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## RESEARCH ARTICLE

# Heterogeneity in association of remote herpesvirus infections and pediatric MS

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## Abstract

**Objective:** While prior Epstein–Barr virus (EBV) infection has been consistently associated with subsequent risk of developing multiple sclerosis (MS), the association with other common herpesviruses has been more controversial. Our objectives were to determine whether remote infection with EBV and other common herpesviruses affect the susceptibility to pediatric MS and if there are interactions between genetic and demographic factors and viral infections. **Methods:** Cases with pediatric-onset MS or clinically isolated syndrome within 4 years of disease onset, and controls were recruited from 16 American pediatric MS centers. Logistic regression models adjusted for potential confounders assessed the association between case status and serological evidence for past infection with EBV, cytomegalovirus (CMV), Herpes Simplex viruses-1 (HSV-1) and -2. We determined the heterogeneity of the effect of viral infection on the risk of having MS according to race, ethnicity and *HLA-DRB1:1501* status. **Results:** A total of 356 pediatric cases and 493 controls were recruited. In multivariable models, EBV-viral capsid antigen (VCA) seropositivity was associated

Michael Waltz and T. Charles Casper conducted the statistical analysis.

with increased odds of having MS by 7.4 times (95% CI: 4.5–12.0,  $P < 0.001$ ). Seropositivity for HSV-1 was also associated with increased odds of having MS (OR 1.54, 95% CI: 1.06–2.25,  $P = 0.025$ ) but this increase was seen only in Whites (OR = 2.18, 95% CI 1.35–3.52,  $P < 0.001$ ) and those negative for *HLA-DRB1\*1501* (OR = 1.89, 95% CI 1.17–3.03,  $P = 0.009$ ). The effect of remote EBV infection on the risk of pediatric MS depended on race and *HLA-DRB1\*15:01* status. **Interpretation:** EBV seropositivity is strongly associated with pediatric MS, as is HSV-1 seropositivity in subjects negative for *HLA-DRB1\*15:01*. Our report of interactions between select viral exposures, and age, race and *DRB1* status suggests a complex effect of environmental and genetic risk factors on MS development.

## Introduction

Genetic and environmental risk factors and their interactions determine an individual's risk of developing multiple sclerosis (MS).<sup>1</sup> Approximately 200 common genetic variants that alter the risk of MS onset have been described in white adults<sup>2</sup> and *HLA-DRB1\*15:01* has the largest effect. Environmental risk factors consistently associated with MS susceptibility include prior infection with EBV, exposure to cigarette smoking, vitamin D deficiency and obesity.<sup>1</sup> Exposures to commonly encountered viruses have also been linked to MS susceptibility. The most well-studied ones include Epstein–Barr virus (EBV), cytomegalovirus (CMV) and Herpes Simplex virus (HSV). While prior infection with EBV is reported in most patients with adult or pediatric-onset MS,<sup>1</sup> a few studies have suggested that remote CMV exposure might decrease the risk of having MS.<sup>3,4</sup>

Although interactions between genetic and environmental factors have been suspected to modulate MS risk, only a few have been reported<sup>5–8</sup> in part due to limited study sample sizes and extent of available data. Patients with pediatric-onset MS may have a higher burden of genetic susceptibility variants and/or a greater dose of environmental exposures, resulting in much earlier disease onset and as such, finding gene-environment interactions may be easier in this age group.

We have reported the association between prior exposure to several herpesviruses and MS susceptibility in pediatric MS<sup>4</sup> and suggested evidence for an interaction between *HLA-DRB1\*15:01* status and HSV-1. Although several other studies investigated the association of prior viral infections and pediatric MS risk,<sup>9–13</sup> very few studies examining the risk of MS in relation to previous viral exposures and the possible interactions with age, race and ethnicity have been conducted.<sup>14</sup> Using a large case-control study, we aimed to assess the heterogeneity of association between remote infections with common herpesviruses and the risk of pediatric MS in children with a diverse racial and ethnic background.

## Methods

### Participating sites

This is a case-control study of risk factors in pediatric MS (R01NS071463, PI Waubant). The participating centers include University of California San Francisco, State University of New York at Buffalo, Massachusetts General Hospital for Children, Mayo Clinic Rochester, Stony Brook University Medical Center, Texas Children's Hospital Baylor, Loma Linda University, Children's Hospital of Philadelphia, Ann & Robert H. Lurie Children's Hospital of Chicago, Children's National Medical Center, Children's Hospital of Colorado, University of Texas Southwestern/Children's Medical Center Dallas, Boston Children's Hospital, University of Alabama, Cleveland Clinic and Washington University School of Medicine in St. Louis. Parents completed a comprehensive environmental questionnaire and data including medical history, demographics and environmental exposures (such as parental smoking) were entered into a central database. Institutional review boards at all participating centers reviewed and approved the study. Assent and consent forms were signed by the participants and one of the parents/guardians before enrollment in the study. Participants were recruited between November 2011 and September 2015.

### Study participants

Patients with relapsing MS or clinically isolated syndrome (CIS) with high risk for MS whose initial attack occurred before 18 years of age, whose disease duration was less than 4 years and who had at least two silent MRI lesions were recruited to the study. Case status was confirmed by a review panel of at least two pediatric MS experts. Control subjects included pediatric patients seen at general and specialty pediatric clinics at the same institutions during the same period. They were less than 22 years of age, did not have autoimmune disorders except asthma or eczema or severe health conditions and were never

treated with immunosuppressive medications. Their parents did not have MS. Of note, none of the cases or controls in this study had participated in the research previously published by this group.<sup>4</sup>

Race and ethnicity were self-reported, based on NIH categories. We categorized the racial subgroups as whites, African-Americans and others. Ethnicity was characterized as Hispanic or non-Hispanic. Socio-economic status (SES) was defined as the highest level of education attained by the participant's mother. Participants answered an environmental questionnaire and provided blood for serum and DNA.

### Viral studies

Batched EBV-viral capsid antigen (VCA), CMV, HSV-1, and HSV-2 assays (serum IgG) were tested at the Oklahoma Medical Research Foundation by commercially available, standardized enzyme-linked immunosorbent assay (ELISA) (Wampole Laboratories, Princeton, NJ) as described previously.<sup>15,16</sup> Quality control requirements included calibration and having positive and negative controls that met predefined measures. We did not analyze Epstein-Barr nuclear antigen 1 (EBNA-1) seropositivity as the manufacturing source of EBNA-1 antigen changed during the study resulting in inconsistent results.

### HLA-DRB1\*15:01 and 15:03 genotyping

DNA samples of cases and control subjects were tested by single-nucleotide polymorphisms (SNPs) for the presence of *HLA-DRB1\*15:01/15:03* and copy number. We used a validated TaqMan polymerase chain reaction (PCR), as previously described.<sup>4</sup>

### Genetic ancestry inference

In a subset of cases and controls, genetic ancestry was determined. Whole-genome single-nucleotide polymorphism (SNP) analysis was performed on Illumina OmniExpress BeadChip or Illumina Infinium Human OmniExpress Exome.<sup>17</sup> Standard quality control (QC) was performed. We used SNPWEIGHTS for ancestry inference (<https://www.hsph.harvard.edu/alkes-price/software/>). The details are described previously.<sup>18</sup> Briefly, whole-genome data of the study participants were strand aligned and merged and following QCs were performed before inferring genetic ancestry: SNPs with more than 10% missing or with MAF <0.01 were excluded and individuals with less than 90% genotyping rate were removed. These analyses were performed in PLINK v.1.9. Then, SNPWEIGHTS was run on the merged/quality-controlled data using the weights for four major ancestral

populations (European, West African, East Asian and Native American). More than 200,000 SNPs were used for inference. The output included three predicted principal components and the inferred percentage ancestry.

### Statistical analysis

All analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC). Descriptive statistics for participants' demographic and clinical data were presented as percentage (%), using mean  $\pm$  standard deviation (SD) or median (range). Significant differences between cases and controls were tested using Chi-square tests of no association for categorical variables and Kruskal–Wallis tests for continuous variables.

Multivariable logistic regression adjusted for age, sex, race, ethnicity, and SES was used to test for an association between viral serostatus and pediatric MS. Because our research questions focused on seroconversion, EBV-VCA, CMV, HSV-1, and HSV-2 serostatus were treated as binary variables (seropositive or seronegative). In a subanalysis involving the subset of participants who underwent genotyping for ancestry markers, we substituted the race variable with the genetic ancestry data. *P*-values smaller than 0.05 were considered statistically significant.

In separate models, we explored the multiplicative interaction between age, race, ethnicity and *DRB1* status (*HLA-DRB1\*15:01/15:03* positive or negative) and the viral serostatus, as well as the interaction between different viral infections (such as interaction between seropositivity of EBV and CMV, EBV and HSV, and HSV and CMV). If the *P*-value of the interaction term was less than <0.15; we stratified the analysis based on that variable. Age analyses used mean age to stratify the cohort.

## Results

### Characteristics of cases and controls

We recruited 356 pediatric MS or CIS cases with short mean disease duration (<1 year) and 493 control subjects who provided blood samples at the time of enrollment. Demographics, frequency of seropositivity for EBV-VCA, CMV, HSV-1, and -2 and *HLA-DRB1* status of cases and controls are presented in Table 1. Cases were older and the proportion of females among cases was higher than controls.

### Viral seropositivity and the risk of pediatric MS

A higher proportion of cases than controls had evidence of prior infection with HSV-1, HSV-2 and EBV (Table 1).

**Table 1.** Baseline characteristics of the cases and control subjects.

	Control N = 493	Case N = 356	P-value
Average enrollment age (SD)	14.3 (3.8)	15.2 (3.2)	<0.001 <sup>1</sup>
Average disease duration in days (SD)	–	347 (319)	
Sex			<0.001 <sup>2</sup>
Male	237 (48.1%)	127 (35.7%)	
Female	256 (51.9%)	229 (64.3%)	
Race			0.848 <sup>2</sup>
White	321 (69.5%)	224 (67.7%)	
Black	80 (17.3%)	62 (18.7%)	
Other	61 (13.2%)	45 (13.6%)	
Ethnicity			<0.001 <sup>2</sup>
Hispanic or latino	84 (18.0%)	108 (31.8%)	
Not hispanic or latino	383 (82.0%)	232 (68.2%)	
Mother's education			<0.001 <sup>2</sup>
None	26 (6.0%)	35 (11.3%)	
High school or Associate's	203 (47.0%)	176 (56.6%)	
Bachelor's or Graduate	203 (47.0%)	100 (32.2%)	
Positive for HSV-1	97 (20.0%)	93 (26.4%)	0.029 <sup>2</sup>
Positive for HSV-2	61 (12.6%)	65 (18.7%)	0.015 <sup>2</sup>
Positive for CMV	156 (32.0%)	134 (38.0%)	0.071 <sup>2</sup>
Positive for EBV-VCA	284 (58.2%)	325 (91.6%)	<0.001 <sup>2</sup>
Positive for <i>DRB1</i> *15:01 or 15:03	109 (22.1%)	141 (40.2%)	<0.001 <sup>2</sup>

<sup>1</sup>Kruskal–Wallis test.<sup>2</sup>Chi-squared test of no association.

In multivariable logistic regression models adjusted for sex, age, race, ethnicity and SES, seropositivity for EBV-VCA was associated with increased odds of having MS by more than sevenfold (OR: 7.36, 95% CI: 4.50–12.04, *P*-value <0.001). Serological evidence of prior infection with HSV-1 was also associated with modestly increased odds of developing MS (OR: 1.54, 95% CI: 1.06–2.25, *P*-value = 0.025), while for the association with HSV-2 was only borderline significant (OR: 1.54, 95% CI: 1.00–2.38, *P*-value = 0.053). There was no association between CMV serostatus and having pediatric MS (OR: 1.29, 95% CI: 0.92–1.80, *P*-value = 0.14) (Table 2). Additional adjustment for *HLA-DRB1* status in the models did not substantially change the results (data not shown). No significant interaction was found between seropositivity of EBV and CMV, EBV and HSV, or HSV and CMV (data not shown).

## Race and ethnicity

There was a statistically significant multiplicative interaction between race and HSV-1, HSV-2 and EBV-VCA serostatus on the risk of MS (*P*-value for the interaction term: 0.006, 0.013 and 0.005, respectively) (Table 3).

**Table 2.** Multivariable association between serostatus and the odds of pediatric MS.

Effect for positive vs. negative*	Odds ratio	95% CI	<i>P</i> -value
HSV-1	1.54	1.06–2.25	0.025
HSV-2	1.54	1.00–2.38	0.053
CMV	1.29	0.92–1.80	0.142
EBV-VCA	7.36	4.50–12.04	<0.001

\*Adjusted for age, sex, race, ethnicity, and mother's highest level of education as a measure of socio-economic status. Further adjusting for *DRB1* status did not change the results significantly.

**Table 3.** Multiplicative interaction test between serostatus and race, ethnicity, age, and *DRB1* status.

Interaction term	<i>P</i> -value
HSV-1*race	0.006
HSV-2*race	0.013
CMV*race	0.677
VCA*race	0.005
HSV-1*ethnicity	0.241
HSV-2*ethnicity	0.160
CMV*ethnicity	0.238
VCA*ethnicity	0.363
HSV-1*age	0.711
HSV-2*age	0.367
CMV*age	0.516
VCA*age	<0.001
HSV-1* <i>DRB1</i>	0.076
HSV-2* <i>DRB1</i>	0.176
CMV* <i>DRB1</i>	0.478
VCA* <i>DRB1</i>	0.151

HSV-1 and -2 seropositivity were associated with increased risk of MS only in whites (Table 4). The effect of EBV-VCA seropositivity was stronger in whites, as opposed to African-Americans or others (OR = 10.64, 95% CI: 5.77–19.62, *P*-value <0.001 in whites; OR = 6.75, 95% CI = 1.42–31.91, *P*-value = 0.016 in African-Americans; OR = 1.96, 95% CI = 0.52–7.37, *P*-value = 0.32 in others) (Table 4).

The *P*-value for the interaction between Hispanic ethnicity and viral serostatus was >0.15 and so this was not explored further (Table 3).

## Age

There was a significant interaction between EBV-VCA serostatus and age in changing the odds of having MS (*P*-value of the interaction terms <0.001) (Table 3). We then analyzed the effect of viral status for this marker in children younger than 15 years of age (the mean age of participants in the study) versus those who were 15 years of age and older. Seropositivity for EBV-VCA was more

**Table 4.** Stratified analysis based on variables in which the *P*-value of the interaction with the serostatus was <0.15.

Effect	Odds ratio	95% CI	<i>P</i> -value
Race = White			
HSV-1: positive vs. negative	2.18	1.35–3.52	0.001
HSV-2: positive vs. negative	2.30	1.32–3.99	0.003
VCA: positive vs. negative	10.64	5.77–19.62	<0.001
Race = Black			
HSV-1: positive vs. negative	1.43	0.63–3.26	0.395
HSV-2: positive vs. negative	1.43	0.57–3.59	0.453
VCA: positive vs. negative	6.75	1.43–31.91	0.016
Race = Other			
HSV-1: positive vs. negative	0.36	0.11–1.20	0.096
HSV-2: positive vs. negative	0.23	0.04–1.26	0.089
VCA: positive vs. negative	1.96	0.52–7.37	0.319
Stratified analysis based on age			
Age < 15			
VCA: positive vs. negative	3.29	1.86–5.84	<0.001
Age ≥ 15			
VCA: positive vs. negative	**		
Stratified analysis based on <i>DRB1</i> status			
<i>DRB1</i> *15:01 or 15:03 = positive			
HSV-1: positive vs. negative	0.93	0.47–1.84	0.828
<i>DRB1</i> *15:01 or 15:03 = negative			
HSV-1: positive vs. negative	1.89	1.17–3.03	0.009

\*\*173 of 174 cases in the older age group were positive for EBV-VCA antibody, compared to 127 of 200 for controls. Since there was only one negative case, the model was not stable in this age group.

strongly associated with MS in those who were 15 years of age or older (Table 4).

### ***DRB1* interaction**

Using multiplicative interaction terms (between carrying *HLA-DRB1* allele and viral serostatus) and considering the *P*-value of <0.15 for further exploration in stratified analysis, the presence of *HLA-DRB1* modified the effect of seropositivity for HSV-1 on the risk of pediatric MS (Table 3). HSV-1 positive serostatus was associated with increased odds of having MS only in *HLA-DRB1* negative subjects. (Table 4).

### **Subanalysis of participants with available genetic ancestry data**

Genetic ancestry inferences were available for 263 cases and 296 controls. (Tables S1 and S2).

In multivariable logistic regression models adjusted for sex, age, genetic ancestry, ethnicity and socio-economic status, seropositivity for EBV-VCA increased the odds of having MS by more than eight times (OR: 8.26, 95% CI: 4.69–14.53, *P* <0.001). Although there was no statistically significant association between HSV-1, HSV-2 and CMV

serostatus and the risk of pediatric MS in this subset of participants (OR: 1.30, 95% CI: 0.85–2.01, *P* = 0.23; OR: 1.37, 95% CI: 0.83–2.24, *P* = 0.22; OR: 1.32, 95% CI: 0.86–1.97, *P* = 0.17, respectively), the effect sizes were similar to the main analyses and the lack of statistical significance is likely due to the smaller sample size.

### **Discussion**

Environmental exposures explain a large proportion of MS risk.<sup>19</sup> In this large, multicenter, multi-racial and multi-ethnic case-control study, we demonstrated that race, genetic background and age change the effects of prior infections with common herpesviruses on the risk of pediatric MS.

Prior EBV infection is thus far the strongest and most consistent environmental factor associated with pediatric and adult-onset MS.<sup>9–13,20,21</sup> We observed that this association with pediatric MS is seen regardless of race and ethnicity, although the association is stronger in whites, as recently reported in adult-onset MS.<sup>14</sup> Differences in the magnitude of the association of EBV infection with the risk of MS among different races and ethnicities point to potential environment-environment or gene-environment interactions.

We report several additional meaningful interactions that modify the association between EBV and pediatric MS. First, in line with previous reports, we observed that prior EBV infection is a stronger risk factor in carriers of *HLA-DRB1*\*15:01.<sup>7,22</sup> Although, this synergistic effect suggests possible causality of EBV infection in MS development, there is also a possibility that carrying *HLA-DRB1*\*15:01 affects both the risk of MS and susceptibility to EBV infection. Second, EBV seropositivity is a much stronger risk factor in older versus younger children which raises the possibility of a different pathophysiology in this age group.

Whether HSV affects MS risk remains controversial.<sup>23–25</sup> In this study, HSV infection was modestly associated with MS risk according, especially in whites raising the possibility that the disparity between previous reports might be related to the racial make-up of study populations. We also confirm an interaction between *HLA-DRB1* status and HSV infection and the risk of MS.<sup>4</sup> Although *DRB1* is more frequent in whites, a remote HSV infection was associated with increased MS risk only in *HLA-DRB1*\*15:01 negative subjects, which is the reverse interaction we identified for prior EBV infection. Although the underlying reasons for these differences remain to be clarified, these results highlight the complexity of gene-environment interactions as infections with viruses from the same family may have opposing effects on MS risk in individuals with the same genotype.

We did not find any association between CMV and odds of pediatric MS. This is in line with a prior report in adults<sup>26</sup> but in contrast with two others in children.<sup>4,27</sup> Two small groups of controls were used in our former study which could explain differences in the findings.<sup>4</sup> One control group included subjects with inflammatory neurological diseases while the other was recruited during a different time period and from different sites compared with cases. In contrast, this study was a larger, multicenter study in which cases and controls were selected from the same populations and during the same time frame, and as such is more likely valid. In a recent case–control study in adult MS,<sup>14</sup> CMV seropositivity was negatively associated with MS risk only in Hispanics, but not in Blacks or whites. This observation was interpreted as a support for the hygiene hypothesis, in which lack of exposure to common pathogens in childhood increases the risk of autoimmune disease later in life.

When using genetic ancestry data rather than self-reported race, the associations between viral serostatus and risk of pediatric MS remained unchanged except for HSV possibly because the genetic ancestry data were available for only a subset of participants, resulting in a 34% decreased sample size.

The strengths of our study include the large number of cases and controls with diverse racial and ethnic backgrounds recruited at several US centers, the relatively short disease duration of cases (mean < 1 year) and the adjusted analyses for several potential confounders. The study also has several limitations. Serological testing was done after disease onset and our sample is not population-based, that is, unknown selection bias may have influenced the results. Similar to all observational studies, unmeasured or unknown confounders might have affected the results. As mentioned previously, we did not analyze EBNA-1 seropositivity because the manufacturing source of the antigen changed during the study and the results were not consistent. While antibody responses against VCA are universally present in EBV exposed persons, EBNA-1 IgG is not produced in about 5% of patients after EBV infection and in some individuals, it may disappear over time.<sup>28</sup> Hence, the anti-VCA IgG is a more reliable indicator of previous EBV exposure.

Although this study does not explain how environmental factors affect MS risk in children at the molecular level, it highlights the complexity of factors at play. Larger case–control studies will allow further modeling of the interactions between viral infections, and other environmental and genetic factors.

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## Author Contributions

Bardia Nourbakhsh: study design, interpretation of data, manuscript composition. Alice Rutatangwa: manuscript composition. Michael Waltz: analysis and interpretation of data, manuscript composition. Mary Rensel: acquisition of data and manuscript composition. Manikum Moodley: acquisition of data and manuscript composition. Jennifer Graves: acquisition of data and manuscript composition. T. Charles Casper: analysis and interpretation of data, manuscript composition. Amy Waldman: acquisition of data and manuscript composition. Anita Belman: acquisition of data and manuscript composition. Benjamin Greenberg: acquisition of data and manuscript composition. Manu Goyal: acquisition of data and manuscript composition. Yolanda Harris: acquisition of data and manuscript composition. Ilana Kahn: acquisition of data and manuscript composition. Timothy Lotze: acquisition of data and manuscript composition. Soe Mar: acquisition of data and manuscript composition. Teri Schreiner: acquisition of data and manuscript composition. Gregory Aaen: acquisition of data and manuscript composition. Janace Hart: acquisition of data and manuscript composition. Jayne Ness: acquisition of data and manuscript composition. Jennifer Rubin: acquisition of data and manuscript composition.

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## Conflict of Interest

The authors report no disclosure relevant to the manuscript.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Number of subjects with available genetic ancestry data.

**Table S2.** Summary of the genetic ancestry data for the subset of participants with available data.