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TITLE: A Multidisciplinary Approach to Study the Role of the Gut Microbiome in Relapsing and Progressive MS

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14. ABSTRACT
Multiple sclerosis (MS) is a chronic, inflammatory disease of the central nervous system (CNS) and a common cause of progressive neurological disability in young adults. The typical disease course (85% of patients) is relapsing or bout-onset MS (RMS) but 15% of patients do not have relapses and experience progression of disability from disease onset or primary progressive form of MS (PPMS). We hypothesize that the gut microbiota in RMS and PPMS patients is different, thus potentially influencing disease course. In this project we recruited 381 subjects (149 RMS patients, 84 PPMS and 148 healthy) and performed fecal (bacterial) DNA by 16S ribosomal RNA gene sequencing, and high-resolution HLA typing. In addition, we transferred microbiota from RMS (n=2) and PPMS (n=2) patients into germ free mice before inducing experimental allergic encephalomyelitis (EAE). Despite the heterogeneous and modest sample size, we observed increased frequency of Enterobacteriaceae in RRMS patients and a reduction of Prevotella Copri in PPMS subjects. Transplantation of fecal microbiota from RMS patients into germ free mice resulted in milder neurological disability (EAE) compared to mice receiving microbiota from PPMS patients. While results need to be replicated, this suggests that gut microbiota from PPMS is less inflammatory than that of RMS subjects, thus shedding some light into disease pathogenesis.

15. SUBJECT TERMS
microbiome, multiple sclerosis, progressive, relapsing

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1. INTRODUCTION:

We hypothesize that specific human GI microbiota can alter the balance of inflammatory and regulatory immune cell populations thus leading to disease in genetically susceptible hosts. Furthermore, we hypothesize that gut microbiota from subjects with RMS and PPMS is fundamentally different and can elicit distinguishable effects when transferred into susceptible animal models of the disease. To test these hypotheses, we will conduct a series of experiments including high-throughput bacterial DNA sequencing, immunological profiling of patients and controls, and live microbiota transfer from patients into mice followed by their immuno-molecular characterization.

2. KEYWORDS:

microbiome,
multiple sclerosis
progressive
relapsing

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**

Specific Aim 1: To compare the gut microbiome of subjects with RMS and PPMS.	**Timeline**
Major Task 1: Identification and recruitment of research subjects	Months
Major Task 2: Sample collection and initial processing.	
Milestone #1: Recruitment and processing samples from 150 RMS, 150 PPMS and 150 healthy controls.	1-24
Major Task 3: 16S ribosomal gene sequencing and initial bioinformatics analysis.	
Milestone #2: Sequencing of the MS microbiome.	12-28
Major Task 4: Data integration and advanced bioinformatics analysis.	
Specific Aim 2: To test the effect of human MS microbiota in a spontaneous and induced EAE mouse model	
Major Task 1: microbiota transfer into germ-free mice and EAE induction	
Major Task 2: Immuno-pathological characterization of experimental mice	
Milestone #3: Co-authored manuscript.	28-36

- **What was accomplished under these goals?**

Specific Aim 1: To compare the gut microbiome of subjects with RMS and PPMS.	**Timeline**	**Responsible PI**	**Status**
Major Task 1: Identification and recruitment of research subjects	Months		
Subtask 1: Chart reviews to identify eligible patients from MS clinic at UCSF and Mt Sinai. Participating teams: • Dr. Cree (including a clinical coordinator) • Dr. Katz Sand (subcontract 1: Mt Sinai)	1-24	Cree (Katz-Sand)	Completed
Subtask 2: Clinical evaluation and invitation to participate in the study • Neurological evaluation of prospective subjects. • Invitation to participate in study • Signed informed consent • Blood collection	1-24	Cree (Katz-Sand)	Completed
Major Task 2: Sample collection and initial processing.			
Subtask 1: Preparation of collection mailing kits	1-12	Baranzini (Casaccia)	Completed
Subtask 2: Bacterial DNA extraction from stool material. • DNA purification • Gel electrophoresis • Spectrophotometric quantification	6-24	Baranzini (Casaccia)	Completed
Subtask 3: Genotyping and HLA characterization of host DNA. • DNA genotyping with known MS susceptibility variants • High-resolution HLA typing	6-24	Baranzini	Completed
Milestone #1: Recruitment and processing samples from 150 RMS, 150 PPMS and 150 healthy controls.	1-24		Completed
Major Task 3: 16S ribosomal gene sequencing and initial bioinformatics analysis.			
Subtask 1: sequencing of 16S ribosomal RNA gene in all DNA samples from MS patients and controls	12-24	Knight	Completed
Subtask 2: bioinformatics analysis. • Quality control of sequencing runs • Alignment of reads to Greengenes database • Unifrac distance computation and principle coordinate analysis visualization	24-28	Knight	Completed
Milestone #2:Sequencing of the MS microbiome.	12-28	Knight	Completed

Major Task 4: Data integration and advanced bioinformatics analysis.			
Subtask 1: Integration of microbiome and genomic data	24-32	Baranzini	Ongoing
Specific Aim 2: To test the effect of human MS microbiota in a spontaneous and induced EAE mouse model			
Major Task 1: microbiota transfer into germ-free mice and EAE induction			
Subtask 1: re-derivation of Tob1/2D2 mice into a GF line	1-12	Mazmanian	incomplete
Subtask 2: Transfer of live microbiota from select patients into germ-free mice, EAE induction and follow-up	12-18	Mazmanian	Partially complete
Major Task 2: Immuno-pathological characterization of experimental mice			
Subtask 1: tissue dissection, harvesting and pathological analysis • Dissect lymph nodes, brains and spinal cords • Analyze CNS tissues for H&E and myelin stainings	18-24	Mazmanian	Partially complete
Subtask 2: Flow cytometry • Dendritic cells (CD11c+, CD11b+) • B cells (CD19+) • MHC class II expression (CD80, CD86)	18-24	Baranzini (Mazmanian, Casaccia)	incomplete
Subtask 3: Immunohistochemistry • Immunostaining for CC1/APC, NG2, Caps-3, SMI31, NFH, MBP (axonal damage)	18-24	(Casaccia)	incomplete
Subtask 4: Molecular characterization • Gene expression profiling • DNA methylation profiling	18-24	Baranzini (Casaccia)	Partially complete
Milestone #3: Co-authored manuscript.	28-36		In progress

Specific aim 1 was a complete success. Identification and recruitment of 381 subjects was accomplished (major task 1), and although it took longer than anticipated, the quality of data and biological samples obtained resulted in a valuable resource (Major task 2). Furthermore, sequencing of the bacterial DNA from stool and HLA typing from the host DNA was also successful (Major task 3).

Recruited participants

149 relapsing-remitting MS patients, 84 primary progressive MS patients and 148 healthy participants were recruited from UCSF and Mount Sinai (Table 1, Excel File 1). Patients and controls were age matched, while as a consequence of the uneven sex distribution of MS, 63.5% of the MS participants were female, compared with 43.2% of controls. As more males are controls, a higher body mass index

(BMI) was observed in the control group. More than half (57.1%) MS patients were treated, and specifically a higher proportion of RRMS patients (69.1%) were treated than PPMS patients (35.7%).

Table 1. Participants' characteristics

	Control	MS	RRMS (4 CIS)	PPMS
Number	148	233	149	84
Site				
San Francisco	93	129	69	60
New York	55	104	80	24
Age (y)	51.3(42,62)	53.8(40,60)	45.5(36,56)	68.4(52,65)
Female (%)	64 (43.2%)	148(63.5%)	111(74.5%)	37(44%)
BMI	29.6(23.1,29.5)	25.7(22,28.9)	25.5(21.6,28.3)	26.1(22.9,29.6)
Disease duration(y)		12.4(5,18)	12.6(4.75,20)	12(5,16)
EDSS		2.8(1,4)	1.5(0,2.5)	5.0(3.5,6.4)
Treated(%)		133 (57.1%)	103(69.1%)	30(35.7%)
Treatment				
Tecfidera		33	29	4
Copaxone		29	28	1
Ocrevus/Rituxan		32	11	21
Gilenya		17	15	2
Interferon		11	10	1
Tysabri		11	10	1

Data present as mean (Interquartile range, IQR). y, year; EDSS, Expanded Disability Status Scale.

Microbiome diversity is impacted by confounding factors

Amplicon sequencing variants (ASVs) were resolved from the 16S rRNA sequencing data and classified based on the Greengenes reference dataset (Excel File 2). To understand how the microbial composition differs between patients and healthy controls, we compared the diversity of microbial composition characterized by ASVs within (α-diversity) and between individuals (β-diversity). As expected, no significant difference in α-diversity was observed between MS and healthy control groups as measured by Shannon and Chao1 index, neither among the disease course (Fig.1).

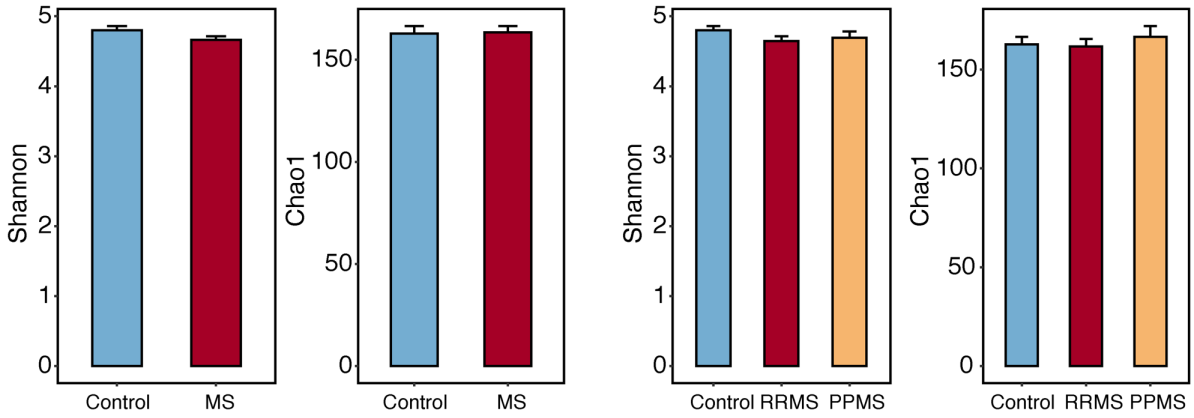

Figure 1. Microbiome diversity. Microbiome α-diversity measured by Shannon and Chao1 index was compared by disease status and disease course. Data are presented as mean ± SEM (ANOVA, not significant).

A significant difference of β-diversity among healthy control, RRMS and PPMS was observed even though the samples were not clearly clustered by disease status (Fig. 2A). Given the heterogeneity of human samples, we applied PERMANOVA to test how much microbiome differences are influenced by demography, medication use and physiology (i.e. confounders). Besides disease course, three other confounders were also identified to be significantly associated with microbiome diversity measured by inter-individual weighted uniFrac distance (Fig. 2B). Recruitment site accounted for the largest variation and a PCoA of the microbiome beta-diversity showed the significant difference of samples from San Francisco and New York (adonis R^2=0.0465, Fig. 2C). We also observed the difference of microbiome diversity associated to sex (Fig. 2D), which has to be adjusted in the disease associated analyses as more MS patients are females while more controls are males (Fig .2E). The impact of these factors on shaping gut microbiome raised the possibility that the largest difference given by recruitment site was potentially co-contributed by a biased distribution of disease status or sex, we then quantified the sample size by disease course and sex in each site (Fig. 2E). No uneven distribution of disease or sex was observed in the two recruitment sites, hence the impact of geographic location had to be adjusted. Body mass index (BMI) also exerted significant effects (Fig. 2A), but the effect could be reduced by the sex adjustment as a consequence of the observed higher BMI of males compared to that of females (Fig. 2F).

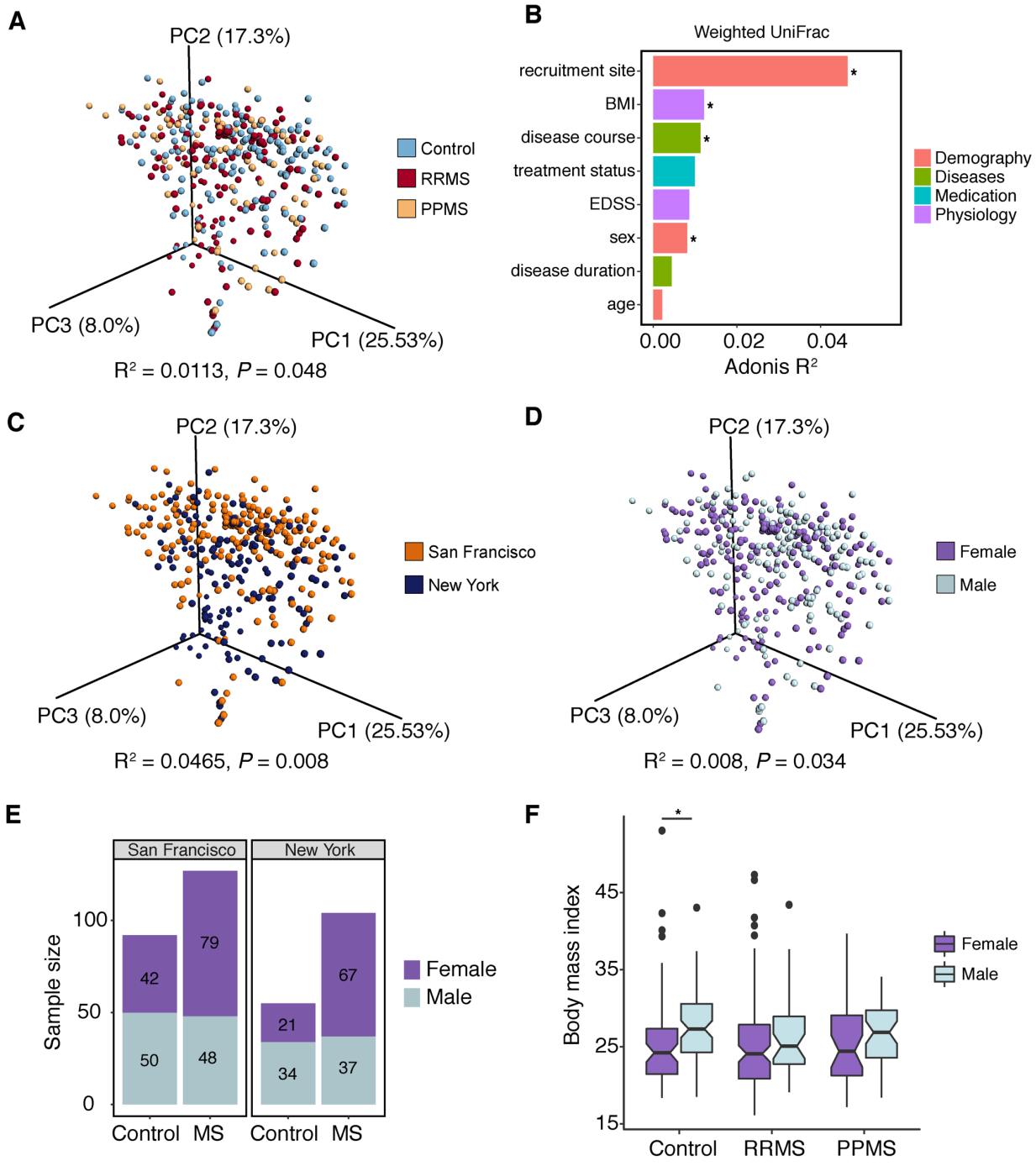

Figure 2. Effect size of confounders. (A) PCoA of weighted UniFrac community distance compared by disease status. (B) Bar plot showing the size effect (Adonis R^2) of confounders associated with gut microbial variations (weighted UniFrac distance). Confounders showing significant impact on gut microbiome were labeled (Multiple testing correction P value < 0.05). BMI, body mass index; EDSS, expanded disability status scale. (C) PCoA of weighted UniFrac community distance compared between recruitment sites. (D) PCoA of weighted UniFrac community distance by sex. (E) Sample size summarized by disease course, sex in two recruitment sites. (F) Body mass index compared between female and male by disease course. Statistical significance was determined by ANOVA (multiple testing corrected P value,*$P \leq 0.05$).

Individual microbes associated with MS

To identify the individual microbes associated with MS disease course, we performed differential analyses to compare the microbial community between MS patients and heathy controls by controlling the impact of recruitment site and sex as mentioned above. Only one microbe, belonging to the *Enterobacteriaceae* family, was identified to be different between RRMS and controls subjects in this study (Fig. 3A). The abundance of *Ruminococcus torques*, *Bacteroides caccae* and *Eubacterium dolichum* were increased in PPMS patients compared to healthy controls while *Provetella Copri* was reduced in PPMS patients (Fig. 3B). Both *R. torques* and *B. cacca* are mucin degradation bacteria. Another mucin degradation bacterium, *Akkermansia muciniphila*, has also been reported in MS by our previous studies [1, 2]. *R. torques* was also enriched in patients with autism spectrum disorder and inflammatory bowel disease (IBD) [3]. A TonB-linked outer membrane protein, termed OmpW, was identified from *B. caccae* and shown to induce adaptive immune responses in pediatric IBD patients more frequently than in healthy controls [4]. These data suggest the possibility that these mucin degradation bacteria could induce proinflammatory responses in MS. *Eubacterium dolichum* was reported to associate with propionate production in human intestine, and predominant in patients with relapsing polychondritis [5] and individuals consuming a high-fat diet. There is no significant correlation between this bacterium and BMI in our study. Further studies are needed to identify the roles of these bacteria in the pathogenesis of MS. Patients with RRMS had more *P. copri* compared to PPMS as a result of the reduction in PPMS (Fig. 3C), but it was decreased in RRMS patients shown in a related study [6]. By stratifying the comparisons based on treated status, we still observed an increase of *R. torques* in both treated PPMS and untreated PPMS compared to healthy controls, and increase of *B. caccae* in treated PPMS (Fig. 3D). *P. copri* was only significantly reduced in untreated PPMS patients, which was also reported in another study [Intestinal microbiota in multiple sclerosis: influence of treatment with interferon β-1].

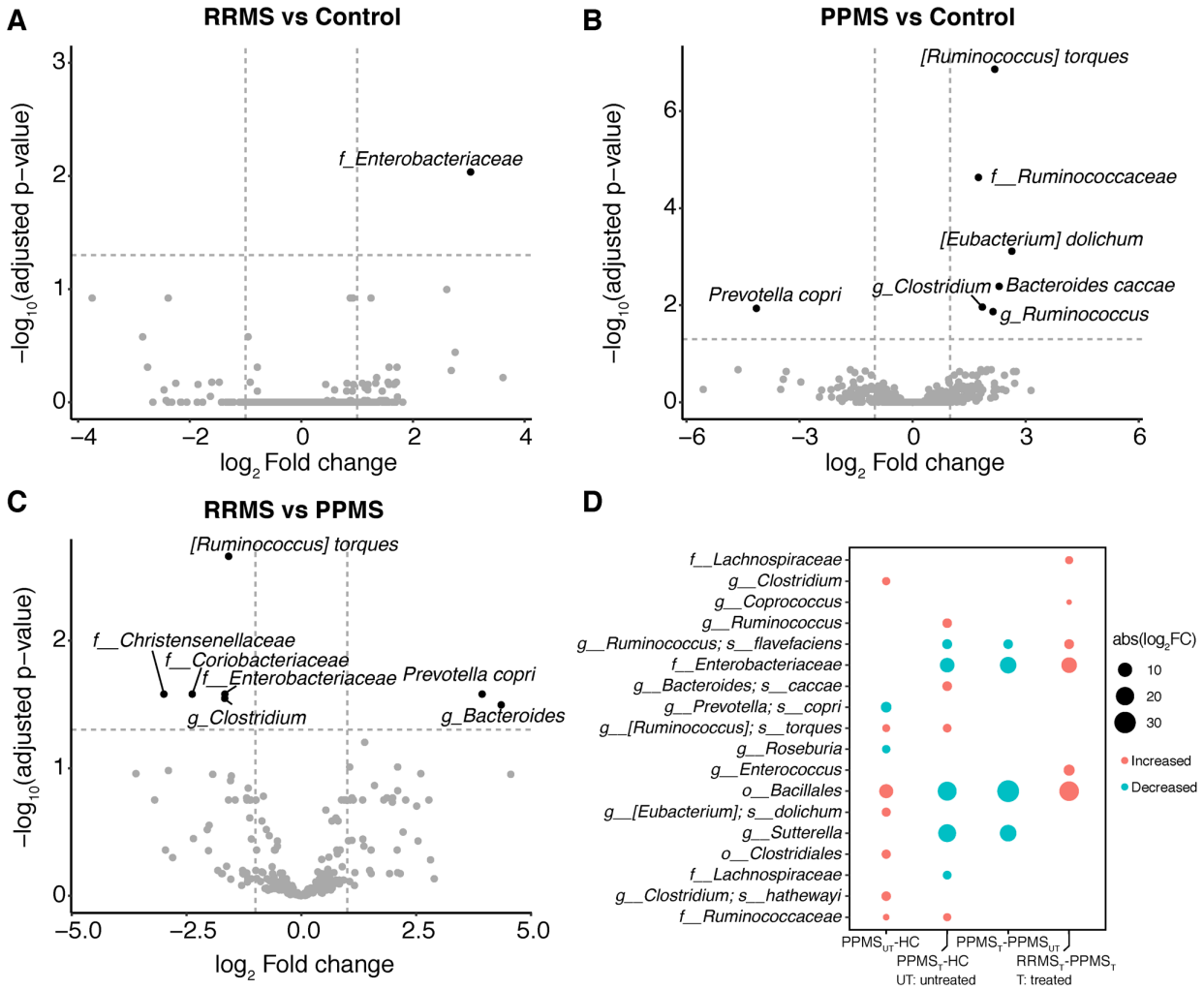

Figure 3. (A-C) Significantly differential ASVs associated with MS disease course by controlling the impact of recruitment site and sex (Fold change ≤ 2 and FDR ≤ 0.05). Only ASVs classified to genus/species level were labeled. (D) Significantly differential ASVs compared between HC and MS stratified by treated status controlled by recruitment site and sex (Fold change ≤ 2 and FDR ≤ 0.05). Dot size is proportional to the absolute log fold change at base 2, red dots represent an increase of microbial abundance and yellow ones represent a decrease of the abundance.

Association between HLA genotype and gut microbes characterized by 16S and shallow shotgun sequencing

To understand how host genetics impact on the gut microbial composition, we applied the Chi-squared statistics to test whether there is an association between HLA genotype and the structure of a specific bacterium. The HLA genotyping reported 735 alleles in 22 HLA genes (Excel File 3), we first truncated the alleles from four sets of digits to two sets of digits covering only the difference in one or more nucleotide substitutions that change the amino acid sequence of the encoded protein, which identified 367 HLA alleles (Excel File 4). The presence/absence of each bacterium and the status (positive/negative) of each HLA allele were quantified among controls samples, MS patients and all samples, respectively, and then calculated for the association. We observed multiple significant

associations between HLA alleles and gut microbes either characterized by 16S rRNA sequencing (Excel File 2) or shallow shotgun sequencing (Excel File 5) but only few or none remained significant after multiple test correction for all HLA alleles and bacteria (Excel File 6). We did not observe significantly associated bacteria with the MS-associated allele DRB1:15:01.

Further effort is needed to stratify the samples into high MS risk or low risk groups based on the HLA alleles carried. A differential comparison of the microbes in these groups would identify the bacterial that associated with MS susceptibility rather than one allele.

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- **What opportunities for training and professional development has the project provided?**

During this project, several opportunities for training and professional development were realized.

At the beginning of the project Keith Place (Baranzini Lab) visited the Mazmanian Lab in Caltech to learn from their senior rodent technician on sterile technique to keep mice germ-free within the gnotobiotic facility.

Elva Kogl (Baranzini lab) was trained in rederivation of germ-free mice by members of the Mazmanian (Caltech) and Turnbaugh (UCSF) Labs.

Xiaoyuan Zhou (Baranzini Lab) visited the Knight lab (UCSD) to learn about processing and analysis of 16S ribosomal RNA gene sequencing data.

- **How were the results disseminated to communities of interest?**

Dissemination mostly occurred via scientific presentation in academic institutions. Since the analysis is still ongoing, most of the dissemination has been among the team members and closely related collaborators.

- **What do you plan to do during the next reporting period to accomplish the goals?**

This is the final report.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**

This project generated the largest microbiome dataset ever described in MS to date. In particular, this is the largest dataset of Primary progressive MS patients.

In addition, we have generated preliminary evidence suggesting that transfer of stool microbiota from PPMS patients into germ-free mice has lower impact than transferring stool microbiota from RRMS patients.

- **What was the impact on other disciplines?**

This project was focused in MS.

- **What was the impact on technology transfer?**

N/A

- **What was the impact on society beyond science and technology?**

This project addressed a major question in the field. While it is difficult to measure societal impact, patients suffering from MS and their relatives have shown enthusiasm and curiosity to learn about our results.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

No changes to the original project were needed.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

The major challenge associated with this project was the time-consuming nature of performing experiments in germ-free conditions. While we were successful in setting up and maintaining our

colonies, we were only able to conduct two successful microbiota transfer experiments. This in part was due to the long breeding period needed to bring germ-free mice to adulthood with control littermates. The slow breeding was compounded by the small size of our germ-free facility at UCSF, which only allowed us to house mice in the ISO-cage system (which offers higher flexibility but lower scale operations). A persistent challenge of any germ-free facility is bacterial contamination, which can occur at any cage change and jeopardize or invalidate an experiment. Unfortunately, 2 of our experiments needed to be terminated due to bacterial contamination.

In addition, due to time constraints, we were unable to rederive germ-free and experiment with the Tob1-/- mice.

- **Changes that had a significant impact on expenditures**

None.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

None.

- **Significant changes in use or care of human subjects**

None.

- **Significant changes in use or care of vertebrate animals.**

None.

- **Significant changes in use of biohazards and/or select agents**

None.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

- **Journal publications.**

Since analysis is still ongoing, no publications have yet resulted from this work.

- **Books or other non-periodical, one-time publications.**

None.

- **Other publications, conference papers, and presentations.**

None.

- **Website(s) or other Internet site(s)**

None.

- **Technologies or techniques**

None

- **Inventions, patent applications, and/or licenses**

None

- **Other Products**

None.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Personnel	Role	Person month
Sergio Baranzini	PI	10
Bruce Cree	PI	5
Rob Knight	PI	5
Ilana Katz-Sand	Patient evaluation and recruitment	5
Patrizia Casaccia	Molecular biology, immunology	2
Sneha Singh	Clinical Coordinator	15
Adam Santaniello	Database manager	5
Xiaoyuan Zhou	Post Doc	8
Tomasz Piotr Kosciolek	Post Doc	5
Jon Sanders	Post Doc	5
Stefan Janssen	Post Doc	5
Daniel McDonald	Bioinformatics Programmer, PhD	7
Jeffrey E Dereus	Lab Technician - Programmer	8

Sergio Baranzini: Submitting PI. Oversaw and coordinated all scientific and budgetary aspects of this proposal.

Bruce Cree: Clinical PI. Directed patient evaluation and recruitment efforts at UCSF.

Rob Knight: oversight and management of sequencing phase of project overall.

Ilana Katz-Sand: Directed patient evaluation and recruitment efforts at UCSF.

Patrizia Casaccia: Conducted molecular and immune profiling of mouse tissues derived from the fecal transfer experiments.

Adam Santaniello: Built and oversaw the sample and patients database resulting from this project.

Xiaoyuan Zhou: Analyzed all 16S and shotgun sequencing data from the Knight lab. Also conducted statistical analysis on metadata and HLA.

Amnon Amir: Developed fast algorithms for OTU picking that were used in the 16S analysis. Amnon left UC-SD in May 2017.

Stefan Maximilian Janssen: Developed methods to analyze exRNA sequences to assess interkingdom communication.

Tomasz Piotr Kosciolek: Developed methods to identify ncRNAs in the genomic and metagenomic data.

Daniel McDonald: Conducted computational studies of sequenced genomes, extracting and integrating biological information and data. Daniel joined the project in May 2017.

Jon Sanders: Developed a genome annotation pipeline and fast matching for separating human reads from microbial that will be used in the shotgun metagenomics.

Jeffrey Dereus: Managed the software team, setup of project tracking and LIMS for this project.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

No change from last reporting period.

- **What other organizations were involved as partners?**

- **Organization Name: N/A**
- **Location of Organization:**
- **Partner's contribution to the projec**t:
- **Financial support;**
- **In-kind support:**
- **Facilities**:
- **Collaboration**:
- **Personnel exchanges:**
- **Other:**

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**
- **QUAD CHARTS:**

9. APPENDICES: