

Epstein-Barr Virus Antibodies and Risk of Multiple Sclerosis

A Prospective Study

Alberto Ascherio, MD, DrPH

Kassandra L. Munger, MSc

Evelyne T. Lennette, PhD

Donna Spiegelman, ScD

Miguel A. Hernán, MD, DrPH

Michael J. Olek, DO

Susan E. Hankinson, ScD

David J. Hunter, ScD

THE ETIOLOGY OF MULTIPLE sclerosis (MS) is largely unknown, but evidence supports an autoimmune process triggered by infection or other environmental factors.¹⁻³ Epstein-Barr virus (EBV), a herpesvirus, infects more than 90% of the human population, establishing a persistent and highly immunogenic infection of B lymphocytes.⁴ Antigen-specific cytotoxic T cells are massively expanded in response to primary infection and persist at high levels for several years.^{5,6} Autoimmunity could result if some of these cells carry T-cell receptors that recognize self-peptides. Epstein-Barr virus has been related to nasopharyngeal carcinoma, Burkitt lymphoma, and Hodgkin disease,⁷ and a relation to autoimmune diseases has been proposed⁸ but remains unproven.

Infection with EBV is usually asymptomatic in childhood but frequently causes infectious mononucleosis in ado-

Context Epidemiological studies suggest an association between infection with Epstein-Barr virus (EBV) and risk of multiple sclerosis (MS).

Objective To determine whether elevation in serum antibody titers to EBV viral capsid antigen (VCA), nuclear antigens (EBNA, EBNA-1, and EBNA-2), and diffuse and restricted early antigen (EA-D and EA-R) as well as to cytomegalovirus (CMV) precede the occurrence of MS.

Design, Setting, and Subjects Prospective, nested case-control study. Of 62439 women participating in the Nurses' Health Study (aged 30-55 years in 1976) and Nurses' Health Study II (aged 25-42 years in 1989) who gave blood samples in 1989-1990 and 1996-1999, respectively, and were followed up through 1999, 144 women with definite or probable MS and 288 healthy age-matched controls were included in the analysis.

Main Outcome Measure Serum antibody titers to the specific EBV and CMV antigens, compared between cases and controls.

Results We documented 18 cases of MS with blood collected before disease onset. Compared with their matched controls, these women had higher serum geometric mean titers (GMTs) of antibodies to EBV but not CMV. Elevations were significant for antibodies to EBNA-1 (GMT, 515 vs 203; $P=.03$), EBNA-2 (GMT, 91 vs 40; $P=.01$), and EA-D (15.9 vs 5.9; $P=.04$). The strongest association was found for antibodies to EBNA-2; a 4-fold difference in titers was associated with a relative risk (RR) of MS of 3.9 (95% confidence interval [CI], 1.1-13.7). The corresponding RRs were 1.6 (95% CI, 0.7-3.7) for VCA, 2.5 (95% CI, 1.0-6.3) for EBNA, 1.8 (95% CI, 1.0-3.1) for EA-D, and 1.0 (95% CI, 0.6-1.7) for CMV. Significant but generally weaker elevations in anti-EBV antibodies were also found in analyses of 126 cases of MS with blood collected after disease onset and their matched controls.

Conclusions Our results support a role of EBV in the etiology of MS.

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lescents and adults. The similarity in the epidemiology of MS and infectious mononucleosis led to the proposition that MS could be caused by infection with EBV during or after adolescence in genetically susceptible individuals.⁹ This hypothesis is supported by observations suggesting an increased risk of MS following infectious mononucleosis,¹⁰⁻¹⁵ the rarity of MS among individuals without serum anti-EBV

Author Affiliations: Departments of Nutrition (Dr Ascherio and Ms Munger), Epidemiology (Drs Ascherio, Spiegelman, Hernán, Hankinson, and Hunter), and Biostatistics (Dr Spiegelman), Harvard School of Public Health, Center for Neurological Diseases—Multiple Sclerosis Unit (Dr Olek), and Channing Laboratory, Department of Medicine (Drs Hankinson and Hunter), Harvard Medical School and Brigham and Women's Hospital, Boston, Mass; and ViroLab Inc (Dr Lennette), Berkeley, Calif.

Corresponding Author and Reprints: Alberto Ascherio, MD, DrPH, Harvard School of Public Health, Nutrition Department, 665 Huntington Ave, Boston, MA 02115 (e-mail: Alberto.Ascherio@channing.harvard.edu).

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antibodies,^{16,17} and the higher serum titers of anti-EBV antibodies in MS patients than in controls. Elevated titers have been reported against both the EBV viral capsid antigen (VCA), which is expressed in the viral replicative cycle, and the Epstein-Barr nuclear antigen (EBNA), which is expressed in latently infected B lymphocytes.^{18,19} These elevations in antibody titers are consistent with an association between infection with EBV and risk of MS¹⁶ but could also simply reflect the immune dysregulation in MS.²⁰ To clarify the temporal relation between antibody titers and MS, antibody status should be assessed before clinical evidence of disease exists.

To address the possible role of EBV in the etiology of MS, we prospectively examined the association between serum anti-EBV antibody titers and risk of developing MS in 2 large cohorts of US women.

METHODS

Study Population

The study base for this investigation comprised the subsets of participants who provided a blood sample in 2 large ongoing US cohorts, the Nurses' Health Study (NHS) and the Nurses' Health Study II (NHS II). The NHS was established in 1976, when 121 700 female registered nurses aged 30 to 55 years from 11 states responded to a mailed questionnaire about disease history and lifestyle items. The NHS II was established in 1989, when 116 671 female registered nurses aged 25 to 42 years from 14 states responded to a similar questionnaire. All participants in both cohorts were invited to provide a blood sample to investigate several biomarkers of chronic disease. Blood was collected from 32 826 participants in the NHS between 1989 and 1990 and from 29 613 women in the NHS II between 1996 and 1999. Follow-up questionnaires are mailed to the participants of both studies every 2 years to update information on potential risk factors for chronic diseases and to ascertain whether major medical events have occurred.

Case Ascertainment and Control Selection

Details on the documentation of MS cases in these cohorts have been reported previously.²¹ Briefly, participants who reported a new diagnosis of MS in 1 of the follow-up questionnaires were asked permission to contact their neurologists and review their medical records. After obtaining permission, we sent the neurologists a questionnaire addressing the certainty of the diagnosis (definite, probable, possible, or not MS), the date of onset of neurological symptoms related to MS, other aspects of the clinical history, and laboratory test results. Since 93% of all definite and probable diagnoses appeared to conform to the Poser criteria²² for diagnosis of MS when applied to the clinical and laboratory data provided in the questionnaire,²³ we classified as cases women who had a diagnosis of definite or probable MS according to their neurologists.

The date of onset of the disease was determined by asking both women with MS and their neurologists for the date of first neurological symptoms. When the 2 dates were discordant, the earliest date was considered valid. A total of 149 incident cases of definite and probable MS were documented between baseline and December 1999 among women with available blood samples. For each case, we randomly selected 2 women without MS, matched by year of birth and study cohort. One case was excluded because no serological results were obtained and 4 because of missing dates of onset; thus, 144 women with MS and 288 controls were included in the analysis.

In 18 of the women with MS, the onset of neurological symptoms occurred after blood collection (median, 1.9 years; range, 2 months–6.5 years). The diagnoses of MS in these women were all made by neurologists and supported by magnetic resonance imaging; the diagnosis was reported as definite MS in 12 women and probable MS in 6. Because of the age distribution of the cohorts at the time of blood collection, most of these women had a late

onset of MS (median age, 52 years; range, 39–66 years).

Blood Collection and Laboratory Analyses

Blood was obtained using collection kits that were returned to our laboratory via overnight courier. Approximately 97% of the samples arrived within 26 hours of being drawn. On arrival in our laboratory, the blood samples were centrifuged and the blood components aliquotted into cryotubes and stored in liquid nitrogen freezers until laboratory analysis. Serum samples from MS cases and controls were sent to the laboratory (ViroLab Inc, Berkeley, Calif) in triplets containing the case and the 2 matched controls in random order. The laboratory was blind to case-control status and unidentified triplets were included for quality control.

Immunoglobulin G antibodies to EBV VCA and anti-early antigen complex (diffuse [EA-D] and restricted [EA-R]) were determined by indirect immunofluorescence according to methods of Henle et al.²⁴ Immunoglobulin G antibodies against the EBNA family and 2 of its individual components, EBNA-1 and EBNA-2, were determined using anticomplement immunofluorescence, as previously described.²⁵ Antibodies to the VCA and the EA-D components emerge during the late incubation period or in the course of the acute phase of infectious mononucleosis, whereas antibodies to EBNA and EA-R arise only weeks or months after onset of the disease.²⁴ Antibodies to EBNA-2 arise before those to EBNA-1 and usually decline over several months, whereas antibodies to EBNA-1 persist indefinitely. Persistently high anti-EBNA-2 titers and low anti-EBNA-1 titers have been associated with chronic infectious mononucleosis.²⁴ Antibody titers against cytomegalovirus (CMV) were also determined to assess the specificity of any association that may be found between MS and EBV serology. The methods used for CMV serology have been previously described.²⁶ The intra-assay coefficients of variation were

VCA, 8.6%; EBNA, 13.9%; EBNA-1, 9.8%; EBNA-2, 9.1%; EA-D, 13.0%; EA-R, 9.2%; and CMV, 7.3%.

Assessment of Covariates

We considered in the analyses age, latitude of birthplace (northern, middle, or southern), ancestry (Scandinavian, Southern European, other white, or nonwhite), and smoking (never, past, or current), data on which were collected from all participants as part of the cohort follow-up. The associations of birthplace latitude, ancestry, and smoking history with risk of MS in these cohorts have been previously reported.^{23,27}

Statistical Analyses

Antibody geometric mean titers (GMTs) were compared between cases and controls using generalized linear models that take into account the matched design of the study and the use of clustered measurements.²⁸ We used conditional logistic regression to estimate the relative risk (RR) of MS associated with a 4-fold difference in specific antibody titers. In these regression models, we included the base 2 logarithm of the reciprocal of the dilution of the antibody titers as a continuous variable. On this scale, the regression coefficient estimates the logarithm of the RR associated with a 2-fold difference in titers; we doubled and exponentiated this value to estimate the RR associated with a 4-fold difference.

The main analyses included only women with MS who provided blood samples before onset of the disease compared with their matched controls or with all controls combined. However we also presented results for women with blood collected after onset of MS to assess whether there is any effect of the disease on antibody titers. Finally, we classified women according to whether they had evidence of past infection with EBV or CMV, to estimate the risk of MS associated with prior infection. For this analysis, women were considered EBV-positive if the antibody titer to VCA was at least 1:20 or that to EBNA was at least 1:5; women

Table 1. Relative Risk of MS Corresponding to a 4-Fold Increase in Anti-EBV and Anti-CMV Serum Antibody Titers*

| Antibodies | Cases With Blood Collected Before MS Onset | | | | Cases With Blood Collected After MS Onset | |
|------------|--|---------|------------------|---------|---|---------|
| | vs Matched Controls† | | vs All Controls‡ | | vs Matched Controls§ | |
| | RR (95% CI) | P Value | RR (95% CI) | P Value | RR (95% CI) | P Value |
| VCA | 1.6 (0.7-3.7) | .20 | 1.5 (0.8-2.8) | .20 | 1.7 (1.3-2.3) | <.001 |
| EBNA | 2.5 (1.0-6.3) | .06 | 2.8 (1.3-5.6) | .007 | 1.6 (1.2-2.1) | <.001 |
| EBNA-1 | 2.7 (1.0-7.2) | .06 | 2.5 (1.3-4.9) | .006 | 1.7 (1.3-2.1) | <.001 |
| EBNA-2 | 3.9 (1.1-13.7) | .03 | 2.9 (1.4-6.1) | .005 | 1.6 (1.2-2.1) | <.001 |
| EA-D | 1.8 (1.0-3.1) | .05 | 2.7 (1.7-4.3) | <.001 | 1.4 (1.0-1.9) | .03 |
| EA-R | 0.9 (0.5-1.5) | .06 | 0.9 (0.6-1.4) | .60 | 1.2 (1.0-1.5) | .03 |
| CMV | 1.0 (0.6-1.7) | >.99 | 1.0 (0.7-1.6) | .90 | 0.9 (0.7-1.1) | .20 |

*MS indicates multiple sclerosis; EBV, Epstein-Barr virus; CMV, cytomegalovirus; RR, relative risk; CI, confidence interval; VCA, viral capsid antigen; EBNA, Epstein-Barr nuclear antigen; EA-D, diffuse early antigen complex; and EA-R, restricted early antigen complex.

†Conditional logistic regression; 18 cases and 36 matched controls.

‡Unconditional logistic regression; 18 cases and 288 controls; adjusted for age at blood collection.

§Conditional logistic regression; 126 cases and 252 controls.

were considered CMV-positive if the antibody titer to CMV was at least 1:10. The association between seropositivity and MS was estimated using the Mantel-Haenszel method for matched data²⁹ or conditional logistic regression for multivariate analyses. All *P* values are 2-tailed. The statistical software SAS (SAS Institute Inc, Cary, NC) was used for all analyses.

RESULTS

The GMTs of serum antibodies to EBV were consistently higher among women with MS compared with their matched controls. In analyses including only the 18 cases with blood collected before disease onset and their matched controls, these differences were significant for antibodies to EBNA-1 (GMT for cases, 515 vs for controls, 203; *P* = .03), EBNA-2 (GMT, 91 vs 40; *P* = .01), and EA-D (GMT, 15.9 vs 5.9; *P* = .04). The GMTs to VCA and EBNA were also higher among cases than controls (GMT for VCA, 1613 vs 1036; GMT for EBNA, 667 vs 333), but these differences only achieved statistical significance in analyses of the 126 cases with blood collected after onset of MS. There was no difference between cases and controls in the GMTs of antibodies to EA-R or CMV. The case-control differences in antibody titers to EBV were of similar magnitude whether the blood was collected before or after dis-

ease onset, although the larger number of cases with blood collected after disease onset conferred a stronger degree of statistical significance. A direct comparison of these associations stratified by time of blood collection is shown in TABLE 1. In matched analyses, the RRs of MS associated with a 4-fold difference in antibody titers before onset of MS ranged from 1.6 (95% confidence interval [CI], 0.7-3.7) for antibodies to VCA to 3.9 (95% CI, 1.1-13.7) for antibodies to EBNA-2. Significant positive associations with antibodies to EBNA-1, EBNA-2, and EA-D were also observed in age-adjusted analyses including all controls (Table 1). A plot of this association for EBNA-1 is shown in FIGURE 1; results for EBNA-2 were similar. The corresponding RR for antibody titers collected after onset of MS were smaller, except for VCA (1.7 vs 1.6). The RRs associated with antibodies to CMV were close to 1.0 for all comparisons.

To address the possibility that the increased anti-EBV antibody titers among the cases with blood collected before onset were due to preclinical disease, we calculated the mean antibody titers among 12 women with MS who provided a blood sample at least 1 year before the onset of MS and plotted the antibody titers of women with MS according to the temporal relation between blood collection and disease

onset (FIGURE 2). Serum antibody titers to EBV antigens were already elevated among women who gave blood at least 1 year before onset of MS and were unrelated to the time of blood collection, except for a decline in anti-EBNA-1 after MS onset (Figure 2).

The 18 women with MS who gave blood before onset of disease were all positive for both VCA and EBNA, whereas 2 of their matched controls were negative for VCA and 1 was negative for EBNA ($P = .30$ and $P = .50$, respectively). Using the combined sample of MS subjects, only 1 of 144

women with MS had negative anti-VCA titers vs 18 of 287 controls (OR, 9.0; 95% CI, 1.8-45.2; $P = .008$) (TABLE 2). Results for anti-EBNA titers were similar. In contrast, the proportions of women with serum anti-CMV antibodies were similar in the case and control groups (Table 2). These associations were not materially changed in multivariate analyses.

COMMENT

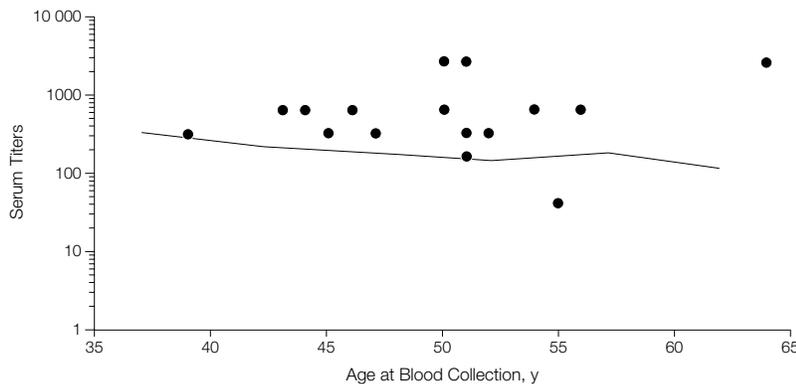
In this prospective study, we found that significant elevations in serum anti-EBV antibody titers were present be-

fore onset of MS. Elevations were observed for antibodies against antigens expressed during replication of the virus (EA-D) as well as in latency (EBNAs). The strongest association was found with antibodies to EBNA-2; a 4-fold difference in titers of antibodies to this antigen was associated with a 4-fold increase in risk of MS.

The nested case-control design of our investigation makes a biased selection of controls unlikely. Some degradation of the IgG antibodies or desiccation of blood samples may have occurred during shipping and storage, but these are probably modest because most samples reached our serum bank within 26 hours and were kept in closely monitored liquid nitrogen freezers. Most importantly, blood samples from cases and controls were handled in the same manner throughout the study, and triplets composed of a case and 2 matched control samples were assayed in the same run and in random order by technicians who were blind to disease status. Under these conditions, any laboratory error should be unrelated to disease status and would attenuate the difference in antibody titers between cases and controls.

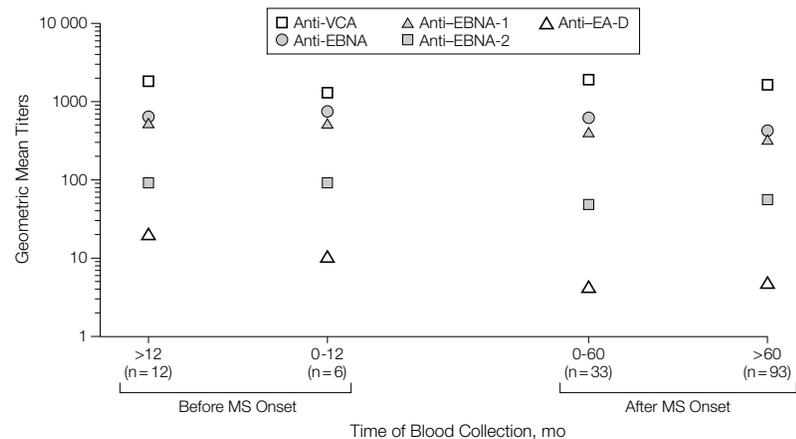
A potential limitation of our study is the relatively short period between blood collection and onset of MS (median, 1.9 years). The onset of the autoimmune process that leads to demyelination may precede the recognition of neurological symptoms by several months and so may the immune dysregulation that accompanies the disease. Thus, the elevation in anti-EBV titers could be an early manifestation of the preclinical phase of the disease. The fact that antibody titers in serum samples collected 1 or more years before onset of symptoms were similar to those in serum samples collected in women with fully clinical disease provides some evidence against this explanation, but confirmation of these results in a larger number of serum samples collected years before MS onset will be important. The late age of onset of MS among women in our study is atypical, but it is consistent with the

Figure 1. Anti-EBNA-1 Antibody Titers in Serum Samples Collected Before Onset of Neurological Symptoms From 16 Women With Multiple Sclerosis



Serum titers are the reciprocal of dilution. The line indicates the mean antibody titers among all control women. Epstein-Barr nuclear antigen 1 (EBNA-1) titer values were missing for 2 cases.

Figure 2. Temporal Relation Between Anti-EBV Serum Antibodies and MS Onset in 144 Cases



EBV indicates Epstein-Barr virus; MS, multiple sclerosis; VCA, viral capsid antigen; EBNA, Epstein-Barr nuclear antigen; and EA-D, diffuse early antigen complex.

age distribution of our cohorts at the time of blood collection.

A causal association between EBV and MS was originally suggested 20 years ago,⁹ primarily because of the similarities in the epidemiology of infectious mononucleosis and MS and of the increased risk of MS among individuals with a history of infectious mononucleosis.¹⁰⁻¹⁵ If EBV had a critical role in the etiology of MS, then it would be expected that few or no MS cases would occur among individuals who are not infected with EBV. This expectation is indirectly supported by the results of several case-control studies. In a meta-analysis of published investigations, we estimated that the odds of disease are more than 10 times higher among EBV-positive than EBV-negative persons.¹⁶ Since primary EBV infection is rare among patients with MS,^{17,30} this finding suggests that either EBV itself increases the risk of MS or that they both are related to some common factor. A microorganism transmitted in a manner similar to EBV could be involved, but the EBV-MS association is so strong that it is unlikely to be fully explained by confounding. A common genetic predisposition may be more plausible, because of the known association between HLA class II polymorphism and risk of MS and the recent observation that the HLA class II protein acts as a cofactor in EBV infection of B lymphocytes.³¹ A study of long-term EBV-negative adults, however, revealed a distribution of the DR2 alleles commonly associated with MS similar to that of EBV-seropositive adults,³² and higher titers of anti-EBV antibodies have been reported in individuals with MS than in HLA-DR2-

matched controls.³³ These observations suggest that genes are unlikely to explain the strong associations reported herein. Further evidence that EBV has an active role in MS recently has been provided by the observation that active viral replication occurs more commonly in MS patients with exacerbations than in patients with stable disease.¹⁷

The elevation of anti-EBV antibody titers before onset of MS that we found in this study provides further support for an important role of EBV in the etiology of MS. The observation of elevated anti-EBV serum antibody titers before diagnosis has contributed to establishment of the causal link between EBV and Burkitt lymphoma³⁴ and has been reported in other EBV-related diseases, including nasopharyngeal carcinoma³⁵ and Hodgkin disease.³⁶ The pattern of antibody response that we observed among women with MS is different from that observed in immunocompromised hosts or in chronic infectious mononucleosis, which is characterized by elevated anti-EBNA-2 and reduced anti-EBNA-1 titers.²⁵ The simultaneous elevation of titers to VCA, EBNA-1, EBNA-2, and EA-D rather suggests a more severe or more recent primary infection among women who developed MS than in women who remained healthy.

Evidence on potential mechanisms by which EBV could be causally related to MS is limited. The failure to demonstrate EBV in MS plaques by in situ hybridization³⁷ or polymerase chain reaction³⁸ suggests that direct central nervous system infection is not involved. Rather, the T-cell response to EBV infection could include clones that are poten-

tially cross-reactive with self-antigens. In acute infectious mononucleosis, there is a massive expansion of activated circulating T cells that are specific for EBV latent and lytic antigens.⁶ A high proportion of these cells is committed to a single viral epitope,⁵ and populations of cells that are specific for both latent and lytic antigens are still present at frequencies of more than 1% up to 3 years after recovery.⁶ Other potential mechanisms proposed to explain a potential role of EBV in MS include cross-reactions of anti-EBNA antibodies with epitopes of a neuroglial antigen³⁹ and the EBV-induced expression in B lymphocytes of α - β -crystallin, a small stress protein that has been reported to be an immunodominant myelin antigen in MS patients.⁴⁰ It has recently been reported that variant A of human herpesvirus 6 is more commonly reported in MS patients than in controls.⁴¹⁻⁴³ Unlike the more common human herpesvirus 6 variant B, variant A may infect EBV-positive B-cell lines and activate the latent EBV genome.^{44,45} These observations suggest that interactions between herpesviruses may have a role in the pathogenesis of MS.

In conclusion, our results, in conjunction with those of case-control studies, offer evidence that EBV infection may increase the risk of MS. Because few individuals infected with EBV develop MS, other cofactors are required. These may include genetic predisposition and, perhaps, age at primary infection or infection with other microbes. Final proof of causality could come from the demonstration that a suitable vaccine prevents MS. Available antiviral drugs have little effect on

Table 2. Relative Risk of Multiple Sclerosis According to Presence of Anti-EBV or Anti-CMV Serum Antibodies in 144 Cases vs 288 Controls*

| Antibodies | Cases, No. | | Controls, No. | | OR (95% CI)‡ | P Value |
|------------|--------------------|-------------------|-------------------|-------------------|----------------|---------|
| | Antibody-Positive† | Antibody-Negative | Antibody-Positive | Antibody-Negative | | |
| VCA§ | 143 | 1 | 269 | 18 | 9.0 (1.8-45.2) | .008 |
| EBNA | 141 | 1 | 266 | 16 | 8.3 (1.6-43.8) | .01 |
| CMV | 72 | 72 | 161 | 127 | 0.8 (0.5-1.2) | .24 |

*EBV indicates Epstein-Barr virus; CMV, cytomegalovirus; OR, odds ratio; CI, confidence interval; VCA, viral capsid antigen; EBNA, Epstein-Barr nuclear antigen; and EA-D, diffuse early antigen complex.

†Serum samples were considered positive for the dilutions VCA \geq 20, EBNA \geq 5, EBNA-1 \geq 5, EBNA-2 \geq 5, EA-D \geq 5, and CMV \geq 10.

‡By Mantel-Haenszel log-rank test.

§Titers missing for 1 control.

||Titers missing for 2 cases and 6 controls.

the number of infected B lymphocytes and may thus be ineffective in MS treatment.⁷ However, a better understanding of the mechanism that relate EBV to MS may also lead to novel therapeutic approaches.

Author Contributions: Study concept and design: Ascherio.

Acquisition of data: Ascherio, Lennette, Hernán, Olek, Hankinson, Hunter.

Analysis and interpretation of data: Ascherio, Munger, Spiegelman, Olek, Hankinson, Hunter.

Drafting of the Manuscript: Ascherio.

Critical revision of the manuscript for important intellectual content: Ascherio, Munger, Lennette, Spiegelman, Hernán, Olek, Hankinson, Hunter.

Statistical expertise: Ascherio, Spiegelman, Hunter.

Obtained funding: Ascherio, Hankinson.

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Study supervision: Ascherio, Olek.

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