Humoral response to EBV is associated with cortical atrophy and lesion burden in patients with MS

ABSTRACT

Objective: Because dysregulated Epstein-Barr virus (EBV)-infected B cells may induce meningeal inflammation, which contributes to cortical pathology in multiple sclerosis (MS), we investigated associations between antibody responses to EBV and development of cortical pathology in MS.

Methods: We included 539 patients with MS (369 with relapsing-remitting MS, 135 with secondary progressive MS, and 35 with primary progressive MS), 66 patients with clinically isolated syndrome (CIS), 63 patients with other neurologic diseases (OND), and 178 age- and sex-matched healthy controls (HC). All participants were scanned on 3T MRI. Serum samples were analyzed for IgG antibodies against EBV viral capsid antigen (VCA) and EBV nuclear antigen-1 (EBNA-1), and their quartiles were determined on the whole study sample. Differences between the study groups were assessed using analysis of covariance adjusted for multiple comparisons.

Results: More than 30% of patients with MS and CIS presented with the highest quartile of anti-EBV-VCA and -EBNA-1 status compared to ≤10% of HC (p < 0.001). The figures were 9 (14.3%) and 7 (12.3%) for patients with OND. Patients with MS with the highest quartile of anti-EBV-VCA showed significantly increased T2 lesion volume (p = 0.001), T1 lesion number (p = 0.002), and T1 lesion volume (p = 0.04) and decreased gray matter (p = 0.041) and cortical (p = 0.043) volumes compared to patients with MS with lower quartiles. No significant differences of MRI outcomes in patients with CIS, patients with OND, and HC with lower or highest quartiles of anti-EBV-VCA and -EBNA-1 were detected.

Conclusions: Humoral response to anti-EBV-VCA and -EBNA-1 is associated with more advanced cortical atrophy, accumulation of chronic T1 black holes, and focal white matter lesions in patients with MS. *Neural Neuroimmunol Neuroinflamm* 2016;3:e190; doi: 10.1212/NXI.0000000000000190

GLOSSARY

ANCOVA = analysis of covariance; CE = contrast-enhancing; CIS = clinically isolated syndrome; CMV = cytomegalovirus; EBNA-1 = Epstein-Barr virus nuclear antigen-1; EBV = Epstein-Barr virus; FLAIR = fluid-attenuated inversion recovery; GM = gray matter; HC = healthy controls; LV = lesion volume; MS = multiple sclerosis; NBV = normalized brain volume; NCV = normalized cortical volume; NGMV = normalized gray matter volume; NLLV = normalized lateral ventricle volume; NWMV = normalized white matter volume; OND = other neurologic diseases; PMS = primary progressive MS; RRMS = relapsing-remitting MS; SE = spin-echo; SET study = Observational Study of Early Interferon beta 1-a Treatment in High Risk Subjects after CIS; SPMS = secondary progressive MS; TE = echo time; TI = inversion time; TR = repetition time; VCA = viral capsid antigen; WM = white matter.

One of the scientific challenges of investigating environmental risk factors in patients with multiple sclerosis (MS) is that they represent common exposures in the general population. For example, Epstein-Barr virus (EBV) is a common environmental exposure and >90% of the world’s population is seropositive.1

In patients with clinically isolated syndrome (CIS), anti-EBV nuclear antigen-1 (EBNA-1) antibody levels are associated with progression to clinically definite MS and with the formation
of new inflammatory lesions.2-4 Cross-sectional and longitudinal studies in patients with MS suggest that exposure to EBV may increase the likelihood of disease progression, as evidenced by clinical and MRI outcomes of disease severity.2,2-11

In the last decade, it has been established that cortical gray matter (GM) pathology is strongly associated with the presence of cortical subpial lesions,12 meningeal inflammation in the form of ectopic lymphoid-like structures,13 and retrograde wallerian degeneration of neurons.14 Clusters of meningeal inflammatory cells may act to sustain the intrathecal immune response and engender subpial cortical lesions.15,16 It has been hypothesized that dysregulated EBV-infected B cells17 may induce meningeal inflammation that could contribute to GM pathology. Our previous work suggested that higher levels of EBV antibodies are associated with increased MRI lesion activity and greater brain atrophy, particularly of the GM.2,7-9

In this large cohort study, we aimed to examine the association of cortical pathology and IgG anti-EBV antibodies, as measured by cortical atrophy. We focused our attention on cortex because subpial cortical lesions and meningeal inflammation are emerging from pathology and imaging studies as the most important pathologic substrate for cognitive and physical disease progression in MS12,15,18,19 and EBV-infected B cells may play role in its pathogenesis.17,20

METHODS Study population. This study used baseline data from an ongoing prospective study of cardiovascular, environmental, and genetic risk factors in MS that enrolled more than 1,000 patients with CIS, MS, and other neurolologic diseases (OND) and healthy controls (HC).21-23 The inclusion criteria for this substudy of the association of EBV and cytomegalovirus (CMV) and MRI outcomes were (1) having a valid serum sample for this substudy of the association of EBV and cytomegalovirus, EBNA-1 IgG, and anti-CMV antibodies, as previously described.2-7

MRI acquisition and analysis. All participants were examined on a 3T GE Signa Excite HD 12.0 Twin Speed 8-channel scanner (General Electric, Milwaukee, WI). MRI sequences included the axial dual fast spin-echo (SE) T2/proton density-weighted image, 3D spoiled gradient-recalled T1-weighted image, SE T1-weighted image pre- and postcontrast, and fluid-attenuated inversion recovery (FLAIR) scans. Pulse sequence characteristics for 3T MRI were as follows. All scans were acquired with a 256 × 256 matrix and a 25.6-cm field of view (FOV) for an in-plane resolution of 1 × 1 mm² with a phase FOV of 75% and one average. Sequence-specific parameters were as follows. For the T2/proton density-weighted image: 3-mm-thick slices with no gap, TE1 (echo time)/TE2/TR (repetition time) = 12/95/3,000 milliseconds, echo train length = 14, flip angle = 90°; for the FLAIR scans: 3-mm-thick slices with no gap, TE/TI (inversion time)/TR = 120/2,100/8,500 milliseconds, flip angle = 90°; for 3D T1-weighted image: 1-mm-thick slices with no gap, TE/TI/TR = 2.8/900/5.9 milliseconds, flip angle = 10°; and for SE T1-weighted image: 3-mm-thick slices with no gap, TE/TR = 16/600 milliseconds, flip angle = 90°. The SE T1-weighted image sequence was obtained after injection of a single-dose IV bolus (0.1 mMol/kg gadolinium-diethylenetriaminepentacetate) 5 minutes after administration of contrast agent only in patients with MS and CIS.

MRI analyses were blinded to the participants’ physical and neurologic condition. The MRI measures included in the analysis were contrast-enhancing (CE) T1 and T2 lesion number and lesion volumes (LVs), assessed by a semi-automated edge detection contouring/thresholding technique,23 and measures of brain atrophy, including normalized brain volume (NBV), normalized GM volume (NGMV), normalized cortical volume (NCV), normalized white matter (WM) volume (NWMV), and normalized lateral ventricle volume (NLVV),23 assessed by the SIENAX 2.6 cross-sectional software tool.4-2

Statistical analysis. Statistical analysis was performed using SPSS version 21.0 (IBM, Armonk, NY). The data were investigated separately by disease group (HC, CIS, MS, and OND) and by MS disease subtype (relapsing-remitting MS [RRMS], secondary progressive MS [SPMS], and primary progressive MS [PPMS]).

The analyses were focused on MRI associations with anti-EBV antibody levels rather than antibody positivity, because the percentage of participants positive for anti-EBV-VCA and anti-EBV-EBNA-1 antibodies was very high (>99%) because of the ubiquity of exposure to EBV. Given that relative concentrations of the anti-VCA and anti-EBNA-1 antibodies are not normally distributed, these were categorized into lower and the highest quartiles based on the whole study sample regardless of clinical group, as previously reported.2,7 We also performed a
Demographic and clinical characteristics of the study groups. Demographic and clinical characteristics of the study groups are shown in table 1. A total of 846 consecutive participants who fulfilled inclusion and exclusion criteria entered this sub-study of EBV and CMV and MRI outcomes in MS. The study population consisted of 178 HC, 539 patients with MS, 66 patients with CIS, and 63 patients with OND (25 with neurodegenerative, 18 with autoimmune, 10 with vascular, and 10 with neuromuscular origin). Patients with MS, HC, and patients with OND were age- and sex-matched, whereas patients with CIS were significantly younger, had shorter disease duration, and had a lower level of disability, as expected (table 1 and table e-1 at Neurology.org/nn).

Demographic and clinical characteristics of the patients with MS by subtype are shown in table 2. There were 368 patients with RRMS, 135 patients with SPMS, and 35 patients with PPMS in the study. As expected, patients with SPMS and PPMS were significantly older, had longer disease duration, and had a higher level of disability than those with RRMS.

Eighteen (10.1%) HC, 7 (12.3%) patients with OND, 68 (31.2%) patients with MS, and 22 (33.3%) patients with CIS were grouped into the highest quartile of anti-EBV-VCA antibodies; the corresponding numbers for anti-EBV-EBNA-1 antibodies were 13 (7.3%), 9 (14.3%), 182 (33.8%), and 19 (28.8%), respectively (table 1 and table e-1). The mean titer of anti-EBV-VCA antibodies was significantly greater in patients with MS and CIS than in HC (table 1, \( p < 0.001 \)), and there was a trend for greater mean titer of anti-EBV-EBNA-1 antibodies in patients with MS and CIS (table 1, \( p = 0.068 \)). Frequency of the highest quartile status group of anti-EBV-VCA antibodies was similarly distributed across all MS subtypes. In contrast, more patients with RRMS (36.3%) than those with SPMS (22.2%) and PPMS (28.6%) were found in the highest quartile group of anti-EBV-EBNA-1 antibodies (\( p = 0.001 \)). No significant differences in mean titer of anti-EBV-VCA and -EBNA-1 antibodies were detected between different MS subtypes.

No significant differences between patients with MS and CIS and HC (table 1) or between different OND subtypes (table e-1) were found for anti-CMV antibody positivity or mean titer.

MRI differences according to the anti-EBV-VCA antibodies. Table 3 and figure 1 show group differences of MRI measures between the anti-EBV-VCA highest and lower quartiles across the study groups. Among patients with MS, increased T2 LV (\( p = 0.001 \)), T1 lesion number (\( p = 0.002 \)) and LV (\( p = 0.04 \)) and decreased NGMV (\( p = 0.041 \)) and NCV (\( p = 0.043 \)) were observed in the highest quartile status group. There were no differences between the anti-EBV-VCA highest and lower quartile status groups and MRI outcomes in the HC, OND, or CIS study groups. In Pearson correlation analysis performed in patients with MS, there was a significant association between the greater anti-EBV-VCA titer and increased T1 lesion number (\( r = 0.12, p = 0.008 \)) and decreased NGMV (\( r = 0.11, p = 0.014 \)) and NCV (\( r = 0.1, p = 0.018 \)). No associations between the anti-EBV-VCA titers and MRI outcomes were detected in HC, OND, or CIS study groups.

Group differences of MRI outcomes between anti-EBV-VCA highest and lower quartile status groups within RRMS, SPMS, and PPMS subtypes are shown in table 4 and figure 1. An increased T2 LV (\( p = 0.018 \)) and T1 lesion number (\( p = 0.039 \)) and decreased NGMV (\( p = 0.01 \)) and NCV (\( p = 0.025 \)) were detected in patients with RRMS in the highest quartile status group. Patients with SPMS showed increased T2 LV (\( p = 0.004 \)), T1 lesion number (\( p = 0.017 \)), and LV (\( p = 0.033 \)). No anti-EBV-VCA highest and lower quartile status group MRI outcome differences were found for the patients with PPMS. In Pearson correlation analysis performed in patients with RRMS, there was a significant association between the greater anti-EBV-VCA titer and decreased NGMV (\( r = 0.13, p = 0.019 \)) and NCV (\( r = 0.12, p = 0.034 \)). Patients with SPMS showed a significant association between increased T2 LV and greater anti-EBV-VCA titer (\( r = 0.22, p = 0.011 \)). No associations between the anti-EBV-VCA titers and MRI outcomes were detected in patients with PPMS.

MRI differences according to the anti-EBV-EBNA-1 antibodies. Similar within-group analyses were carried out for anti-EBV-EBNA-1 antibodies. No anti-EBV-EBNA-1 highest and lower quartile status group MRI outcome differences were found for patients with MS, CIS, and OND and HC (table e-2 and figure e-1). In Pearson
correlation analysis, no associations between the anti-EBV-EBNA-1 titers and MRI outcomes were detected in MS, HC, OND, or CIS study groups.

Table e-3 and figure e-1 show anti-EBV-EBNA-1 highest vs lower quartile status group MRI outcome differences for MS subtypes. Patients with RRMS in the anti-EBV-EBNA-1 highest quartile status group showed increased T1 lesion number \((p = 0.035)\) and decreased NGMV \((p = 0.008)\) and NCV \((p = 0.035)\).

Patients with PPMS in the anti-EBV-EBNA-1 highest quartile status group showed increased T2 lesion number \((p = 0.047)\). In Pearson correlation analysis, there was a trend for decreased NGMV \((p = 0.052)\), NCV \((p = 0.074)\), and T1 lesion number \((p = 0.079)\) in patients with RRMS. No anti-EBV-EBNA-1 highest and lower quartile status group MRI outcome differences were found for the patients with SPMS. In Pearson correlation analysis, no associations between the

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### Table 1 Demographic and clinical characteristics of the study groups

|                             | HC \((n = 178)\) | MS \((n = 539)\) | CIS \((n = 66)\) | \(p\) Value |
|-----------------------------|------------------|-----------------|----------------|-------------|
| **Female, n (%)**           | 121 (68)         | 367 (68.1)      | 49 (74.2)      | 0.586       |
| **Age, y, mean (SD)**       | 43.5 (15.7)      | 45.7 (12.2)     | 39.7 (11.0)    | 0.001       |
| **Age at onset, y, mean (SD)** | NA              | 31.5 (10.4)     | 35.2 (10.9)    | 0.008       |
| **Disease duration, y, mean (SD)** | NA              | 14.4 (10.7)     | 1.9 (2.0)      | <0.001     |
| **Presence of DMT, n (%)**  | NA               | 381 (70.7)      | 30 (45.5)      | <0.001     |
| **Interferon \(\beta\)-1a** | 175 (32.5)       | 23 (76.7)       | 110 (20.5)     | 0.110       |
| **Glatiramer acetate**      | 6 (1.1)          | 7 (23.3)        | 2 (0.4)        | 0.004       |
| **Mycophenolate mofetil**   | 76 (14.1)        | 7 (1.3)         | 6 (1.1)        | 0.047       |
| **IV immunoglobulin**       | 4 (0.7)          | 3 (0.6)         | 6 (1.1)        | 0.323       |
| **Azathioprine**            | 15 (8.4)         | 20 (3.7)        | 2 (0.4)        | 0.004       |
| **Mitoxantrone**            | 6 (1.7)          | 2 (0.4)         | 0 (0.0)        | 0.004       |
| **EDSS score, median (IQR)**| NA               | 3.0 (1.5–5.5)   | 1.5 (1.0–2.0)  | <0.001     |
| **Race, n (%)**             |                 |                 |                |             |
| White                       | 155 (87)         | 507 (94)        | 61 (92.4)      | 0.078       |
| Hispanic/Latino             | 2 (1.1)          | 8 (1.5)         | 2 (3)          | 0.338       |
| Black/African American      | 15 (8.4)         | 20 (3.7)        | 2 (3)          | 0.338       |
| Asian                       | 3 (1.7)          | 2 (0.4)         | 1 (1.6)        | 0.338       |
| Other                       | 3 (1.7)          | 2 (0.4)         | 0 (0.0)        | 0.338       |
| **Anti-EBV-VCA, highest vs lower quartiles, n (%)** | | | | |
| Lower quartiles             | 160 (89.9)       | 371 (68.8)      | 44 (66.7)      | <0.001     |
| Highest quartile            | 18 (10.1)        | 168 (31.2)      | 22 (33.3)      | 0.004       |
| **Anti-EBV-EBNA-1, highest vs lower quartiles, n (%)** | | | | |
| Lower quartiles             | 165 (92.7)       | 357 (66.2)      | 47 (71.2)      | <0.001     |
| Highest quartile            | 13 (7.3)         | 182 (33.8)      | 19 (28.8)      | 0.004       |
| **Anti-EBV titer, mean (SD) median** | | | | |
| Anti-EBV-VCA                | 74.7 (46.3)      | 112.1           | 150 (96.1)     | 171 (140)  |
| Anti-EBV-EBNA-1             | 114.7 (27.9)     | 487.1           | 262.7 (131)    | 805 (212.2) |
| **Anti-CMV, n (%)**         |                 |                 |                |             |
| Negative                    | 107 (60.1)       | 311 (57.7)      | 37 (56.1)      | 0.838       |
| Positive                    | 71 (39.9)        | 228 (42.3)      | 29 (43.9)      | 0.838       |
| **Anti-CMV titer, mean (SD) median** | | | | |
| Lower quartiles             | 230 (664)        | 0               | 355 (1,040)    | 0.231       |

Abbreviations: CIS – clinically isolated syndrome; CMV – cytomegalovirus; DMT – disease-modifying therapy; EBNA-1 – Epstein-Barr virus nuclear antigen-1; EBV – Epstein-Barr virus; EDSS – Expanded Disability Status Scale; HC – healthy controls; IQR – interquartile range; MS – multiple sclerosis; NA – not available; VCA – viral capsid antigen. Differences between the groups were tested using \(\chi^2\) test, Mann-Whitney rank-sum, or analysis of variance.
anti-EB- EBNA-1 titers and MRI outcomes were detected in patients with SPMS or PPMS.

MRI differences according to the anti-CMV antibodies. No significant associations between MRI outcomes and anti-CMV antibody positivity or mean titer were found in patients with MS, CIS, and OND or in HC. Because no significant results were found in the MS group, no further analyses regarding MS subtypes were performed.

**DISCUSSION** This cohort study examined association between the humoral response to EBV and MRI outcomes and investigated specifically the effect of this relationship in the cortex of patients with MS in vivo. Moreover, this study investigated whether the association of humoral response to EBV and MRI outcomes is MS-specific. The present study included 63 patients with OND and 178 HC who were age- and sex-matched to the patients with MS and who underwent anti-EBV and anti-CMV antibody determination and standardized 3T MRI with identical study procedures as part of a prospective cardiovascular, environmental, and genetic risk factor study in MS.21,22 We did not find significant associations between humoral response to EBV and CMV and MRI outcomes in patients with OND (even of autoimmune origin) and...
### Differences in MRI measures, according to the anti-EBV-VCA IgG quartile antibody status, in the study groups

|                  | Lower quartiles (n=44) | Highest quartile (n=5) | p Value |
|------------------|------------------------|------------------------|---------|
| **HC (n=178)**   |                        |                        |         |
| No. of CE lesions| 1.8 (1.6)              | 1.5 (1.8)              | 0.219   |
| CE LV            | NA                     | NA                     | NA      |
| T2 LV            | NA                     | NA                     | NA      |
| T1 LV            | NA                     | NA                     | NA      |
| NBV              | NA                     | NA                     | NA      |
| NGMV             | 781.4 (61.1)           | 763.4 (67.2)           | 0.127   |
| NWMV             | 759.9 (43.3)           | 741.9 (64.6)           | 0.172   |
| NCV              | 637.6 (52.9)           | 624.6 (61.9)           | 0.275   |
| NLVV             | NA                     | NA                     | NA      |
| **MS (n=539)**   |                        |                        |         |
| No. of CE lesions| 1.8 (1.9)              | 1.9 (2.0)              | 0.324   |
| CE LV            | NA                     | NA                     | NA      |
| T2 LV            | 0.1 (0.6)              | 0.3 (0.9)              | 0.438   |
| T1 LV            | 2.1 (5.3)              | 3.9 (9.8)              | 0.044   |
| NBV              | NA                     | NA                     | NA      |
| NGMV             | 751.3 (45.0)           | 736.7 (67.2)           | 0.022   |
| NWMV             | 653.7 (67.9)           | 650.4 (68.9)           | 0.972   |
| NCV              | 552 (24.2)             | 552 (24.2)             | 1.000   |
| **CIS (n=66)**   |                        |                        |         |
| No. of CE lesions| 1.8 (1.8)              | 1.9 (2.0)              | 0.653   |
| CE LV            | NA                     | NA                     | NA      |
| T2 LV            | 0.1 (0.4)              | 0.3 (0.9)              | 0.012   |
| T1 LV            | 2.1 (4.6)              | 3.9 (9.8)              | 0.057   |
| NBV              | NA                     | NA                     | NA      |
| NGMV             | 751.3 (45.0)           | 736.7 (67.2)           | 0.022   |
| NWMV             | 653.7 (67.9)           | 650.4 (68.9)           | 0.972   |
| NCV              | 552 (24.2)             | 552 (24.2)             | 1.000   |

**Abbreviations:** CE = contrast-enhancing; CIS = clinically isolated syndrome; EBV = Epstein-Barr virus; HC = healthy controls; MS = multiple sclerosis; LV = lesion volume; NA = not available; NBV = normalized brain volume; NCV = normalized cortical volume; NGMV = normalized gray matter volume; NWMV = normalized white matter volume; VCA = viral capsid antigen.

**Data are presented as mean (SD). All lesion and brain volumes are expressed in cubic milliliters. Differences between groups were tested using analysis of covariance, adjusted for age, sex, and treatment status. The Benjamini-Hochberg correction was used to control the false discovery rate, and p values <0.05 were considered significant (denoted by superscripta).**

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It is well known that mononucleosis, an infection caused by EBV, has been linked to the increased risk of developing MS in susceptible adolescents and young adults. Evidence is mounting that EBV exerts its adverse disease susceptibility effects in MS via interactions with other host-related environmental and genetic factors. Anti-EBV-VCA and -EBNA-1 elevation may indicate a more severe infection among patients with MS. It has been shown that EBV infection–mediated T cell response could lead to cross-reactivity with self-antigens. There is an expansion of activated circulating T cells that are specific for EBV latent and lytic antigens in patients with acute infectious mononucleosis.

The association of EBV-infected B cells located in ectopic follicles and cortical pathology in patients with MS could not be initially replicated. However, more-recent investigations that used sensitive radioactive probes against EBV-encoded RNA have provided further evidence consistent with the presence of EBV-infected cells that are colocalized with innate immune responses characterized by interferon α production in the brain of patients with MS. Higher frequency of CD8+ T cell response to latent and lytic EBV proteins in inactive, untreated patients with RRMS suggests that inability to control EBV infection during inactive MS could set the stage for intracerebral viral reactivation and disease progression. Another study found cells expressing EBV-encoded small RNA of early lytic EBV proteins in the cortical lesion infiltrates of patients with SPMS.

The results of the current study support the hypothesis that EBV-infected B cells may play an important role in the cortical and GM pathology. In fact, we found that the predilection of the brain volume loss in patients with MS was localized specifically to the cortex, as no significant differences were found between patients with MS for the highest and lower quartiles and relative mean titers of anti-EBV-VCA and -EBNA-1 antibodies for the whole brain, WM, or lateral ventricles. Lesions in the GM are frequently recognized in the earliest phases of MS and in patients with radiologically isolated syndrome and could be related to meningeal inflammation and germinal centers in which anti-EBV antibody levels may play an important role. In a previous study,
we reported that GM atrophy was associated with anti-EBV-VCA antibody status in a sample of patients with MS recruited from Italy. The present study confirms these preliminary findings in a 5-fold greater cohort of patients with MS who had different genetic and environment characteristics. It also

Data are presented as mean and SEM. All lesion and brain volumes are expressed in cubic milliliters. The data are displayed for healthy controls (HC) and patients with multiple sclerosis (MS) and clinically isolated syndrome (CIS) on the left and for patients with relapsing-remitting MS (RR), secondary progressive MS (SP), and primary progressive MS (PP) on the right for T2 lesion volume (LV) (A, B), T1 LV (C, D), normalized gray matter volume (NGMV) (E, F), and normalized cortical volume (NCV) (G, H). H = highest quartile; L = lower quartile. *p < 0.05; **p < 0.01.
Table 4 Differences in MRI measures, according to the anti-EBV-VCA IgG quartile antibody status, in patients with MS

|                      | RRMS (n = 342) | SPMS (n = 135) | PPMS (n = 35) |
|----------------------|----------------|----------------|---------------|
|                      | Lower quartile (n = 261) | Highest quartile (n = 108) | p Value | Lower quartile (n = 87) | Highest quartile (n = 48) | p Value | Lower quartile (n = 27) | Highest quartile (n = 8) | p Value |
| No. of CE lesions    | 0.7 (3.1)      | 0.3 (0.6)      | 0.240         | 0.1 (0.5)      | 0.1 (0.4)      | 0.955         | 0.08 (0.3)      | 0 (0)         | 0.479         |
| CE LV                | 0.1 (0.4)      | 0.04 (0.1)     | 0.177         | 0.03 (0.1)     | 0.01 (0.1)     | 0.563         | 0.01 (0.1)     | 0 (0)         | 0.517         |
| No. of T2 lesions    | 28.2 (20.2)    | 28.9 (19.3)    | 0.851         | 32.7 (21.7)    | 36 (21.4)      | 0.363         | 28.9 (18.2)    | 38.8 (36.9)   | 0.312         |
| T2 LV                | 10.8 (12.4)    | 15 (17.5)      | 0.018<sup>a</sup> | 17 (16.6)      | 26.4 (22.9)    | 0.004<sup>a</sup> | 15 (19.3)      | 28.7 (29.4)   | 0.138         |
| No. of T1 lesions    | 9.3 (10.6)     | 122 (12.5)     | 0.039<sup>a</sup> | 13.4 (11.3)    | 18.9 (15.8)    | 0.017<sup>a</sup> | 10.3 (11.4)    | 18 (6.1)      | 0.255         |
| T1 LV                | 2.3 (5.8)      | 3.0 (4.8)      | 0.352         | 3.8 (6.7)      | 6.5 (7.5)      | 0.033<sup>a</sup> | 2.0 (2.7)      | 2.7 (2.5)     | 0.518         |
| NBV                  | 1,492 (92.0)   | 1,475.7 (83.7) | 0.211         | 1,422.9 (77.7) | 1,425.0 (87.1) | 0.702         | 1,441.7 (86.6) | 1,423.6 (93.6) | 0.420         |
| NGMV                 | 751.2 (66.2)   | 730.0 (60.8)   | 0.01*         | 704.1 (60.4)   | 702.2 (57.5)   | 0.987         | 710.2 (42.7)   | 726.8 (48.3)  | 0.478         |
| NWMV                 | 749.1 (58.7)   | 745.7 (68.2)   | 0.641         | 718.8 (72.4)   | 722.8 (64.1)   | 0.654         | 731.5 (73.7)   | 696.8 (60.1)  | 0.216         |
| NCV                  | 607.8 (55.9)   | 592.1 (49.3)   | 0.025<sup>a</sup> | 569.3 (52.3)   | 566.9 (47.2)   | 0.904         | 577.6 (38.6)   | 582.1 (45.1)  | 0.918         |
| NLVV                 | 444.4 (19.4)   | 491.2 (23.6)   | 0.087         | 572.0 (26.0)   | 61.3 (25.1)    | 0.035         | 571.2 (28.1)   | 488 (2.8)     | 0.507         |

Abbreviations: CE = contrast-enhancing; EBV = Epstein-Barr virus; LV = lesion volume; MS = multiple sclerosis; NBV = normalized brain volume; NCV = normalized cortical volume; NGMV = normalized gray matter volume; NLVV = normalized lateral ventricle volume; NWVM = normalized white matter volume; NA = not available; PPMS = primary progressive MS; RRMS = relapsing-remitting MS; SPMS = secondary progressive MS; VCA = viral capsid antigen.

Data are presented as mean (SD). All lesion and brain volumes are expressed in cubic millimeters. Differences between groups were tested using analysis of covariance, adjusted for age, sex, and treatment status. The Benjamini-Hochberg correction was used to control the false discovery rate, and p values ≤ 0.05 were considered significant (denoted by superscript<sup>a</sup>).25
In order to determine whether antibody responses to another nearly ubiquitous virus (anti-CMV) are unique to patients with MS and CIS, we performed an additional analysis exploring anti-CMV positivity and mean titer differences between the study groups. No anti-CMV positivity or mean titer differences were found between patients with MS, CIS, and OND and HC. No associations between MRI outcomes and anti-CMV variables were found in any study group. These findings strengthen our results, indicating that humoral response to anti-EBV antibodies is MS-specific.

This study used an established, standardized method for determination of curve and the concentration of anti-EBV-VCA, anti-EBV-EBNA-1, and anti-CMV antibodies.\(^2,7\) We used quartiles for anti-EBV antibody titers and concentration determination, as they are not normally distributed, or anti-CMV positivity in the statistical analyses.\(^2,7\) However, we also provided mean titer data and conducted Pearson correlation analyses between anti-EBV-VCA, anti-EBV-EBNA-1, and anti-CMV antibodies and MRI outcomes to allow comparison with the previous literature. The ANCOVA and Pearson correlation analyses for the anti-EBV-VCA antibody status and MRI outcomes yielded similar results. However, there were discrepancies between ANCOVA and Pearson correlation results for the anti-EBV-EBNA-1 antibodies and MRI outcomes in patients with RRMS, possibly due to antibody values that were not normally distributed, which could have affected the correlation results.

The methodologic strengths of this study are that EBV and CMV examinations were carried out by expert physicians on a large cohort of HC and patients with MS and CIS in the setting of a specialized MS center and that MRIs was collected with standardized and sophisticated imaging protocol at 3T. However, the study is not without limitations. Use of more specific MRI sequences such as double inversion recovery could have increased the ability to capture part of lesion GM pathology in vivo.\(^19\) The cross-sectional nature of this study cannot establish a causal relationship between EBV and greater cortical atrophy in patients with MS, although findings from the control groups indicated that these associations are MS-specific. A longitudinal 5-year examination of the present cohort is under way in our center to determine the cause–effect relationship of humoral response to EBV and progression of MRI and clinical outcomes.

Our results demonstrate that humoral immune responses to herpesviruses such as EBV are associated with more advanced cortical atrophy and accumulation of chronic T1 white matter lesions in patients with MS. The results provide evidence for further research aimed at modulating the response of patients with MS to these viruses via drug and vaccine strategies.

**AUTHOR CONTRIBUTIONS**

Robert Zivadinov, Bianca Weinstock-Guttman, and Murali Ramanathan substantially contributed to the concept and design of the study. Robert Zivadinov drafted the article, and all authors revised it critically for important intellectual content. Nicole Cerza and Jesper Hagemeier performed statistical analysis. All authors had access to the data.

**STUDY FUNDING**

This study was funded by internal resources of the Buffalo Neuroimaging Analysis Center and Department of Neurology, University of Buffalo. In addition, we received support from the National Multiple Sclerosis Society (RG4836A5, RR2007A2) and Department of Defense (MS090112).

**DISCLOSURE**

R. Zivadinov served on the scientific advisory board for Genzyme and Novartis; received speaker honoraria from Biogen Idec, Claret, EMD Serono, Inc, Novartis, Sanofi-Genzyme, and Teva; has consulted for Biogen, Claret, EMD Serono, Inc, Novartis, Sanofi-Genzyme, and Teva; and received research support from Biogen Idec, Claret, EMD Serono, Inc, Novartis, Sanofi-Genzyme, and Teva. N. Cerza, J. Hagemeier, E. Carl, D. Badgett, and D.P. Ramasamy report no disclosures. B. Weinstock-Guttman served on the scientific advisory board for National Multiple Sclerosis Society; served on the advisory board and speaker engagements for Biogen, Teva, EMD Serono, Pfizer, Novartis, Acorda, Genzyme & Sanofi, and Questcor; is on the editorial board for Multiple Sclerosis International, BMJ Neurology, and Journal of Multiple Sclerosis; has consulted for Biogen, Teva, EMD Serono, Novartis, Genentech, Questcor, and Genzyme & Sanofi; and received research support from Biogen, EMD Serono, Teva, Novartis, Genzyme & Sanofi, Questcor, NIH, NMSS, DOD, National MS Society, and Clinical grant for Pediatric MS Center of Excellence DOD. M. Ramanathan is on the editorial board for AAPS Journal, receives publishing royalties from Pinnacle, Summit, and Zenith; and received research support from Serono, Novartis, Pfizer, Biogen, Department of Defense, and National Multiple Sclerosis Society. Go to Neurology.org/nn for full disclosures.

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Robert Zivadinov, Nicole Cerza, Jesper Hagemeier, et al.

Neurol Neuroimmunol Neuroinflamm 2016;3; DOI 10.1212/NXI.0000000000000190

This information is current as of January 7, 2016