Analyzing β-glucuronidase expression in gut microbial populations of MS patients and healthy controls

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Abstract

Multiple sclerosis (MS) is a chronic and progressive autoimmune disorder of the central nervous system. Studies have observed specific differences in the microbiota of MS patients and some have established a clear link between estrogen variants and MS by observing a gender bias and demonstrating that estrogens have several immunoprotective and anti-inflammatory properties. β-glucuronidase is an exogenous enzyme that deconjugates estrogens into their active form and as such, may contribute to protection against MS. There has not yet been a study that characterizes the interplay between estrogen variants, β-glucuronidase, and the gut microbiome but doing so may help elucidate hormonal and immunological mechanisms underlying MS pathogenesis. We hypothesize that the relative gut microbial β-glucuronidase gene counts for healthy controls will be greater compared to that for MS patients. To test this hypothesis we assessed the estrobolome to analyze the levels of β-glucuronidase gene expression in control and MS patients. Using data obtained from previous clinical studies, we characterized microbiota populations through the QIIME2 pipeline and performed a predictive metagenomic analysis using PICRUSt2 plugin to generate β-glucuronidase gene counts and allow for pairwise comparison between MS patients and healthy controls. We were unable to confirm our hypothesis that gut microbial β-glucuronidase gene counts for healthy controls are greater than that of MS patients. We were however, able to qualitatively identify several microbial populations that could be correlated with MS pathogenesis which may prove worthwhile to further investigate.

Introduction

Multiple sclerosis (MS) is a chronic and progressive autoimmune disorder of the central nervous system (CNS). Studies have observed that there are specific microbial taxonomic differences in the gut microbiome of MS patients when compared to healthy controls. The factor driving the gender bias characteristic of RRMS and its onset overall may be attributed to the influence of gut microbes and gender-based autoimmunity. There is clinical evidence that identifies a clear link between estrogen variants and MS.[1] 17β-estradiol (E2) and estriol (E3) are endogenous estrogens produced in humans. E2 inhibits pro-inflammatory cytokines and E3 has been shown to reduce EAE severity in mouse models.[1] A randomized, placebo controlled, double-blind trial conducted with female RRMS patients and E3 found that confirmed relapse rate was lower in the E3-treated RRMS group.[1] As such, lower concentrations of active circulating estrogens may promote a pro-inflammatory breeding ground for MS pathogenesis.

Here, we focus on a subset of the microbiome called the estrobolome which concerns bacteria that are capable of modulating or metabolizing the body's circulating estrogens. Bacteria in the estrobolome regulate circulating estrogen levels by secreting β-glucuronidase, an enzyme that deconjugates estrogen into their active form.[2] Subsequent dysbiosis of the gut microbiome reduces deconjugation and decreases circulating estrogens. This may contribute to the development of MS because estrogen variants, as previously mentioned, have been shown to have several immunoprotective and anti-inflammatory properties.

There has not yet been a study that characterizes the interplay between estrogen variants, and the gut microbiome but doing so may help elucidate hormonal and immunological mechanisms underlying MS pathogenesis.

Hypothesis - The relative gut microbial β-glucuronidase gene counts and by extension, gene expression, for healthy controls will be greater compared to that for MS patients.

Specific Aims - Aim 1: Identify what microbial populations are associated with RRMS. Aim 2: Identify what microbial populations in the estrobolome express the ß-glucuronidase gene by assessing DNA counts in case vs. control.

Materials & Methods

Table 1: Comparison of the Methods of the Clinical Studies

Title of Publication	Alterations of the human gut microbiome in multiple sclerosis [3]	Gut microbiome of treatment-naive MS patients of different ethnicities early in disease course [4]
Experimental Design and Exclusion Criteria	Untreated patients were treatment naïve or received no steroid treatment in the previous month. Subjects were to have had no other treatments in the past six months including antibiotic or probiotic use and corticosteroids. They were also required to have no history of gastroenteritis, irritable bowel syndrome, bowel surgery, inflammatory bowel disease or other autoimmune diseases where immunosuppressive medications were taken. Subjects could not be pregnant nor could they have travelled to outside countries in prior months..	None of the MS patients had active relapse and treated patients received beta-interferon or glatiramer acetate for at least six months leading up to the study.
Number of Subjects	60 MS patients (57 Caucasian, 1 Hispanic and 2 African American) and 43 healthy controls. In the context of our study, we only analyzed the untreated MS patients (n=27), excluding patients undergoing beta-interferon or glatiramer acetate treatment	45 MS subjects (15 Caucasian, 16 Hispanic and 14 African American) and 44 healthy controls
Sequencing Method	16s Sequencing	Whole Genome Sequencing
Sample Type	Stool	Stool
Notable Points	The MS cohort had significantly more males. Most of the subjects were Caucasian; there were only two Black MS patients and one Hispanic MS patient.	Three stool samples from African American MS patients were collected more than six months after diagnosis.

We used the QIIME2 pipeline to identify microbial populations present in the datasets retrieved from the two clinical studies, which contained raw 16s rRNA sequences.[3,5] We imported the sample data and screened them using DADA2 for demultiplexing and denoising before utilizing the pipeline to align the resulting sequences with the GreenGene database. This enabled us to quantify core metrics like alpha and beta diversity while also developing a phylogenetic tree to identify observed OTUs as well as their differential abundance. For alpha diversity, we considered the Faith, Shannon and Observed Features metrics to a sequencing depth of 50,000 base pairs. For beta diversity, the UniFrac method we used is a good fit because it measures the phylogenetic distances between sets of taxa based on mutual lineages while taking into account degrees of similarity between 16s rRNA sequences.

With the alignment data, we then used the PICRUSt plugin in QIIME2 to perform a predictive analysis on the raw 16s rRNA sequences.[6] Both of these analyses generate β-glucuronidase gene counts specific to each microbial population present for pairwise comparison between MS patients and healthy controls.

Results

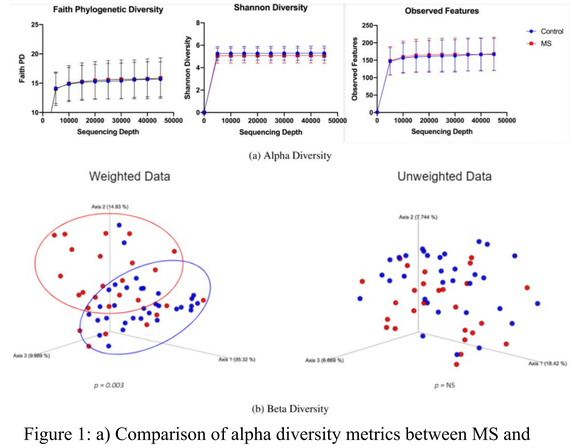

Figure 1: a) Comparison of alpha diversity metrics between MS and control patients. b) Difference in phylogenetic distances between MS patients and controls regarding community structure.

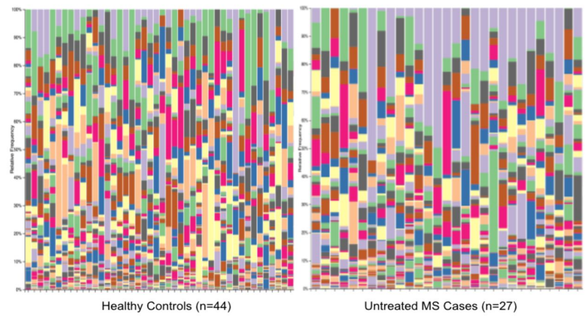

Figure 2: Microbial species present and their differential abundance in healthy controls and untreated MS cases

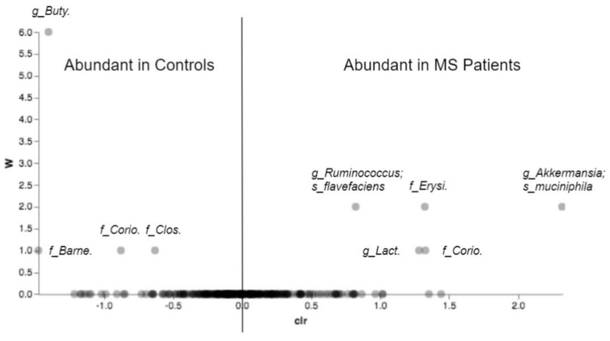

Figure 3: L7 Volcano Plot of the differential abundance of OTUs present in MS and control subjects

Discussion

In this study, we explored and characterized the estrobolome in RRMS and control patients and the impact of the expression of the ß-glucuronidase gene on MS pathogenesis. We identified microbial populations associated with RRMS and those that express the ß-glucuronidase gene by performing a pairwise analysis of ß-glucuronidase gene count between healthy controls and MS patients. This was done using datasets from two previous clinical studies and by taking advantage of bioinformatics platforms like QIIME2 to assess differences in microbial communities and structure and other state-of-the-art pipelines like PICRUSt for predictive metagenomic analysis of the ß-glucuronidase gene.

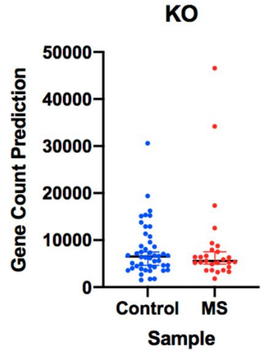

Figure 4: β-glucuronidase gene count metagenome prediction comparison between control and MS patients

We first assessed the alpha diversity of our samples obtained from the two clinical studies, one dataset generated from 16s sequencing and the other from whole genome sequencing, using the Faith, Shannon, and Observed Features metrics up to a sequencing depth of 50,000 and were able confirm observations made by other studies that similar microbial populations are present in the gut microbiomes of MS patients and healthy controls (Figure 1a). And in assessing beta diversity, we observed a significant phylogenetic difference in microbial community structure between the microbiota of MS patients and that of healthy controls than the microbiota of healthy controls with that of other healthy controls (Figure 1b), $p<0.05$.

Since there were differences in microbial community structure, we then assessed the microbial composition of each subject to identify specific taxonomic groups present in each subject and their differential abundance (Figure 2). With this and a further analysis on the differential abundance of these OTUs, we were able to qualitatively and quantitatively identify several microbial populations that were more abundant in either MS patients or healthy controls (Figure 3). Notable OTUs abundant in healthy controls include microorganisms in the family Butyricimonas and notable OTUs abundant in MS patients include *Akkermansia muciniphila* and *Ruminococcus flavefaciens*.

Finally, we assessed β-glucuronidase gene count through PICRUSt2 metagenome prediction analysis and performed an unpaired, nonparametric Kolmogov-Smirnov test on our gene counts, obtaining an insignificant p-value (Figure 4). We were not able to confirm our hypothesis that gut microbial β-glucuronidase gene counts for healthy controls are greater than that for RRMS patients which suggests that β-glucuronidase gene expression may not be as directly correlated to MS pathogenesis as we had predicted.

A caveat of our analyses is that we were limited by the computing power of our personal computers and bottlenecked by downloading large files for other analyses. We were thus unable to perform whole genome analysis of the alignment data using MetaPhlAn and HUMAnN on the confirmed microbial populations. A potential future direction for this study would be to secure increased computing power in order to consider more subjects during predictive analyses, analyze the whole genome data sets, and further analyze the impact of selected OTUs. It may prove worthwhile to study these microbial populations in greater detail to better understand their physiological significance in the human body, particularly in the estrobolome, as they may be correlated with MS pathogenesis or confer protection against MS.

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See full paper for a comprehensive list of references.

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