for ALS. Further investigations are warranted, preferably earlier in the disease with corresponding dietary collection and a longitudinal design.

Keywords: Amyotrophic lateral sclerosis, gut microbiota, risk factors

Introduction
Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting primarily motor neurons. The disease is likely a result of a complex gene-environment interplay (1), and while the etiology remains elusive, much of the disease pathophysiology has been explored in detail. Neuroinflammation clearly plays a role in the disease in animal models and can also be detected in people with ALS (PALS) (2–4). This inflammation appears to drive ALS progression and could be associated with the onset of the disease (4).
Activation of glial cells can lead to neuroinflammation that promotes motor neuron loss (5), while levels of regulatory T-cells (T-regs) capable of suppressing inflammation are inversely correlated with the rate of ALS disease progression (6).

The gut microbiota modulates pathways likely to be involved in ALS, including inflammation and energy metabolism (7,8), raising the hypothesis that it could be involved in the development of the disease. SOD1G93A transgenic mice, a commonly used animal model of ALS, have increased intestinal permeability and lower levels of bacteria producing butyrate (9), an anti-inflammatory short-chain fatty acid (SCFA) that is the end product of bacterial fermentation of indigestible fiber in the colon (10,11). In humans, SCFA production is directly correlated with a higher body mass index (BMI) (12), which is associated with a lower risk and slower progression of ALS (13,14). In a later study of SOD1G93A transgenic mice, oral supplementation of butyrate improved the gut integrity, prevented weight loss, and was associated with improved survival (15), suggesting that the gut microbiota may affect the course of ALS and could be modifiable. This is consistent with findings from a recent study on C9orf72-mutant mice, where modification of the gut microbiota ameliorated systemic inflammation and improved survival (16).

Only a few studies have examined the role of the gut microbiota in ALS, and the results are conflicting. Lower diversity and/or an altered composition in the gut microbiota were reported in three smaller studies (17–19). While one study reported alterations in the microbial composition in 50 ALS patients compared to 50 healthy controls (20), no significant differences in the gut microbiota was detected in a study comparing 25 PALS and 32 age- and sex-matched controls (21). The conflicting results could suggest that the gut microbiota is not a strong risk factor for ALS, or could be the result of methodological limitations, such as small sample size and sequencing approaches with insufficient taxonomic resolution. Thus, it remains unclear whether the gut microbiota is involved in the etiology of ALS.

Our objective was to compare the gut microbiota in PALS, healthy controls (HC), and neurodegenerative controls (NDC). We used 16S rRNA sequencing in a subset of participants, and metagenomic shotgun analysis, which provides higher taxonomic resolution, in all participants.

**Methods**

**Study participants**

The study was approved by the human ethics committee of the Partners Human Research Office and informed consent, including consent for human DNA analysis, was obtained from each subject prior to participation. Participants were recruited both in-person and remotely over a 23 month period. In-person recruitment occurred from Massachusetts General Hospital (MGH). Remote recruitment drew from other US multidisciplinary ALS clinics and web advertisement. For participants enrolling in-person, the study visit occurred in the clinical research suite at MGH. Remote enrollment occurred over the phone and via a research portal. Medical records were reviewed by an ALS physician, and diagnostic El Escorial Criteria were verified to confirm eligibility for sample collection. Caregivers and other healthy individuals were invited to participate in the study as controls.

Participants were over 18 years of age, did not have active inflammatory bowel disease, gastrointestinal (GI) cancer, irritable bowel syndrome, or other GI illness requiring treatment (excluding gastroesophageal reflux), had not used antibiotics or immune suppressants within the previous 3 months, or probiotics for 14 days. This study was conducted prior to the approval of Radicava in the USA. People with familial and sporadic ALS were permitted to participate. PALS were required to carry a clinical diagnosis of ALS given by an ALS specialist and to meet El Escorial Criteria for at least suspected ALS. Including suspected ALS allowed the inclusion of participants very early in the disease course. To maximize recruitment of patients to the study, we did not make any restrictions on disease duration or current treatment. The NDC group comprised of seven patients with other motor neuron and neuromuscular disorders, which included primary lateral sclerosis (n=2), progressive muscular atrophy (n=1), inclusion body myositis (n=1), carpal tunnel syndrome (n=1), radiculopathy (n=1), and undefined motor neuron disorder (not ALS, n=1), and five patients with other neurological conditions, which included Tourette Syndrome (n=1) and migraine (n=4). Further details on the NCD group are provided in Table 1.

**Clinical data collection**

Clinical information included demographics, medical and exposure history, medications and supplements, and family history of neurological disease. All participants answered questions regarding bowel movement frequency and associated abdominal discomfort on the Wexner constipation scale (minimum score: 0, maximum score: 30) to quantify constipation and assess for the presence of irritable bowel syndrome as a potential confounder (22). For PALS, a detailed ALS history was collected, and the presence or absence of gastrostomy tube was noted. ALS Functional Rating Scale-Revised (ALSFRS-R) was administered by trained
clinic staff. Vital capacity was collected from people participating at MGH when possible. Only a few participants had missing values for covariates included in the statistical models. Specifically, one case had missing information on time from first symptom and one case had missing information on time since diagnosis. Further, one case did not have information on ALSFRS-R. Information on vital capacity was missing for 20 cases (30.3%), as this was only collected from participants recruited at MGH.

### Stool sample collection

We used a validated protocol for a one-time stool sample self-collection for microbiome analysis (23). Participants were provided with a simple kit with clear instructions for sample collection and shipping. The stool samples were collected in tubes made of polypropylene. Samples arrived at the laboratory for analysis within 48 hours of collection to ensure no alterations in the bacterial composition occurred during shipment (23). The samples were processed and frozen at −80°C on the day of receipt at the Broad Institute (Cambridge, MA).

### Nucleic acid extraction

The DNA and RNA extraction was performed as per previously described protocol (23). In short, the frozen samples were thawed on ice and centrifuged at maximum speed to remove the excess of RNAlater. Lysozyme and proteinase K were then added to the sample, which was then incubated for 10 min, followed by 3 min of bead beating to extract the nucleic acids. QiaGen AllPrep DNA spin columns were used for RNA and DNA extraction following the manufacturer’s protocol.

### Metagenomic shotgun profiling and sequencing

The Illumina HiSeq was used for shotgun sequencing. The sequence reads were analyzed using the bioBakery metagenomics workflow (24), which includes the KneadData quality control pipeline ([http://huttenhower.sph.harvard.edu/kneaddata](http://huttenhower.sph.harvard.edu/kneaddata)) implementing the Trimmomatic (25) and BMTagger (26) filtering and decontamination algorithms to remove low-quality reads and reads of human origin, respectively. The metagenomic data was taxonomically profiled using Metagenomic Phylogenetic Analysis (MetaPhlAn2) (27) with the default settings as implemented in the bioBakery.
16s profiling and sequencing

The Illumina MiSeq platform was also used for 16S ribosomal RNA (rRNA) gene amplicon sequencing targeting the V4 variable region (28). 16S rRNA sequencing data were processed using the bioBakery (24) UPARSE (29) workflow, which includes sample demultiplexing and operational taxonomic units (OTU) picking (at 97% identity with default parameters) and taxonomic assignment against Greengenes v13.5 (30).

Statistical analysis

We used principal coordinate analyses (PCoA) to obtain ordination plots using Bray-Curtis distances, as implemented in the phyloseq R package (31). We also estimated beta diversity using weighted and unweighted UniFrac distances and compared the three groups using PERMANOVA, as implemented in the adonis function in the vegan R package. We estimated the Shannon Index as a measure of alpha diversity and compared the groups using pairwise Wilcoxon rank-sum tests. To quantify the variance in the microbial composition explained by covariates and inter-individual variation, we used PERMANOVA as described above. The variance was calculated for each covariate separately to avoid issues related to variable ordering. For these calculations, missing values on clinical ALS covariates were imputed with the median value in the remaining ALS population.

To reduce the effect of zero-inflated microbiome data, we removed taxonomic features with zero abundance in more than 90% of the samples in all subsequent statistical analyses. The relative abundance was used in the statistical models. We used linear discriminant analysis effect size (LEfSe) (32) to detect taxonomic features with significantly different abundances in ALS compared to controls. LEfSe combines the non-parametric Kruskal–Wallis rank-sum test to detect taxonomic differences followed by an optional pairwise test among subclasses using the Wilcoxon rank-sum test. Finally, an effect size is computed using linear discriminant analysis (LDA). We used LEfSe without the subclass option, and defined features as significantly different at an alpha level of 0.05 and a logarithmic LDA score of 2. To evaluate whether clinical covariates were jointly associated with taxonomic features, we used Microbiome Multivariable Association with Linear Models 2 (MaAsLin2) (33), an R package that evaluates multivariable associations between clinical metadata and microbial omics features using general linear models.

To evaluate whether a subset of bacterial species was associated with ALS, we conducted L1 penalized regression (Lasso) analyses (34). We arcsine square-root transformed and standardized the
relative abundances (mean = 0, standard deviation = 1) and included them as predictors in a logistic Lasso regression model. To estimate the best tuning parameter for the penalty term, we used 10-fold cross-validation. We further used logistic regression to evaluate the influence of pre-defined potential confounders, including age, sex, and level of constipation.

To examine whether the total abundance of butyrate-producing species was associated with ALS, we calculated the total relative abundance of dominant species capable of butyrate production (35). The levels in ALS vs. HC and ALS vs. NDC were first compared using the Wilcoxon rank-sum test. Further, we used logistic regression to estimate odds ratios (ORs) and 95% confidence intervals (CIs). To explore a possible nonlinear relationship in the association, we categorized the participants into quartiles based on the distribution of butyrate-producing species in the HC. We tested for a linear trend across the quartiles by assigning the median value to each quartile and modeled this as a continuous variable.

To evaluate the robustness of our findings, we conducted several sensitivity analyses, including restricting the analyses to 1) samples collected within the first year of ALS diagnosis; 2) participants not using riluzole; and 3) participants not using gastrostomy tube.

To account for multiple testing, we corrected nominal p values using the Benjamini Hochberg false discovery rate (FDR) approach (36). Q-values lower than 0.2 were considered significant.

**Results**

**Baseline characteristics**

We enrolled 98 PALS, 88 HC, and 12 NDC. Of these, 139 provided stool samples: 66 PALS, 61 HC, and 12 NDC. Metagenomic analysis was performed on the entire cohort providing stool samples.

The ALS group was more predominantly male and reported more constipation than the HC and NDC groups (Table 1). Other characteristics were similarly distributed in the three groups.

ALS and HC gut microbiota differ on metagenomic shotgun sequencing.

Using metagenomic shotgun sequencing, a total of 303 microbial species were identified in the samples. The overall composition of these
species was similar across ALS and controls (Figure 1(A)). We observed similar results using unweighted ($p$ value from permutation test: 0.59) and weighted UniFrac distances ($p$ value from permutation test: 0.20). Further, there were no significant differences in alpha diversity in the three groups. None of the clinical ALS covariates captured a large proportion of the overall variation in the microbial composition (Figure 1(B)). After filtering taxonomic features with zero abundance in more than 90% of the samples, 137 species were included in the differential abundance analyses.

Comparison of specific metagenomic species composition between ALS and HC participants demonstrated differences in 15 bacterial species (Figure 2), including two dominant butyrate producers Roseburia intestinalis ($p < 0.001$) and Eubacterium rectale ($p < 0.001$). Both of these species remained significantly inversely correlated with ALS after correction for multiple comparisons. Adjustment for age and sex in a multivariable model did not alter the results.

Further adjustment for constipation scores in each group attenuated the association between Roseburia intestinalis and ALS ($p = 0.018$), while the association for Eubacterium rectale remained similar to the univariable model ($p = 0.004$). In analyses comparing the metagenomic species composition in ALS and NDC, none of the observed differences in species abundance ($n = 9$; Figure 3) reached statistical significance after FDR correction.

Because only certain bacteria produce butyrate (35), we compared the total relative abundance of major butyrate producers across diagnostic groups. The total relative abundance of the eight dominant butyrate producers was significantly lower in ALS compared to HC ($p < 0.001$; Figure 4), and there was a non-significant trend toward reduction of these species in ALS compared to NDC ($p = 0.077$). In a multivariable model adjusted for age and sex, a higher abundance of butyrate-producing bacteria was associated with a significantly lower risk of ALS (OR top vs. bottom quartile: 0.20, 95% CI: 0.06–0.64, $p$-trend = 0.002). Adjustment for constipation did not markedly alter the results (OR top vs. bottom quartile: 0.24, 95% CI: 0.07–0.80, $p$-trend = 0.011).

**Paucity of correlation of gut microbiota with ALS clinical data**

We explored whether the relative abundance of these bacteria correlated with any clinical features of ALS. The relative abundance of 7 species correlated positively with the constipation score (Escherichia coli, Eggerthella unclassified, Bifidobacterium bifidum, Coprobacillus unclassified, Clostridium symbiosum, Flavonifractor plautii, Ruminococcus gnavus). None of the bacterial species were significantly associated with other clinical features after we accounted for multiple comparisons.

**Groups of bacterial species associated with ALS**

When we used Lasso for feature selection based on the relative abundance profile of bacterial species, a total of 9 bacterial species were selected in the final model comparing PALS and HC. These included both Roseburia intestinalis and Eubacterium rectale, both with lower abundance in ALS compared to HC. Among the other species selected by the Lasso, the relative abundance of Bilophila...
(unclassified), Clostridiaceae bacterium JC118, Coprobacter fastidiosus, Eubacterium eligens, and Ruminococcus sp 5 1 39 BFAA was also lower in ALS compared to HC, while the relative abundance of Escherichia (unclassified) and Streptococcus salivarius was higher in ALS compared to HC.

Sensitivity analyses recapitulate the overall analysis

We repeated the main analyses, including only subsets of PALS to evaluate the robustness of the results. First, to limit the influence of changes that come late in ALS (e.g. bulbar weakness, dietary modification, low BMI, altered metabolism), we compared only PALS with samples collected within the first year of their diagnosis ($n = 33$) to HC. Like in the main analysis, the relative abundance for both Roseburia intestinalis ($p = 0.018$) and Eubacterium rectale ($p = 0.025$) was significantly lower in this subgroup of patients compared to HC. The abundance of total butyrate-producing bacteria was also lower in this subgroup compared to HC, but the difference was not statistically significant ($p = 0.068$).

Second, we only included ALS patients not using riluzole at the time of sample collection ($n = 25$). The results recapitulated the main analyses ($p = 0.007$, $p < 0.001$, $p = 0.002$, for Roseburia intestinalis, Eubacterium rectale, and total abundance of butyrate-producing bacteria, respectively).

16S rRNA gene sequencing did not clearly discriminate between ALS and HC.

In a subset of those with metagenomic data, 16S rRNA gene amplicon-based taxonomic profiles were available. This included 48 ALS, 56 HC, and 8 NDC with both sequencing datasets, and one individual with missing metagenomic data. The relative abundance of the family Lachnospiraceae, which includes several important butyrate producers, including Roseburia intestinalis and Eubacterium rectale (35), was lower in ALS compared to controls (median proportion of 0.076, 0.099, and 0.087 for ALS, HC, and NDC, respectively), but the difference was not statistically significant ($p = 0.09$ and 0.39 comparing ALS to HC and NDC, respectively). None of the taxonomic features were differently abundant in the three groups after correction for multiple comparisons.

Discussion

We conducted this study of the gut microbiota in ALS patients to test the hypothesis that we would find reduced levels of anti-inflammatory SCFA-producing bacteria in the gastrointestinal systems of PALS. As we had hypothesized, we found lower relative abundance of these bacteria in PALS.
compared to HC, which included the two dominant butyrate-producing species *Roseburia intestinalis* and *Eubacterium rectale*. Further, the total relative abundance of 8 dominant species capable of producing butyrate was also lower in ALS compared to HC. These results were robust across several sensitivity analyses. The results suggest that changes in the gut microbiota are associated with ALS.

Previous research on the gut microbiota in ALS is sparse. Although several case–control studies have been conducted, two of these included only five (18) and six (17) cases, and one only reported preliminary results based on selected bacterial species (20). In a fourth study, which included 25 individuals with ALS and 32 controls, no overall difference was found between the microbiome of cases and controls using 16S rRNA gene sequencing (21). However, the taxonomic resolution of this approach may be insufficient to determine the relative abundance of the butyrate-producing species that we found to be depleted in individuals with ALS, as demonstrated by the fact that using data from 16S sequencing alone we could not demonstrate significant differences between ALS cases and controls in our own study.

Butyrate and the other SCFAs produced in the gut may impact systemic inflammation. Butyrate acts as a ligand on different G protein-coupled receptors (GPCRs), such as GPR41 and GPR109A, which are expressed in a variety of tissues and cells, including immune cells (37). While most of the evidence on butyrate is limited to its function in or close to the gastrointestinal tract, butyrate is also present in the plasma and does appear to have systemic immunological effects (37). It can induce the differentiation of regulatory T-cells (10) and inhibit the activation of NF-κB (38). Derangements in both of these inflammatory pathways have been shown to contribute to ALS (39).

Our study has some limitations. Because of the cross-sectional design, we cannot evaluate the direction of the association. Many people alter their diet after ALS onset, both unconsciously due to dysphagia and consciously to increase caloric intake and reduce weight loss (40), which could affect the composition of the gut microbiota. We did not have access to detailed dietary records, and could therefore not account for dietary differences among the participants in our analyses. As we invited caregivers to participate as HC, there was a higher proportion of females in this group compared to the ALS group. Further, use of caregivers as controls can lead to overmatching, i.e. in controls that are too similar to the cases for the exposure of interest, which may have reduced the statistical power in our analyses. The NDC group included a spectrum of disorders and consisted of only 12 patients, which limited the statistical power in analyses including this group. There was heterogeneity in the ALS group in terms of disease duration, but the results remained similar in a sensitivity analysis restricted to ALS patients within the first year of their diagnosis, suggesting that our findings cannot be fully explained by differences in the patient population. Lastly, as is inherent to any observational study, we cannot exclude the possibility that the results may be affected by residual or unmeasured confounding that we cannot account for.

In conclusion, several important butyrate-producing bacteria were depleted in ALS compared to controls. These changes in the gut bacteria could be an important part of ALS pathophysiology, might have a role as a disease biomarker, and could even act as a therapeutic target. Further validation of these results is needed, ideally with a large longitudinal evaluation, careful dietary analysis, and assessment of inflammatory markers in the blood.

**Declaration of interest**

Dr. Katharine Nicholson reports relevant grants from ALS Finding a Cure, the funding source of this study. Dr. Kjetil Bjornevik reports no relevant disclosures. Dr. Galeb Abu-Ali reports no relevant disclosures. James Bjornevik reports no relevant disclosures. Dr. Marianna Cortese reports no relevant disclosures. Brixilda Dedi reports no relevant disclosures. Dr. Curtis Huttenhower reports no relevant disclosures. Dr. Ramnik Xavier reports no relevant disclosures. Dr. Curtis Huttenhower reports no relevant disclosures. Dr. James D. Berry reports relevant grants from ALS Finding a Cure, the funding source of this study.

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