



Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration

ISSN: (Print) (Online) Journal homepage: <https://www.tandfonline.com/loi/iafd20>

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To cite this article: Vicki S. Hertzberg, Harinder Singh, Christina N. Fournier, Ahmed Moustafa, Meraida Polak, Claire A. Kuelbs, Manolito G. Torralba, Malú G. Tansey, Karen E. Nelson & Jonathan D. Glass (2021): Gut microbiome differences between amyotrophic lateral sclerosis patients and spouse controls, *Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration*, DOI: [10.1080/21678421.2021.1904994](https://doi.org/10.1080/21678421.2021.1904994)

To link to this article: <https://doi.org/10.1080/21678421.2021.1904994>



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RESEARCH ARTICLE

Gut microbiome differences between amyotrophic lateral sclerosis patients and spouse controls

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Abstract

Objective: Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that is incurable and ultimately fatal. Few therapeutic options are available to patients. In this study, we explored differences in microbiome composition associated with ALS. **Methods:** We compared the gut microbiome and inflammatory marker profiles of ALS patients (n = 10) to those of their spouses (n = 10). Gut microbiome profiles were determined by 16S rRNA gene sequencing. **Results:** The gut microbial communities of the ALS patients were more diverse and were deficient in *Prevotella* spp. compared with those of their spouses. In contrast, healthy couples (n = 10 couples of the opposite sex) recruited from the same geographic region as the patient population did not exhibit these differences. Stool and plasma inflammatory markers were similar between ALS patients and their spouses. Predictive analysis of microbial enzymes revealed that ALS patients had decreased activity in several metabolic pathways, including carbon metabolism, butyrate metabolism, and systems involving histidine kinase and response regulators. **Conclusions:** ALS patients exhibit differences in their gut microbial communities compared with spouse controls. Our findings suggest that modifying the gut microbiome, such as via amelioration of *Prevotella* spp. deficiency, and/or altering butyrate metabolism may have translational value for ALS treatment.

Keywords: Inflammation, neurodegeneration, butyrate, amyotrophic lateral sclerosis, *Prevotella*, epidemiology, biomarker, neurophysiology

Introduction


Studies using animal models of amyotrophic lateral sclerosis (ALS) suggest that gut microbiome dysregulation may play a role in ALS pathophysiology. The murine ALS model that expresses a mutant form of human superoxide dismutase 1 (SOD1^{G93A}) exhibits damaged gut epithelial cell tight junctions, increased gut permeability, and shifted gut microbiome profiles compared with wild-type mice (1). In particular, these animals

have proportionately fewer microbes that produce butyrate, a short-chain fatty acid. Butyrate dietary supplementation appears to be protective and lengthens both the onset time of weight loss and survival in these mice (2). Moreover, reductions in immune-stimulating bacteria appear to be protective from premature mortality in a *c9orf72*-mutant mouse model (3). However, evidence regarding the gut microbiome in humans with ALS is less clear.

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 Supplemental data for this article can be accessed [here](#).

(Received 7 December 2020; revised 8 February 2021; Accepted 8 March 2021)

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DOI: [10.1080/21678421.2021.1904994](https://doi.org/10.1080/21678421.2021.1904994)

A small study reported altered gut microbiome profiles in ALS patients ($n=6$), again with proportionately fewer butyrate-producing microbes, compared with healthy controls ($n=5$) (4). Another study found that ALS patients ($n=5$) had a less diverse microbiota than healthy controls, but the source and number of controls were not specified (5). Most recently, a large study of 66 people with ALS and 61 healthy controls found that the relative abundance of butyrate-producing bacteria was significantly lower in ALS patients compared to healthy controls (6). In contrast, a larger study showed that ALS patients ($n=25$) exhibited higher microbiome species richness, as quantified by 16S rRNA gene amplicon sequence variants (ASVs), indicating a higher number of total species compared with age- and sex-matched controls. However, there was no difference in species evenness, as indicated by the Shannon and Simpson's indices (7). Additionally, ALS patients had a significantly higher proportion of sequences from the *Ruminococcaceae* family (genus and species unknown), but no other differences in the relative composition of the microbiome were observed compared with the controls (7). Similarly, no differences associated with functional predictions were observed (7). Mazzini et al. (8) also compared ALS patients with age- and sex-matched healthy controls ($n=50$) and found that the ALS patients had a higher abundance of *Escherichia coli* and *Enterobacter* spp. as well as a lower abundance of yeasts. The apparent contradictions of these studies may be due to differences in the control groups (age- and sex-matched (7,8) vs. unmatched), sequencing technology (pyrosequencing (4,7) vs. PCR (5,8)) and platforms, bacterial genome sequencing targets (V3-V4 region of the 16S rRNA gene (4) vs. V2-V3 region of the 16S rRNA gene and D1 region of the 26S rRNA gene (8)), reference databases (Silva123 (7), NCBI (5), or unspecified), and bioinformatics software (QIIME v1 (4), ClustalW2 (5), or unspecified) used. Furthermore, various methodological differences among these previous human ALS studies prevent an inference of stronger evidence supporting one study's findings over the others.

Another study examined the gut microbiota in both the ALS mouse model and human ALS patients (9). SOD1^{G93A} mice exhibited gut microbial dysbiosis that was exacerbated after antibiotic administration. Symptom severity was associated with 11 distinct strains of bacteria. Of these, *Akkermansia muciniphila* ameliorated ALS symptoms, whereas *Ruminococcus torques* and *Parabacteroides distasonis* exacerbated them. *A. muciniphila* administration resulted in central nervous system nicotinamide accumulation, and nicotinamide dietary supplementation improved motor symptoms in the mice. Similarly, ALS patients

($n=37$) had different microbiome compositions and lower levels of nicotinamide compared with body mass index- and age-matched family members ($n=29$).

Here, we examined the gut microbiota of ALS patients using a novel study design in which the patients' spouses served as respective controls. Several reports have established gut microbial composition similarities among cohabitating people due to environment and diet commonalities. This has been particularly observed with physically intimate partners (10–13). Thus, the spouses of the ALS patients provided a natural control group, thus establishing the null hypothesis of a lack of difference in gut microbial composition and effectively excluding any differences due to environment, diet, and physical intimacy. We conducted comparisons between the ALS patients and their spouses with respect to gut microbiome compositional profiles and a comprehensive set of stool and plasma inflammatory biomarkers.

Materials and methods

Participants and setting

A total of 40 participants were recruited into this study. The "ALS group" consisted of 20 individuals, 10 patients who were recruited from the ALS Center of Emory University, and their spouses were recruited as controls. The "healthy volunteer group" included 10 healthy couples (20 individuals) who volunteered to serve as geographical controls. Inclusion criteria included: (1) patients with live-in spouses who were willing and eligible to participate, and (2) participants between the age of 18–79 years. Patients receiving enteral nutrition as well as those with a history of bowel disease other than constipation, malignancy, dementia/other cognitive disorders, or Parkinson's disease (PD)/other neurodegenerative diseases were excluded. The study was approved by the Emory University Institutional Review Board (IRB00078771).

Participant recruitment

Patients diagnosed with ALS were identified by research staff at the Emory ALS Center, who reviewed the lists of individuals with clinic appointments over the 4 months in which we recruited. Research staff sought to identify patients who were within 2 years of symptom onset and who had spouse caregivers. About one-third of eligible couples were not approached due to lack of research staff for this unfunded study, and approximately half of couples who were approached agreed to participate. Most of the prospective participants who declined did so because of concerns about the rectal swab or blood draw.

Biological sample collection

One rectal swab was obtained from each participant in the healthy volunteer group, and two rectal swabs were obtained from each participant in the ALS group. Each participant received verbal and pictorial instructions on how to easily obtain a rectal swab using Sterile Catch-All Sample Collection swabs (Epicentre Biotechnologies, Madison, WI) with little or no discomfort. Swabs were stored in MoBio garnet bead tubes (MoBio Laboratories, Inc., Carlsbad, CA) and frozen upright on dry ice prior to transport to the laboratory. They were then stored at -80°C until shipment on dry ice to the J. Craig Venter Institute in La Jolla, CA. The second swabs obtained from the ALS group participants were sealed in Parafilm and stored at room temperature prior to processing to determine stool inflammatory marker levels. In addition, the research coordinator collected blood samples via venipuncture from the ALS group participants, which were aliquoted into EDTA-treated tubes and frozen at -80°C .

16s rRNA gene sequencing

DNA was extracted using the Qiagen DNeasy PowerSoil Kit, according to the manufacturer's protocol (Catalogue # 12888-100; Qiagen, Inc. Germantown, MD). Using approximately 100 ng of sample DNA, the V4 variable region of the 16S rRNA gene was amplified by PCR using adaptor- and barcode-ligated primers (14). The 515-533F forward (GTGCCAGCMGCCGCGGTAA) and 806-787R reverse (GGACTACHVGGGTWTCTAAT) primers were used. Library amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Inc.) and then quantified using the SYBRGold TECAN assay (ThermoFisher, Inc., Waltham, MA). Quantified libraries were normalized, pooled, and sequenced at a loading density of 10 pM on the Illumina MiSeq using the MiSeq Reagent kit v2 500 cycles supplemented with 20% PhiX, according to the manufacturer's specifications. Mock community positive controls and negative quality control samples were processed in the same batch as the participant samples. Standard Illumina post-sequencing quality control software (cassava 1.8) was used to bin sequences according to dual indices and to remove poor quality sequences ($>Q30$) prior to data analysis.

Inflammatory biomarker analysis

Inflammatory biomarkers were measured in stool and plasma samples. Plasma supernatants were aliquoted and batch analyzed. The rectal swabs that were stored at room temperature were placed in 1 mL phosphate-buffered saline (PBS [pH 7.4]), and the stool was extracted using Qiagen TissueLyser at 4°C . After centrifugation at $260 \times g$

for 10 min to clear debris, supernatants were aliquoted and batch analyzed. Plasma and stool samples were transported to the Emory Multiplexed Immunoassay Core for processing. Lipopolysaccharide (LPS) was measured in plasma using an enzyme-linked immunosorbent assay (ELISA) kit (Pierce LAL Chromogenic Endotoxin Quantitation Kit, Thermo-Fisher Scientific, Inc., Waltham, MA). Cytokines (interleukin [IL]-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, tumor necrosis factor [TNF], and interferon [IFN]- γ) were measured in stool and plasma samples using a V-PLEX Pro-inflammatory Panel 1 Human Kit in a 96-well plate on a QuickPlex instrument (Meso Scale Diagnostics, Rockville, MD). LPS-binding protein (LBP) and high-sensitivity C-reactive protein (hsCRP) were measured in stool and plasma samples using the Meso Scale Human LBP and CRP plates, respectively. All samples were processed in duplicate.

Bioinformatics processing

ASVs were generated from raw Illumina sequence reads using UPARSE (15) and mothur (16) open-source bioinformatics tools. Through this pipeline, paired-end reads were trimmed of adaptor sequences, barcodes, and primers, after which low-quality reads were discarded. After a de-replication step, sequences were filtered for chimeras using the UCHIME2 tool from UPARSE (15,17). Next, sequences were clustered with UPARSE set at 100% identity, thus producing ASVs (18). We used mothur to predict taxonomy using the Wang classifier, bootstrapping 100 iterations. We reported taxonomies only when 80 or more of the 100 iterations were identical. Taxonomies were assigned to ASVs with mothur, using version 123 of the SILVA 16S ribosomal RNA database as the reference database (19). We used DESeq2 to test for differential community composition (20,21).

Microbial community analyses

Microbial community analyses were performed using R statistical software (22). To avoid the inclusion of possible sequencing artifacts, ASVs that were either found in fewer than five individuals or fewer than five times in the overall dataset were excluded from further analyses. Principal coordinate analyses were performed on Bray-Curtis distance matrices after transforming raw counts into proportions using the ape (23) and vegan (24) packages. The vegan package was used to calculate alpha diversity.

Statistical analyses

Group identity (ALS patient or spouse control) was not provided for initial group comparisons, but the pairing was preserved (i.e. the only group

identity was A or B). To prevent unblinding, no demographic data were released during this initial analysis. Alpha diversity index comparisons between patients and their spouses were performed using the Student's paired *t*-test or Wilcoxon signed-rank test, as appropriate. Group identities were revealed only after preliminary comparisons regarding alpha and beta diversity and relative ASV abundances were agreed upon. Principal coordinate analyses were also performed for the ALS group (ALS patients vs. spouse controls) and healthy volunteer group (males vs. females). We applied a Wald test (25) to test for differential community composition. We used the Benjamini-Hochberg correction (26) for multiple testing to identify genera, species, and inflammatory markers that differed between ALS patients and their spouse controls.

Predicted function comparisons

We used the SILVAngs pipeline (19) with tax4fun (27) to predict microbial enzyme abundance in the ALS patient and control samples. We performed *t*-tests to determine predicted differential enzyme abundances between the two groups and then selected enzymes with *p* values ≤ 0.05 . The selected enzymes from each group were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database to obtain functional profiles of each group.

Software

R version 3.6.2 and phyloseq version 1.30.0 were used to conduct statistical analyses and generate figures. Additional packages used are listed in the supplementary information.

Results

Ten pairs of patients (mean age: 50.8 ± 12.9 years, 70% male, 100% Caucasian, mean disease duration: 1.7 ± 1.1 years, mean ALS functional rating scale score—revised: 39.9 ± 3.6 points) and their opposite sex spouses (mean age: 47.5 ± 14.2 years, 30% male, 90% Caucasian) were enrolled. All ALS patients had a history of sporadic disease and negative testing for the C9orf72 mutation, the most common gene associated with ALS. No patients were taking riluzole, the most common treatment for ALS patients that can prolong survival for 2–3 months. None of the spouse controls had a history of chronic disease. The healthy volunteer group included 10 opposite sex couples (mean age: males, 47.2 ± 11.7 years; females, 49.4 ± 14.0 years). All were Caucasian. There were no significant differences with respect to age and race among the four groups: ALS patients, their

spouses, and the males and females of the healthy volunteer group.

One patient/spouse pair was excluded from further 16S rRNA gene ASV analysis due to a lower number of reads from the patient sample (<1000) compared with the others. Rectal swab gut microbiome profiles from the nine ALS patients and their spouses and the 10 healthy control couples revealed 952 unique ASVs, clustered at 100% 16S rRNA gene sequence similarity (average number of reads per participant: 11,263). ASVs that were not detected more than three times in at least 20% of the samples across the 38 participants were eliminated, and 143 ASVs remained. These ASVs clustered into 85 genera and were used for further analyses.

Principal coordinate analysis showed no differences in beta diversity between male and female spouses of the healthy volunteer group (by PERMANOVA: $p=0.362$ for Bray-Curtis distance; $p=0.429$ for unweighted UniFrac distance; $p=0.304$ for weighted UniFrac distance) (Figure 1). However, ALS patients differed from their spouses with respect to beta diversity (by PERMANOVA; $p=0.002$ for Bray-Curtis distance; $p=0.107$ by unweighted UniFrac distance; $p=0.004$ for weighted UniFrac distance) (Figure 2). Group centroids with the labeled pairs were plotted for the healthy volunteer (Supplementary Figure S1) and ALS (Supplementary Figure S2) groups. ALS patients had higher species richness and evenness than their spouses (Chao1 diversity: $p=0.03$; Shannon diversity: $p=0.004$; Wilcoxon signed-rank test; Figure 3).

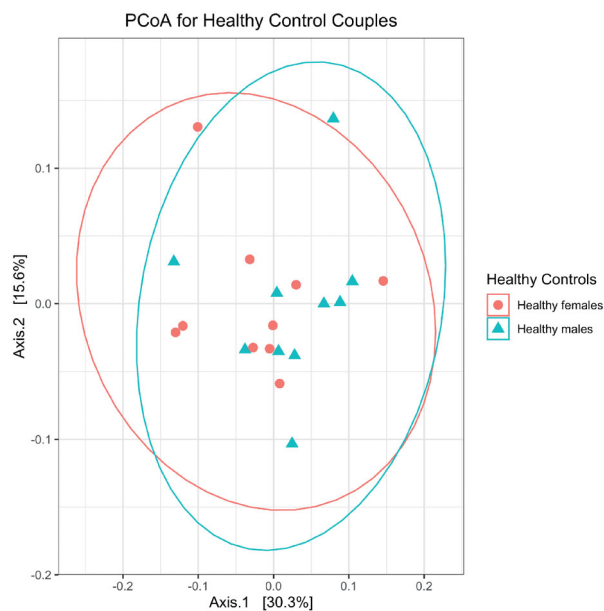


Figure 1. Gut microbiome profiles did not differ between the males ($n=10$) and females ($n=10$) of the healthy volunteer group, as assessed by permutational analysis of variance ($p=0.4$). PCoA: Principal coordinate analysis.

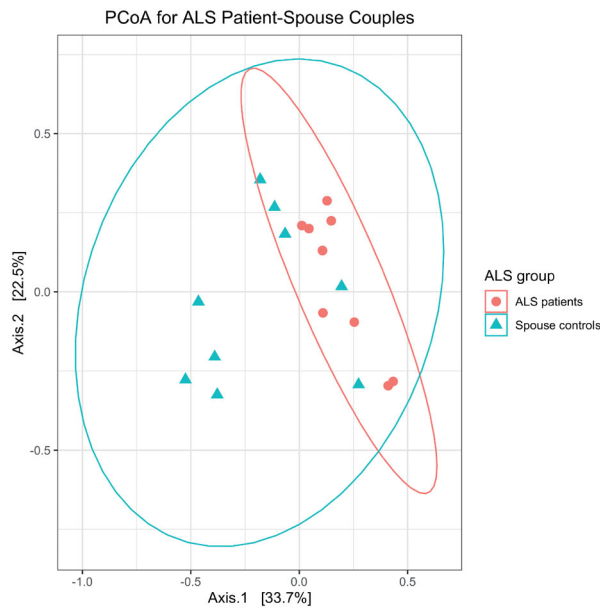


Figure 2. Gut microbiome profiles were different between ALS patients ($n=9$) and their spouses ($n=9$), as assessed by permutational analysis of variance ($p=0.003$).

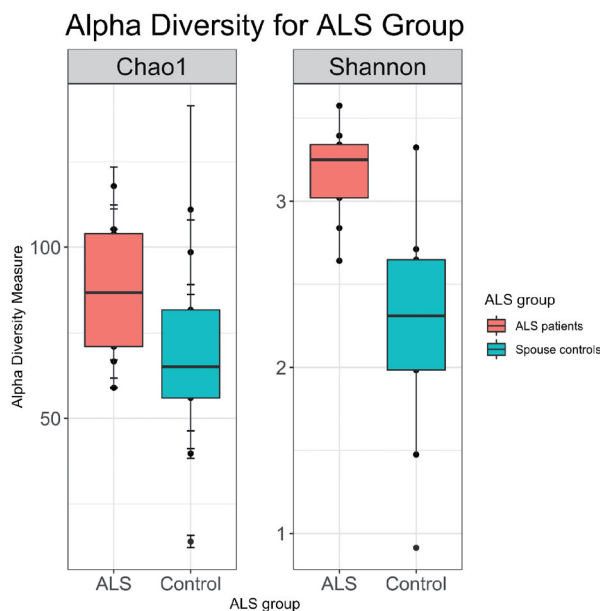


Figure 3. ALS patient ($n=9$) gut microbiomes were more diverse than those of their spouses ($n=9$) with respect to species richness ($p=0.03$) and species evenness ($p=0.004$), which were measured using the Chao1 and Shannon indices, respectively.

Compared with their spouses, ALS patients lacked ASVs of the genus *Prevotella* (adjusted $p=0.002$; Figure 4). A particularly disparate ASV from the genus *Prevotella* in the two groups was *P. timonensis* (adjusted $p=9 \times 10^{-18}$; Figure 5). However, none of the 25 comparisons of stool and plasma inflammatory biomarkers were statistically significant after correcting for multiple comparisons (Table 1).

Predicted functional profile differences between the microbiomes of ALS patients and their spouses were determined (Figure 6). The gut microbiomes

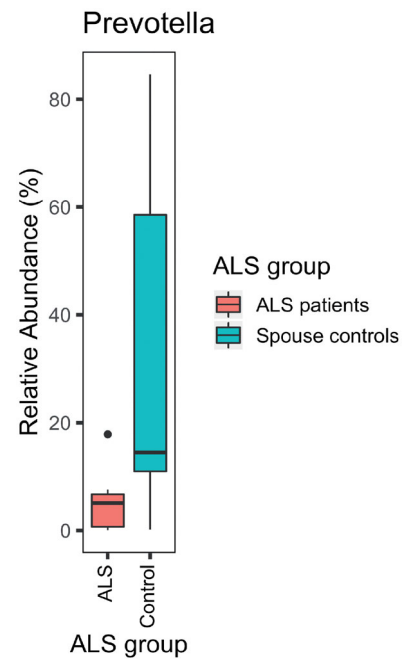


Figure 4. ALS patients ($n=9$) were deficient in *Prevotella* spp. compared with their spouses ($n=9$; $p=0.02$).

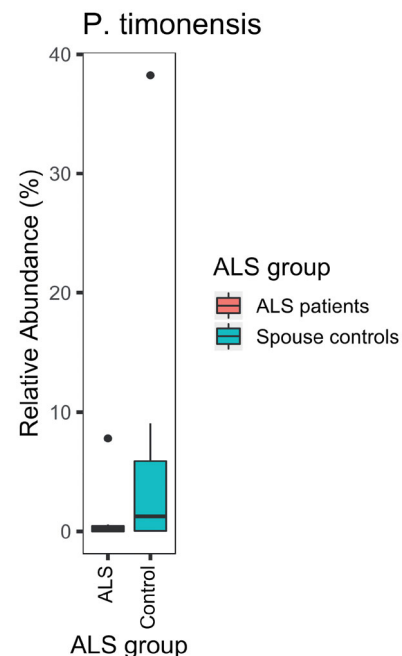


Figure 5. ALS patients ($n=9$) were deficient in *P. timonensis* compared with their spouses ($n=9$; $p=0.04$; sign test).

of ALS patients exhibited lower levels of enzymes involved in some metabolic pathways, including carbon metabolism and systems involving histidine kinase and response regulators. Additionally, ALS patients completely lacked butanoate metabolic pathway enzymes, essential for butyrate production.

Discussion

In this study, we found differences in the gut microbiomes of ALS patients compared with their

Table 1.. Average (Avg.) and standard deviation (SD) values of inflammatory biomarkers in plasma and stool for ALS patients as well as for partner/caregiver controls, and the average and standard deviation of the difference between patient and control.

Group	Medium	Analyte ^a	ALS patients (n = 10)		Partner/caregivers (n = 10)		Δ (n = 10)		p Value*	Adjusted p value**
			Avg.	SD	Avg.	SD	Avg.	SD		
Plasma		CRP ($\times 10^{-6}$)	1.73	1.67	3.25	2.83	-1.52	3.19	0.17	0.46
		IFN- γ	3.94	1.63	7.14	5.26	-3.19	6.33	0.15	0.46
		IL-10	0.35	0.12	0.47	0.44	-0.12	0.43	0.41	0.60
		IL-1 β	0.05	0.05	0.06	0.12	-0.01	0.10	0.73	0.79
		IL-12p70	0.16	0.17	0.23	0.20	-0.07	0.27	0.44	0.61
		IL-13	0.76	0.68	0.58	0.41	0.19	0.85	0.50	0.66
		IL-2	0.26	0.12	0.39	0.14	-0.13	0.21	0.08	0.38
		IL-4	0.03	0.01	0.03	0.01	0.01	0.01	0.19	0.46
		IL-6	0.71	0.43	0.78	0.73	-0.07	0.87	0.81	0.81
		IL-8	2.25	0.79	3.05	2.12	-0.80	1.98	0.23	0.46
		LBP	3125	893	3684	1469	-558	1819	0.36	0.56
Stool		LPS	6.01	0.72	6.10	1.08	-0.09	1.04	0.79	0.81
		TNF	3.19	0.46	3.40	1.41	-0.20	1.35	0.65	0.77
		CRP ($\times 10^{-6}$)	449	651	1093	1296	-644	1610	0.24	0.46
		IFN- γ	29.7	58.6	55	111.12	-25.32	133.6	0.56	0.70
		IL-10	0.20	0.16	0.64	0.63	-0.45	0.57	0.03	0.38
		IL-1 β	39.0	34.0	90.5	92.7	-51.5	93.9	0.12	0.43
		IL-12p70	0.95	0.70	1.14	1.18	-0.19	1.42	0.68	0.77
		IL-13	11.39	19.98	23.09	31.63	-11.9	37.0	0.34	0.56
		IL-2	26.2	56.9	65.4	82.3	-39.2	109	0.28	0.50
		IL-4	0.16	0.10	0.23	0.14	-0.06	0.09	0.06	0.38
		IL-6	0.25	0.23	0.52	0.54	-0.26	0.44	0.09	0.38
	IL-8	179	226	806	934	-627	902	0.06	0.38	
	LBP	0.42	0.50	2.92	6.09	-2.49	6.21	0.24	0.46	
	TNF	1.29	0.88	2.89	3.03	-1.61	2.58	0.08	0.38	

p Values were calculated by paired t-test.

^aUnits are pg/ml except for LPS, which is EU/ml.

*p Value for paired t-test.

**p Value adjusted for multiple comparisons using Benjamini–Hochberg correction.

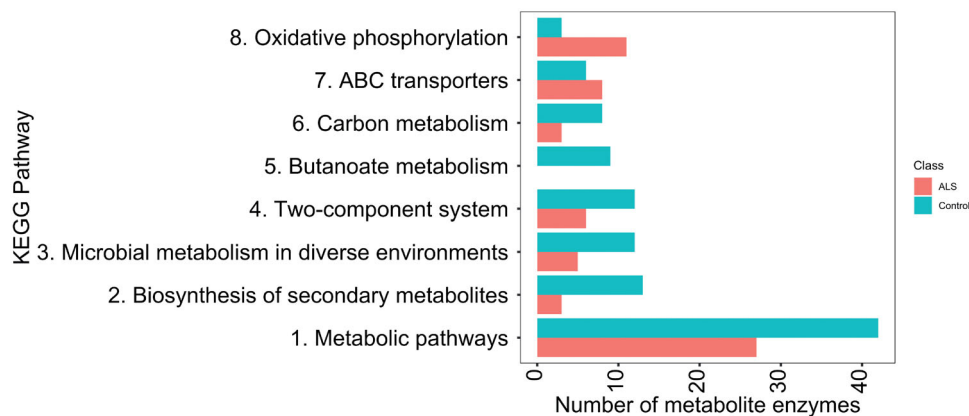


Figure 6. KEGG pathway analysis revealed a differential abundance of enzymes in ALS patients (upper bar) and their spouses (lower bar).

healthy spouses. ALS patient microbiomes were significantly more diverse with a higher number of taxa. ALS patients were also deficient in *Prevotella* spp. These differences were not found between couples in the healthy volunteer group.

Several human studies have previously reported an association between the gut microbiome and ALS pathophysiology (3–6,8); however, another study has suggested the contrary (7). Gut microbiome differences have been described for several

diseases, including irritable bowel syndrome (28), psoriatic arthritis (29), celiac disease (30), atopic dermatitis (31), Crohn’s disease (32), and obesity (33). In each case (28–33), diseased patient microbiome profiles had lower alpha diversity (species richness and evenness) than those of controls, suggesting that diversity indicates a healthy gut. Thus, our finding of increased diversity with ALS was surprising. This finding might be due to stress-related responses in the spouses (34–36).

However, the lack of differences in key inflammatory cytokines indicated that either stress was exculpatory or that patients and spouses were equally stressed, albeit for different reasons.

A few studies have investigated gut microbiome profiles in PD patients and their spouses. Like ALS, PD is a neurodegenerative disease. Hill-Burns et al. (37) reported that PD patients had significantly altered abundances of *Bifidobacteriaceae*, *Christensenellaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Pasteurellaceae*, and *Verrucomicrobiaceae* compared with controls, including some patient spouses and other community controls. In this same cohort, a secondary multivariate analysis of 37 immune and angiogenic factors in stool samples from patient/spouse pairs revealed that PD patients exhibited significantly higher levels of IL-1 α , IL-1 β , CXCL8, and CRP than their spouses, after controlling for sex, body mass index, smoking history, and probiotic use (38).

The implications from these findings are critical for microbiome study design to meet the unbiased standard for the validity of a null hypothesis of no difference. The optimal study design should incorporate a spouse control group whenever possible because of influential environmental factors shared between patient/spouse pairs. Standard epidemiological parameter matching, including sex, age, and race, is not enough. Regardless of the matching process rigor, non-cohabitating case/control pairs do not share these same factors, thus affecting the study. We do note that use of spouse controls heightens the possibility of overmatching. In addition, the ALS patient was male for the majority of our ALS patient-spouse couples. In larger studies, comparisons of couples in which the patient is male to those in which the patient is female may address this possibility.

Our finding of lower *Prevotella* spp. proportions in ALS patients was consistent with other gut microbiome studies regarding neurodegenerative diseases. Gut microbiome dysbiosis has been associated with PD (37,39–45), multiple sclerosis (46–50), and autism spectrum disorder (51,52). Some reports have indicated *Prevotellaceae* deficiencies in PD (42–44), multiple sclerosis (46–49), and autism spectrum disorder (51). Again, inconsistencies among conclusions regarding the association of disease and lower levels of *Prevotella* spp. may have resulted from differences in technologies or control groups.

Prevotella spp. predominance in the gut microbiome has been associated with dietary fiber consumption (53). Additionally, it has been shown that *P. timonensis* induced strong pro-inflammatory immune responses via dendritic cells, inducing dendritic cell maturation and Th1 cell differentiation (54). Thus, *P. timonensis* predominance may have contributed to the lack of difference in the pro-inflammatory cytokines in ALS patients compared with their spouses.

Our functional predictions revealed that ALS patients lacked microbial enzymes associated with butyrate metabolism compared with their spouses. However, a deficiency in butyrate-producing microbes, as reported by others (4,6), was not observed. Nevertheless, our finding of the predicted lack of enzymes associated with butyrate metabolism along with a previously reported finding that butyrate supplementation delayed disease progression in SOD1^{G93A} mice and the finding of deficiency of the butyrate-producing microbes *Eubacterium rectale* and *Roseburia intestinalis* in ALS patients relative to healthy controls in a larger human ALS study strongly suggest an association of butyrate availability in the gut and disease progression. Further studies of the association of ALS with butyrate-producing microbes are warranted.

Although Brenner et al. (7) found no microbiome compositional differences between ALS patients and age- and sex-matched controls, they did find that ALS patients had higher species richness. This was consistent with our current findings. Results of the initial diversity analysis came to the principal investigators (V.S.H. and J.D.G.), who were blinded to the group identity (i.e. labeled as group A or B). The finding of higher species richness in ALS patient microbiomes was concerning because it has been believed that a diverse microbial community is associated with health. However, despite a decade of research, the definition of a healthy microbiome remains unclear.

A key strength of our study was use of spouses as controls. Additionally, we used a second set of healthy volunteer couples. Limitations of the study included the small sample size, thus restricting our ability to investigate the relationship of differential microbial communities with ALS characteristics such as site of onset as well as our ability to determine differential expression of markers of inflammation in plasma and stool between ALS patients and spouse controls. Moreover, 70% of the ALS patients were male, so gut microbiome differences due to sex may have dominated the case/control comparison. However, given the clustering of all ALS patients seen in the principal coordinate analysis (Figure 2), this conclusion seems unlikely. Another limitation is the lack of detailed diet information. Finally, because of the cross-sectional nature of our study we were unable to investigate the relationship of differential microbial communities with temporal ALS characteristics such as disease progression rates.

In this study, we presented evidence of an altered gut microbiome in ALS patients relative to their spouses, which may have a role in ALS pathophysiology. Our findings of higher microbial diversity, *Prevotella* spp. deficiency, and differential compositional profiles in ALS patients compared with their spouses warrant further investigation to understand the role of gut microbes in ALS

pathophysiology. Shotgun or whole-metagenome sequencing is necessary to determine the species and strains of *Prevotella* that differ in ALS patients, and further exploration of short-chain fatty acids, including butyrate, is needed to better understand its role in ALS pathophysiology. Our findings may have implications for developing new therapeutics for ALS that target the gut microbiome.

Acknowledgements

The authors are grateful for the participation of the patients and their spouses in this study.

Ethics approval

This study was approved by the Emory University Institutional Review Board (IRB00078771).

Informed consent

All participants provided written informed consent.

Author contributions

Conceptualization (V.S.H., M.G.T., and J.D.G.); clinical investigation (C.N.F., M.P., and J.D.G.); laboratory investigation (C.A.K. and M.G.T. [JCVI]); 16S rRNA gene sequencing (K.E.N.); stool and plasma cytokine measurement (M.G.T. [EU]); data curation and formal analysis (V.S.H. [lead], H.S., and A.M.); funding acquisition (V.S.H. and K.E.N.); software and visualization (H.S. [lead], A.M., and V.S.H.); writing—original draft (V.S.H.); and writing—review and editing (V.S.H. [lead], H.S., C.N.F., A.M., M.P., C.A.K., M.T., M.G.T., K.E.N., and J.D.G.).

Declaration of interest

The authors declare no competing interests.

Funding

This research was conducted without sponsored funding.

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Data availability statement

The 16S sequence data have been deposited in the National Center for Biotechnology Information (NCBI) database on BioProject accession number

PRJNA566436. Sample metadata are maintained at dx.doi.org/10.15139/S3/84SGKO.

Code for processing and analyses are maintained at dx.doi.org/10.15139/53/845GKO.

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