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Teriflunomide's effect on humoral response to Epstein-Barr virus and development of cortical gray matter pathology in multiple sclerosis

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ABSTRACT

Background: Teriflunomide has been shown to slow cortical gray matter (GM) atrophy in patients with multiple sclerosis (MS). Previous work showed that higher levels of Epstein-Barr virus (EBV) are associated with greater development of cortical pathology in MS.

Objectives: To investigate whether the effect of teriflunomide on cortical volume loss in relapsing MS patients may be associated with the change in humoral response to EBV.

Methods: This was a prospective, observational, single-blinded, longitudinal study of 30 relapsing MS patients, who started treatment with teriflunomide, and 20 age- and sex-matched healthy controls (HCs). Subjects were assessed at baseline, 6 and 12 months with clinical, MRI and EBV examinations. MRI outcomes included percent changes in cortical, GM, deep GM and whole brain volumes. Serum samples were analyzed for IgG antibodies titers against EBV viral capsid antigen (VCA) and nuclear antigen-1 (EBNA-1).

Results: There were no significant differences in anti-VCA and anti-EBNA-1 IgG titers between MS patients and HC at baseline. However, over the 12-month follow-up, MS patients experienced a greater decrease in anti-EBNA-1 (-35.1, $p = .003$) and anti-VCA (-15.9, $p = .05$) IgG titers, whereas no significant changes were observed in HCs (-3.7 and -1.6, respectively). MS patients who showed the highest decrease in anti-EBV VCA and EBNA-1 IgG titers from baseline to follow-up, developed less cortical ($p < .001$ and $p = .02$) and GM volume loss ($p = .004$ for both), respectively.

Conclusions: Teriflunomide's effect on slowing cortical and GM volume loss may be mediated by its effect on altering humoral response to EBV.

1. Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system, but many patients also undergo progressive atrophy, especially in the gray matter (GM) (Fisher et al., 2008). GM atrophy plays a particularly prominent role in cognitive and physical decline in MS patients (Fisher et al., 2008). Cortical GM pathology in MS is characterized by the presence of cortical subpial lesions (Kutzelnigg et al., 2005) and leptomeningeal inflammation (Absinta et al., 2015) in the form of ectopic lymphoid-like structures (Magliozzi et al., 2007; Serafini et al., 2004). The leptomeningeal inflammation may act to sustain the intrathecal immune response and engender subpial cortical lesions (Gilmore et al., 2009; Lucchinetti et al., 2011).

It has been hypothesized that dysregulated Epstein-Barr virus (EBV)-infected B cells may induce leptomeningeal inflammation that could contribute to subpial lesions and GM pathology in MS (Serafini et al., 2007; Guan et al., 2019). Previous work has suggested that higher levels of EBV antibodies are associated with increased MRI lesion activity (Buljevac et al., 2005; Farrell et al., 2009; Horakova et al., 2013;

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Zivadinov et al., 2016a) and greater development of brain atrophy, particularly of the cortical GM (Zivadinov et al., 2016a, 2014, 2009; Jakimovski et al., 2019). Therefore, we hypothesized that disease modifying therapies (DMTs) that ameliorate cortical pathology in MS may exert this effect by interacting with the change in humoral response to EBV.

Teriflunomide (Aubagio®) is an oral immunomodulatory therapy approved for treatment of relapsing forms of MS that selectively and reversibly inhibits dihydroorotate dehydrogenase, a mitochondrial enzyme essential for de novo pyrimidine synthesis (Bar-Or, 2014). Teriflunomide modulates glutamatergic dysregulation and microglial density in the cortex-basal ganglia-thalamus circuit, thereby reducing possible excitotoxicity, inflammation, and axonal damage (Modica et al., 2017; Pol et al., 2019). Moreover, in a preclinical Theiler's Murine Encephalitis Virus model of MS, the drug demonstrated an increased rate of viral clearance versus the vehicle placebo (Gilli et al., 2017). A recent *in-vitro* study showed that teriflunomide, at a clinically-relevant dose, may inhibit lytic EBV infection both by preventing the initial steps of viral reactivation and by blocking viral DNA replication, via its impact on host pyrimidine metabolism (Bilger et al., 2017). Teriflunomide demonstrated significant efficacy in the reduction of whole brain atrophy development, compared to placebo (Radue et al., 2017; Zivadinov et al., 2018b) and dimethyl-fumarate, (Zivadinov et al., 2019b) and evidence is mounting that this effect can be primarily driven by its effect on cortical pathology (Zivadinov et al., 2019a,2018a, 2016b).

Against this background, we hypothesized that the effect of teriflunomide on cortical GM pathology may be related to its anti-viral effect on altering humoral response to EBV. Therefore, this study aimed to explore whether the effect of teriflunomide on cortical GM volume loss in relapsing MS patients is associated with the change in humoral response to EBV over 12 months.

2. Methods

This was a prospective, observational, single-blinded, longitudinal, 12-month study of 30 relapsing MS patients, who started treatment with teriflunomide 14 mg orally once daily, and 20 healthy controls (HCs), group-matched for age and sex (ClinicalTrials.gov: NCT01881191). The main results of this study were reported previously, (Zivadinov et al., 2018a) while the current analysis focused on examining the relationship between the humoral response to EBV and development of cortical GM volume loss in the same study subjects. The main inclusion criteria were: a) age 18–65, b) MS according to the McDonald criteria, (Polman et al., 2011) c) relapsing MS, d) Expanded Disability Status Scale (EDSS) scores $\leq$5.5, e) disease duration < 30 years, f) signed informed consent, and g) none of the exclusion criteria. The main exclusion criteria were: a) MS patients with hepatic impairment, b) nursing mothers or pregnant women, c) women of childbearing potential not using reliable contraception, d) patients previously treated with leflunomide, e) clinically significant infectious or neurological illness (for HC only), f) other pathology related to brain MRI abnormalities, and g) abnormal kidney function (creatinine clearance < 59 mL/min) (patients only).

Subjects were assessed at baseline, 6 and 12 months with clinical, 3T MRI and laboratory examinations. Neurologic examinations were not blinded. The MRI and laboratory analyses were rater-blinded. All relapsing MS patients who fulfilled inclusion criteria started teriflunomide 14 mg orally once daily and were followed as per prescribing information recommendations.

The protocol was approved by the University at Buffalo Health Sciences Institutional Review Board and all subjects provided informed consent.

2.1. MRI acquisition

All scans were carried out on a 3T GE Signa Excite HD 12.0 (General Electric, Milwaukee, WI, USA), using a multi-channel head and neck (HDNV) coil. The following scans covering the entire brain were acquired at all time points: 2D multi-planar dual fast spin-echo (SE) proton density (PD) and T2-weighted images (WI): (TE)1/TE2/repetition time (TR) = 9/98/5300 ms, flip angle (FLIP) = 90°, echo train length (ETL) = 14, voxel size $1 \times 1 \times 3\,mm^3$ with no gap; 2D SE T1-WI: TE/TR = 16/600 ms, FLIP = 90°, voxel size $1 \times 1 \times 3\,mm^3$ with no gap; Fluid-Attenuated Inversion-Recovery: TE/TI/TR = 120/2100/8500 ms (TI-inversion time), FLIP = 90°, ETL = 24, voxel size $1 \times 1 \times 3\,mm^3$ with no gap, and 3D high resolution T1-WI using a fast, spoiled, gradient echo with magnetization-prepared inversion recovery pulse (IR-FSPGR), TE/TI/TR = 2.8/900/5.9 ms, FLIP =10°, voxel size $1 \times 1 \times 1\,mm^3$.

2.2. MRI analyses

T2, T1 and gadolinium (Gd) lesion volumes (LVs) were measured using a semi-automated edge detection contouring-thresholding technique, as previously described (Zivadinov et al., 2012). The number of new/enlarging brain T2 lesions was determined using subtraction imaging.

Brain volume measures were determined on 3D T1-WI that were modified by using an inpainting technique to avoid tissue misclassification (Gelineau-Morel et al., 2012). At baseline, whole brain, cortical, GM, WM and ventricular volumes, normalized for head size, were calculated using the SIENAX method, (Smith et al., 2002) whereas for longitudinal changes, the SIENA method was used to calculate the percentage brain volume change (PBVC), (Smith et al., 2002) and the SIENAX multi-time point method (Dwyer et al., 2014) was used to obtain percentage volume changes of cortical (PCVC), GM (PGMVC), WM (PWMVC) and ventricles (PVVC). The deep GM and thalamus volumes and their percentage changes were estimated using FMRIB's Integrated Registration and Segmentation Tool (FIRST) (Patenaude et al., 2011).

2.3. Determination of anti-EBV VCA and EBNA-1 IGG antibodies

Enzyme-linked immunosorbent assay (ELISA) kits from Diamedix Corporation (Miami, FL) were used to quantify anti-EBV viral capsid antigen (VCA) and nuclear antigen (EBNA-1) IgG antibodies on serum samples, as previously described (Horakova et al., 2013, Zivadinov et al., 2016a). Serial dilutions of positive control samples provided with each kit were used to generate standard curves. The EBNA-1 and VCA IgG levels were normalized to the manufacturer's cut-off calibrator standard, which represents a sample that is just positive. The changes in normalized anti-EBV EBNA-1 and VCA IgG levels were calculated. The anti-EBV VCA and EBNA-1 quartiles were determined on the whole study sample.

2.4. Statistical analyses

Analyses were conducted using SPSS for Windows version 24.0 (IBM Corp., Armonk, NY). The demographic and clinical characteristics at baseline were compared using chi-square and Student's *t*-test. Differences between MS patients and HCs were compared using analysis of covariance (ANCOVA), adjusted for age and sex and Mann Whitney rank sum test. Within subject change in anti-EBV VCA and EBNA-1 IgG antibodies were tested using Wilcoxon rank sum test. Spearman correlation analysis was used to test the correlations between anti-EBV VCA and EBNA-1 IgG antibodies at baseline and their absolute changes with longitudinal changes in MRI measures over the follow-up. Nominal p values < 0.05 were regarded as significant, and < 0.1 as a trend, using two-tailed testing.

Table 1
Demographic and clinical characteristics of MS patients and healthy controls.

	HC (n = 20)	MS (n = 30)	P value
Age at baseline, mean (SD) 95% CI	51.3 (9.3) 47.2–55.4	50.9 (8.5) 47.5–53.9	.86
Age of MS onset at baseline, mean (SD) 95% CI	–	26.2 (10.2) 22.5–29.9	–
Disease duration at baseline, mean (SD) 95% CI	–	17.7 (9.1) 14.4–21	–
Sex, n (%)			
Female	17 (85)	23 (76.7)	.37
Male	3 (15)	7 (23.3)	
BMI at baseline, mean (SD) 95% CI	28.8 (6.1) 26.1–31.5	28.9 (4.6) 27.3–30.5	.93
Race, n (%)			
Caucasian	20 (100)	27 (90)	.21
African-American	0 (0)	3 (10)	
EDSS at baseline, median (IQR)	–	3.5 (3.0–5.5)	–
EDSS at month 12 follow-up, median (IQR)	–	3.5 (3.0–5.5)	–
Relapses in previous 12 months to baseline, mean (SD) 95% CI	–	.31 (0.47) 0.14–0.48	–
Relapses in previous 24 months to baseline, mean (SD) 95% CI	–	.35 (0.56) 0.15–0.55	–
New relapses over 12 months follow-up, mean (SD) sum (95% CI)	–	.05 (0.21) 1 (−0.03–0.13)	–
Dropout at 12-month follow-up, n (%)	4 (20%)	8 (26.7%)	.90
DMT in previous 12 months to baseline, n (%)	–		–
Interferon-beta 1a I.M.		15 (50)	
Interferon-beta 1a S.C.		4 (13.3)	
Glatiramer acetate		8 (26.7)	
Natalizumab		3 (1)	

Legend: HC-healthy controls; MS-multiple sclerosis. BMI; body mass index; EDSS-Expanded Disability Status Scale; IQR-interquartile range; DMT-disease modifying therapy; I.M.-intramuscular; S.C.-subcutaneous; 95% CI-95% confidence interval.
P-values derived from chi-squared test and Student's t-test.

3. Results

3.1. Demographic, clinical and MRI characteristics at baseline and over follow-up

Table 1 shows demographic and clinical characteristics of 30 relapsing MS patients and 20 HCs. There were no differences between the groups in age (p = .86), sex (p = .37) or race (p = .21).

The mean disease duration was 17.7 years (SD 10.2) and patients had moderate disability (median EDSS 3.5, interquartile range 3.0–5.5). There was a mean of 0.31 (SD 0.47) relapses in a year previous to the study enrollment. Nineteen (19) of the 30 enrolled patients were on previous DMT with interferon-beta 1a, 8 were on glatiramer-acetate and 3 were on natalizumab (Table 1). The average time between stopping previous DMT and starting teriflunomide was 2.5 (SD 1.3) months. Of the 30 patients, who started teriflunomide, 15 patients switched because they decided to start an oral medication, 8 patients switched because of clinical/radiological progression, and 7 had tolerability/adverse events issues with previous DMT.

Table 1 also shows clinical characteristics of MS patients over the 12-month follow-up. At 6-month follow-up, 4 (13.4%) of MS patients and 2 (10%) HCs dropped from the study (p = .51). At the 12-month follow-up, 4 more MS patients and 2 more HCs dropped from the study, for a total dropout of 8 (26.7%) MS patients and 4 (20%) HCs (p = .90). The reason for the drop-out in all cases lost to follow-up was due to decision of the subjects to not proceed with study schedule requirements. Over the 12-month follow-up, only one MS patient presented a relapse and EDSS remained stable. No serious adverse events were recorded during the 12-months of the study.

Supplement Table shows demographic and clinical characteristics of 22 relapsing MS patients and 16 HCs who completed the 12-month follow-up. The figures were comparable to the entire study population.

3.2. Anti-EBV VCA and EBNA-1 IGG quartile antibody status, at baseline and over follow-up

Table 2 shows baseline and follow-up differences in anti-EBV VCA or EBNA-1 IgG titers or their quartile's status between MS and HCs. MS patients showed numerically higher anti-EBV VCA or EBNA-1 IgG titers at baseline, compared to HC, but these differences did not reach significance. Similar number of MS patients and HC showed the highest quartile status. At baseline, there were no differences in anti-VCA (195.8 vs. 75, p = .25) and anti-EBNA-1 (93.1 vs 126.8, p = .57) IgG titers of MS patients who were lost (n = 8) or completed (n = 22) the follow-up.

Over the follow-up, MS patients experienced a significant decrease in anti-EBNA-1 (−35.1, p = .003) and anti-VCA (−15.9, p = .05) IgG titers, whereas no significant changes were observed for HCs in anti-EBNA-1 (−3.7, p = .86) and anti-VCA (−1.6, p = .74). There was a significantly higher decrease in anti-VCA between MS patients and HCs (p = .05) and a trend for decrease in anti-EBNA-1 (p = .09). In addition, there was a trend for fewer MS patients being categorized in the highest quartile of anti-EBNA-1 compared to HC at the 12-month follow-up (p = .08).

3.3. Lesion and brain volume changes between baseline and follow-up, according to the anti-EBV quartile's status

Table 3 shows baseline lesion and brain volumetric characteristics between MS patients and HCs, according to the anti-EBV quartile's status. At baseline, MS subjects with the highest quartile of anti-EBV VCA showed significantly lower total deep GM (p = .03) volumes and increased T1-LV (p = .01). No significant differences in MRI measures according to the anti-EBV VCA quartile's status was found in HCs, or for EBNA-1 neither in MS patients nor HCs.

No significant PBVC, PCVC, PGMVC, PVVC, deep GM or thalamic percent volume changes were found between baseline to 12 months between HCs and MS patients (Table 4) (Zivadinov et al., 2018a).

3.4. Relationship between anti-Epstein-Barr virus IGG titer and longitudinal MRI measures in multiple sclerosis patients

Table 5 shows the correlation analysis between anti-EBV VCA and EBNA-1 IgG antibodies at baseline and their absolute changes, and longitudinal changes in MRI measures in MS patients over the follow-up. There were no significant associations between baseline anti-VCA or anti-EBNA-1 IgG titers status and changes in MRI measures over the 12-month follow-up.

A decrease in anti-EBV VCA and EBNA-1 IgG titers from baseline to follow-up was associated with less cortical (r = −0.72, p < .001 and

Table 2
Anti-Epstein-Barr virus viral capsid antigen IgG quartile antibody status, in healthy controls and multiple sclerosis patients at baseline and over the follow-up.

	HC	MS	P value
Anti-EBV VCA at baseline, mean (SD) 95% CI	84.7 (98.8) 41.4–128 *n* = 20	104 (145.8) 52.8–156.2 *n* = 30	.63
Anti-EBV VCA at 6 months, mean (SD) 95% CI	90.5 (110.3) 39.5–141.6 *n* = 18	82 (87.8) 48.3–115.6 *n* = 26	.81
Anti-EBV VCA at 12 months, mean (SD) 95% CI	99 (125) 37.8–160.3 *n* = 16	59.7 (49.4) 39.1–80.3 *n* = 22	.19
Anti-EBV VCA absolute change between baseline to 12 months, mean (SD) 95% CI	−1.6 (15.9) −9.4–6.2 *n* = 16	−15.9 (36.3) −31.1- −0.7 *n* = 22	**.05**
Anti-EBV EBNA-1 at baseline, mean (SD) 95% CI	79.6 (114.4) 29.5–129.7 *n* = 20	118.6 (134.3) 70.5–166.7 *n* = 30	.26
Anti-EBV EBNA-1 at 6 months, mean (SD) 95% CI	66.3 (111.5) 14.8–117.9 *n* = 18	103.6 (125.5) 55.4–151.8 *n* = 26	.48
Anti-EBV EBNA-1 at 12 months, mean (SD) 95% CI	76.7 (160.6) 2.5–150.9 *n* = 16	66.9 (57.6) 42.8–90.9 *n* = 22	.80
Anti-EBV EBNA-1 absolute change between baseline to 12 months, mean (SD) 95% CI	−3.7 (69.1) −37.6–30.2 *n* = 16	−35.1 (48.2) −55.2- −15 *n* = 22	*.09*
Subjects with highest anti-EBV quartiles at baseline, n (%)			
Anti-EBV VCA	4 (20)	6 (20)	.99
Anti-EBV EBNA-1	3 (15)	7 (23.3)	.47
Subjects with highest anti-EBV quartiles at 12 months, n (%)			
Anti-EBV VCA	5 (31.3)	2 (9.1)	*.08*
Anti-EBV EBNA-1	3 (18.8)	4 (18.2)	.96

Note: HC-healthy control; MS-multiple sclerosis; EBV-Epstein-Barr virus; VCA-Viral capsid antigen; EBNA-1-Epstein-Barr nuclear antigen 1; 95% CI-95% confidence interval.
P-values derived from analysis of covariance, adjusted for age and sex for anti-EBV mean and Mann Whitney rank sum test for absolute changes between subject groups. P-values derived from chi-squared between subject groups for anti-EBV quartiles. In bold are displayed p values < 0.05 and in italic p values < 0.1.

$r = -0.49$, $p = .02$) and GM volume loss in MS patients ($r = -0.58$ and $r = -0.59$, $p = .004$ for both), respectively (Fig. 1).

4. Discussion

This is one of the first studies to show that altering humoral response to EBV by teriflunomide may result in slowing down of cortical GM volume loss in MS patients. Our study suggests that future strategies toward slowing down cortical GM pathology in MS should also consider evaluation of the effect on humoral response to EBV. In addition, we found that treatment with teriflunomide significantly decreased IgG titer of anti-EBNA-1 and anti-VCA over the 12-month period in MS patients, while the levels of these antibodies remained stable in HC, suggesting a potential anti-viral effect of teriflunomide.

The etiology of MS is poorly understood but is likely a result of a combination of genetic and environmental factors, among which viral exposure appears to play an important role (Guan et al., 2019). Exposure to EBV has been linked to an increased risk of MS, and can be assessed in the clinical setting using anti-EBV antibody panels and by measuring viral load (Guan et al., 2019). Anti-EBV EBNA-1 antibodies appear 2–4 months after infection, while anti-EBV VCA antibodies develop early in EBV infection, and both persist for the remainder of the host's life. Elevated titers in MS patients were found against both the anti-VCA, which is expressed in the viral replicative cycle, and the anti-EBNA-1, which is expressed in latently infected B lymphocytes (Guan et al., 2019; Lucas et al., 2011). About 40% of MS patients have their anti-EBV VCA and EBNA-1 IgG titers in the highest quartiles, (Zivadinov et al., 2016a) especially in those individuals who have increased genetic susceptibility for MS, (Guan et al., 2019) indicating a different immune response. The discovery of tertiary lymphoid follicles within leptomeninges of MS brain containing large proportion of EBV-activated B-cells and T-cells, suggested an alternative route of entry for

Table 3
Baseline differences in MRI measures, according to the anti-Epstein-Barr virus viral capsid antigen IgG quartile antibody status, in healthy controls and multiple sclerosis patients.

	HC (*n* = 20)			MS (*n* = 30)		
	Lower quartiles (*n* = 16)	Highest quartile (*n* = 4)	P value	Lower quartiles (*n* = 24)	Highest quartile (*n* = 6)	P value
Number of CE lesions	–	–	–	.4 (1.2) −0.08–0.9	0 (0)	.50
CE-LV	–	–	–	33.0 (100.9)	0 (0)	.40
Number of T2 lesions	9.6 (15.9) 1.9–17.4	13.0 (14.6) −1.3–14.6	.304	22.6 (13.8) −7.4–73.4	27.2 (12.5) 17.2–37.2	.62
T2-LV	1.9 (4.8) −0.5–4.3	1.3 (1.9) −0.5–3.2	.826	9.9 (16.2) 3.5–16.4	24.6 (18.3) 10–39.2	*.08*
Number of T1 lesions	–	–	–	7.2 (6.9) 4.4–10	12.8 (7.0) 7.2–18.4	*.06*
T1-LV	–	–	–	1.3 (2.0) 0.5–2.1	3.5 (2.5) 1.5–5.5	**.01**
NBV	1514.5 (65.5) 1482.4–1546.6	1542 (67.7) 1475.7–1608.3	.442	1463 (98.8) 1423.5–1502.5	1455 (114.4) 1363.5–1546.5	.39
NGMV	758 (24.2) 746.1–769.9	792 (56.9) 736.2–847.8	.506	730 (55.1) 707.9–752	716.6 (69.6) 660.9–772.3	.17
NWMV	757 (50.0) 732.5–781.5	749 (25.5) 724–774	.212	732 (48.6) 712.6–751.4	740.3 (55.7) 695.7–784.9	.85
NCV	615 (24.5) 603–627	642 (37.9) 604.9–679.1	.422	596 (47.2) 577.1–614.9	580.1 (61.3) 531.1–629.2	.13
NLVV	39.1 (16.3) 31.1–47.1	27.7 (5.5) 22,3–33.1	.451	465 (18.5) 457.6–472.4	53.4 (19.6) 37.7–69.1	.36
Total DGM	60.4 (3.9) 58.5–62.3	61.7 (7.3) 54.5–68.9	.431	56.9 (6.7) 54.2–59.6	50.6 (6.9) 45.1–56.1	**.03**
Thalamus	20.5 (1.3) 19.9–21.2	21.1 (1.9) 19.2–23	.740	19.0 (2.4) 18–19.9	17.8 (2.4) 15.9–19.7	.16

Legend: MS- multiple sclerosis; CE – contrast enhancing; LV – lesion volume; NBV – normalized brain volume; NGMV - normalized gray matter volume; NWMV - normalized white matter volume; NCV – normalized cortical volume; NLVV – normalized lateral ventricle volume; 95% CI-95% confidence interval.
Data are presented as mean, (standard deviation) and 95% CI. All lesion and brain volumes are expressed in milliliters.
P-values derived from analyses of covariance, adjusted for age and sex. In bold are displayed p values < 0.05 and in italic p values < 0.1.

Table 4
Change in lesion and whole brain volume measures from baseline to 12 months between MS patients and healthy controls.

	HC (n = 16)	MS (n = 22)	P value
T2 total new lesions	–	.15 (0.37) 3 −0.005–0.3	–
T1 total new lesions	–	.21 (0.42) 3 0.03–0.4	–
Abs change in T2-LV	–	.11 (1.35) −0.5–0.7	–
Abs change T1-LV	–	.36 (0.70) 0.07–0.7	–
PBVC	−0.40 (0.5) −0.6- −0.2	−0.56 (0.91) −0.9- −0.2	.31
PGMVC	−0.42 (1.7) −1.3–0.4	−0.77 (1.2) −1.27- −0.3	.52
PWMVC	.05 (1.7) −0.8–0.9	−0.23 (1.2) −0.7–0.28	.76
PCVC	−0.1 (0.3) −0.25–0.05	−0.97 (1) −1.39- −0.6	*.06*
PLVVC	1.48 (3.2) −0.9–3.1	3.22 (4.7) 1.3–5.2	.26
Percent DGM volume change	.75 (2.9) −0.68–2.2	−0.76 (2) −1.6–0.08	*.09*
Percent thalamus volume change	.67 (1.6) −0.1–1.5	.36 (2.6) −0.7–1.5	.34

Legend: HC-healthy control; MS-multiple sclerosis; Abs-absolute; PBVC- percent brain volume change; PGMVC- percent gray matter volume change; PWMVC- percent white matter volume change; PCVC- percent cortical volume change; PLVVC: percent lateral ventricle volume change; DGM-deep gray matter; 95% CI-95% confidence interval.
Lesion numbers are present as mean (SD) sum (95% CI). Absolute (abs) and percent changes are presented as mean (SD), 95% CI. Absolute volume changes are presented in milliliters.
P-values derived from analyses of covariance, adjusted for age and sex. In bold are displayed p values < 0.05 and in italic p values < 0.1.

lymphocytes into the cortical regions (Magliozzi et al., 2007; Serafini et al., 2007). In fact, self-antigen presentation by EBV-immortalized B-cells may be important for the reactivation of T-cells found in the subarachnoid space and may potentially contribute to cortical subpial lesion pathology (Louveau et al., 2016; Magliozzi et al., 2013). The association between altered humoral response to EBV and GM pathology in MS was previously corroborated in clinical and MRI studies. In a 3-year longitudinal study, the increased anti-VCA IgG titer was negatively correlated with decrease of GM volume (Zivadinov et al., 2009). In a large cohort cross-sectional study, MS patients with the highest anti-VCA IgG titer showed the lowest cortical GM volume, while patients with the highest anti-EBNA-1 quartile showed increased T1 hypointense lesion number and decreased GM volume compared to the lower quartile group (Zivadinov et al., 2016a). In a recent cross-sectional study of 101 MS patients, increased anti-EBNA-1 titer was associated with decreased magnetization transfer ratio of T1 hypointense lesions and of the normal appearing GM, indicating that greater humoral EBV response is associated with greater demyelination of the GM and focal destructive pathology in MS patients (Jakimovski et al., 2019). This leads us to hypothesize that DMTs that slow down cortical pathology in MS may exert this effect by interacting with the change in humoral response to EBV. In order to test this hypothesis, we used teriflunomide, an MS treatment that inhibits a key enzyme (mitochondrial dihydroorotate-dehydrogenase) in the de-novo pyrimidine synthesis pathway, a mechanistic pathway that may be involved in preventing proliferation of EBV-immortalized B-cells and development of cortical pathology in MS.

The extent of cortical pathology in MS patients can be indirectly assessed by measuring cortical atrophy (Absinta et al., 2015, Zivadinov et al., 2016a, 2009, 2018a). Increasing evidence suggests that teriflunomide can slow down cortical GM volume loss from the earliest clinical stages (Zivadinov et al., 2019a, 2018a, 2016b). In the post-hoc analysis of the phase-III TOPIC study, (Miller et al., 2014) it has been shown that teriflunomide reduced significantly cortical GM volume loss versus placebo over 24 months in patients with clinically isolated syndrome, (Zivadinov et al., 2016b) and that this effect was even more robust compared to its effect on whole brain volume, which was also significant. (Zivadinov et al., 2018b) In a recent head-to-head retrospective, single-center, observational study that followed 60 teriflunomide- and 60 dimethyl fumarate-treated MS patients over 24 months, teriflunomide showed significantly lower rates of cortical and GM volume loss compared to dimethyl fumarate, suggesting that teriflunomide may have a protective effect against cortical GM atrophy. (Zivadinov et al., 2019a) In the present study, MS patients treated with teriflunomide showed only a trend for more cortical GM volume loss compared to HC over 12 months. (Zivadinov et al., 2018a) It is unknown whether the potential anti-viral effect of teriflunomide, detected in preclinical and *in-vitro* studies, (Modica et al., 2017; Gilli et al., 2017; Bilger et al., 2017) could contribute to slowing the development of cortical pathology in MS patients by altering humoral response to EBV.

Table 5
Relationship between anti-Epstein-Barr virus IgG titer and longitudinal MRI measures in multiple sclerosis patients using Spearman correlation coefficients.

	Anti-EBV VCA at baseline	Anti-EBV VCA absolute change	Anti-EBV EBNA-1 at baseline	Anti-EBV EBNA-1 absolute change
T2 total new lesions	0	.38	.09	.10
Abs change T2-LV	.07	.11	.15	.17
Abs change T1-LV	.28	.07	.30	.15
Abs change CE-LV	−0.09	−0.002	−0.30	.05
PBVC	−0.01	−0.39	−0.40	−0.28
PGMVC	.05	**−0.58****	.21	**−0.59****
PWMVC	−0.21	.21	−0.46	.33
PCVC	−0.06	**−0.72****	−0.12	**−0.49***
PLVVC	.15	.15	.28	.24
Percent DGM volume change	.02	−0.22	.06	−0.13
Percent thalamus volume change	.18	−0.10	.08	−0.10

Legend: EBV-Epstein-Barr virus; VCA-Viral capsid antigen; EBNA-Epstein-Barr nuclear antigen; Abs-absolute; LV-lesion volume; CE- contrast enhancing; PBVC-percent brain volume change; PGMVC- percent gray matter volume change; PWMVC- percent white matter volume change; PCVC- percent cortical volume change; PLVVC: percent lateral ventricle volume change;.
P-values derived from Spearman correlation.
** $p < .01$,.
* $p < .05$. In bold are displayed significant p values.

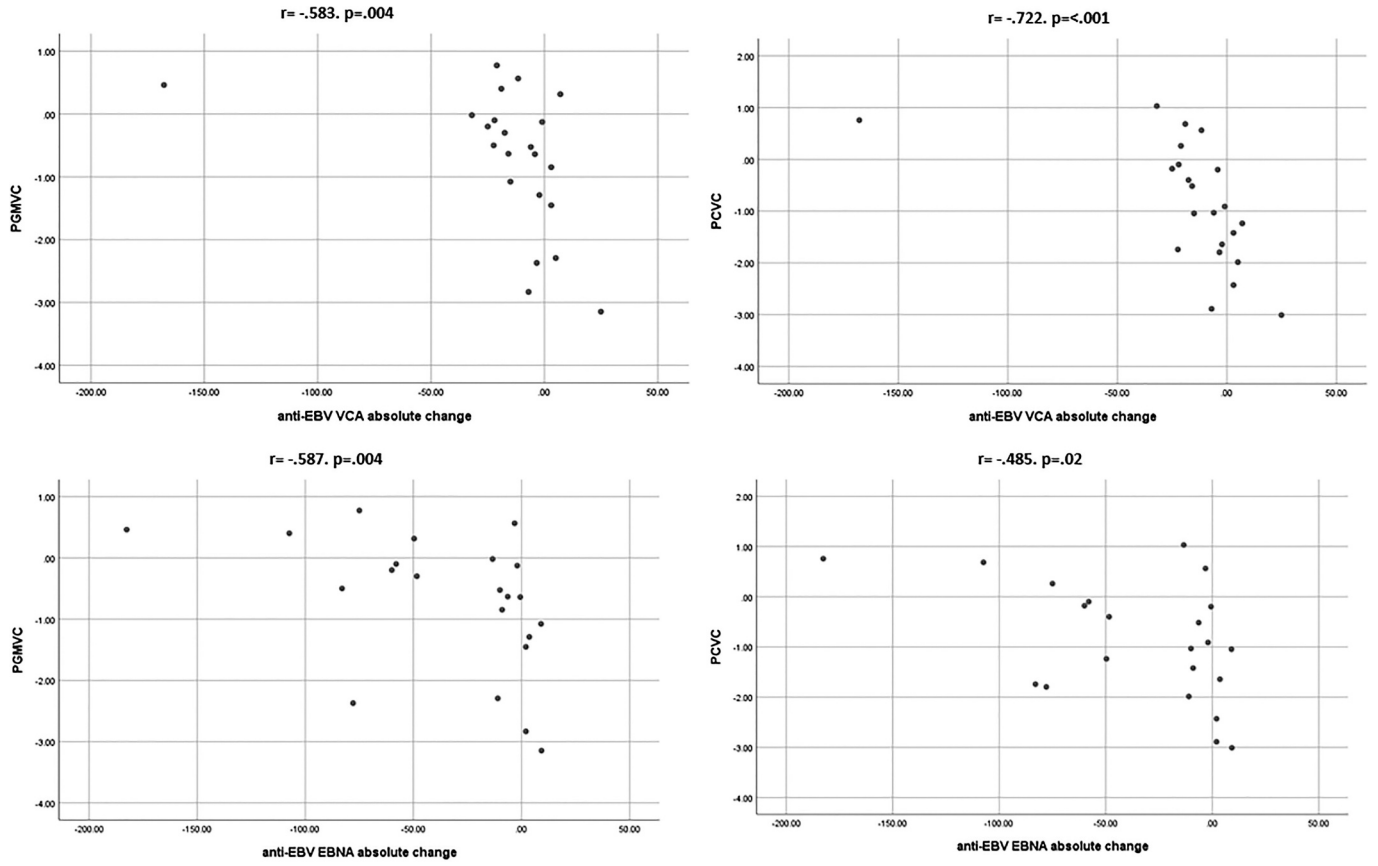

Fig. 1. Relationship between percent gray matter and cortical volume changes and absolute changes in anti-Epstein-Barr virus viral capsid and nuclear 1 antigen IgG titers in multiple sclerosis patients over 12 months. **Legend:** EBV-Epstein-Barr virus; VCA-Viral capsid antigen; EBNA-Epstein-Barr nuclear antigen; PGMVC- percent gray matter volume change; PCVC- percent cortical volume change. P-values derived from Spearman correlation.

In the present study, we found in the correlation analysis that decreases in anti-EBV VCA and EBNA-1 IgG titers from baseline to follow-up were associated with less cortical and GM volume loss in MS patients (Fig. 1). However, although we detected a significant relationship between reduced anti EBV VCA and anti EBNA titers, reduced cortical/GM atrophy and teriflunomide treatment, we are not able to infer on the basis of these findings that the impact of teriflunomide on EBV is uniquely related to the observed reduced atrophy rate. In order to prove this hypothesis, serial cerebrospinal fluid EBV viral load measurement would be necessary to determine if teriflunomide cleared EBV from the CNS. The decrease in anti EBV immune globulin levels could also reflect impairment of immune globulin synthesis as a direct effect of impaired de novo pyrimidine synthesis. Therefore, the potential anti-viral effect of teriflunomide on development of cortical pathology and its effect on EBV clearance needs to be explored further.

In addition, we found a significant decrease of anti-EBV EBNA-1 and VCA IgG titers over 12 months in MS patients treated with teriflunomide, while the levels of these antibodies remained stable in HCs. Also, there was a trend for fewer MS patients being categorized in the highest quartile of anti-EBNA-1 compared to HC at the 12-month follow-up. While the mechanism of action of teriflunomide leading to the anti-viral effect against EBV is not clear at this time, a recent *in-vitro* study (Bilger et al., 2017) showed that teriflunomide inhibits cellular proliferation, and promotes apoptosis in EBV-transformed B cells at a clinically relevant dose. In addition, teriflunomide prevented the development of EBV-induced lymphomas in both a humanized mouse model and a xenograft model by probably inhibiting lytic EBV infection in vitro, both by preventing the initial steps of lytic viral reactivation (an effect not rescued by uridine supplementation), and by blocking lytic viral DNA replication (largely rescued by addition of uridine). (Bilger et al., 2017) In support of this possible anti-viral effect of teriflunomide, it has been also shown that its predecessor, leflunomide, decreased EBV DNA viral load over 6 months in patients with rheumatoid arthritis. (Valleala et al., 2010) Evidence from other preclinical and clinical studies indicate that leflunomide or teriflunomide inhibited the replication of a broad range of viruses, including herpes simplex virus-1, polyoma BK virus and cytomegalovirus (Josephson et al., 2006; Knight et al., 2001).

DMTs may interfere with the rate and extent of the persistent EBV infection in MS (Guan et al., 2019). A number of previous studies with interferon-beta, glatiramer acetate and natalizumab explored whether these DMTs may regulate humoral response against EBV, without conclusive findings (Guan et al., 2019). The present study contributes to this increasing body of evidence, which shows that DMTs may alter humoral response to EBV in MS.

The present study has some limitations to be considered. A major limitation was the small sample size and limited follow-up time of 12 months. As such, the results have to be interpreted with caution as there is an increased risk for type II error. The study was not powered to examine differences in anti-EBV humoral response respect to HCs or MS patients treated with other DMTs, however the observed absolute change differences in IgG titers, can be used to better power future studies in this direction. Furthermore, 26.7% of the MS patients and 20% of HCs were lost to follow-up at 12 months of the study. However, the post-hoc analysis of baseline subject characteristics of those patients who completed or dropped during the study, showed no difference in EBV, clinical and MRI outcomes.

In conclusion, our findings suggest that the effect of teriflunomide on slowing cortical GM atrophy may be mediated by its effect on altering humoral response to EBV.

Declarartion of Competing Interest

None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2019.101388.

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