MICB 475 Project Proposal | Team 10

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**Investigating How Aging and Smoking Shape the Gut Microbiome in the Context of Progressive Multiple Sclerosis**

**Abstract**

1. Background on MS and its relationship with the gut microbiome
2. The role of aging and smoking as factors influencing microbiome composition
3. Objectives/ aim
4. Results found
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**Introduction & Background**

Multiple Sclerosis (MS) is a chronic autoimmune condition characterized by immune infiltration of the central nervous system (CNS) and subsequent demyelination of axons [1, 2]. This results in impaired action potential propagation, leading to cognitive and physical disability [1, 2]. Though the cause of MS has yet to be agreed upon, it is hypothesized that T lymphocytes primed by mimotopes from pathogens confuse endogenous cells of the nervous system as foreign and mount an immune response against them [3]. Hence, T lymphocytes primed against myelin oligodendrocyte glycoprotein (MOG) attack the myelin sheath, a functional component of nerve fibers, resulting in the formation of lesions in the white and gray matter [4]. As a result, the ensuing sclerosis inhibits conduction of electrical impulses from the CNS to the periphery of the body and vice-versa, causing patients to present with a variety of neurological symptoms, varying in severity [4]. Initially, symptoms can manifest as blurred vision and paresthesia, but upon clinical progression of disease, paralysis, pain, and difficulty controlling bowel movements are often observed [4]. The sudden onset of one or more of the aforementioned symptoms is commonly referred to as a demyelinating attack [4].

Depending on the severity and duration of these attacks, there are three clinical courses of MS, with two representing patients that have experienced significant disease progression. Firstly, relapsing-remitting MS (RRMS) is characterized by alternating periods of symptom incidence and symptom regression whereby the patient is clinically stable [4]. In contrast, the clinical progression of MS entails non-remitting symptoms with worsening disability over time in the absence of distinguishable attacks and is categorized into primary progressive MS (PPMS) and secondary progressive MS (SPMS) [4]. The difference between the two disease courses is whether or not the patient had RRMS prior to disease progression which would be the case for a patient subsequently diagnosed with SPMS, whereas PPMS patients did not experience cycles of relapsing and remitting MS [5]. Additionally, PPMS and SPMS can be grouped into an umbrella category called progressive MS (pMS)[6]

Additionally, there is growing evidence that age may play a crucial role in the transition from RRMS to SPMS as older patients have been shown to experience a shorter period of disease latency prior to progression [7]. This may be due to the increased inflammation observed with aging, or inflammaging, which is characterized by significant changes to the gut microenvironment, including shifts in the gut microbiome composition and increased leakage of intestinal contents through the gut barrier [8,9,10]. Miyauchi et al. [11] demonstrated that by co-colonizing germ-free mice with OTU0002 and Lactobacillus reuteri which has MOG-mimicking peptides and then inducing a murine model of MS, CD4+ T cells co-localized with microbes in the small intestine and inflammatory biomarkers, along with clinical scores, were all increased. Therefore, by altering the microbiome composition and skewing it in favor of pro-inflammatory species, disease progression can be observed. Contrastingly, species of the Akkermansia genus have been identified as increasing in abundance alongside increasing age; this interaction is not frequently observed when considering disease-associated confounders [12]. Studies have observed that elevated Akkermansia in MS may promote tolerance [13, 14]. The genus was associated with a decrease in IL-17-producing γ/δ T cells [15]. This evidence supports the anti-inflammatory nature of Akkermansia which has been associated with the regression of neurological disease in an animal model of amyotrophic lateral sclerosis (ALS), alongside the anti-seizure effects of a ketogenic diet [12]. These findings suggest a relationship between the members of the gut microbiome and encephalomyelitis.With an increase in human microbiome research, researchers have observed changes in the composition of the gut microbiota in patients with chronic diseases such as MS [16]. The human gut hosts a variety of commensal microbes that influence physiological functions and immune responses [6] such that perturbations of these interactions may result in dysbiosis, causing increased inflammation and promoting various inflammatory bowel diseases [16]. In addition, the iMSMS Consortiumobserves that specific bacterial species and families residing in the gut contribute to an increased risk of encephalomyelitis, as well as disease progression [17]. Bacterial species such as *Akkermansia muciniphila*, *Ruthenibacterium lactatiformans*, *Hungatella hathewayi*, and *Eisenbergiella tayi* were in higher abundancein MS patients [17]. Furthermore, there was a decrease in species such as *Faecalibacterium prausnitzii* and *Blautia* species [17]. However, potential factors for why these species are observed in higher or lower abundance in the gut microbiota of MS patients remains yet to be elucidated as certain factors such as therapeutic interventions, history of smoking, geographic location, age, and other lifestyle choices were not controlled for during the investigation.

The literature is sparse on research that studies the impact of smoking on the composition of the gut microbiome despite increasing evidence by neuromodulation of the CNS via metabolic byproducts of commensal bacteria traveling up the gut-brain axis. The gut microenvironment, along with other mucosal surfaces, require homeostasis to regulate metabolism and promote tolerance, while repeated exposure to environmental stressors such as smoking promote inflammation [18]. The mucosal surface plays a crucial role in barrier exclusion of pathogens. In the respiratory tract, smoking introduces aerosol particles which upon inhalation, result in disruption of mucociliary clearance, increasing the susceptibility of smokers to mucocutaneous infection [19]. Although the connection between smoking and the gut microbiome remains unclear, it has been observed that the effects of inhaled aerosols extend to the gut mucosal surfaces and mucin expression, inciting inflammatory responses [20]. In addition, nicotine, found readily in smoking products, is a neuromodulator of the CNS and has been observed to alter the gut-brain axis [21]. Considering this, changes in the gut-brain axis may further promote dysbiosis of the gut microbiome, warranting additional investigation on whether smoking impacts the gut taxa composition and specifically, the establishment of proinflammatory species.

The literature has established a connection between the gut microbiome and CNS, yet there remains a critical need to investigate the factors shaping the gut microbiome and the role of dysbiosis in MS progression. This study aims to address this gap by examining how age and smoking history relate to changes in microbiome composition and MS disease progression. By characterizing and comparing gut microbial taxa in individuals across diverse ages, smoking histories, and disease stages, we aim to identify unique taxa to individuals with pMS. . We hope that this project will provide clinically relevant insights for healthcare providers and researchers to prevent disease progression and treatment resistance.

**Research Objectives**

Research question: How does age and smoking history influence the composition of the gut microbiome in progressive multiple sclerosis patients?

We seek to investigate two variables: age and smoking, and how these factors influence the composition of the gut microbiome in pMS patients as well as healthy controls.  **We hypothesize a taxonomical difference of the gut microbiome between groups defined by disease status, age groups, and smoking status .Specifically, we aim to analyze the diversity and functionality of the gut microbiota across these distinct groups within patients in San Francisco, examining how age and smoking may influence microbial profiles and their implications for disease progression and health outcomes in pMS. We expect that these factors - alone and in combination - will be associated with distinct microbial communities, potentially revealing links between microbiome characteristics and disease progression in pMS.**

Differences in the gut microbiota composition can be observed amongst individuals with varying age, health, and lifestyle. Certain taxa abundant in older individuals, such as Desulfobiviro, Enterobacteriaceae, and some disease-associated Clostridium species, are often regarded as pathobionts [22, 23, 24, 25]. Older adults have half the abundance of Dorea longicatena, Rosburia faecis, and Blautia luti compared to younger adults [26]. This indicates that age is an important influence on an individual’s microbiome composition. In addition to age, there are reduced levels of Dorea formicigenerans and unclassified *Blautia* in pMS patients compared to healthy controls [27]. It is uncertain whether age or pMS has a greater effect on the composition of the gut microbiota as well as the taxa present within these groups of interest. The observed age-related changes in gut microbiome composition not only highlight the potential role of changing microbial diversity with age, but also raises the possibility that specific taxa may contribute to increase susceptibility to inflammation and disease in older individuals. . . Within older adults, higher numbers of Ruminococcus species are present compared to the youth [28]. Ruminococcus species as well as Bacteroides, Lachnospira, and Prevotella, are pro-inflammatory genuses of bacteria that could populate the gut microbiome as a result of an elevated intestinal pH [29]. One of the environmental factors contributing to this pH imbalance may be due to exposure to cigarette smoke [29]. The connection between smoking, inflammation, and alterations of the gut microbiome presents a plausible route through which smoking may select for a proinflammatory gut microbiome, thereby indirectly contributing to increased systemic immune activation. It would be interesting to investigate whether smoking in younger age groups can disrupt intestinal pH, resulting in a microbiota similar to those who are older in both healthy controls and pMS patients.

While smoking may exacerbate MS symptoms [30, 31], it remains unclear how this occurs and whether it contributes to disease progression. Smoking may promote systemic inflammation, impairing immune function, and potentially play a crucial role in MS pathogenesis. Microbial changes due to smoking have been associated with inflammatory states that could contribute to the deterioration of the CNS in MS patients [32, 33]. Interestingly, the role of Ruminococcus gnavus, in intestinal and neurological conditions, was associated both positively and negatively with the pathogenesis of MS [34, 28], thus the impact of the Ruminococcus genus on MS remains unclear. There is a need for further research to clarify whether its presence indicates inflammation-promoting activity in the context of MS [28]. This gap in our understanding of the Ruminococcus genus highlights the need to investigate if there are similar microbial changes associated with smoking and pMS in order to further clarify the role of this microbe. We aim to investigate the differences in gut microbiome composition between younger and older pMS patients, as well as those with a smoking history, by comparing them to their healthy counterparts. This will allow us to identify unique and prominent taxa within the gut microbiome due to aging and smoking within the context of pMS.

We have discussed how aging promotes the establishment of pathobionts in the gut microbiome and the alterations observed in those with pMS, alongside how smoking increases systemic inflammation and similarly changes the gut microbial composition. However, the relationship between these microbial shifts due to aging and smoking and whether or not these factors pMS patients have yet to be elucidated. We expect to observe differences in gut microbiome composition between healthy controls and pMS patients, amongst different age groups. We further expect that a history of smoking will accentuate these differences, particularly in pMS individuals. Older pMS patients with a history of smoking are expected to display more pronounced microbiome alterations compared to controls, younger individuals, and non-smokers. Based on current literature, we anticipate identifying unique taxa that are proinflammatory microbial species specific to pMS. Addressing this knowledge gap between gut microbiome research and lifestyle factors potentially contributing to disease progression, will inform clinical interventions for treatment-resistant pMS and improve health outcomes for patients.

**Results**

***1. Results found from Aim 1***

This study investigates differences in alpha and beta diversity of the gut microbiome across age groups in pMS patients. The literature has identified differences in the gut microbiome composition between healthy age-matched and older individuals with comorbidities [10]. In addition to the gut microbiome changing due to chronic diseases [16], it undergoes changes due to age, as well as in an attempt to accommodate lifestyle adjustments [35,10]. Knowing this, investigated differences observed with increasing age in pMS patients when compared to healthy controls to better understand why patients progress faster to pMS when older.

We will establish the pMS patient category by binning together SPMS and PPMS patients as these MS courses are similar in their pathology and clinical presentation [17]. Additionally, we will bin data together based on age groups (25 - 55 vs 56+), then performed \_\_ statistical analysis for:

Figure 1A results: alpha diversity

Figure 1B results: beta diversity

Figure 1C results: indicator diversity

***2. Results found from aim 2***

Continuing from the first aim, we want to identify, if any, differences exist between non-smokers and pMS patients with a history of smoking. We grouped smokers and former smokers together as previous cohort studies (Nurses’ Health Study I and II) with 121,700 women (age 30-55 years) and 116,671 women (age 25-42 years) respectively, reported a relative increase in MS case documentation in both current smokers and previous smokers when compared to non-smoker controls [36].

Although smoking is a general risk factor, the association between smoking and MS susceptibility has been a topic of debate in literature. In some cases, it has been shown that the majority of MS patients were smokers prior to MS onset [37], while other surveys suggest no association between smoking and MS when comparing MS patients to healthy controls [38]. To address this inconsistency, we analyzed the impact of smoking on the gut microbiome diversity as a third factor in pMS patients, focusing on alpha and beta diversity metrics.

To achieve this, we analyzed experimental groups categorized by age, pMS status, and smoking history: young-pMS-smoker, young-pMS-nonsmoker, young-healthy-smoker, young-healthy-nonsmoker, old-pMS-smoker, old-pMS-nonsmoker, old-healthy-smoker, and old-healthy-nonsmoker.

Figure 2A: alpha diversity

Figure 2B: beta diversity

Figure 2C: ISA

Our analysis revealed significant differences (p=0.06, p= ) in beta diversity between \_\_ and \_\_. Specifically. This finding highlights the potential role for smoking in shaping the gut microbiome by .

***3. Results found from aim 3***

Building on the findings of Aim 1 and 2, Aim 3 investigates how age group, smoking and disease course contribute to differences in both taxonomic composition and microbial function in the gut microbiome.

Figure 3A: PICRUST2

***4. Results found form aim 4***

Based on the microbial taxa identified ( ) and function pathways ( ) as potential predictors for pMS diagnosis, we integrated the three considered variables (age, pMS status, and smoking) into a predictive model. Unlike indicator species analysis, which evaluates taxa indiviually, Random Forest (CITATION) assess the combined influence

**Discussion**

**Main conclusion 1**

**Main conclusion 2**

**Main conclusion overall**

**Methods**

**Dataset Source and Initial Preparation**

The dataset for this study was obtained from Zhou et al.’s *Gut microbiome of multiple sclerosis patients and paired household healthy controls reveal associations with disease risk and course* [17]. It consists of 1152 samples collected from multiple cities. To minimize confounding variables such as dietary and environmental microbial variations associated with geographical location [17], only samples from San Francisco were included. Additional filtering criteria were applied to focus on progressive multiple sclerosis (pMS) patients, excluding relapsing-remitting MS (RRMS) samples. Samples with incomplete metadata for smoking status (NA values) were excluded, as well as nonbacterial sequences (mitochondrial and chloroplast DNA). These steps reduced the dataset to 145 samples.

**Sequence Processing and Quality Control**

Raw sequence data was denoised using the DADA2 plugin in QIIME2. Base quality scores were evaluated using QIIME2 View, revealing a median PHRED score ≥32 across all bases (Figure 1), corresponding to a base call accuracy of >99.9 [47]. Based on these high-quality scores, no truncation was applied during denoising.

**Sampling Depth Determination**

To account for server limitations while ensuring robust downstream analyses, sampling depth was selected to retain as many samples as possible, particularly focusing on current smokers, the smallest subgroup in the dataset (n=6). A sampling depth of 6154 was chosen, which retained 140 samples, distributed as follows: 6 current smokers, 43 former smokers, and 91 nonsmokers. Sampling depth adequacy was confirmed using an alpha rarefaction curve generated in R with the *vegan* package [48] The curve plateaued beyond the selected sampling depth, indicating sufficient sequencing depth for diversity analyses. This rigorous preprocessing pipeline ensured a high-quality, appropriately stratified dataset for subsequent analyses.

**Data Binning and Grouping**

For all aims, data was stratified into age groups (25–55 vs. 56+), disease status (pMS vs. controls), and smoking status (smoking history vs. never smoked). These binned datasets served as the basis for subsequent taxonomic, functional, and diversity analyses.

**Diversity Metrics**

Alpha diversity (within-sample) and beta diversity (between-sample) metrics were computed for all binned groups. Statistical tests for alpha diversity included Kruskal-Wallis rank-sum and log-transformed ANOVA tests, while Adonis tests were applied for beta diversity analysis. All statistical analyses were conducted in R.

**Differential Abundance and Functional Analysis**

Differential abundance analysis was performed using ALDEx2 in R, with log2 fold change visualization created in ggplot. Taxa showing statistically significant differences were compiled for use in predictive modeling (Aim 4). Functional analysis was conducted using PICRUSt2 in Qiime2 to generate a metabolomics table, and results were visualized using the ggpicrust package in R.

**Indicator Taxa and Core Microbiome Analysis**

Indicator taxa analysis was conducted in R to identify taxa characteristic of specific age and disease status groups. Core microbiome analyses assessed taxonomic overlaps between smoking and non-smoking cohorts within these groups.

**Predictive Modeling**

Key pathway data and differentially abundant taxa from previous analyses were filtered to create input files for random forest modeling. Predictive models were developed in Python, with visualizations highlighting variable importance and model performance (AUC values).

**Statistical and Visualization Tools**

R/RStudio served as the primary platform for statistical analyses, including Kruskal-Wallis, ANOVA, and Adonis tests. Visualization tools included ggplot for differential abundance data and Python for predictive model performance. All software versions and dependencies were specified according to reproducibility standards. This comprehensive approach integrates robust statistical methods, diversity metrics, functional assessments, and machine learning to investigate the relationship between the gut microbiome and pMS across key demographic and lifestyle factors.

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