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Assessing the effects of combinations of genetic and environmental factors on multiple sclerosis using genomic and computational approaches

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Assessing the effects of combinations of genetic and environmental factors on multiple sclerosis using genomic and computational approaches

By
Mary Katherine Horton

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Committee in charge:

Professor Lisa F. Barcellos, Chair
Professor Lexin Li
Professor Priya Moorjani

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Abstract

Assessing the effects of combinations of genetic and environmental factors on multiple sclerosis using genomic and computational approaches

by

Mary Katherine Horton

Doctor of Philosophy in Epidemiology

Designated Emphasis in Computational and Genomic Biology

University of California, Berkeley

Professor Lisa F. Barcellos, Chair

Multiple sclerosis (MS) is a chronic, often disabling disease characterized by neurodegeneration and inflammation. It is the most common non-traumatic neurological disorder among young adults. What triggers MS pathology and symptoms over time remains largely unknown. Evidence suggests genetic and environmental factors contribute to risk of MS and there are at least 230 known genetic risk variants. However, much less is known about the effect of multiple environmental and/or genetic risk factors, which can co-occur in the same individual at the same or different times, on MS risk and clinical manifestations. It is important to consider combinations of risk factors because their joint effects may differ from individual effects. Additionally, it might not be possible to tease apart individual effects from highly inter-related variables, so clustering or other methods should be considered. In this dissertation, I utilize computational, statistical, genomic, and epidemiologic approaches to study the role of combinations of genetic and environmental/behavioral risk factors on MS risk and clinical outcomes in humans. Chapter one introduces MS and background relevant for proceeding chapters. Chapter two shows that individual and clusters of co-occurring gut microbes are associated with new brain lesions on MRI and relapses among individuals with pediatric-onset MS. Chapter three shows that adverse childhood experiences, assessed as individual events and combinations of events, are not associated with MS risk or clinical outcomes in our data. Chapter four suggests that MS and migraine (a common comorbidity of MS) may co-occur because they share several genetic variants. Altogether, this dissertation advances our knowledge of risk factors for MS onset and clinical features of disease and will inform future work of gut microbe, comorbidities, and stress research.

For my family. Especially, Mom and Dad.

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CHAPTER 1 - INTRODUCTION

Multiple sclerosis (MS) is a chronic, often disabling disease characterized by neurodegeneration and inflammation.[1] It is the most common non-traumatic neurological disorder among young adults with a mean age of onset at ~30 years.[2] In recent decades, incidence and prevalence of MS have increased and clinical management has improved, resulting in more individuals living longer with MS.[3] Overall long-term prognosis for individuals remains poor and little is known regarding factors contributing to its highly heterogeneous nature, which results in varying symptoms, severity, and long-term disability. Twenty years after onset, up to 60% of individuals with MS require ambulatory assistance.[3] Additionally, as many as 60% of individuals with MS experience cognitive impairment, primarily consisting of processing speed and episodic memory deficits.[3] MS is not often lethal; however, death from resulting disability and co-morbid conditions is observed, and overall quality of life is significantly impacted in most MS cases. On a global level, estimated years lived with disability for MS are nearly identical to Parkinson's disease, a more prevalent condition than MS, underscoring the significant disease burden of MS due to its earlier onset, progressive nature, and impact on quality of life.[4]

MS susceptibility involves a substantial genetic component.[5] HLA class II genes within the major histocompatibility complex (MHC) region confer a major portion of the risk. The primary MHC susceptibility locus is the *HLA-DRB1*15:01* allele.[6] Recent genome-wide association studies (GWAS) have begun to unravel the polygenic etiology of MS, and there are now >200 established non-MHC MS risk variants and at least 30 independent risk loci within the MHC.[7] Despite the strong genetic contribution of MS, these variants only partially explain the genetic risk of developing MS and are responsible for only about 20% of MS heritability.[8] This suggests much of the genetic risk responsible for MS has yet to be discovered.

In addition to genetic factors, a substantial portion of MS risk is from environmental and/or behavioral factors. This is clear through the only moderate concordance rate in MS for monozygotic twins (30-40%) along with results from studies of migrants which show that migrants who move from an area where MS is common to an area where MS is rarer show a decrease in the rate of MS.[9,10] To date, environmental/behavioral factors that have been consistently identified as risk factors for MS include exposure to tobacco smoke [11,12], Epstein-Barr virus infection [13], childhood/adolescence obesity [14,15], residing in latitudes farther away from the equator [16], and vitamin D levels.[17] Notably, studies have shown that several of these are most pertinent during the first two decades of life, in particular during adolescence.[9,18,19] Despite these consistent findings, it remains largely unknown how or why individuals develop MS. Much work is left to determine additional environmental/behavioral risk factors for MS and how they are interconnected to already-established risk factors.

Once individuals are diagnosed with MS, symptoms, rate of progression, number of relapses, new lesions on magnetic resonance imaging (MRI,) comorbidities, and disability vary greatly from person-to-person. Little is known about genetic or environmental/behavioral factors that may contribute to this variation. The only consistent findings are of tobacco smoke, which is associated with poor prognosis and progressive disease.[20] This underscores the importance of identifying

both genetic and environmental risk factors for clinical manifestations of MS, which can directly impact an individual's quality of life.

This dissertation aims to add critically missing knowledge regarding novel risk factors for both risk of MS and clinical manifestations of MS. We use computational, genomic, statistical, and epidemiologic approaches that consider multiple risk factors together. It is important to consider combinations of risk factors because their joint effects may differ from individual effects. For example, the risk of MS among smokers is 1.6 times the risk of MS among non-smokers [21,22], but the risk of MS among smokers who also have the highest risk genotype (*HLA-DRB1*1501* positive and *HLA-A*02* negative) is 14 times the risk of MS among non-smokers without the high risk genotype.[23] Additionally, it might not be possible to tease apart individual effects from highly inter-related variables, so methods that use clustering or other approaches should be utilized.

First, in the second chapter, we examine the role of an emerging set of risk factors, gut microbes, on MS clinical activity. Using 16S ribosomal RNA sequencing profiles from the stool of 55 pediatric-onset MS cases (diagnosed before 18 years old), we investigated whether individual and clusters of gut microbes were associated with time to three separate disease activity outcomes: clinical relapses, new gadolinium-enhancing lesions (representing areas of active inflammation), and new or enlarging T2 hyperintense lesions (markers of overall disease burden). Considering clusters or networks of microbes was important because they exist in specific niches, often co-occur, and have complex interactions which are challenging to interpret individually. This was the first study to assess the role of gut microbes on measures of MS activity on MRI. Using pediatric-onset cases to investigate these associations was advantageous because symptom onset was likely closer to the biological onset of disease. Further, children and youth have higher disease activity, compared to adults, making it more feasible to study relapses and MRI activity over time.[24,25]

Second, despite overwhelming knowledge that adverse childhood experiences (ACEs) are associated with worse physical and mental health outcomes in adulthood[26], few studies have investigated whether ACEs contribute to risk of MS. Even less is known about their effects on clinical aspects of MS including physical disability and cognition. In the third chapter, we use multiple approaches to assess the association between ACEs and MS risk and clinical outcomes. Because ACEs are complex and often inter-related, it was important to consider them in multiple ways including individual events, any/none, counts, and weighted linear combinations of variables.

Third, in the fourth chapter, we test hypothesized mechanisms for the co-morbidity of MS and migraine. Approximately 1/3 of MS patients experience migraine.[27] It is a substantial contributor to reduced quality of life and it is unknown whether migraine causes MS, genetic variants are shared between the disorders, or, in the context of MS, migraine is a symptom of MS and not an independent disorder. Our approach was to use publicly available GWAS data and deeply phenotyped and genotyped data from a cohort of MS patients to test these mechanisms. We utilized methods that tested for genetic correlation across the entire genome and in local regions, using millions of GWAS variants, to assess sharing of genetic variants across MS and migraine. This framework may be helpful for additional studies of MS co-morbidities and co-morbidities of other conditions, given the limited knowledge we have of why certain conditions co-occur.

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CHAPTER 2- GUT MICROBIOME IS ASSOCIATED WITH MULTIPLE SCLEROSIS ACTIVITY IN CHILDREN

ABSTRACT

Objective: Identify features of the gut microbiome associated with multiple sclerosis activity over time.

Methods: We used 16S ribosomal RNA sequencing from stool of 55 recently diagnosed pediatric-onset multiple sclerosis patients. Microbiome features included abundance of individual microbes and networks identified from weighted genetic correlation network analyses. Prentice-Williams-Peterson Cox proportional hazards models estimated associations between features and three disease activity outcomes: clinical relapses and both new/enlarging T2 lesions and new gadolinium-enhancing lesions on brain MRI. Analyses adjusted for age, sex, and disease-modifying therapies.

Results: Participants were followed, on average, 2.1 years. Five microbes were nominally associated with all three disease activity outcomes after multiple testing correction. These included butyrate-producers *Odoribacter* (relapse hazard ratio=0.46, 95% confidence interval: 0.24, 0.88) and *Butyricicoccus* (relapse hazard ratio=0.49, 95% confidence interval: 0.28, 0.88). Two networks of co-occurring gut microbes were significantly associated with a higher hazard of both MRI outcomes (gadolinium-enhancing lesion hazard ratios (95% confidence intervals) for Module 32 and 33 were 1.29 (1.08, 1.54) and 1.42 (1.18, 1.71), respectively; T2 lesion hazard ratios (95% confidence intervals) for Module 32 and 33 were 1.34 (1.15, 1.56) and 1.41 (1.21, 1.64), respectively). Metagenomic predictions of these networks demonstrated enrichment for amino acid biosynthesis pathways.

Interpretation: Both individual and networks of gut microbes were associated with longitudinal multiple sclerosis activity. Known functions and metagenomic predictions of these microbes suggest the important role of butyrate and amino acid biosynthesis pathways. This provides strong support for future development of personalized microbiome interventions to modify multiple sclerosis disease activity.

INTRODUCTION

Multiple sclerosis (MS) is a chronic, inflammatory disease of the central nervous system with symptoms and disease activity that vary greatly from person to person. Despite recent advances in treatment, there is no cure for MS, and it remains largely unknown what factors contribute to disease activity over time. Smoking, obesity, Epstein-Barr virus infection, low vitamin D, and over 200 genetic variants are established risk factors for developing MS. However, with the exception of low vitamin D, they have not been convincingly or consistently shown to contribute to MS outcomes such as clinical relapse or lesion activity on brain MRI.[1,2] Thus, it is critical to investigate novel drivers of MS activity that might inform interventions designed to attenuate disease course.

Recently, a growing body of experimental and observational studies have suggested that microbes in the gut contribute to MS pathogenesis.[3] Several potential biological mechanisms include direct and indirect interactions of microbes and microbial metabolites with immune cells and pro-inflammatory chemokines and cytokines, all of which can influence the central nervous system.[4–6] However, it remains unknown which, if any, features of the gut microbiome contribute to disease activity in MS. In animal models of MS, a germ-free environment has been associated with lower disease activity, and perturbations to the gut microbiota have been associated with changes in disease activity.[7–9] Additionally, oral administration of *Bacteroides fragilis* have been associated with lower "clinical" scores in relapsing mouse models of MS.[10] One small observational study of the gut microbiome and disease activity in persons with MS investigated clinical relapse as the outcome.[11] After adjusting for age and disease-modifying therapy use, relative absence of *Fusobacteria* was associated with a higher chance of relapse (hazard ratio=3.2; 95% confidence interval: 1.2, 9.0). This study was limited in size, did not investigate the role of specific microbial taxa (such as genus or species) or co-occurring networks of microbes, and did not include other clinical outcomes. No studies have investigated the association between gut microbes and direct measures of disease activity assessed by brain MRI, which is sensitive to lesion formation, more common than clinical relapses,[12] and can serve as a biomarker of active inflammation.

In this study, we utilized 16S ribosomal RNA sequencing profiles from the stool of 55 pediatric-onset MS cases to investigate whether specific features of the gut microbiome were associated with time to three separate disease activity outcomes: clinical relapses, new gadolinium-enhancing lesions (representing areas of active inflammation), and new or enlarging T2 hyperintense lesions (markers of overall disease burden). Using pediatric-onset cases (individuals with MS symptom onset before 18 years of age) to investigate these associations was advantageous because symptom onset was likely closer to the biological onset of disease. Further, children and youth have higher disease activity, compared to adults, making it more feasible to study relapses and MRI activity over time.[13,14]

MATERIALS AND METHODS

Study population

Between 2012 and 2018, 60 individuals with MS onset before 18 years old were enrolled and provided stool samples that could be analyzed. Six enrollees did not have clinical follow-up (leaving 54 in the "clinical cohort") and 14 did not have subsequent MRI scan data available

(leaving 46 in the "MRI cohort"). Participants were recruited from seven sites in the United States (US) Network of Pediatric MS Centers including the University of California San Francisco, State University of New York at Buffalo, University of Alabama at Birmingham, Boston Children's Hospital, Stony Brook University Medical Center, Children's Hospital of Philadelphia, and New York University. At stool sample collection ("baseline"), all participants were within 24 months of symptom onset and met the 2010 McDonald criteria for MS.[15] Exclusion criteria included: participant's banked serum tested positive for myelin oligodendrocyte glycoprotein antibodies, participant had been exposed to a systemic antibiotic, probiotic, or steroid within one month prior to stool sample collection; or participant had previously used a cytotoxic immunosuppressant.
All parents and participants provided written informed consent and assent. Ethical approval for the study was obtained from each institution's Institutional Review Board.

Clinical relapses and MRI outcomes
During the study period, participants were seen for regular care at the enrolling clinic, which usually included a visit every six months, with additional visits if the participant experienced a relapse or other clinical reason. MRI scans were ordered at study visits (per the primary neurologist) and conducted using each site's scanner and local protocol. Data from follow-up visits (including the dates of relapse onset, use of disease-modifying therapies, and MRI) were prospectively entered into a web-based registry. The Data Coordinating and Analysis Center at the University of Utah managed the data and performed quality control.

Three outcomes, which could recur over the study period, were assessed separately: clinical relapse(s), development of new gadolinium-enhancing brain lesion(s), and development of new or enlarging T2 hyperintense brain lesion(s). These outcomes were defined previously.[16] We considered a lesion new or enlarging relative to the previous MRI.

Gut microbiota profiling
A parent collected the participant's first stool of the day and shipped overnight on ice to the University of California, San Francisco, where it was stored at −80°C before processing. DNA was extracted and the V4 region of the 16S rRNA gene was amplified for sequencing, as previously described.[17]

Forward and reverse reads were processed separately, and quality filtered using the DADA2 package version 1.9.0. in R.3.5.2.[18,19] Reads having more than two expected errors or ≤ 150 base pairs in length were removed. Error rates of the filtered dereplicated reads were estimated using 100,000 sequences. Paired sequencing reads with a minimum overlap of 25 base pairs were merged to obtain the full denoised sequences. Chimeras and any sequences abnormally short or long were removed. Amplicon sequence variants (ASVs) were inferred exactly, resolving variants that differ by as little as one nucleotide. Taxonomy was assigned using the naïve Bayesian classifier method (Kingdom to Family) and exact string matching (Genus and Species) utilizing the SILVA v132 reference database.[18,20,21] It is important to note that while an ASV has a unique nucleotide sequence, it might not be assigned a unique species or taxonomy due to limitations of 16S sequencing in determining strain-level differences among species and missing microbial genomes in reference databases. Using the *decontam* package, ASVs with a contaminant classification threshold $p<0.1$ were removed.[22] ASVs containing less than 1/1000th of a percent of total reads were removed. Sequencing reads were representatively rarefied to the minimum sequencing depth

(84,818 reads/sample) 100 times, and the rarefied sample profile closest to the sample-specific centroid was selected, as described previously.[17] The resulting tables included 1,482 ASVs.

Covariates
Upon enrollment, participants completed a questionnaire including age, symptom onset, race, ethnicity, and sex. Medication history was obtained, and subsequent medication use was tracked over the follow-up period. Disease-modifying therapies included those previously described.[16] For relapse-related analyses, time varying disease-modifying therapy use was defined as "yes" if the subject used a disease-modifying therapy within three months prior to the respective relapse and "no" if otherwise. For MRI analyses, time varying disease-modifying therapy use was defined as "yes" if the subject was using a disease-modifying therapy during the period between the respective MRI and the previous MRI and "no" if otherwise.

Statistical analyses

Alpha and beta diversity
All statistical analyses were completed using R and the *phyloseq* package.[23] Alpha (within sample) diversity was evaluated using a rarefied ASV table with richness (Chao1 and Faith's phylogenetic diversity) and evenness (Pielou) estimators. To test for the association between each alpha diversity metric and time to each disease activity outcome, we used Prentice-Williams-Petersen time-to-event models.[24] These are an extension of Cox proportional hazard models and are appropriate for outcomes that can recur over the study period and are not independent.

For relapse analyses, clinical cohort members were followed from baseline to the earlier of the final clinic visit or occurrence of a third relapse (Fig. 1 panel A). Relapses were truncated after the first three to prevent estimation of hazard ratios (HRs) in event strata with few individuals. Time to each relapse (or final clinic visit) was defined as the total time from baseline to each respective event. Because, by definition, a new relapse cannot occur until at least 30 days after the previous relapse, a 30-day period was discounted from the follow-up time at risk for each subsequent relapse.

For brain MRI analyses, MRI cohort members were followed from baseline to the earlier of the final MRI or occurrence of a second new/enlarging lesion (gadolinium-enhancing and T2 lesions were evaluated separately) (Fig. 1 panel B). Data were truncated after the first two new/enlarging lesions. Since a new or enlarging lesion was relative to a past MRI, we defined a "baseline MRI" as the MRI that occurred closest, but previously, to stool sample collection. Time to each new/enlarging lesion (or final MRI visit) was defined as the gap time between a new/enlarging lesion and the previous new/enlarging lesion (or baseline if first new/enlarging lesion). We used the midpoint of time between a MRI with a new/enlarging lesion and prior MRI (with or without new/enlarging lesion) as an estimate of when the new lesion developed.[16,25] Between baseline and a new/enlarging lesion, the midpoint was halfway between the baseline MRI and the new/enlarging lesion, and time between stool collection and the midpoint was used for the respective at-risk interval. If the midpoint between the baseline MRI and first MRI with a new/enlarging lesion after baseline occurred before stool was collected, it was excluded from analyses.

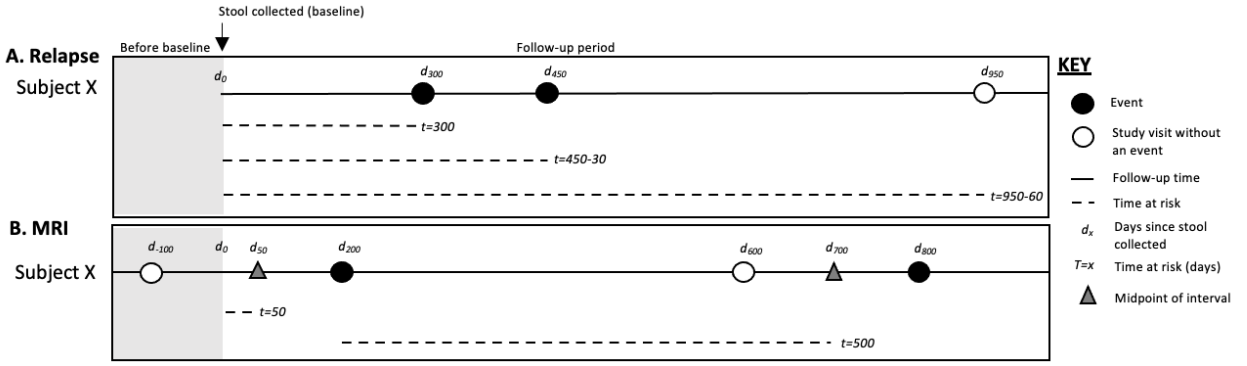

Figure 1 Example of survival analyses for relapse and MRI outcomes. **(A)** For relapse analyses, time to each relapse (an "event" in panel A) started on the day stool was collected (d0) and ended on the day of each respective relapse or the last study visit where relapse status was known (d_x). A 30-day period was subtracted from the at-risk period following a relapse because, by definition, a new relapse must be at least 30 days after the previous. **(B)** For MRI analyses, an "event" was defined as a brain MRI that indicated a new or enlarging lesion compared to the prior MRI. Specifically, we used two MRI outcomes considered separately: new gadolinium-enhancing lesion and new or enlarging T2 lesion. Because the timing of MRI varies in clinical practice and the specific time of lesion activity is unknown, midpoint survival analyses were used. For the first MRI event after stool was collected, time at risk started on the date stool was collected and ended on the midpoint between the first MRI event and the prior MRI where an event did not occur (or the MRI that preceded baseline). For subsequent MRI events, time at risk started on the date of the previous MRI with an event and ended on the midpoint between the respective MRI event and the prior MRI where an event did or did not occur. Individuals were censored at the date of their last MRI (with or without an MRI event) before the study end.

For all Prentice-Williams-Petersen models, robust variance was computed, and HRs and 95% confidence intervals (CIs) were estimated for each alpha diversity metric and each MS activity outcome, adjusting for sex, age at event, and disease-modifying therapy use. The proportional hazard assumption for each model was assessed using the *cox.zph* function in the *Survival* package. All alpha diversity metrics met the proportional hazard assumption.

For beta (between sample) diversity, weighted and unweighted UniFrac distance matrices were constructed.[26] Relationships between each beta diversity metric and whether a participant had a clinically meaningful relapse rate (annual rate ≥0.5, i.e. more than one relapse over two years), had any new gadolinium-enhancing lesions, or had any new or enlarging T2 hyperintense lesions over the follow-up period were assessed using permutational multivariate analysis of variance (PERMANOVA) using *adonis2*. Models adjusted for sex, age at stool collection, and whether or not a participant was using a disease-modifying therapy when stool was collected.

ASV-level relative abundance

To identify whether specific gut microbes were associated with subsequent disease activity, we used Prentice-Williams-Petersen models described above to estimate HRs and 95% CIs for each ASV and each disease activity outcome, adjusted for age, sex, and disease-modifying therapy use. ASVs identified in <20% of a respective analytic cohort (clinical or MRI) were excluded to reduce potentially spurious taxa and reduce the burden of multiple testing with a small sample. Rarefied counts of each ASV were dichotomized according to prevalence. ASVs in 20% to <80% of samples were categorized as "present" or "absent" if any or no taxa reads were in the sample. ASVs in ≥80% of samples were categorized as "high" or "low" depending on whether samples had ≥ or < the median number of taxa reads. This resulted in 271 ASVs available for individual-level analyses for the clinical cohort and 256 ASVs for the MRI cohort. For each disease activity outcome, observations were corrected for false discovery rate (FDR) using the Benjamini-Hochberg method.[27] ASVs with FDR *q-value*<0.05 were considered significant.[27] The

proportional hazard assumption for significant ASVs was assessed the same as above, and all met the proportional hazard assumption.

Microbial network analysis

Co-occurrence networks of ASVs in at least 10% of samples (resulting in 437 ASVs available for the clinical cohort and 426 for the MRI cohort) were constructed from an unrarefied ASV table using *SPIEC-EASI* and *WGCNA* packages.[28,29] A correlation matrix was generated using *SPIEC-EASI*, transformed to an adjacency matrix using soft thresholding, and a topology overlap matrix was generated. The topology overlap matrix was hierarchically clustered using *hclust* and the resulting dendrogram was cut using *dynamicTreeCut* in the *stats* package to generate modules (clusters). Modules needing at least three ASVs to be retained. Correlated modules ($r \geq 0.5$) were combined, generating a dissimilarity matrix for further hierarchical clustering. Quantitative values of each module were calculated for each participant from module eigengenes, defined as the first principal component of the abundance matrix of a respective module. Each module eigengene was tested for its association with time to relapse, new gadolinium-enhancing lesions, and new or enlarging T2 hyperintense lesions using the Prentice-Williams-Petersen models described above, adjusting for age, sex, and disease-modifying therapy use. To improve interpretability of results, we presented beta coefficients and HRs from regression coefficients and 95% CIs scaled to a 0.1-unit increase in module eigengenes. Analyses were corrected for FDR, and modules with an FDR q-value<0.05 were considered significant. The proportional hazard assumption for significant ASVs was assessed the same as above. One significant module did not meet the proportional hazard assumption, so a time by eigengene interaction term was added to the model. The interaction term did not have $p<0.05$, so the HR and 95% CI for the module eigengene from the non-interaction term model was presented.

Metagenomic prediction

Conserved functional genes of microbes within each significant module were predicted using PICRUSt 2.[30] For each significant module, predicted gene counts were grouped into MetaCyc metabolic pathways.[31] We estimated the association between predicted metabolic pathways in at least 20% of samples and the disease activity outcome(s) previously identified as associated with the respective module. Pathway abundances were dichotomized as > or ≤ the respective pathway's median abundance. HRs and 95% CIs were estimated using Prentice-Williams-Petersen models, adjusted for age, sex, and disease modifying use, and corrected for FDR.

RESULTS

Characteristics of pediatric-onset multiple sclerosis microbiome cohort

Among all 55 cohort members, the average age at baseline was 15.9 years (IQR=2.5), 72.7% were female, 67.2% identified as white, and 36.3% identified as Hispanic (Table 1). The distribution of these characteristics match the sex, age, race, and ethnicity distribution of pediatric-onset MS in the US.[32] Approximately half were using a disease-modifying therapy at baseline, of which 25.0% were using interferon beta and 64.3% were using glatiramer acetate. The proportion of individual's ASVs belonging to a particular taxonomic class did not significantly differ by baseline DMT use categories (none, glatiramer acetate, interferon beta, or other DMT) except for *Melainabacteria* (p=0.0002) and *Verrucomicrobiae* (p=0.049) (data not shown, Supplementary Fig. 1). Among all

55 participants, 54 were prospectively followed and evaluated for the presence (or absence) of clinical relapses ('clinical cohort') and 46 had at least one MRI scan available ('MRI cohort'). Characteristics of these cohorts were similar, albeit a higher proportion of girls were in the clinical cohort relative to MRI cohort (Table 1). For the relapse analyses, participants were followed for an average of 2.4 years (IQR=2.1) after baseline during which time 44.4% experienced a relapse. Of the relapses that occurred, 75.5% were using a disease-modifying therapy in the three months prior. Participants were followed for a similar amount of time for the gadolinium-enhancing (mean=2.0 years, IQR=1.7) and T2 hyperintense lesion (mean=1.9 years, IQR=15) analyses. Over the follow-up period, approximately half of participants had a new or enlarging T2 hyperintense lesion (54.3%) while 40.0% had a new gadolinium-enhancing lesion.

Table 1 Cohort characteristics of pediatric-onset multiple sclerosis cases at baseline and during follow-up

Characteristics	Combined cohorts	Clinical cohort	MRI cohort
Baseline (stool sample collection)			
N (%)	55 (100.0)	54 (98.2)	46 (83.6)
Age (mean, IQR)	15.9 (2.5)	15.9 (2.5)	15.8 (2.6)
Age at disease onset, years (mean, IQR)	14.7 (2.7)	14.7 (2.8)	14.6 (2.7)
Sex (female) (n, %)	40 (72.7)	39 (72.2)	33 (89.2)
Race (n, %)			
Asian	4 (7.3)	4 (7.41)	3 (6.52)
Black	6 (10.9)	6 (11.1)	6 (13.0)
White	37 (67.2)	36 (66.7)	32 (69.6)
Other	6 (10.9)	6 (11.1)	5 (10.9)
Not reported	2 (3.6)	2 (3.7)	0 (0.0)
Hispanic (n, %)	20 (36.3)	19 (35.2)	16 (34.8)
Expanded Disability Status Scale (mean, IQR)	1.1 (1.5)	1.1 (1.5)	1.2 (1.3)
Disease-modifying therapy exposed (n, %)	28 (50.9)	28 (51.9)	24 (52.2)
Interferon beta (n, %)	7 (25.0)	7 (25.0)	6 (25.0)
Glatiramer acetate (n, %)	18 (64.3)	18 (64.3)	16 (66.7)
Over follow-up period for the clinical cohort			
Follow-up time after stool collection, years (mean, IQR)		2.4 (2.1)	
Experienced relapse over follow-up period (n, %)		24 (44.4)	
Number of relapses (mean, IQR)		0.9 (2.0)	
Time to first relapse after stool collection, days (mean, IQR)		297.8 (458.5)	
Relapse preceded by DMT use within 90 days prior (n, %)		37 (75.5)	
Over follow-up period for the MRI cohort			
Time between baseline MRI and stool collection, days (mean, IQR)			89.2 (70.8)
Time to first MRI after stool collection, days (mean, IQR)			189.9 (184.0)
Gadolinium-enhancing lesions:			
Follow-up time, years (mean, IQR)			2.0 (1.7)

Had a new lesion over follow-up period (n, %)	17 (40.0)
Number of new lesions (mean, IQR)	1.4 (1.0)
DMT used between MRI with new lesion and prior MRI (n, %)	52 (92.9)
T2 hyperintense lesions:	
Follow-up time, years (mean, IQR)	1.9 (1.5)
Had a new/enlarging lesion over follow-up period (n, %)	25 (54.3)
Number of new/enlarging lesions (mean, IQR)	1.4 (1.0)
DMT used between MRI with new/enlarging lesion and prior MRI (n, %)	33 (97.1)

Abbreviations: disease modifying therapy, DMT; interquartile range, IQR

Gut microbiome alpha and beta diversity were not associated with multiple sclerosis activity

Alpha diversity was not significantly associated with relapse (p_{chao1}=0.56, p_{faith}=0.29, $p_{evenness}$=0.67), new gadolinium-enhancing lesions (p_{chao1}=0.16, p_{faith}=0.15, $p_{evenness}$=0.58), or new or enlarging T2 hyperintense lesions (p_{chao1}=0.84, p_{faith}=0.77, $p_{evenness}$=0.95) (Fig. 2). For beta diversity, relapse and MRI outcomes did not explain the observed variance in microbiota composition in fecal samples (Fig. 3). Over the study period, irrespective of whether a weighted or unweighted UniFrac distance matrix was employed, variance in fecal microbiota composition was not related to MS activity outcomes: annualized relapse rate ≥0.5 (weighted UniFrac PERMANOVA R^2=0.01, p=0.78; unweighted UniFrac PERMANOVA R^2=0.02, p=0.38), having any new gadolinium-enhancing lesions (weighted UniFrac PERMANOVA R^2=0.01, p=0.78; unweighted UniFrac PERMANOVA R^2=0.02, p=0.42), or having any new T2 hyperintense lesions (weighted UniFrac PERMANOVA R^2=0.02, p=0.43; unweighted UniFrac PERMANOVA R^2=0.02, =0.63).

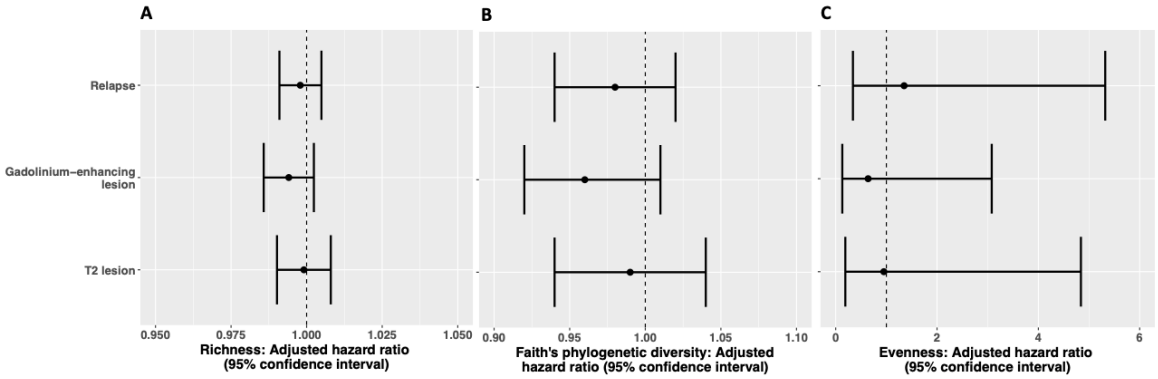

Figure 2 Microbial alpha diversity was not associated with clinical relapses or MRI outcomes in pediatric-onset multiple sclerosis. (A) The Chao1 microbial richness estimator was not associated with relapse (HR= 1.00; 95% CI: 0.99, 1.00; p=0.56), new gadolinium-enhancing lesion on MRI (HR= 0.99; 95% CI: 0.99, 1.00; p=0.16), or new or enlarging T2 hyperintense lesion on MRI (HR= 1.00; 95% CI: 0.99, 1.01; p=0.84). **(B)** The Faith's phylogenetic diversity microbial richness estimator was not associated with relapse (HR= 0.98; 95% CI: 0.94, 1.02; p=0.29), new gadolinium-enhancing lesion on MRI (HR= 0.96; 95% CI: 0.92, 1.01; p=0.15), or new or enlarging T2 hyperintense lesion on MRI (HR= 0.99; 95% CI: 0.94, 1.04; p=0.77). **(C)** Microbial evenness (Pielou estimator) was not associated with relapse (HR= 1.35; 95% CI: 0.34, 5.32; p=0.67), new gadolinium-enhancing lesions on MRI (HR= 0.64; 95% CI: 0.13, 3.08; p=0.58), or new or enlarging T2 hyperintense lesions on MRI (HR= 0.95; 95% CI: 0.19, 4.84; p=0.95). Beta coefficients and related HRs and 95% CIs for evenness were scaled to represent a 0.1-unit change in evenness. Regression models adjusted for sex, age, and disease-modifying therapy use.

Five gut microbes were nominally associated with all three multiple sclerosis activity outcomes

A lack of relationship between MS activity and variance in overall fecal microbiota composition does not preclude the possibility that specific microbes may contribute to MS pathogenesis. For this reason, we tested whether specific ASVs were associated with pediatric-onset MS outcomes. No ASVs were significantly associated with disease activity outcomes using a conservative threshold of FDR q<0.05 (Fig. 4, see Supplementary Table 1 for full results). Using a less stringent cut-off of FDR q<0.2, we identified three ASVs associated with disease activity. Two of these were associated with higher hazard of relapse: *Blautia stercoris* (HR: 3.19, 95% CI: 1.72, 5.92) and an unidentified species within the genus *Catabacter* (HR: 2.81, 95% CI: 1.51, 5.22). One ASV was associated with a lower hazard of new gadolinium-enhancing lesions, *Odoribacter splanchnicus* (HR: 0.25, 95% CI: 0.12, 0.54).

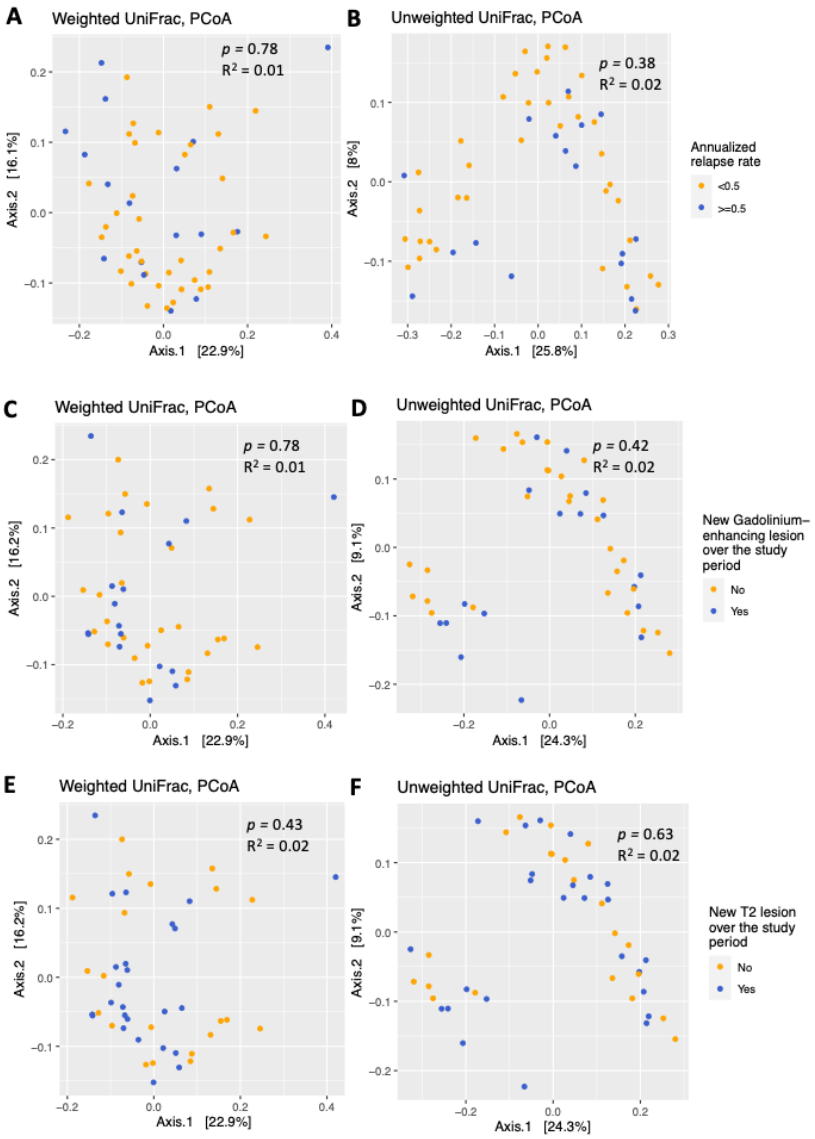

Figure 3 Variance in fecal microbiota composition was not associated with pediatric-onset multiple sclerosis clinical relapse and MRI outcomes. Having, on average, more than 0.5 relapses per year was not associated with beta diversity using **(A)** weighted UniFrac (PERMANOVA R^2=0.01, p=0.78) or **(B)** unweighted UniFrac distance matrices (PERMANOVA R^2=0.02, p=0.38). Having any new gadolinium-enhancing lesions over the study period was not associated with beta diversity using **(C)** weighted UniFrac (PERMANOVA R^2=0.01, p=0.78) or **(D)** unweighted UniFrac distance matrices (PERMANOVA R^2=0.02, p=0.42). Having any new or enlarging T2 hyperintense lesions over the study period was not associated with beta diversity using **(E)** weighted UniFrac (PERMANOVA R^2=0.02, p=0.43) or **(F)** unweighted UniFrac distance matrices (PERMANOVA R^2=0.02, p=0.63). PERMANOVA models adjusted for sex, age, and disease-modifying therapy use. The first two principal coordinates from principal coordinates analysis were plotted.

To explore whether there may be microbes associated with all three disease activity outcomes, we compared the effect sizes of ASVs across all three outcomes if the ASV was associated with at least one outcome at p<0.05. While several ASVs were not tested in both the relapse and MRI analyses because they were not in at least 20% of both samples, we identified five ASVs associated with all three disease activity outcomes (Fig. 5). Four of these showed protective effects across all outcomes, meaning having any of the respective ASV

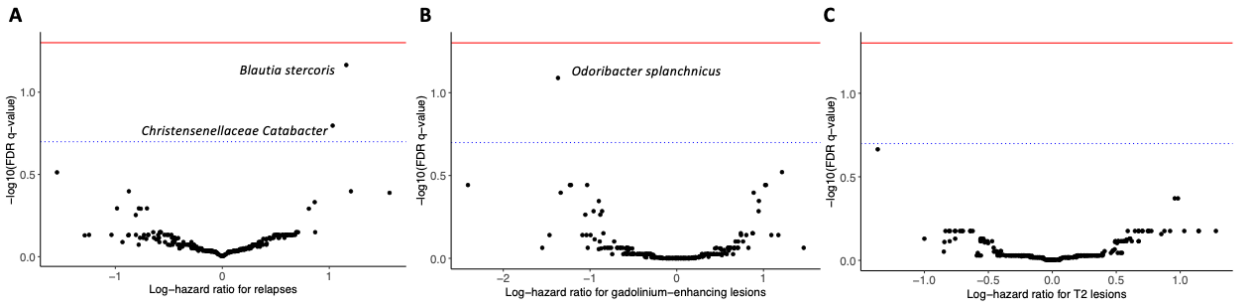

Figure 4 No species of gut microbes were significantly (FDR q<0.05) associated with pediatric-onset multiple sclerosis activity outcomes. The long-dashed line indicated an FDR q cut-off of 0.05 and the small dotted line indicated a less conservative threshold FDR q=0.2. Each point was an ASV. The genus and species (or lowest known taxonomy) of ASVs associated with a respective outcome with FDR q<0.2 were labeled. Regression models adjusted for sex, age, and disease-modifying therapy use. **(A)** Adjusted log-hazard ratios for relapse; **(B)** adjusted log-hazard ratios for new gadolinium-enhancing lesions on MRI; and **(C)** adjusted log-hazard ratios for new or enlarging T2 hyperintense lesions on MRI.

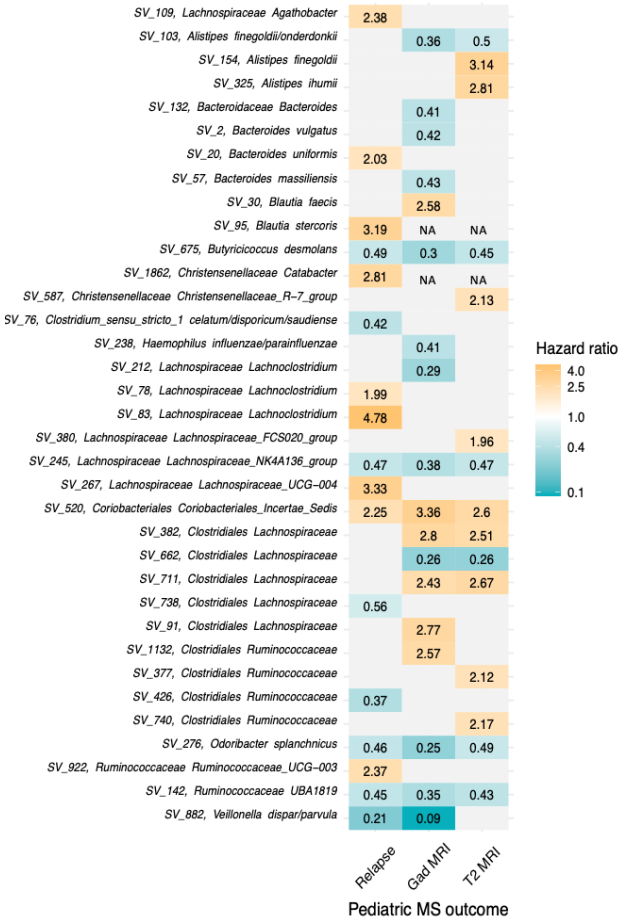

Figure 5 Five gut microbes were associated with all three pediatric-onset multiple sclerosis activity outcomes. Each row was an ASV that was associated with either relapse, new gadolinium-enhancing lesions, or new or enlarging T2 hyperintense lesions at p<0.05. Hazard ratios, adjusted for sex, age, and disease-modifying therapy use, were shown for each significant (p<0.05) ASV-outcome association. Grey indicated an ASV-outcome association was not significant. "NA" indicated an association was not estimated because the ASV was not in at least 20% of the respective sample. Rows were labeled with ASV ID and the lowest known taxonomic classification. Rows were arranged by taxonomic *order*.

(or above the median number of reads) was associated with a lower hazard of relapses, gadolinium-enhancing lesions, and T2 hyperintense lesions. These included *Butyricicoccus desmolans* ($HR_{relapse}$= 0.49, 95% CI: 0.28, 0.88), *Odoribacter splanchnicus* ($HR_{relapse}$= 0.46, 95% CI: 0.24, 0.88), an unidentified species in the *Lachnospiraceae NK4A136* group ($HR_{relapse}$= 0.47, 95% CI: 0.24, 0.89), and *Ruminococcaceae* ($HR_{relapse}$= 0.45, 95% CI: 0.22, 0.91). For these ASVs, similar HRs were observed for MRI outcomes (Fig. 5 and Supplementary Table 1). In contrast, having any reads of SV_520, an unspecified member of *Coriobacteriales*, was associated with more than double the hazard for all three disease activity outcomes ($HR_{relapse}$= 2.25, 95% CI: 1.12, 4.49; HR_{Gad}= 3.36, 95% CI: 1.54, 7.35; HR_{T2} = 2.60, 95% CI: 1.34, 5.08). The abundance of each of these five ASVs did not significantly differ by baseline DMT status (data not shown).

Gut microbial networks were associated with MRI outcomes

Gut microbes exist in complex, inter-connected communities, so we tested the association between networks of co-occurring microbes and each disease activity outcome. Gut microbes were classified into 33 (M1-33) and 27 (M34-60) modules (or clusters/networks) using the MRI and clinical

cohorts, respectively (Supplementary Fig. 2). The ASVs constituting each module for MRI and clinical cohorts were shown in Supplementary Tables 2 and 3. Among the 33 modules identified from subjects within the MRI cohort, five (M7, 10, 11, 32, 33) were significantly associated (FDR q<0.05) with new gadolinium-enhancing lesions (Fig. 6). Two of these modules were protective, where higher module values were associated with a lower hazard of new gadolinium-enhancing lesions: M7 (HR=0.37, 95% CI: 0.18, 0.76) and M10 (HR=0.20, 95% CI: 0.06, 0.63). For the other three significant modules, higher module values were associated with a higher hazard of new gadolinium-enhancing lesions: M11 (HR=1.26, 95% CI: 1.12,1.42), M32 (HR=1.29, 95% CI: 1.08, 1.54), and M33 (HR=1.42, 95% CI: 1.18, 1.71). Higher M32 and M33 module values were also significantly associated with higher hazard of new or enlarging T2 hyperintense lesions (HR_{M32}=1.34, 95% CI: 1.15 1.56; HR_{M33}=1.41, 95% CI: 1.21, 1.64). No other modules were significantly associated with new or enlarging T2 hyperintense lesions, and no modules were significantly associated with relapse(s) (see Supplementary Table 4 and 5 for full results). Interestingly, only one of the five ASVs shown to be individually associated with all three disease activity outcomes was a member of a significant module. This was SV_245, an unidentified member of the *Lachnospiraceae NK4A136* group (which showed a protective effect for all three disease activity outcomes) and a member of the M10 module (associated with a lower hazard for the MRI outcomes).

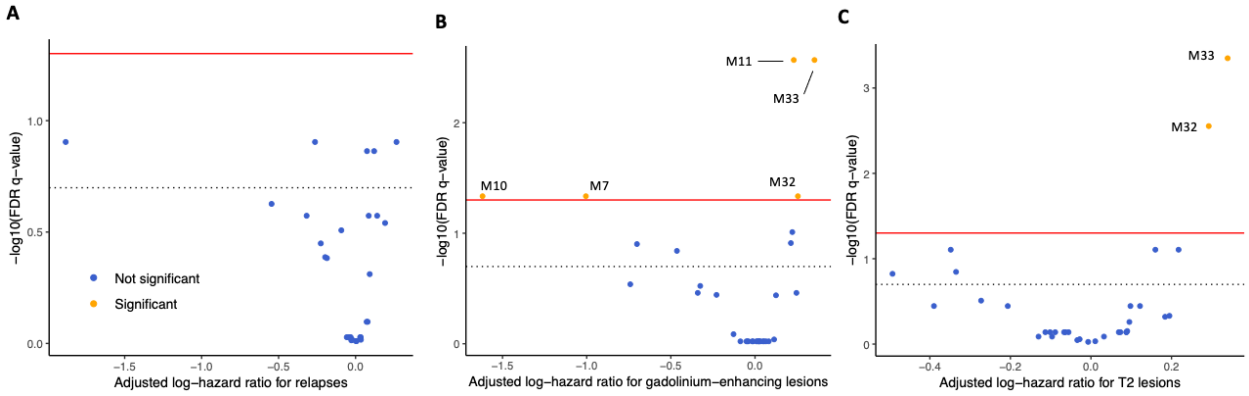

Figure 6 Five networks of gut microbes were significantly associated with MRI-related multiple sclerosis activity. The long-dashed line indicated an FDR q cutoff of 0.05 and the small dotted line indicated an FDR q cutoff of 0.2. Significant modules were labeled with their respective module name. Regression coefficients were scaled to a 0.1-unit increase in module eigengenes because the standard 1-unit increase would represent nearly the entire range of eigengene values. Regression models adjusted for sex, age, and disease-modifying therapy use. **(A)** Adjusted log-hazard ratios for relapse; **(B)** adjusted log-hazard ratios for new gadolinium-enhancing lesions on MRI; and **(C)** adjusted log-hazard ratios for new or enlarging T2 hyperintense lesions on MRI.

Several of the modules significantly associated with MRI outcomes could be mapped to the clinical modules. Notably, all four ASVs within the significant M33 MRI module were in the M56 clinical module. While not statistically significant, the effect size between the M56 module and relapse ($HR_{relapse}$= 1.15, 95% CI: 0.98, 1.35) was similar to the effect sizes between the M33 module and MRI outcomes (HR_{Gad}=1.42 and HR_{T2}=1.40). Additionally, ASVs in the significant M32 MRI module overlapped with the M38 clinical module. The effect size between the M38 module and relapse ($HR_{relapse}$= 1.21, 95% CI: 0.96, 1.53) was similar to the effect sizes between the M32 module and MRI outcomes (HR_{Gad}=1.29 and HR_{T2}=1.34).

Predicted functional pathways from gut microbial networks were associated with multiple sclerosis

Metabolic pathways identified in each significant fecal microbial module were shown in Supplementary Tables 6-10. No modules were significantly associated with relapse, so pathway-relapse associations were not assessed. Metagenomic predictions indicated that modules significantly associated with MRI outcomes encoded amino acid biosynthesis pathways, including the superpathways of L-arginine and L-tryptophan biosynthesis (Supplementary Tables 6-10). Filtering pathways to those only associated with an outcome at $p<0.05$, several pathways were specific to modules associated with a lower hazard (M10 module) or higher hazard (M32 and M33 modules) for the MRI outcomes (Supplementary Fig. 3). Notably, the superpathways of L-tyrosine ($p=0.01$) and L-phenylalanine ($p=0.01$) biosynthesis were associated with a lower hazard of MRI outcomes.

DISCUSSION

In this longitudinal study of subjects with pediatric-onset MS, we identified several individual gut microbes and networks of co-occurring microbes associated with a higher or lower hazard of clinical relapse and MRI-related disease activity. Known functions and metagenomic predictions of these microbes suggest the important role of butyrate and amino acid biosynthesis pathways. The protective, anti-inflammatory effects of butyrate, which have previously been observed in MS studies, provides a potential target for future microbiome interventions intended to modify disease activity in MS.

Three microbes were associated with subsequent disease activity in pediatric-onset MS at FDR $q<0.2$ and warrant further functional investigation. These included *Blautia stercoris* and *Christensenellaceae catabacter,* whereby having any of these bacteria nearly tripled the hazard of relapse, and *Odoribacter splanchnicus*, where having no copies increased the hazard (four times) of a new gadolinium-enhancing lesion. In one small case-control study, higher abundance of *Blautia* was found among MS cases compared to controls.[33] In line with our findings, a higher abundance of *Blautia* and lower abundance of *Odoribacter* have been found among individuals with active lupus disease, another autoimmune disorder, compared to controls.[34] *Odoribacter* has also been found to be lower among individuals with cystic fibrosis, inflammatory bowel disease, and Crohn's disease.[35–38] The potential benefits of *Odoribacter* were largely attributed to its production of butyrate, a short-chain fatty acid that can help maintain gut homeostasis and suppress proinflammatory cytokines.[39,40] Presence of *Odoribacter* was also identified in our study as associated with lower hazard of relapse and T2 hyperintense lesions, but results were not significant after multiple testing correction. In addition to *Odoribacter,* four gut microbes were found to be associated with all three disease activity outcomes before multiple testing correction. These included three microbes that may also be beneficial in higher amounts: *Butyricicoccus desmolans* (another butyrate-producing microbe), an unidentified species in the genus *Lachnospiraceae NKA136 group*, and an unidentified species in the family *Ruminococcaceae*. In contrast, having any abundance of an unspecified species belonging to the *Coriobacteriales* order more than doubled the hazard of disease activity outcomes. All of these microbes had consistent effect sizes across all three MS activity outcomes. Together, these findings suggest the role of butyrate-producing microbes in reducing the risk (hazard) of MS relapses and new/enlarging MRI lesions. This agrees with other studies that have shown oral administration of butyrate decreased demyelination in mice, serum butyric acid concentration was lower among MS cases compared to controls, and gut butyrate (assessed via metagenomics and stool metabolites) was reduced among individuals with relapsing-remitting MS.[41–43]

Individual microbes are unlikely to work independently, and for the first time, unsupervised machine learning has identified networks (or modules/clusters) of co-occurring gut microbes associated with disease activity outcomes in MS. We identified five networks of co-occurring gut microbes associated with an altered risk of new gadolinium-enhancing lesions, of which two were also associated with T2 hyperintense lesions. Across these five modules, pathways involving aromatic amino acid biosynthesis were predicted to be enriched. Namely, phenylalanine and tyrosine biosynthesis pathways were enriched in the M10 module (a module significantly associated with a lower hazard of MRI-related outcomes), while tryptophan was enriched in the M10, M32, and M33 modules (M32 and 33 were associated with a higher hazard of MRI-related outcomes). This relationship of tryptophan with both increased and decreased risk may relate to differences in expression of genes in these microbial modules or their differential catabolism to bioactive metabolic products e.g., kynurenine. Tryptophan, specifically, has been identified as a modulator of central nervous system inflammation and associated with MS risk and course.[44–46] Interestingly, serum metabolite studies of MS activity have identified shifts in aromatic amino acid metabolism among individuals with worse disease activity.[47,48] Our findings suggest that networks of gut microbes associated with MS activity may contribute to the concentration of amino acids, specifically aromatic amino acids that serve as potent CNS and immunomodulatory signaling molecules.

There are several notable strengths of this study. We were able to include individuals with MS shortly after disease onset and follow them prospectively. This captured clinically relevant relapses and MRI data, the latter of which are considered highly sensitive and useful when assessing changes in disease activity over time. Further, our participants were well characterized and were either not using a disease-modifying therapy or using drugs with low effectiveness (in terms of relapse prevention) at baseline. Pediatric-onset cases are useful because it allows for the examination of disease processes much closer to biological onset compared to adults in individuals with very few confounding comorbidities.[13,49–51] While the gut microbiome does undergo significant changes in very early childhood, it is relatively stable in adolescence, with functional capacity similar to adults.[52] Because our sample was almost entirely enrolled as adolescents, this potential source of variation was limited.

While a pediatric-onset cohort represents a unique opportunity for studying modifiers of MS, its rarity limited our sample size and ability to account for other potential confounders or modifiers, such as study site, race, ethnicity, body mass index, diet, vitamin D status, and specific disease-modifying therapies. The small sample size and large number of multiple tests also made it particularly challenging for a result to achieve statistical significance, despite potential biological significance. Because this study is the first of its kind, we reported individual ASV findings with FDR $q<0.2$ despite not reaching statistical significance (FDR $q<0.05$). It is possible these may be false positive findings and should thus be conservatively interpreted. They should be considered candidates for future functional studies and hypotheses and replicated in future work. Another limitation was that all MRI scans were performed without a centralized or standardized imaging protocol and the timing of scans was not at pre-determined intervals (as part of routine clinical practice). Finally, metagenomic predictions cannot be interpreted as true functions or pathways. However, our findings warrant further investigation, including microbes that influence butyrate and amino acid synthesis pathways. We do not have metabolomic or metagenome data to confirm

predicted findings, which should be the focus of future work. Additionally, it would be useful for future studies to collect stool samples repeatedly over time to assess how changes in gut composition due to treatment, diet, and other factors might be associated with relapses and MRI outcomes over time.

In summary, we identified several individual gut microbes and networks of co-occurring microbes that were associated with an altered risk of clinical relapse and activity on brain MRI among pediatric-onset MS patients. Known functions and metagenomic predictions of these organisms suggests the roles of butyrate and amino acid biosynthesis as potential modifiers of MS activity. Further research is needed to confirm the functional and clinical implications of these findings so personalized microbiome interventions may be designed to decrease MS activity.

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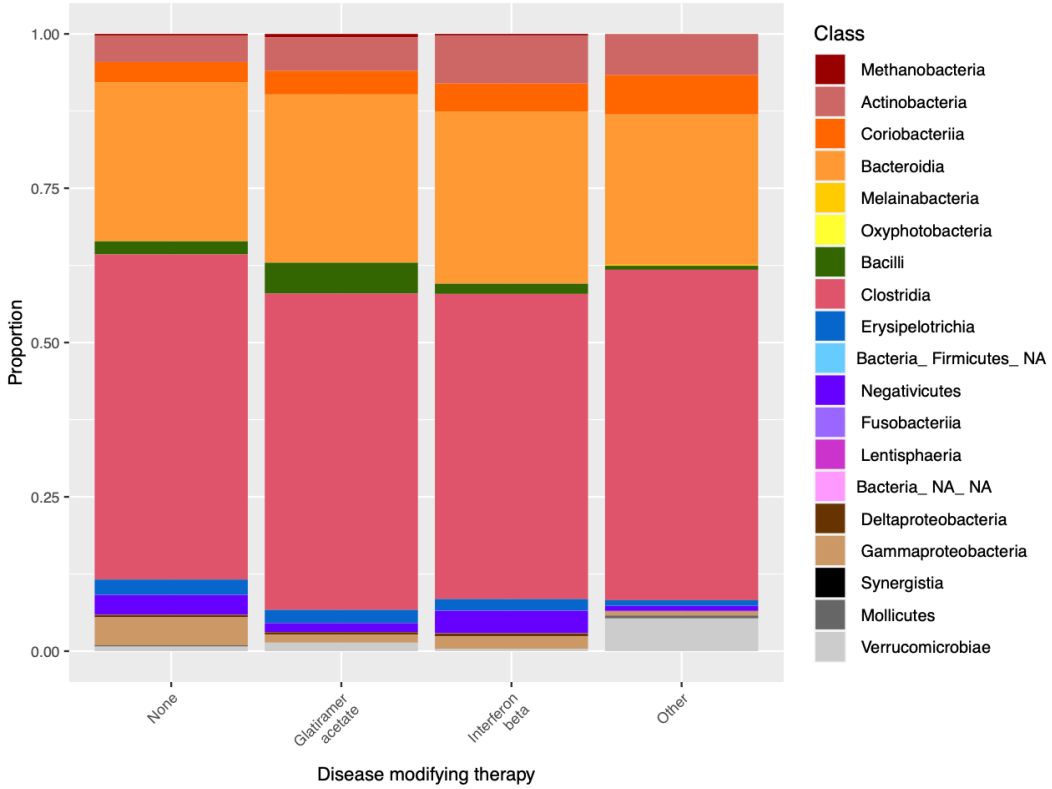
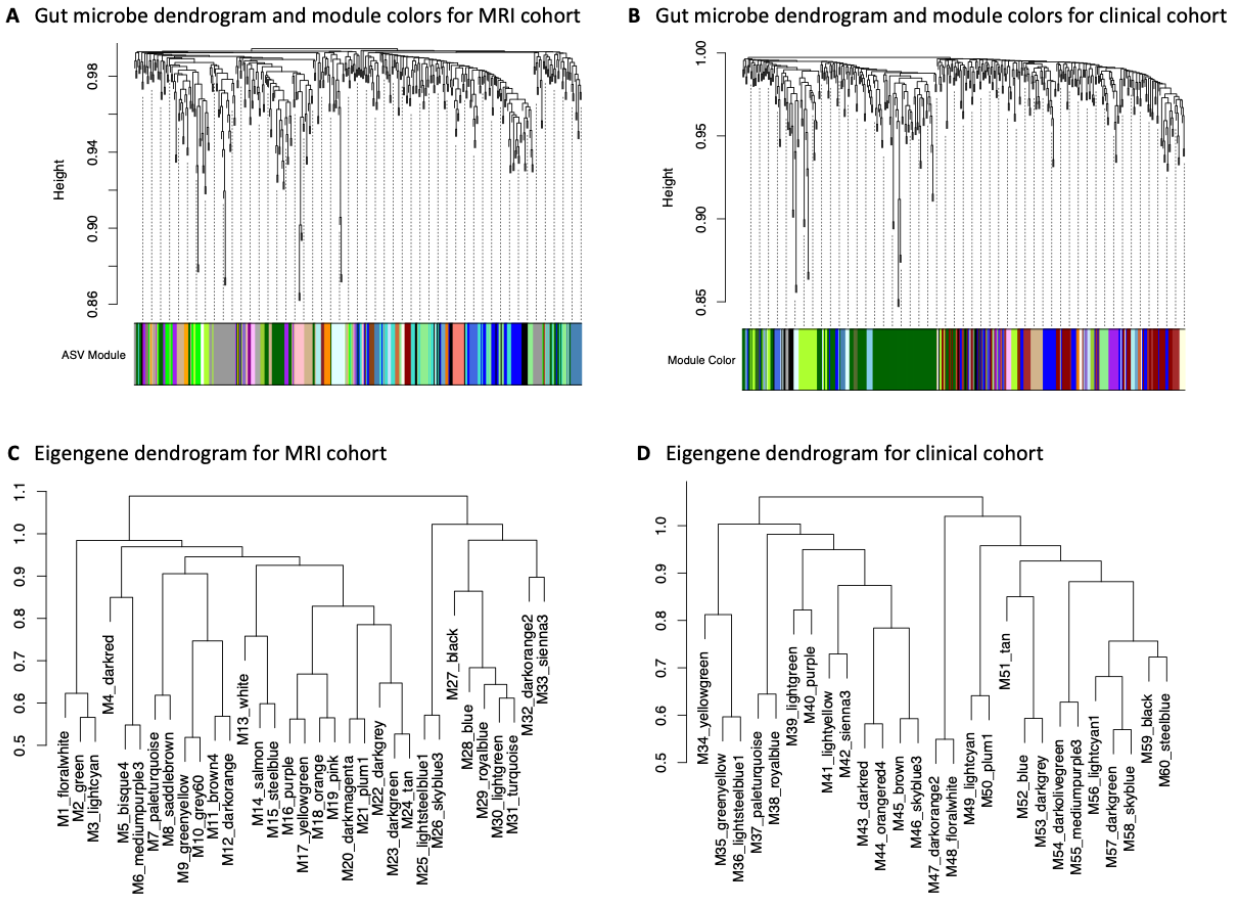
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Total proportion of abundance of microbes (by class) according to baseline disease modifying therapy status. The proportion of individuals ASVs belonging to a particular taxonomic class did not significantly differ by baseline DMT use categories (none, glatiramer acetate, interferon beta, or other DMT) except for Melainabacteria (p=0.0002) and Verrucomicrobiae (p=0.049). P-values determined from F-test.



Supplementary Figure 2. Weighted genetic correlation network analysis identified 33 modules of co-occurring microbes for the MRI cohort and 27 modules for the clinical cohort. **(A and B)** Bacterial taxa dendrogram and module names (colors) for MRI and clinical cohorts, respectively. Each node was an ASV. Taxa that co-occur were positioned closer together and the module for which an ASV was a member was plotted in a vertical band below. **(C and D)** Clustering of module eigenvalues for MRI and clinical cohorts, respectively, with corresponding names (number and color).

Supplementary Figure 3. Pathways predicted to be associated with significant gut microbial modules from PICRUSt2. Each row was a MetaCyc pathway that was associated with a pediatric-onset multiple sclerosis outcome at $p<0.05$ in at least one of the five significant microbial modules (M7, 10, 22, 32, 33). Colors indicated the magnitude of hazard ratios, adjusted for sex, age, and disease modifying therapy use, which represented the association between a pathway and pediatric-onset MS activity outcome, per module (columns). Hazard ratios were only estimated for disease activity outcomes previously identified as associated with a respective module. Grey indicated the module-specific pathway-outcome association had $p\geq0.05$. Hazard ratios were estimated for pathways present in at least 20% of the respective cohort and module. No pathways within the M7 or M11 modules were significant, so were not shown. Abbreviations: Gad = gadolinium, MS = multiple sclerosis.

CHAPTER 3 – CASE-CONTROL STUDY OF ADVERSE CHILDHOOD EXPERIENCES AND MULTIPLE SCLEROSIS RISK AND CLINICAL OUTCOMES

ABSTRACT

Background: Adverse childhood experiences (ACEs) are linked to numerous health conditions but understudied in multiple sclerosis (MS). This study's objective was to test for the association between ACEs and MS risk and clinical outcomes.

Methods: We used a sample of adult, non-Hispanic MS cases (n=1,422) and controls (n=1,185) from Northern California. Eighteen ACEs were assessed including parent divorce, parent death, and abuse. Outcomes included MS risk, age of MS onset, and several indicators of disease severity. Logistic and linear regression estimated odds ratios (ORs) (and beta coefficients) and 95% confidence intervals (CIs) for ACEs operationalized as any/none, counts, individual events, and latent factors/patterns.

Results: Overall, more MS cases experienced ≥1 ACE compared to controls (54.5% and 53.8%, respectively). After adjusting for sex, birthyear, and race, this small difference was attenuated (OR=1.01, 95% CI: 0.87, 1.18). There were no trends of increasing or decreasing odds of MS across ACE count categories. Consistent associations between individual ACEs between ages 0-10 and 11-20 years and MS risk were not detected. Factor analysis identified five latent ACE factors, but their associations with MS risk were approximately null. Age of MS onset and indicators of disease severity were not associated with ACEs after multiple testing correction.

Conclusion: Despite rich data and multiple approaches to operationalizing ACEs, no consistent and statistically significant effects were observed between ACEs with MS. This highlights the challenges of studying sensitive, retrospective events among adults that occurred decades before data collection.

INTRODUCTION

Adverse Childhood Experiences (ACEs) are potentially traumatic events that occur in childhood and can include physical, emotional, and sexual abuse and/or neglect and household disfunction [1]. They are common in the U.S. - occurring in about 58% of the population - and an important social determinant of health [2]. Childhood represents a particularly vulnerable period when body systems are developing. Excessive activation of stress response systems during this period can impact brain development, immunity, metabolic regulatory systems, and the cardiovascular system [3]. A large body of literature has linked ACEs to physical and mental health conditions in adulthood including heart disease, obesity, type 2 diabetes, cancer, and depression [4].

One particularly relevant downstream effect of excessive activation of stress response systems is dysregulation of the immune system. Numerous studies have shown in experimental and observational settings that psychosocial stressors can cause persistent inflammation and suppression of anti-inflammatory compounds [5–7]. Dysregulation of the immune system can lead to many serious health conditions, including autoimmune conditions such as multiple sclerosis (MS), lupus, and rheumatoid arthritis. The literature regarding the effects of ACEs on autoimmune disorders is limited but suggests an increased numbers of ACEs are associated with increased risk of autoimmune conditions overall and individually [8]. More studies are needed to fully understand this relationship, particularly among individual autoimmune conditions.

MS is one autoimmune condition where more work on this topic is needed. MS is a chronic, inflammatory autoimmune condition of the central nervous system and is the second most common neurological disorder among young adults [9,10]. Diagnosis is common relatively early in adulthood (ages 20 to 40 years) and among women (3:1 female-to-male ratio). Several studies have shown that risk factors (e.g., obesity, concussion, Epstein-Barr virus infection, and vitamin D/sun exposure), particularly during adolescence (ages 11-20 years), are associated with increased MS risk [11,12]. Given the relatively young age of MS diagnosis, support for adolescent exposures being important for MS risk, and the critical involvement of inflammation in MS disease processes, determining whether a role exists for ACEs in MS risk is important. Of the few studies that have examined the association between ACEs and MS risk, their results are inconsistent. Findings from the U.S.-based Nurses' Health Study, which asked adult participants to quantify "physical or sexual abuse in childhood or adolescence", suggested MS risk was not significantly associated with abuse [13]. A Danish study found that parent divorce, but not parent or sibling death, was associated with risk of MS [14]. A German study using a 28-item self-report questionnaire of childhood maltreatment found an increased risk of MS among domains of physical abuse, sexual abuse, emotional neglect, and severe abuse [15]. Inconsistencies in these findings are likely due to differences in specific ACEs and how they were quantified, as well as differing cultural and social contexts in each population, underscoring the challenges of this important work and need for further investigation.

There is even less knowledge about whether ACEs affect clinical outcomes of MS, such as disease severity and age of onset, which may be influenced by early life stress and inflammation. The largest study, to date, to investigate this association utilized 217 MS cases and determined that physical abuse, emotional neglect, and severe abuse were associated with higher relapse rates but not age of onset or other indicators of physical or cognitive outcomes [16]. The only other (smaller) study to investigate this found that more ACEs were associated with younger age of MS onset and

worse reading cognition [17]. Understanding the relationship between ACEs and MS risk and clinical outcomes may strengthen the argument for childhood screening of ACEs and interventions that prevent or modify the effects of ACEs and improve our understanding of MS etiology.

Our approach to studying ACEs was to interrogate how they might affect MS risk and clinical outcomes using multiple methodologies. For our study, ACEs included death of a parent or sibling, victim of a violent crime, loss of a home, and significant physical or verbal abuse or neglect, among others. It is common to analyze ACEs as individual events or summarized into any/none or count variables; however, these have several limitations. It is possible that individual ACEs (such as parent divorce shown by Riise et.al) may have different effects on MS risk, but ACEs (and social exposures more broadly) often co-occur and are not necessarily independent [18]. This limits the interpretability of assessing single events that are highly inter-related. In addition, single events may be rare and limit our power to examine associations with MS in all but very large studies. Quantifying ACEs as any/none may be meaningful if the hypothesis is that any adverse event impacts health. However, this dichotomy fails to consider the relative importance of different types of ACEs with varying impacts on chronic stress or behaviors and thus MS. The use of counts assumes the cumulative burden of ACEs affects health, rather than particular type, combination, or chronicity. These limitations highlight the challenges in studying ACEs and the need to consider them in multiple ways in order to understand their complex, nuanced relationships with health outcomes, particularly MS.

The aim of the current study was to estimate the association between ACEs and MS risk and clinical outcomes in a case-control sample of 2,607 adults in Northern California using multiple approaches including quantifying ACEs as individual events, any/none, and counts. We also included a factor analysis to evaluate variance of ACEs in order to identify "latent factors", which are weighted linear combinations of variables, that represent patterns of ACEs that tend to co-occur. Collectively, this approach may help identify how ACEs are associated with MS.

METHODS

Study population

Data were from the Kaiser Permanente Northern California (KPNC) MS Research Program which recruited non-Hispanic MS cases and controls from the KPNC Health Plan between 2006 and 2014. This membership includes over four million people, representing 25-30% of the 22-county service area population in Northern California. The broad goal of this study was to assess risk factors for MS across hundreds of genetic and environmental exposures. To achieve sufficient power for genome-wide analyses, the sample was limited to the largest subgroup of KPNC members which were largely non-Hispanic whites. Recruitment details are explained elsewhere [19]. Briefly, eligible cases were diagnosed with MS by a neurologist (*International Classification of Diseases, Ninth Revision,* code 340.x), aged 18-69 years old, and a KPNC member at initial contact. For our analyses, cases were excluded if age of onset occurred before age 21 years to minimize the potential for reverse causality or MS onset occurring before ACEs (assessed up to age 20). Age of onset was determined by review of electronic health records and comprehensive interview data. Controls were KPNC members without a MS diagnosis or related condition (optic neuritis, transverse myelitis, or demyelinating disease) and were matched to cases by sex, age, and zip code. A total of 2607 participants (1422 cases and 1185 controls) were available for analyses.

Study protocols for participants were approved by the Institutional Review Boards of KPNC and the University of California, Berkeley. Written informed consent was obtained from all study participants.

Adverse Childhood Experiences (ACEs)

Participants were administered a comprehensive computer-assisted telephone interview (CATI) including hundreds of self-reported demographic, clinical, environmental, and lifestyle questions, as described elsewhere [20]. The CATI included nine ACE questions modified from Coddington's Life Event Record [21] (Table 1). Not all questions were included to reduce the length of the extensive CATI. Events included broadly overlap with the original Centers for Disease Control and Prevention-Kaiser ACE Study [1], but there are several differences. Our study does not ask about sexual abuse, household substance abuse, or incarcerated household members. It also combines physical and verbal abuse and adds questions about parent/sibling death and foster care/adoption. Participants indicated yes/no as to whether they experienced any of the events in either of two age periods: 0-10 and 11-20 years old (a total of 18 ACEs). These two time periods were chosen because studies have shown that relationships between several risk factors and MS differ in adolescence and childhood [11,22].

Table 1. Definition and baseline prevalence of adverse childhood experiences reported by adult multiple sclerosis (MS) cases and controls in the Kaiser Permanente Northern California MS Research Program, 2006-2014 (n=2607).

		Total		MS Cases		Controls	
	Adverse Childhood Experience	No.	%	No.	%	No.	%
Remembering back to your early childhood through the age of 10, did you experience any of the following list of events?							
1	Death of parent or sibling	97	3.7	44	3.1	53	4.5
2	Divorce of parents	275	10.5	145	10.2	130	11.0
3	Remarriage of parents	183	7.0	95	6.7	88	7.4
4	Placed in foster care or adoption	57	2.2	34	2.4	23	1.9
5	Went to live with other family members	149	5.7	86	6.1	63	5.3
6	Serious (life-threatening) illness of parent or sibling (including psychiatric illness or substance abuse problem)	312	12.0	170	12.0	142	12.0
7	You experienced significant physical or verbal abuse or neglect	315	12.1	143	10.1	172	14.5
8	Your family lost their home or had to move	196	7.5	85	6.0	111	9.4
9	You were the victim of a violent crime	58	2.2	32	2.3	26	2.2
Remembering back to when you were a teenager, between 11 until you turned 20 years old, did you experience any of the following list of events?							
10	Death of parent or sibling	156	6.0	93	6.5	63	5.3
12	Divorce of parents	266	10.2	140	9.9	126	10.6
13	Remarriage of parents	232	8.9	119	8.4	113	9.5
13	Placed in foster care or late adoption	44	1.7	31	2.2	13	1.1
14	Went to live with other family members	205	7.9	117	8.2	88	7.4
15	Serious (life-threatening) illness of parent or sibling (including psychiatric illness or substance abuse problem)	367	14.1	203	14.3	164	13.8
16	You experienced significant physical or verbal abuse or neglect	389	14.9	200	14.1	189	15.9
17	Your family lost their home or had to move	184	7.1	98	6.9	86	7.3
18	You were the victim of a violent crime	130	5.0	71	5.0	59	5.0

Abbreviations: MS, multiple sclerosis

MS clinical outcomes

As part of the CATI, MS cases were asked the year of their first MS symptom (i.e., "onset"), the type of MS they currently have (relapsing-remitting, secondary progressive, primary progressive, or relapsing-progressive), and an indication of their walking ability in the past four weeks. For each MS case, we calculated the Multiple Sclerosis Severity Scale (MSSS), which is an indicator of disease severity that uses the Expanded Disability Severity Scale and disease duration (time from onset to EDSS) [23]. We also created an indicator of whether a case had severe or mild MS based on MSSS scores ($\geq$7.5 was severe and <3 was mild).

Covariates

Demographic and clinical data collected from the CATI and considered confounders included sex, birth year, race, and years since MS onset. Race was categorized as white or non-white, noting 98.5% of non-whites identified as African American. Additional confounders considered in sensitivity analyses (see below) included education-level (bachelor's degree or not), parent's homeowner status when participant was 10 years old (rent vs own/other), and family history of MS (parent or sibling). These were not included in primary/secondary analyses to preserve statistical power and prevent over-stratification of models with already low frequency substrata (including rare ACEs, men, and non-whites).

Statistical analysis

Factor analysis was conducted among all participants to determine the latent factor structure of 18 total ACEs (nine ACEs at two time points). A tetrachoric correlation matrix, appropriate for binary data, was constructed. Zero-count cells were corrected by adding 0.1. Factors were extracted using maximum likelihood estimation in the *polycor* package and *factanal* in R Version 3.5 [24]. VARIMAX (orthogonal) rotation was used to increase interpretability of factors. Number of factors to extract was based on optimal coordinates and reduced if any factor loading was ≥1.0 [25]. Factor scores were calculated and standardized to a mean of zero and standard deviation of one [26].

Primary analyses tested the association between ACEs and MS risk using logistic regression to estimate odds ratios (ORs) and 95% confidence intervals (CIs). ACEs were expressed as: 1) any/none at each time period and overall, 2) 0, 1, 2, 3, or 4 or more at each time period and overall, 3) individually at each time period and overall, and 4) continuously for each factor score. The associations between individual ACEs and MS risk were only estimated for ACEs that occurred in at least 5% of the sample in order to achieve sufficient statistical power. To improve interpretability of ORs from models using continuous factor scores (where a 1-unit increase in respective factor score would represent nearly the entire range of values), beta coefficients and their standard errors were divided by ten. All models adjusted for sex, birthyear, and race. Multiple testing corrected false discovery rate (FDR) *q* values are presented for primary analyses [27]; they account for all primary models assessing MS risk simultaneously. All analyses used R Version 3.5 [24].

Secondary analyses investigated the association between ACEs and clinically relevant MS outcomes including MSSS, age of onset, progressive MS subtype, and current walking ability. We also included a sub-analysis comparing ACEs among individuals with mild and severe MS. For MSSS and age of onset outcomes, linear regression models were used to estimate beta coefficients and 95% CIs. Both models adjusted for sex and race. MSSS models additionally adjusted for birthyear. For MS subtype, type of MS was categorized as relapsing (relapsing remitting or secondary progressive) (reference) or progressive (primary progressive or relapsing progressive). For current walking ability, individuals were classified according to whether they did or did not (reference) regularly use a walking aid (such as cane, walker, or wheelchair). Additionally, a sub-analysis utilized individuals only at the extreme ends of the MSSS scale (n=818) where the outcome was severe or mild (reference) illness. For all binary outcomes, ORs and 95% CIs were estimated using logistic regression and adjusted for birthyear, sex, and race. Walking ability models additionally adjusted for years since MS onset. For all MS outcome models, ACEs were

considered the independent variable and expressed as count categories (0, 1, 2, 3, or 4 or more) over the entire exposure period (0 through 20 years of age). Additional ACE classifications were not included to minimize the impact of multiple testing corrections on a reduced sample size (1422 MS cases). Results from secondary analyses were corrected for FDR and account for all secondary clinical outcome tests.

Sensitivity analyses

To evaluate whether socioeconomic factors independent of race might confound the observed primary associations between ACEs and MS risk, we included two additional logistic regression models which adjust for covariates in the original models plus 1) participant's educational level or 2) parent's homeowner status when participants were 10 years old and family history of MS. Family history was considered a potential confounder because the risk of MS is ~seven times higher among those who have a first degree relative with MS [28] and it may be a cause of parent or sibling illness or death (an ACE in our assessment).

RESULTS

Baseline characteristics were described in Table 2. Among MS patients, 79.0% identified as female (81.5% for controls). The average years since MS onset was 17.1 (sd=11.8), and the majority of MS cases had mild illness (MSSS <3) (47.6%). Cases had higher frequency of family history of MS (6.8%) compared to controls (1.6%), as expected. When participants were 10 years old, fewer parents of MS cases owned a home compared to controls (78.0% and 81.9%, respectively), as previously reported [20].

Table 2. Baseline characteristics among multiple sclerosis (MS) cases and controls in the Kaiser Permanente Northern California MS Research Program, 2006-2014 (n=2,607).

Characteristic	MS Cases (n=1,422)		Controls (n=1,185)	
	No.	%	No.	%
Birth year (mean, sd)	1958 (8.8)		1958 (8.9)	
Sex (female)	1,124	79.0	966	81.5
Parent Homeowner Status at 10 years old				
Own	1,109	78.0	970	81.9
Rent/Other Arrangement	298	21.0	210	17.7
Not available	15	1.0	5	0.4
Race[a]				
White	1,288	90.6	1,114	94.0
African American	134	9.4	72	6.0
American Indian or Alaskan native	3	0.2	0	0.0
Family history of MS[b] (yes)	97	6.8	19	1.6
ACEs, count (mean, sd)	1.3 (1.8)		1.4 (1.9)	
Any ACEs (yes)	775	54.5	637	53.8
Years since MS onset (mean, sd)	17.1 (11.8)			
MSSS (mean, sd)	3.8 (2.5)			
MSSS <3	677	47.6		
MSSS ≥7.5	141	9.9		
MS subtype				
Relapsing remitting	938	66.0		
Primary progressive	113	7.9		
Secondary progressive	221	15.5		
Relapsing progressive	51	3.6		
Unknown	99	7.0		

Abbreviations: ACEs, adverse childhood experiences; MS, multiple sclerosis; MSSS, Multiple Sclerosis Severity Score

[a]Two individuals reported American Indian/Alaskan Native and white race, one reported American Indian/Alaskan Native and African American race, and one reported African American and white race

[b]Defined as having a parent or sibling with MS

The proportion of participants who experienced ≥1 ACE was higher among cases (54.5%) compared to controls (53.8%) (Table 2). Among the entire sample, the most common ACE during ages 0-10 years was significant physical abuse/neglect (12.1%); it was also the most common ACE during ages 11-20 year (14.9%) (Table 1). The distribution of individual ACEs was similar among

cases and controls although fewer cases reported significant physical abuse/neglect or home loss during ages 0-10 years.

Overall, individuals who reported at least one ACE between ages 0-20 years did not have a significantly higher odds of MS compared to individuals who experienced none (OR=1.01, 95% CI: 0.87, 1.18) (Table 3). A similar non-significant effect was also observed for each age category separately. When ACE counts were categorized into 0, 1, 2, 3, or 4 or more, none of the categories were significantly associated with MS and there were no consistent trends where increased ACEs increased or decreased odds of MS. No individual ACEs were significantly associated with MS at an FDR q<0.05 except abuse (OR=0.66, 95% CI: 0.52, 0.84) and home loss (OR=0.61, 95% CI: 0.45, 0.82) between ages 0-10 years. These effect sizes were attenuated and not statistically significant at ages 11-20 years (OR_{abuse} = 0.87, 95% CI: 0.70, 1.08 and $OR_{home\ loss}$ = 0.96, 95% CI: 0.71, 1.30). For secondary analyses pertaining to ACEs and clinical outcomes of MS, no associations were significant at FDR q<0.05 (Table 4). Before adjusting for multiple testing comparisons, two associations were significant at p <0.05. These included a two year younger age of onset, on average, for MS cases who experienced at least four ACEs compared to those who experienced no ACEs (β= -1.99, 95% CI: -3.62, -0.37, p=0.02), and a higher odds of needing to regularly use a walking aid among MS cases who experienced at least four ACEs compared to MS cases who experienced no ACEs (OR= 1.52, 95% CI: 1.03, 2.24, p=0.03).

Table 3. Results from multivariable logistic regression models of the effect of adverse childhood experiences (ACEs) during two age periods on odds of multiple sclerosis.

	Overall			Ages 0-10 years			Ages 11-20 years		
Model	OR	95% CI	FDR q	OR	95% CI	FDR q	OR	95% CI	FDR q
At least one ACE (ref=none)	1.01	0.87, 1.18	0.96	0.86	0.73, 1.01	0.34	1.03	0.88, 1.21	0.95
Count category									
0 ACEs (ref)	1.00	-	-	1.00	-	-	1.00	-	-
1 ACE	1.29	1.04, 1.61	0.17	1.02	0.83, 1.24	0.96	1.12	0.93, 1.35	0.59
2 ACEs	0.99	0.79, 1.24	0.97	0.70	0.54, 0.92	0.12	0.87	0.67, 1.13	0.63
3 ACEs	0.78	0.57, 1.05	0.38	0.59	0.39, 0.89	0.12	0.96	0.67, 1.38	0.96
4 or more ACEs	0.86	0.67, 1.10	0.59	0.87	0.55, 1.41	0.83	1.05	0.68, 1.63	0.96
Individual events									
Parent/sibling death	0.95	0.73, 1.25	0.95	-	-	-	1.22	0.88, 1.71	0.59
Parent divorce	0.86	0.70, 1.05	0.48	0.88	0.68, 1.14	0.63	0.91	0.71, 1.18	0.78
Parent remarries	0.86	0.69, 1.08	0.59	0.88	0.65, 1.19	0.69	0.87	0.66, 1.14	0.63
Live elsewhere	1.13	0.89, 1.43	0.63	1.12	0.80, 1.57	0.78	1.10	0.82, 1.47	0.78
Parent/sibling illness	1.02	0.84, 1.23	0.96	1.02	0.80, 1.29	0.96	1.04	0.84, 1.30	0.95
Abuse	0.82	0.67, 1.01	0.31	0.66	0.52, 0.84	0.02	0.87	0.70, 1.08	0.59
Home lost	0.79	0.62, 1.01	0.31	0.61	0.45, 0.82	0.02	0.96	0.71, 1.30	0.96
Violent crime	1.00	0.74, 1.38	0.98	-	-	-	0.98	0.69, 1.40	0.96
Latent variables									
Factor 1	0.98	0.95, 1.02	0.76	-	-	-	-	-	-
Factor 2	0.99	0.96, 1.01	0.63	-	-	-	-	-	-
Factor 3	0.99	0.97, 1.01	0.63	-	-	-	-	-	-
Factor 4	1.07	1.01, 1.14	0.20	-	-	-	-	-	-
Factor 5	0.97	0.92, 1.00	0.36	-	-	-	-	-	-

All models adjusted for birthyear, sex, and race. ORs for individual ACEs that did not occur in at least 5% of samples were not estimated. Beta coefficients, standard errors, and their respective ORs and 95% CIs were scaled to 0.1-unit increases for factor scores.
Abbreviations: ACEs, adverse childhood experiences; CI, confidence interval; FDR, false discovery rate; OR, odds ratio

Optimal coordinates analysis identified five factors of co-occurring ACEs which explained 57.0% of the variance in 18 reported ACEs (S1 Table). For each factor, the following ACEs contributed the largest loadings: lost home or moved ages 0-10 and 11-20 years (Factor 1), parent divorce and parent remarriage ages 0-10 (Factor 2), physical or verbal abuse or neglect ages 0-10 and 11-20 years (Factor 3), placed in foster care and parents divorced ages 11-20 years (Factor 4), and parent

or sibling death ages 0-10 years (Factor 5). Logistic regression using continuous factor scores did not yield statistically significant results (Table 3). For all factors, a 0.1-unit increase in factor score had very small or null association with MS risk (e.g., Factor 1 OR= 0.98, 95% CI: 0.95, 1.02).

Table 4. Results from multivariable regression models of the effect of adverse childhood experiences (ACEs) during ages 0-20 years on clinical outcomes of multiple sclerosis

	1 vs 0 ACEs			2 vs 0 ACEs			3 vs 0 ACEs			4 or more vs 0 ACEs		
Outcome	Beta/ OR	95% CI	FDR *q*	Beta/ OR	95% CI	FDR *q*	Beta/ OR	95% CI	FDR *q*	Beta/ OR	95% CI	FDR *q*
MSSS[a]	0.31	-0.03, 0.65	0.27	0.34	-0.04, 0.71	0.27	0.04	-0.49, 0.56	0.91	0.30	-0.13, 0.72	0.44
Age at onset[b]	0.28	-1.03, 1.58	0.90	-0.43	-1.86, 1.00	0.90	-1.03	-3.04, 0.97	0.62	-1.99	-3.62, -0.37	0.27
Progressive course[a]	1.12	0.72, 1.72	0.90	0.96	0.59, 1.53	0.91	0.96	0.46, 1.85	0.91	0.87	0.46, 1.53	0.90
Use of walking aid[c]	1.29	0.94, 1.76	0.33	1.23	0.87, 1.74	0.53	0.86	0.51, 1.42	0.90	1.52	1.03, 2.24	0.27
Severe illness	1.57	0.97, 2.52	0.27	1.59	0.94, 2.66	0.27	1.07	0.46, 2.23	0.91	0.91	0.43, 1.79	0.91

Beta coefficients were presented for continuous outcomes (MSSS, age off onset) while ORs were presented for binary outcomes.
[a]Models adjusted for birthyear, sex, and race.
[b]Model adjusted for sex and race.
[c]Models adjusted for birthyear, sex, race, and years since MS onset.
Abbreviations: ACEs, adverse childhood experiences; CI, confidence interval; FDR, false discovery rate; MSSS, Multiple Sclerosis Severity Score; OR, odds ratio; *q*, *q-value*

Sensitivity analyses for MS risk models yielded ORs and 95% CIs that did not substantially change when models additionally controlled for participant's educational attainment, parent's homeowner status, or family history of MS (S2 and S3 Tables).

DISCUSSION

ACEs are associated with numerous adult health conditions [4], but the relationship between ACEs and MS has remained elusive. Understanding this relationship may be particularly relevant because one hypothesized biological mechanism linking ACEs and general poor adult health is inflammation [29], a key cause of neuronal damage in MS. Despite rich data and multiple approaches for operationalizing ACEs in the current study, no consistent and statistically significant effects were observed between ACEs with MS risk and clinical outcomes after correcting for multiple testing comparisons. This highlights the challenges of studying sensitive, retrospective events among adults that occurred decades before data collection. It also underscores the need for ACE assessments early in the MS disease course to overcome some of these challenges.

Our primary findings, which do not support the role of ACEs in risk of MS, both agree with and contradict past studies of MS and autoimmune disorders. Results from a large cohort study of U.S. nurses did not identify associations between MS and stressful life events, including physical and/or sexual abuse during childhood or adolescence [13]. Corresponding odds ratios ranged from 0.72 to 1.30 but were not statistically significant, which may be due to the small number of MS cases identified from the large cohort (n=369). These findings align with the magnitude and insignificant nature of the current findings. Similar to our results, a Danish study (the largest study to date) found that risk of MS was not associated with parent death (OR=1.04, 95% CI: 0.90, 1.21) or sibling death (OR=1.04, 95% CI: 0.81, 1.32) [14]. However, this study did observe that parent divorce, specifically, was associated with increased risk of MS (OR=1.13, 95% CI: 1.04, 1.23), which is not consistent with our results. Their results are likely highly accurate given that Danish

registries capture all family relations and marital statuses for all Danish residents and capture all MS diagnoses since 1956. However, social structures, levels of inequities, and the demographic make-up of Denmark and the U.S. are very different, so these adverse events might not be expected to have the same effects in both countries. Our findings pertaining to physical abuse (and home loss) demonstrated a significant protective effect during childhood, but there is no reason to believe that physical abuse or home loss, but not other ACEs, would prevent MS. In fact, previous research contradicts this finding which identified an increased risk of MS among those who have experienced severe abuse (OR=1.7) and null associations between physical abuse or neglect and MS risk [15]. Similarly, latent factors 1 or 3 were not associated with MS risk despite being the factors for which childhood abuse and home loss contributed the most.

Among other autoimmune conditions, increasing number of ACEs have been associated with first hospitalization of any autoimmune disease as well as rheumatic, Th1-type and Th2-type immunopathologies, and Systemic Lupus Erythematosus (SLE) [8,30]. In particular, physical and emotional abuse have been shown to be associated with over two times the risk of SLE [8]. These were not found to be associated in our study. The differing results may be a result of different associations between ACEs and specific autoimmune conditions or insufficient statistical power, measurement error, or selection bias within our study or others.

Our findings that a younger age of onset and regular use of a walking aid were more common among MS cases that had at least four ACEs were not significant after correcting for multiple testing comparisons. Current research on this topic is very limited, with only two small studies reporting their findings. Of these, age of onset was found to be inversely correlated with ACEs ($r = -0.30$, $p = 0.04$) [17] or not associated with ACEs [15]. In another autoimmune condition, SLE, higher ACE levels and ACE domains were associated with worse patient-reported disease activity, depression, and health status [31]. Our findings should be explored further in a larger sample size to improve statistical power to identify whether a true relationship exists between clinical features of MS and ACEs.

A major challenge that may have contributed to inconsistencies between our results and other studies, as well as our generally null observed effects, is information bias. Particularly, retrospectively asking adults about ACEs that occurred decades in the past that are sensitive by nature and may be misremembered or repressed from memory could have led to underreporting. Comparing the frequency of several of our study's ACEs to those in the Behavioral Risk Factor Surveillance System (BRFSS) (derived from the Kaiser-CDC ACEs study) provides evidence of this underreporting. For example, 28% and 34% of individuals in the BRFSS had their parents' divorce/separate and experienced emotional abuse while 19% and 18% experienced these ACEs in our sample, respectively [32]. Recall of sensitive events may have been under-reported, specifically, among cognitively impaired MS patients. However, this is not consistent with knowledge that cognitive MS symptoms do not commonly affect recall of memories from the distant past but rather lead to trouble with recall due to deficits in ability to store new knowledge for future recall [33,34]. Alternatively, MS cases may have interpreted questions regarding home loss or abuse more conservatively than controls, not willing to report the event unless they considered it an extreme circumstance. This is unlikely given "recall bias" which often, but not always, leads to more accurate recall of particular events/exposures among case groups than control groups.

In addition to this potential retrospective reporting bias, there are several limitations that should be considered. First, the events utilized in this dataset are not, together, part of a standardized ACE index. Compared to the BRFSS, our events similarly included parent divorce/separation, but did not include substance use, parent incarceration, or sexual abuse. Exclusion of these sensitive, important topics may have contributed to observed null findings. This is particularly relevant given that household substance abuse is one of the more common ACEs in the BRFSS (26.8% reported experiencing this) [2]. Combining physical and verbal abuse into a single category may also have underestimated the impact of ACEs in our sample. We did, however, include important events not part of the BRFSS survey including parent death and life-threatening illness of parent or sibling. Second, using ACEs is an imperfect way of measuring childhood adversity. Individual events tend to be interrelated and the social environment and factors that may influence it are complex and challenging to disentangle. To improve upon individual ACE analyses (which also may suffer from reduced statistical power due to rarity of certain events), we utilized factor analysis to create unobserved "latent" variables to capture the relatedness of ACEs. The observed associations between each latent variable and MS risk were approximately null, but the extent to which these factors might represent true unobserved continuous variables remains unknown. These five factors captured a relatively small amount of variation in ACEs (57%), which also limits the effectiveness of estimating their associations with MS. Last, low income and African American individuals disproportionately experience a number of adverse experiences [32,35]. This demographic is under-represented in the current sample which may lead to limited generalizations of findings to more diverse populations or selection bias. It may also have led to the observed null findings given African Americans tend to have worse MS clinical outcomes compared to Whites [36]. Future studies should further explore relationships between ACEs and MS among African Americans, Hispanics, Asians, and other non-White populations. This work is currently underway. Future studies should also investigate the nuanced synergistic and/or cumulative relationships between ACEs, socioeconomic position, and MS. For example, the effect of ACEs on MS may be stronger among individuals whose parents rented rather than owned a home (indicator of socioeconomic position and associated with MS) or among those who also experienced stressful events as adults later in the lifespan [20].

CONCLUSIONS

Findings from the current study did not support an association between ACEs and development of MS or clinical feature of MS. While we cannot exclude the potential role of ACEs on MS, our results highlight how poor recall or even recall bias for reporting sensitive events in the past may be particularly challenging to overcome in the context of MS. Future studies should consider alternative tools for assessing ACEs and childhood trauma, such as biomarkers of stress, and/or obtain ACE information from MS patients as close to diagnosis as possible to reduce the number of years between exposure and outcome.

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SUPPLEMENTARY TABLES

S1 Table. Factor loadings for a 5-factor model based on adverse childhood experiences data from the Kaiser Permanente Northern California Multiple Sclerosis Research Program cases and controls, 2006-2014 (n=2,607).

	Item	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Ages 0-10 years	Death of parent or sibling	0.16	0.11	0.04	0.02	0.98
	Divorce of parents	0.15	0.96	0.17	0.13	-0.12
	Remarriage of parents	0.02	0.85	0.12	0.10	0.28
	You were placed in foster care (or late adoption)	0.20	0.14	0.04	0.72	-0.08
	Went to live with other family members	0.46	0.28	0.15	0.30	0.19
	Serious (life-threatening) illness of parent or sibling (including psychiatric illness or substance abuse problem)	0.46	0.05	0.12	0.00	0.35
	You experienced significant physical or verbal abuse or neglect	0.29	0.21	0.79	0.18	0.08
	Your family lost their home or had to move	0.60	0.33	0.20	0.11	0.06
	You were the victim of a violent crime	0.49	0.18	0.26	0.17	0.07
Ages 11-20 years	Death of parent or sibling	0.29	0.00	0.01	-0.03	0.07
	Divorce of parents	0.43	0.24	0.13	-0.52	0.01
	Remarriage of parents	0.42	0.57	0.09	-0.13	0.07
	You were placed in foster care (or late adoption)	-0.14	0.09	0.18	0.90	0.12
	Went to live with other family members	0.31	0.27	0.19	0.19	0.01
	Serious (life-threatening) illness of parent or sibling (including psychiatric illness or substance abuse problem)	0.45	0.02	0.16	-0.12	0.04
	You experienced significant physical or verbal abuse or neglect	0.26	0.11	0.95	0.03	0.08
	Your family lost their home or had to move	0.63	0.14	0.19	0.02	-0.03
	You were the victim of a violent crime	0.45	0.16	0.41	-0.02	-0.10
	Proportion of variance explained	0.15	0.14	0.11	0.10	0.07
	Cumulative variance explained	0.15	0.29	0.40	0.50	0.57

S2 Table. Sensitivity analysis of multivariable logistic regression models of the effect of adverse childhood experiences (ACEs) during two age periods on odds of multiple sclerosis accounting for educational attainment.

	Overall		Ages 0-10 years		Ages 11-20 years	
Model	OR	95% CI	OR	95% CI	OR	95% CI
At least one ACE (ref=none)	0.98	0.84, 1.15	0.83	0.71, 0.98	1.00	0.86, 1.18
Count category						
0 ACEs (ref)	1.00	-	1.00	-	1.00	-
1 ACE	1.26	1.02, 1.57	0.99	0.81, 1.21	1.11	0.92, 1.33
2 ACEs	0.96	0.77, 1.21	0.68	0.52, 0.90	0.83	0.64, 1.08
3 ACEs	0.74	0.55, 1.01	0.56	0.47, 0.85	0.92	0.64, 1.32
4 or more ACEs	0.81	0.63, 1.04	0.82	0.50, 1.32	0.99	0.64, 1.54
Individual events						
Parent/sibling death	0.92	0.70, 1.21	-	-	1.18	0.85, 1.65
Parent divorce	0.83	0.68, 1.01	0.85	0.66, 1.09	0.88	0.68, 1.14
Parent remarries	0.84	0.67, 1.05	0.83	0.61, 1.13	0.86	0.65, 1.13
Live elsewhere	1.07	0.84, 1.37	1.07	0.76, 1.50	1.04	0.78, 1.40
Parent/sibling illness	1.02	0.84, 1.24	1.03	0.81, 1.31	1.04	0.83, 1.31
Abuse	0.79	0.65, 0.97	0.64	0.50, 0.81	0.85	0.68, 1.05
Home lost	0.77	0.60, 0.99	0.60	0.44, 0.80	0.93	0.68, 1.26
Violent crime	0.99	0.72, 1.36	-	-	0.97	0.68, 1.40
Latent variables			-	-	-	-
Factor 1	0.98	0.94, 1.02				
Factor 2	0.99	0.96, 1.01	-	-	-	-
Factor 3	0.99	0.97, 1.01	-	-	-	-
Factor 4	1.07	1.00, 1.13	-	-	-	-
Factor 5	0.96	0.93, 1.00	-	-	-	-

Total number of participants without missing covariate data is 2,603. All models adjusted for year of birth, sex, race (white or non-white), and educational attainment. ORs for individual ACEs that did not occur in at least 5% of samples were not estimated. Beta coefficients, standard errors, and their respective ORs and 95% CIs were scaled to 0.1-unit increases for factor scores.
Abbreviations: ACEs, adverse childhood experiences; CI, confidence interval; OR, odds ratio

S3 Table. Sensitivity analysis of multivariable logistic regression models of the effect of adverse childhood experiences (ACEs) during two age periods on odds of multiple sclerosis (MS) accounting for parent homeowner status and family history of MS.

	Overall		Ages 0-10 years		Ages 11-20 years	
Model	OR	95% CI	OR	95% CI	OR	95% CI
At least one ACE (ref=none)	0.99	0.85, 1.17	0.4	0.71, 0.99	1.01	0.86, 1.18
Count category						
0 ACEs (ref)	1.00	-	1.00	-	1.00	-
1 ACE	1.28	1.03, 1.60	0.98	0.80, 1.20	1.10	0.91, 1.33
2 ACEs	0.99	0.79, 1.24	0.70	0.53, 0.92	0.86	0.66, 1.12
3 ACEs	0.75	0.55, 1.01	0.56	0.37, 0.84	0.90	0.62, 1.30
4 or more ACEs	0.82	0.63, 1.05	0.87	0.53, 1.42	0.96	0.62, 1.51
Individual events						
Parent/sibling death	0.91	0.69, 1.19	-	-	1.17	0.84, 1.64
Parent divorce	0.85	0.70, 1.05	0.88	0.68, 1.14	0.91	0.70, 1.18
Parent remarries	0.87	0.70, 1.09	0.89	0.65, 1.21	0.88	0.66, 1.15
Live elsewhere	1.09	0.86, 1.40	1.10	0.78, 1.56	1.07	0.80, 1.44
Parent/sibling illness	0.99	0.82, 1.21	1.00	0.79, 1.28	0.99	0.79, 1.25
Abuse	0.80	0.65, 0.98	0.65	0.51, 0.82	0.83	0.67, 1.04
Home lost	0.80	0.61, 1.00	0.59	0.44, 0.80	0.97	0.71, 1.32
Violent crime	1.00	0.73, 1.38	-	-	0.97	0.68, 1.40
Latent variables			-	-	-	-
Factor 1	0.98	0.95, 1.03				
Factor 2	0.99	0.96, 1.01	-	-	-	-
Factor 3	0.99	0.97, 1.01	-	-	-	-
Factor 4	1.07	1.01, 1.14	-	-	-	-
Factor 5	0.96	0.92, 1.00	-	-	-	-

Total number of participants without missing covariate data is 2,587. All models adjusted for year of birth, sex, race (white or non-white), parent homeownership status (own or rent/other), and family history of MS (parent or sibling with MS). ORs for individual ACEs that did not occur in at least 5% of samples were not estimated. Beta coefficients, standard errors, and their respective ORs and 95% CIs were scaled to 0.1-unit increases for factor scores.
Abbreviations: ACEs, adverse childhood experiences; CI, confidence interval; OR, odds ratio.

CHAPTER 4- A CROSS-TRAIT ANALYSIS OF MIGRAINE AND MULTIPLE SCLEROSIS

ABSTRACT

Background and Objectives: Migraine is common among individuals with multiple sclerosis (MS), but the reason is unknown. This study's objective was to test three hypothesized mechanisms responsible for the comorbidity of MS and migraine including migraine causing MS, shared genetic variants, or MS with migraine being a distinct MS-related symptom.

Methods: Using publicly available genome-wide association study summary statistics, we used two-sample Mendelian randomization to test whether a migraine genetic instrumental variable caused MS. We then used linkage disequilibrium score regression and LOGODetect to ascertain whether MS and migraine shared non-MHC genetic variants across the genome and regionally. We separately tested whether MHC variants were associated with both phenotypes. Last, we estimated odds ratios (ORs) and 95% confidence intervals (CIs) using logistic regression to identify whether MS cases with migraine had distinct clinical characteristics or more frequent MS-specific genetic or environmental risk factors compared to MS cases without migraine.

Results: We did not find evidence of migraine causing MS (p=0.29) using Mendelian randomization. We identified four MHC loci shared between MS and migraine that had not previously been implicated in either disorder. This included a region overlapping *HLA-B* but no other HLA genes. In addition, we observed that MS cases with migraine (compared to non-migraine MS cases) were more likely to have a relapsing-progressive than primary progressive disease course (OR=2.37, 95% CI: 1.26, 4.52) and ever experience depression (OR=1.48, 95% CI: 1.22, 1.80), after adjusting for covariates including sex and interferon beta use. Other clinical features such as age at MS onset and physical and cognitive disability indicators were not associated with migraine status.

Discussion: Our findings did not indicate a causal relationship between migraine and MS. Several genetic variants, particularly those in the MHC, may account for some of the overlap observed between the two conditions. It seems likely that migraine within the context of MS is a symptom of MS, rather than a distinct disorder. Defining "MS-typical headache" might lead to earlier diagnosis, improved treatment, and improvements in related quality of life.

INTRODUCTION

Multiple sclerosis (MS) is a chronic, inflammatory disease of the central nervous system. One common co-morbidity is migraine, which occurs in approximately 31% of individuals with MS (ranging from 2% to 67%).[1] In contrast, the prevalence of migraine among adults in the U.S. is ~12% (17% among women).[2] Similarities between MS and migraine include symptoms (e.g., visual disruptions and sensory loss) and affected populations (predominantly white, female, and diagnosed in early adulthood).[4–6] Despite this, the cause of the overlap remains unknown. Several possible explanations, which will be tested in this study, are described below (Figure 1).

First, migraine may cause MS (Figure 1A). Potential mechanisms include increased permeability of the blood–brain barrier after a migraine attack and increased neuroinflammation triggering MS.[7,8] To date, studies of migraine and MS have either been cross-sectional (not able to discern temporality) or cohort studies limited by the rarity of MS to detect significant effects. These issues may be resolved by mendelian randomization (MR) analyses, whereby genetic variants serve as proxies for an exposure ("instrumental variable" (IV)) and are used to test the causal effect of the exposure on the outcome. Because alleles are randomly assigned at conception, they occur before the respective outcome which avoids reverse causation.

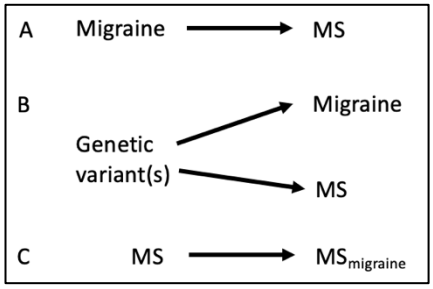

Figure 1. Potential hypotheses for the observed co-occurrence of MS and migraine tested in this study. **A**. Migraine causes MS. **B**. Genetic variants are associated with both MS and migraine (pleiotropy). **C**. Migraine observed in individuals with MS is a symptom of MS, occurring because of MS processes, which could be prodromal or after MS diagnosis.

Second, MS and migraine might share genetic variants (i.e., "pleiotropy") (Figure 1B). Studies have shown strong evidence for genetic factors contributing to MS risk, and genome-wide association studies (GWAS) have identified more than 230 variants that contribute to MS risk.[9] The major histocompatibility complex (MHC) is a region of the genome involved in the adaptive immune response and is widely known to be associated with MS susceptibility, particularly the *HLA-DRB1*15:01* allele.[10,11] GWAS have identified 32 independent loci in the HLA that contribute to MS risk. Over 200 non-MHC variants, predominantly located near genes that regular adaptive and innate immune function, have also been identified in MS GWAS.[12]. The largest migraine GWAS identified 38 independent risk loci, most located near genes involved in neuronal, vascular, and smooth muscle functions.[13] No migraine genome-wide significant variants were identified within the MHC, but results from several non-GWAS suggests immunological dysfunction may be partially responsible for migraine.[14,15] Given the strong role of immune genes in MS and inconclusive role within migraine, it is plausible the two disorders share genetic variants. No studies have explicitly investigated this.

Third, migraine that occurs comorbid with MS might be a symptom of MS rather than a separate disorder with genuine migraine pathology (Figure 1C). This migraine headache symptom could be part of the prodrome for MS or occur after diagnosis. If this is the case, there might be observed differences in clinical features, genetic risk factors, or environmental/behaviors risk factors of MS by migraine status. MS-specific clinical differences by migraine status have been reported in small-to-moderate sized studies but did not account for potential confounders such as interferon

beta use and sex.[16–19] Whether MS-specific genetic or environmental risk factors are more (or less) prevalent among MS cases with migraine has not been assessed.

This study's objective was to test these three hypothesized mechanisms responsible for the comorbidity of migraine and MS using large, publicly available GWAS summary statistics and rich individual-level data from a large MS study from Northern California. Our aims were three-fold: 1) test for evidence of a causal relationship between migraine and MS using MR analysis; 2) test for evidence of pleiotropy between migraine and MS using genetic correlation, globally and locally across the genome; and 3) identify whether individuals with MS and migraine had distinct clinical characteristics or more frequent MS-specific genetic or environmental risk factors compared to MS cases without migraine.

METHODS

Genome-wide association study summary statistics

GWAS summary statistics used for MR and genetic correlation analyses were from multiple publicly available sources (Table 1). For MR analyses, summary statistics for migraine were from the latest migraine GWAS (2016) by the International Headache Genetic Consortium (only summary statistics with $p<1x10^{-5}$ were available) (http://www.headachegenetics.org/content/datasets-and-cohorts).[13], and summary statistics for MS were from the latest 2019 International Multiple Sclerosis Genetic Consortium (IMSGC) GWAS (extracted from the MRC-IEU GWAS database using *TwoSampleMR*).[12,20] For genetic correlation analyses, migraine GWAS summary statistics were from the Neale Lab UKBB pipeline (http://www.nealelab.is/uk-biobank), and MS GWAS summary statistics were from the IMSGC.

Table 1. GWAS summary statistic data sources for statistical analyses

Phenotype	Source	No. cases	No. controls	Analysis	Self-reported (SR) or clinician diagnosed (CD)
Migraine	IHGC	59,674	316,078	MR IV	SR or CD
Migraine	UKBB	10,647	350,494	GC	SR
MS	IMSGC	47,429	68,374	MR outcome, GC	CD

Abbreviations: CD, clinician diagnosed; GC, genetic correlation; GWAS, genome-wide association study; IHGC, International Headache Genetics Consortium; IMSGC, International Multiple Sclerosis Genetics Consortium; IV, instrumental variable; No, number; SR, self-reported; UKBB, United Kingdom Biobank

Kaiser Permanente Northern California MS case subjects

Individual-level data were from the Kaiser Permanente Northern California (KPNC) MS Research Program which recruited two cohorts of individuals: 1) non-Hispanic MS cases (n=1,479) recruited between 2005 and 2012, and 2) MS cases recruited between 2019-2021 (n=515). At enrollment, all were part of the KPNC Health Plan which includes over four million people, representing 25-30% of the 22-county service area population in Northern California. For both cohorts, eligible cases were diagnosed with MS by a neurologist (*International Classification of Diseases, Ninth Revision,* code 340.x) and aged 18-69 years old. Three individuals had missing migraine data, leaving a total of 1,991 MS cases available for analyses.

Standard protocol approvals, registrations, and patient consents

Study protocols were approved by the institutional review boards for human subjects at the University of California Berkeley and KPNC. Informed consent was obtained for all study participants.

Migraine classification

The migraine status of MS cases was identified in two ways: self-report and validated electronic health record (EHR) probability algorithm. Participants were asked during a computer-assisted telephone interview (CATI), "Has a doctor or other health professional ever told you that you have migraine?" or "Have you ever had a migraine?". Additionally, EHR was used to identify individuals who have experienced migraine using a previously validated migraine probability algorithm (MPA).[21] Briefly, codes identified in each participant's EHR were given a point value. For example, one outpatient or emergency room visit with migraine diagnosis equaled 10 points. Participants were considered "migraineurs" if they either self-reported migraine or had an MPA score >10.

Clinical data

For all MS cases, the CATI included MS-specific questions including use of medications, age at first MS symptom and diagnosis, MS subtype, and depression status. Depression was assessed as described previously.[22] Age of onset was confirmed by review of electronic health records. For each MS case, we calculated the Multiple Sclerosis Severity Scale (MSSS), an indicator of disease severity that uses the Expanded Disability Status Scale and disease duration.[23] The CATI included two cognitive assessments: the Modified Telephone Interview for Cognitive Status (TICS-M) and the Perceived Deficits Questionnaire (PDQ). The PDQ was designed for MS to provide a self-report measure of cognitive dysfunction.[24] The total PDQ score was calculated from the abbreviated 5-item version by summing raw scores resulting in a score ranging from 0-20 (higher indicates more perceived impairment). The TICS-M is a widely used 14-item cognitive screening test for mild cognitive impairment and dementia and has been validated for use in MS cases.[22,25] The total TICS-M score was the unweighted sum of correct answers with a maximum of 35 possible points (higher is better).

Environmental/behavioral risk factor data

The CATI asked participants hundreds of questions including behaviors, health conditions, and demographics. Relevant CATI variables for our analyses included: whether or not the participant had ever smoked at least one cigarette per day for one month or more, whether or not a parent or someone living in the participant's home smoked inside the house before the participants was 19 years old, body mass index (BMI) at time of interview, lowest and highest BMI during ages 20-29 years, weight when 10 years old (underweight, about right, little overweight, very overweight), had ever had infectious mononucleosis, had ever been pregnant, age at first pregnancy, whether or not they were breastfed for ≥4 months as a child (no, yes, unknown), and age at menarche.

Genetic data

Single nucleotide polymorphism (SNP) genotyping was performed on samples provided from non-Hispanic white MS cases recruited from 2006 and 2014 using the HumanOmniExpress BeadChip array. Samples with >10% failed genotypes were removed. Related individuals were identified and removed if *pihat* >0.2. SNPs were imputed against haplotypes from Phase I of the 1000 Genomes Project using SHAPEIT and IMPUTE2. Variants were excluding if they had a genotyping success rate <90%, minor allele frequency <1%, Hardy-Weinberg equilibrium $p<0.001$, or info score <0.8. Genetic ancestry proportions were estimated using *snpweights*, and the dataset was restricted to individuals with >80% CEU ancestry to limit the possibility of confounding by genetic ancestry. Principal components analysis (PCA) was conducted to generate

principal components (PCs) to use as covariates in genetic statistical analyses. There were 1,103 MS cases with high quality genotypes available for statistical analyses.

Statistical analyses

Mendelian randomization

To identify whether migraine had a causal effect on MS, we performed two-sample MR using publicly available MS and migraine GWAS summary statistics from European ancestry men and women. Using the *TwoSampleMR* package in R, we created a migraine IV using summary statistics from the 2016 IHGC GWAS. SNPs were included in the IV if genome-wide significant at $p<5x10^{-8}$ in the GWAS and uncorrelated (linkage disequilibrium (LD)-based clumping threshold $r^2<0.001$ within a 10,000kb window). Migraine IV SNPs were then extracted from MS IMSGC GWAS summary statistics. If IV variants were not available in the MS GWAS, proxies (variant with $r^2>0.8$ in European 1000 Genomes population) were selected, if possible. Data were harmonized to ensure estimation of the effect of the migraine IV on MS corresponded to the same alleles, and palindromic and strand ambiguous variants were removed. This resulted in 23 SNPs in the migraine IV.

For the main analysis, we applied inverse variance weighted two-sample MR to obtain effect estimates of the migraine genetic IV on MS. For MR studies, the relevance assumption (all IV SNPs are associated with risk of the exposure), independence assumption (no unmeasured confounding between IVs and outcome), and exclusion restriction (IVs cannot affect the outcome through pathways other than the exposure or "horizontal pleiotropy") are essential for the method to avoid bias and be considered causal. To address these assumptions, several measures were taken. First, we included estimation methods (weighted median, weighted mode, and simple mode) that assume only a portion of the variants in an migraine IV were associated with migraine. We also used MR-Egger regression which provides a causal estimate largely robust to horizontal pleiotropy. Additionally, we measured the degree of heterogeneity across the individual migraine IV SNP effect estimates using the Cochran Q test. A Q statistic much larger than n_{SNP}-1 would suggest violation of either the independence assumption or exclusion restriction. We also conducted a leave-one-out analyses of individual SNPs from the migraine IV to determine whether the estimate was driven by a single SNP.

Genome-wide genetic correlation

Linkage disequilibrium score regression (LDSC) was used to estimate genetic correlation across the genome (excluding the MHC region) between MS and migraine using the *LDSC* tool in Python2.[26,27] We utilized non-MHC summary statistics from the UKBB (migraine) and IMSGC (MS) and a pre-computed LD matrix of European ancestry individuals from the 1000 Genomes Project. GWAS summary statistics were excluded if MAF ≤0.01, on sex chromosomes, not SNPs (e.g., indels), strand ambiguous, or duplicated. Variants were also filtered to HapMap3 SNPs which are well-imputed in most studies. This resulted in a total of 1,171,211 non-MHC SNPs for LDSC analyses. Genetic correlation but not heritability estimates were presented because both source GWAS corrected for genomic control/ancestry which can result in an underestimation of SNP-heritability but not genetic correlation.

Local genetic correlation

We used LOcal Genetic cOrrelation Detector (LOGODetect) to identify non-MHC regions of the genome associated with both MS and migraine, using summary statistics from GWAS (IMSGC for MS and UKBB for migraine).[27–29] LOGODetect achieves this by utilizing partitioned LDSC and a scan statistic approach, as opposed to pre-specifying candidate regions of interest. The scan statistic (Q) was defined as the LD-weighted inner product of two z-score vectors in a respective region. Larger absolute values represent larger genetic correlation in the respective region. Similar to our LDSC analysis, we utilized a pre-computed LD matrix of European ancestry individuals from the 1000 Genomes Project. For each GWAS summary dataset, we removed variants that were indels, strand-ambiguous, or not present (or if MAF<0.01) in the pre-computed LD matrix. We also filtered to variants in HapMap3. We then took the overlapping SNPs from each GWAS and confirmed allele coding schemes were consistent. This resulted in 1,049,383 SNPs for LOGODetect analyses. Within significant regions, we identified independent SNPs were using LD clumping procedures (r^2<0.01 and window size=250kb), used the UCSC Genome Browser to annotate SNPs, used PANTHER to classify gene pathways, and used GTEx to identify whether genes were expressed in specific tissues.

Shared variants in the MHC

The MHC contains the largest genetic risk factors for MS, but its complex LD patterns make it difficult to include in LDSC-based analyses. To test whether variants within the MHC might be associated with both MS and migraine, we first identified all variants in the MHC from the largest MS GWAS (IMSGC) that reached genome-wide statistical significance ($p<5x10^{-8}$). We subset these variants to those in the migraine GWAS summary statistics dataset (UKBB) that had MAF>0.01 and tested whether they were significant in migraine GWAS. To determine the significance threshold for migraine, we used the clumping procedure in Plink (r^2>0.01 and kb=250) to determine the number of independent SNPs in the significant MHC MS SNP set.[30] We used a significance threshold $p < 0.05$/number of independent significant MS MHC SNPs available in migraine GWAS dataset. MS and migraine significant SNPs were input into LocusZoom (http://locuszoom.org/) to identify patterns of LD using a European reference population. LD-based clumping was performed in Plink to identify an independent SNP in each region.

Analyses of individual risk factors and clinical features among MS cases

To determine whether individuals with MS and migraine had distinct clinical characteristics or more frequent MS-specific genetic or environmental risk factors compared to MS cases without migraine, we estimated odds ratios (ORs) and 95% confidence intervals (CIs) using logistic regression (migraine as the outcome) for each risk factor/clinical characteristic among MS cases in the KPNC MS Research Program. Clinical features included MS subtype, age at MS onset, MSSS score, PDQ score, TICS-M score, depression (ever), and ever used interferon beta. For each clinical analysis, we adjusted for interferon beta use (ever), disease duration, sex, and race (white or other). The model for age of MS onset and MSSS did not adjust for disease duration to avoid collinearity. Cognitive outcomes (PDQ and TICS-M) also adjusted for education level (college or not). All environmental/behaviors risk factor logistic regression models, except those pertaining to menarche and pregnancy, adjusted for race, age, and sex. The model for age at menarche adjusted for race only. The model for "ever pregnant" adjusted for race and age. The age at first pregnancy model only adjusted for race. Genetic risk variants included the largest known genetic contributors to MS, *HLA-DRB1*1501* and *HLA-A*02*, and non-MHC and MHC alleles found to

be associated with both MS and migraine in analyses above. For all variants, minor alleles were coded additively, and all logistic regression models adjusted for the first three genetic PCs. In addition to estimating ORs for individual genetic variants, we created polygenic risk scores (PRSs) for MS and migraine and estimated their association with migraine among MS cases, adjusting for the first three genetic PCs. Included in each PRS were genome-wide significant non-MHC variants from the IMSGC GWAS or IHCG GWAS, respectively, that were available in our data and met quality control thresholds. This resulted in 39 migraine SNPs and 158 MS SNPs. GWAS beta coefficients from these variants were used as weights, and PRSs were calculated using the *--score* command in Plink.

RESULTS

No evidence of migraine causing MS

Inverse-variance weighted MR analysis did not reveal a causal association between the migraine IV and MS (beta=0.09, se=0.08, p=0.29) (Table 2). Effect estimates from weighted median, weighted mode, and mode estimation methods did not differ considerably from the inverse-variance weighted method. The MR-Egger regression slope was consistent with the absence of an effect of migraine on MS risk. The Cochran Q test statistic did not reveal large heterogeneity among the individual migraine IV SNP effect estimates in the inverse-variance weighted (Q=38.81) and MR-Egger analyses (Q=37.30). Leave-one-out analyses for migraine IV SNPs indicated consistent effect estimates when one SNP was iteratively removed (Supplementary Figure 1). Together, these results suggest the assumptions of relevance and no horizontal pleiotropy were met and there is no evidence for migraine causing MS.

Table 2. Results of two-sample MR analyses of a migraine genetic IV on MS

Method	beta (se)	p-value
Inverse variance weighted	0.09 (0.08)	0.29
MR Egger	-0.11 (0.23)	0.64
Weighted median	0.13 (0.10)	0.17
Simple mode	0.10 (0.18)	0.60
Weighted mode	0.17 (0.16)	0.29

Abbreviations: IV, instrumental variable; MR, Mendelian randomization; MS, multiple sclerosis; se, standard error

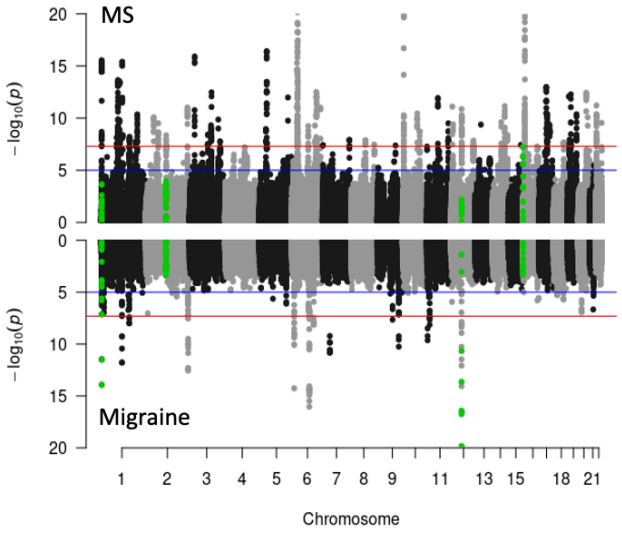

Figure 2. LOGODetect identified four regions across the genome associated with both MS and migraine. Significant regions were highlighted in green. Each point represented a variant from source GWAS (filtered to HapMap3 SNPs) that were input into the LOGODetect program. P-values represent those from source GWAS, not regional p-values identified from LOGODetect.

Four non-HLA genomic regions were associated with both MS and migraine

Using publicly available GWAS summary statistics from the UKBB and IMSGC, we did not find evidence of global genetic correlation between MS and migraine (r_g=0.01, se=0.05, p=0.88). Because estimating global genetic correlation can oversimplify the shared genetic architecture between phenotypes, we sought to identify if local regions of the genome contributed to genetic correlation between MS and migraine using LOGODetect. In total, we identified four genomic regions showing associations with MS and migraine (FDR q< 0.05; Figure 2 and Table 3). The size of the identified genome segments

ranged from 26kb to 94kb. One of the regions (chr12: 57,508,649 – 57,534,64) had a positive test statistic, while the other three regions had negative statistics (indicating negative genetic correlation). Genes identified in or near these regions were involved in RNA polymerase II binding and transcription (GO:0006366, GO:0006357), lipid homeostasis (GO:0055088), fatty acid beta-oxidation (GO:0006635), meiotic cell cycle (GO:0007127 and GO:0007134), and cytokine-mediated signaling pathway (GO:0019221). These genes were not significantly enriched in specific tissue types (Supplementary Figure 2). Comparatively, lung, thyroid, and stomach tissues and EBV-transformed cell lines had upregulated expression of genes in these regions while testis, prostate, hippocampus, and spinal cord tissues had down-regulated expression.

Table 3. Characteristics of four regions of the genome where genetic correlation between MS and migraine was detected (FDR q<0.05)

chr	position	stat	p-value	q-value	Nearby genes[a]	GO biological process
1	3,070,829 – 3,116,620	-4.80	2.0e-4	0.02	*PRDM16*	0006366, 0006357
2	111,854,342 – 111,948,541	-2.95	4.0e-4	0.02	*ACOXL*, *BCL2L11*	0005088, 0006635, 0007127
12	57,508,649 – 57,534,641	4.30	2.0e-4	0.04	*LRP1, STAT6*	0019221, 0042127, 0006366
16	1,048,390 – 1,079,298	-5.66	2.0e-4	0.01	-	-

[a]All genes were within 5kb of the specified region
Abbreviations: chr, chromosome; FDR, false discover rate; GO, gene ontology; stat, statistic; MS, multiple sclerosis

Four HLA regions were associated with both MS and migraine

When assessing whether there were genetic variants in the MHC region shared between MS and migraine, we identified 18,803 MHC variants significantly (p<5x10^{-8}) associated with MS. Of these, 10,896 (40 independent loci) had available summary statistics in migraine GWAS. Ninety-eight variants were significantly associated with migraine (p<1.25x10^{-3}) and were located in four high-LD regions of the MHC (Supplementary Figure 3). These variants clumped into four independent loci (Table 4) in the following genes: *HCG20, HLA-B, MSH5, TNXA,* and *TNXB*. For all four independent loci, the major allele, not minor allele, conferred risk of MS and migraine.

Table 4. Characteristics of four independent MHC variants associated with both MS and migraine

				MS GWAS		Migraine GWAS		
position	rsid	Allele comparison	Minor allele	beta	p-value	beta	p-value	Gene
6:30758126	rs13210146	T:A	T	-1.50x10^{-3}	4.70x10^{-5}	-0.19	1.58x10^{-10}	*HCG20*
6:31249398	rs9264764	C:T	C	-1.26x10^{-3}	4.10x10^{-4}	-0.22	1.14x10^{-37}	*HLA-B*
6:31724219	rs805826	C:G	C	-1.84x10^{-3}	1.20x10^{-5}	-0.15	8.49x10^{-15}	*MSH5*
6:32018573	rs35214850	C:A	C	-2.18x10^{-3}	4.20x10^{-4}	-0.26	2.54x10^{-13}	*TNXA, TNXB*

Abbreviations: GWAS, genome-wide association study; MHC, major histocompatibility complex; MS, multiple sclerosis

*Clinical features and risk factors among MS cases with migrai*ne

To determine whether individuals with MS and migraine had distinct clinical characteristics or more frequent MS-specific genetic or environmental risk factors compared to MS cases without migraine, we utilized a large study of MS cases (n=1,991). Demographic characteristics of MS cases were shown in Table 5. The prevalence of migraine among MS cases was 38.9%. Those who experienced migraine were more likely to be female (88.6%) compared to non-migraine MS cases (70.3%) and had a lower educational attainment (40.4% had a bachelor's degree or higher compared to 48.3% among non-migraine MS cases). Compared to non-migraine MS cases, those with migraine had more frequent relapsing remitting or relapsing progressive disease course (69.4% vs. 74.3%), earlier age at MS onset (32.8 (sd=9.6) vs. 31.5 (sd=10.2)), more frequent experience of depression (35.0% vs. 45.0%), and more frequently had ever used interferon beta treatment (50.7% vs. 54.1%). After adjusting for potential confounders such as age, sex, and interferon beta use, MS cases had a higher odds of migraine if they had a relapsing progressive disease course (compared to primary progressive) (OR=2.37, 95% CI: 1.26, 4.52) or depression (OR=1.48, 95% CI: 1.22, 1.80) (Table 6). When compared to individuals with primary progressive MS, there was not a significant difference in odds of migraine among those with relapsing-remitting (OR=1.28, 95% CI: 0.85, 1.95) or secondary progressive (OR=1.11, 95% CI: 0.70, 1.79) disease courses. No other clinical symptoms, including age at onset, MSSS, and cognitive assessments, were significantly associated with migraine status. The only environmental or behavioral risk factor for MS that was associated with migraine status was smoking, whereby MS cases who had ever smoked had 1.30 times the odds of migraine compared to non-smokers (95% CI: 1.08, 1.57). MHC variants, including *HLA-DRB1*1501*, *HLA-A*02*, and four leading MHC variants identified above as associated with both MS and migraine, were not associated with migraine status among individuals with MS (Table 6). One leading SNP (rs108990) on chromosome 16 identified from significant LOGODetect regions was available in the MS case data; it was not associated with migraine status (OR=1.13, 95% CI: 0.91, 1.41). Neither MS nor migraine PRSs were significantly associated with migraine status among MS cases (OR=0.87, 95% CI: 0.74, 1.04 and OR=1.13, 95% CI: 0.965, 1.96, respectively).

Table 5. Demographic and clinical characteristics of 1,991 MS cases in the Kaiser Permanente Northern California MS Research Program stratified by migraine status

Characteristic	Migraine	No migraine
N	774	1217
Age (mean, sd)	47.4 (9.9)	47.5 (9.9)
Sex, female (n, %)	686 (88.6)	856 (70.3)
Hispanic (n, %)	44 (5.7)	72 (5.9)
Race (n, %)		
White	683 (88.2)	1010 (83.0)
Black	69 (9.0)	126 (10.4)
Other	22 (2.8)	80 (6.6)
Bachelor's degree or higher (n, %)	313 (40.4)	588 (48.3)
MS subtype (n, %)		
Relapsing remitting	539 (69.6)	808 (66.4)
Secondary progressive	99 (12.8)	177 (14.5)
Primary progressive	47 (6.1)	97 (8.0)
Relapsing progressive	36 (4.7)	36 (3.0)
Unknown	53 (6.9)	99 (8.1)
Age at MS onset (years) (mean, sd)	31.5 (10.2)	32.8 (9.6)
MS Severity Score (mean, sd)	3.8 (2.4)	3.9 (2.5)
PDQ score (mean, sd)	6.8 (5.7)	5.5 (5.2)
TICS-M score (mean, sd)	21.5 (3.9)	21.2 (3.8)
Ever depressed (n, %)	348 (45.0)	426 (35.0)
Ever used interferon beta (n, %)	419 (54.1)	617 (50.7)

MS, multiple sclerosis; MSSS, multiple sclerosis severity score; PDQ,

DISCUSSION

Using large, publicly available GWAS summary statistics and rich individual-level data from a large MS study from Northern California, this study tested three mechanistic hypotheses for the co-occurrence of MS and migraine: migraine causes MS, genetic variants were shared between the two disorders, or migraine within the context of MS was a distinct phenotype/symptom of MS. Using two-sample MR, we did not find evidence for a causal relationship between migraine and

MS. We did identify regions, particularly within the MHC, that were shared between MS and migraine. We also identified that MS cases who experienced migraine had a higher odds of ever being depressed and of having a relapsing-progressive disease course. Collectively, this suggests that MS and migraine may co-occur because they share several genetic variants, rather than migraine causing MS. It also highlights that if migraine is a symptom of MS, it does not also occur more frequently with cognitive or physical deficits or result from a higher burden of MS-specific genetic or environmental/behavioral risk factors. This has important implications for treatment of migraine among individuals with MS and even the diagnosis of MS if migraine is indeed a symptom.

Table 6. Results from logistic regression estimating the association between clinical factors and MS risk factors and migraine among individuals with MS

Clinical feature[a]	OR	95% CI
MS subtype (ref=primary progressive)		
Relapsing remitting	1.28	0.85, 1.95
Secondary progressive	1.11	0.70, 1.79
Relapsing progressive	2.37	1.26, 4.52
Age at MS onset (years)	0.99	0.98, 1.00
MSSS	1.01	0.97, 1.05
PDQ score	1.04	1.02, 1.06
TICS-M score	1.02	0.99, 1.05
Ever depressed	1.48	1.22, 1.80
Ever used beta interferon beta	1.08	0.89, 1.32
Environmental risk factor[b]	OR	95% CI
Current BMI	1.02	1.00, 1.03
Highest BMI in 20s	1.00	0.99, 1.02
Weight at 10 years (ref= "about right")		
Underweight	1.25	0.96, 1.76
A little overweight	0.91	0.71, 1.62
Very overweight	1.48	0.91, 1.15
Ever smoker	1.30	1.08, 1.57
Lived with indoor smoker as child	0.95	0.76, 1.18
Ever had infectious mononucleosis	0.85	0.66, 1.10
Breastfed as a child (ref= <4 months)		
>=4 months	0.89	0.67, 1.18
Unknown	0.91	0.74, 1.13
Age at menarche, year	0.98	0.91, 1.04
Ever pregnant	1.12	0.87, 1.45
Age at first pregnancy	0.97	0.95, 0.99
Genetic risk factor[c]	OR	95% CI
*HLA-DRB1*1501*	0.98	0.80, 1.19
*HLA-A*02*	1.14	0.93, 1.39
rs13210146	1.07	0.89, 1.29
rs9264764	0.91	0.77, 1.09
rs805826	0.88	0.72, 1.08
rs35214850	0.91	0.66, 1.24
rs108990	1.13	0.91, 1.41
MS PRS	0.87	0.74, 1.04
Migraine PRS	1.13	0.65, 1.96

[a]For each clinical analysis, we adjusted for beta interferon beta use (ever), disease duration, sex, and race (white or other) unless. The model for age of MS onset and MSSS did not adjust for disease duration to avoid collinearity. Cognitive outcomes (PDQ and TICS-M) also adjusted for education level (college or not).
[b]All environmental/behavioral models except those pertaining to menarche and pregnancy adjusted for race, age, and sex. The model for age at menarche only adjusted for race. The "ever pregnant" model adjusted for race and age, and the age at first pregnancy model only adjusted for race.
Abbreviations: BMI, body mass index; CI, confidence interval; MS, multiple sclerosis; MSSS, multiple sclerosis severity score; OR, odds ratio; PDCS, Perceived Deficits Questionnaire; PRS, polygenic risk score; ref=reference category; TICS-M, Modified Telephone Interview for Cognitive Status.

This study's finding that a causal relationship between migraine and MS was not identified addressed a major, unresolved question regarding MS and migraine. For decades, there have been conflicting observations that migraine often preceded MS by several years and that migraine often occurred after MS relapses.[31] To date, the largest prospective study to investigate this was the Nurses' Health study which separately examined the risk of developing MS among migraineurs and the risk of developing migraine among individuals with MS.[32] They found a higher risk of MS among migraineurs and a higher risk of migraine among participants with MS. Despite having over 116,000 participants, this study was still underpowered due to the rarity of MS.[33] Our use of genetics and MR helped to elucidate this (lack of) cause and effect because inherited genetic variants are not subject to reverse causation. The clear comorbid association of MS and migraine in our study and others but the lack of evidence proving it is a causal relationship suggests that MS and migraine may either share risk factors or migraine in MS may be a separate phenotype.

Our study identified four regions of the MHC significantly associated with both MS and migraine. Two were within the MHC class I region and two were inside the class III region. Within these

significant regions, we identified four independent leading SNPs: rs13210146, rs9264764, rs805826, and rs35214850. For each, the major allele conferred increased risk of both MS and migraine. These MHC variants have not previously been implicated in MS or migraine pathology and might serve as new targets and hypotheses for their shared etiology.

We also identified four regions on chromosomes 1, 2, 12, and 16 exhibiting local genetic correlation between MS and migraine. The region on chromosome 12 was near *LRP1,* which encodes a protein necessary for clearing amyloid precursor protein and beta-amyloid, the main components of amyloid plaques found in Alzheimer patients. This region was also near *STAT6*, a gene involved in IL-4 mediated biological responses (a cytokine with many immune regulatory functions). Interestingly, the family of transcription factors encoded by the STAT family target *BCL2L11* and *ACOXL*, genes near our significant regions on chromosome 2. This pathway might be a particularly interesting target for future research and therapies. Apart from the significant chromosome 12 region, the remaining significant regions all had negative test statistics. This means the loci within those regions were associated with increased prevalence of MS or migraine but reduced prevalence of the other. This is called "antagonistic pleiotropy". For these alleles to confer risk of both phenotypes, there would need to be a mechanism that alters expression of associated genes at different timepoints or scenarios. For example, it may be possible that a variant increases risk of MS while decreasing risk of migraine; however, after MS treatment, the relevant biological process that prevented migraine was affected by the drug and is no longer conferring protection. This antagonistic pleiotropy finding is an important reminder that shared genetic variants can increase the risk of one disorder while reducing the risk of another which has important implications for therapeutic targets of either condition.

Finally, we did not observe that MS cases with migraine had many distinct clinical features apart from a higher prevalence of ever being depressed and a relapsing-progressive disease course. Other studies have also observed that age of MS onset, disease duration, and disability were similar in MS patients with and without migraine.[16–19] Our sample is one of the largest to confirm this and adjusted for sex and interferon beta use, the latter of which has frequently been reported as a trigger for migraine in patients with MS.[34,35] MS cases with migraine also did not have more (or less) frequent environmental risk factors apart from being more likely to have ever smoked. Previous studies have shown that smoking is a risk factor for MS, that smokers experienced a greater severity of headache, and that individuals with migraines tended to smoke more heavily than individuals without migraines.[36,37] Whether migraines are exacerbated by smoking is an important area of future work and potential intervention. MS cases with migraine also did not have more (or less) frequent MS-specific genetic risk factors. This included the strongest genetic risk factors for MS, *HLA-DRB1*1501* and *HLA-A*02*. This suggested that MS risk variants were not driving the occurrence of migraine. It is possible that genetic variants associated with MS progression, rather than risk, play a more active role in migraine symptoms and should be investigated. We also did not observe that four leading MHC variants associated with both MS and migraine were more (or less) frequent among MS cases with migraine. This further indicated that migraine within the context of MS might be a different type of headache without traditional migraine etiology or genetic risk factors.

This study has several notable strengths. First, use of MR allowed for testing hypotheses regarding the temporal relationship of migraine and MS and avoided sources of confounding that might be

present in traditional observational studies. Through use of large GWAS summary statistics, it also allowed for increasing power to detect associations among MS cases. Also, the availability of a large MS cohort with deep phenotyping and genotyping allowed for a unique assessment of characteristics of MS cases with migraine. This study had several limitations. The causal MR method is unbiased if all three causal assumptions are met. While we did use multiple estimation and statistical methods that loosen these assumptions, it was possible that horizontal pleiotropy or confounding existed, or the strength of the genetic instruments were weak. Another limitation, implicated in most migraine-related research, is that migraine may be misclassified. In responding to a self-report, the difference in migraine versus headache might seem ambiguous whereas they are distinct in clinical practice. In this way, migraine prevalence might be over-estimated. Yet, migraine is generally under-reported in the population; an estimated 48% of adult migraineurs in the U.S. have ever received a physician diagnosis of migraine.[38] This makes is more likely that studies of migraine are under-representations of the true distribution of migraine. Therefore, individuals included in GWAS who were classified as not having migraine, might be misclassified. This could lead to null GWAS findings or underestimation of the genetic contribution of migraine. Within the context of MS, it is possible that patients had a higher diagnosis of migraine because they saw a neurologist more frequently than healthy individuals. Given then rarity of MS, this would likely not significantly influence our observed results from GWAS. Finally, there may be additional mechanisms for the comorbidity of MS and migraine not tested in this study. For example, we did not assess shared environmental risk factors between MS and migraine. This should be investigated in the future.

In conclusion, our results did not show a causal relationship between migraine and MS. Several genetic variants, particularly those in the MHC, may account for some of the overlap observed between the two conditions. Given the well-documented strong association between MS and migraine, it seems likely that migraine within the context of MS is a symptom of MS, rather than a distinct disorder. If it were possible to define a MS-typical headache, patients with these headaches might be able to be treated earlier, reducing their risk of MS relapse/progression and related reductions in quality of life.

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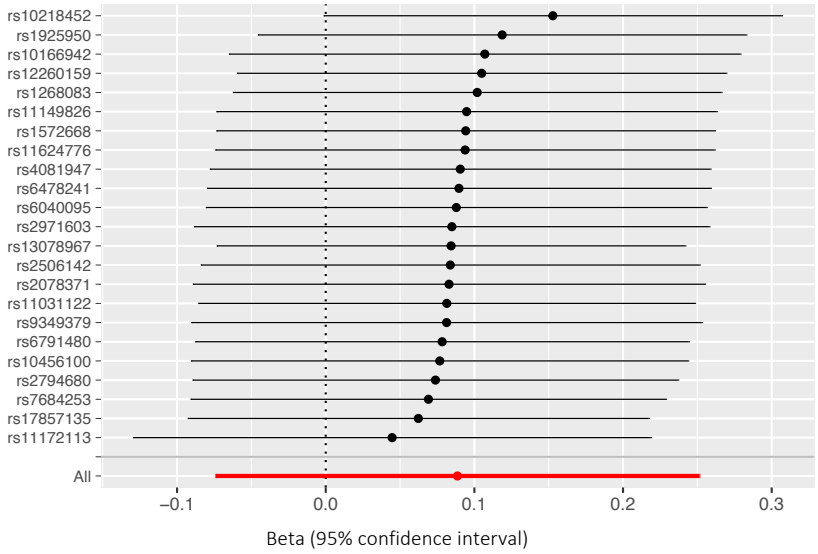
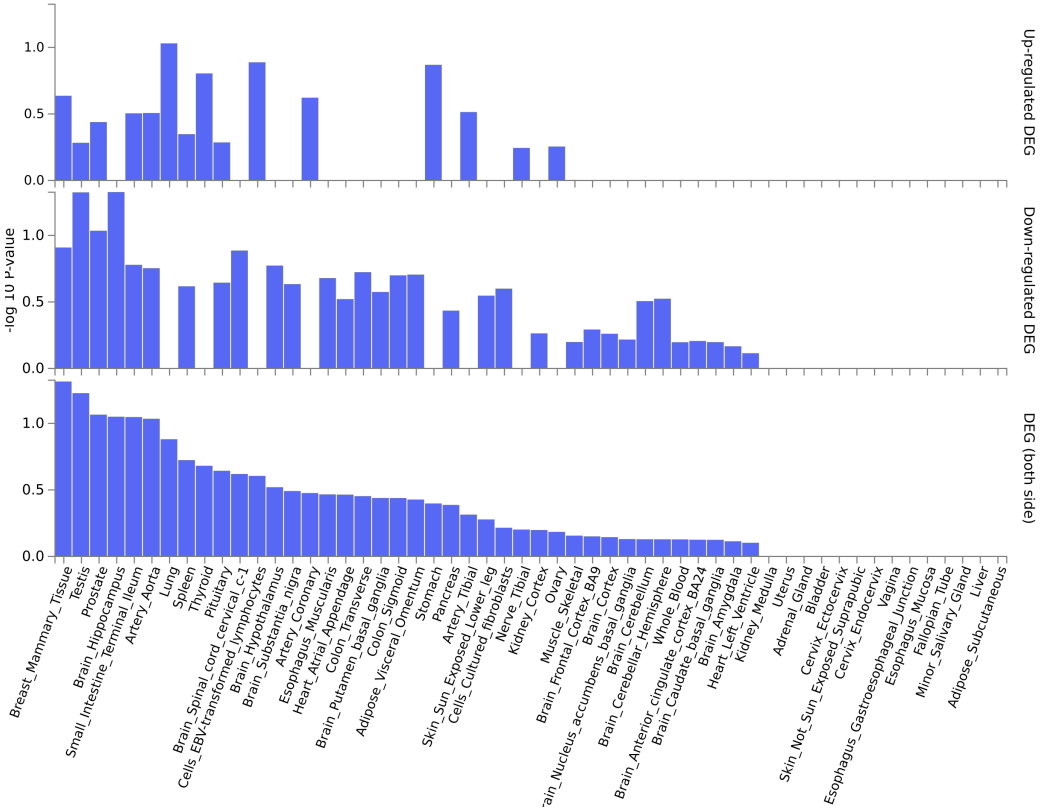
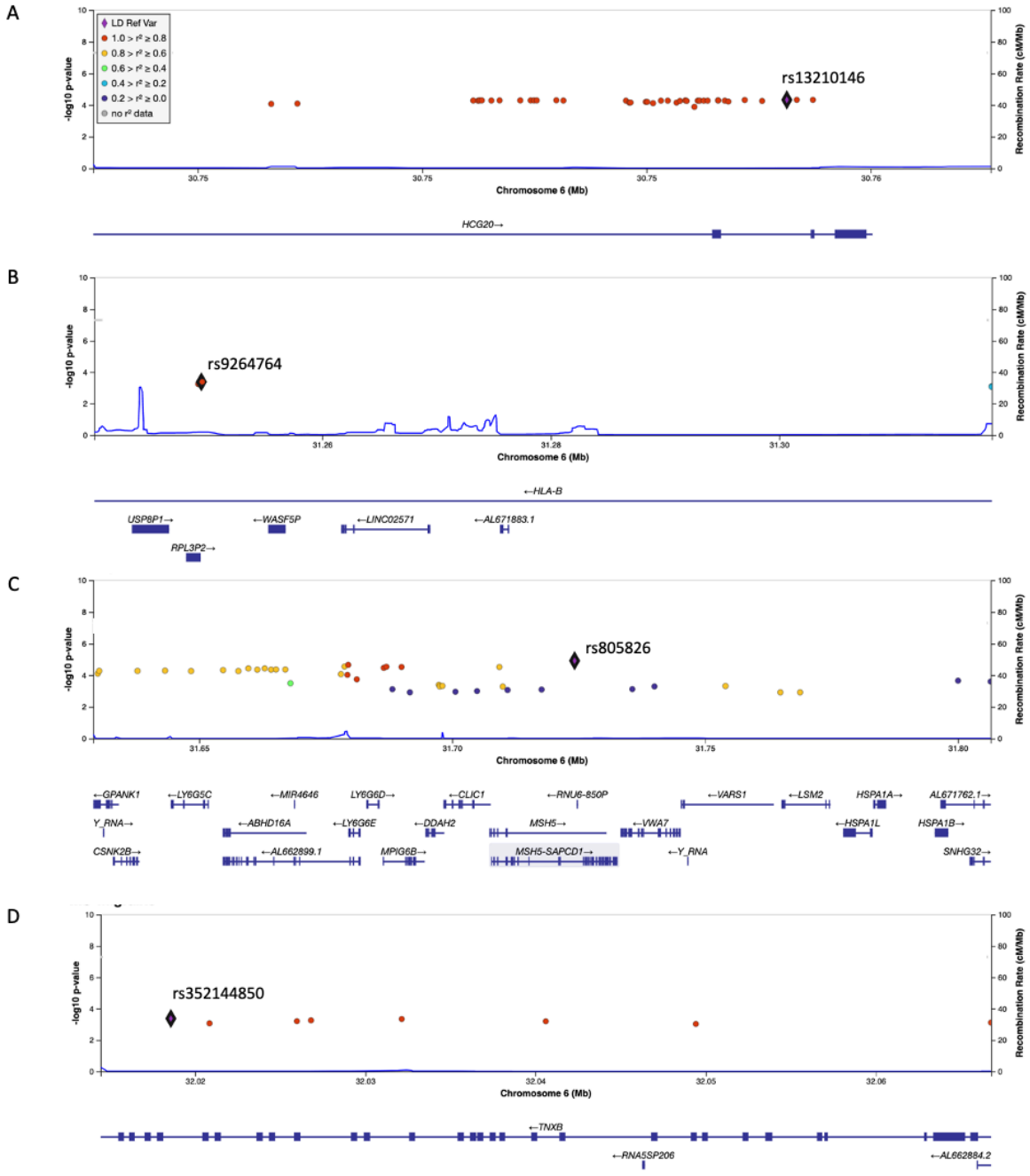
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Leave-one-out analysis testing the effect of migraine genetic instrumental variables on multiple sclerosis with one of 23 migraine variants iteratively excluded (y-axis) from the Mendelian randomization analysis.



Supplementary Figure 2. GTEx tissue expression of genes located within four significant LOGODetect regions.



Supplementary Figure 3. Location and LD patterns of 98 MHC variants associated with both MS and migraine. Significant variants were in four regions of the MHC: a) pos:30,746,633-30,758 ,707; b) 31,240,041-31,318,630; c) 31,629,096-31,806,479; and d) 32,014,456-32,066,765. The independent variant identified from LD-based pruning was considered the LD reference variant (triangle) and the amount of LD with surrounding significant variants was indicated (color). Genes within the selected regions were shown below each plot.