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Differential distribution of antiviral serology across multiple sclerosis phenotypes and its implications for disease pathogenesis

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Multiple sclerosis (MS) is a heterogeneous inflammatory disease of the central nervous system in which environmental factors, particularly viral infections, are thought to contribute to disease susceptibility and progression. However, whether antiviral immune responses differ across clinical phenotypes remains incompletely understood. In this study, we analyzed the distribution of antiviral serology in patients with primary progressive, relapsing–remitting, and secondary progressive MS, comparing them with healthy controls. Serum IgG responses against several neurotropic and non-neurotropic viruses were assessed and evaluated according to disease phenotype, clinical characteristics, and disability measures. We observed distinct serological profiles among MS subtypes, with differential patterns of antiviral antibody prevalence and titers. Notably, specific antiviral responses were associated with progressive forms of the disease and with markers of greater clinical severity. These findings suggest that antiviral immunity is not uniform across MS phenotypes and may reflect divergent pathogenic mechanisms underlying disease progression. Our results support a potential role for virus–host interactions in shaping the clinical course of MS and highlight antiviral serology as a complementary tool to improve disease stratification. Understanding these immune signatures may contribute to refining pathogenic models and identifying novel targets for personalized therapeutic strategies.

Keywords Multiple sclerosis, Epstein-Barr virus, Cytomegalovirus, Human Herpesvirus 6, sNfL, sGFAP

Multiple sclerosis (MS) is a chronic, immune-mediated disease of the central nervous system characterized by inflammation, demyelination, and neurodegeneration. It typically manifests in young adults and more frequently in women. Genetic predisposition and environmental factors such as viruses, vitamin D deficiency, low sun exposure, metabolites from the microbiota, smoking or obesity in adolescence have been implicated in disease risk[1,2].

Among environmental factors, infectious agents, especially viruses, have long been considered potential triggers or modulators of MS. Epidemiological and molecular studies have consistently pointed to several

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viruses, with the strongest evidence for Epstein-Barr virus (EBV)[3]. Additional viruses implicated include human herpesvirus 6 (HHV-6), cytomegalovirus (CMV), and human endogenous retroviruses (HERVs)[4,5]. Mechanisms proposed include molecular mimicry, bystander activation, epitope spreading, direct infection of CNS-resident cells, and immune modulation by latent or reactivated viruses[6,7]. Furthermore, brain tissue studies have shown EBV latent and lytic proteins in MS lesions, suggesting that beyond peripheral immune activation, direct viral involvement in the CNS may contribute to pathology[8].

However, MS is clinically heterogeneous. About 85% of patients present with a relapsing-remitting course (RRMS), characterized by acute inflammatory episodes (relapses) followed by periods of remission, while a smaller proportion have primary progressive MS (PPMS), in which disability accumulates steadily without relapses. Over time some RRMS patients transition to a secondary progressive phase (SPMS), where progression occurs largely independent of relapses. But, despite the existing literature on viruses in MS, few studies reported findings by disease course. Thus, as most of our current knowledge is based on RRMS, there remains a gap in understanding the viruses linked to SPMS or PPMS.

For this reason, we analyzed nearly 700 individuals from the following cohorts: PPMS, RRMS, SPMS, and healthy controls (HC). In these subjects, we assessed the prevalence and titers of EBNA-1 and VCA IgG from EBV, CMV IgG, and HHV-6 A/B IgG and IgM. To better understand the possible role of these viruses in the different clinical forms of the disease, antiviral antibody titers were correlated with two biomarkers of neuronal and glial damage, serum neurofilament light chain (sNfL) and serum glial fibrillary acidic protein (sGFAP). Elevated sNfL reflects acute or ongoing axonal injury, while elevated sGFAP is increasingly linked to astrocytic activation and progressive disease.

Methods

Study design

A multicentric observational cross-sectional study. Samples from PPMS, RRMS, SPMS, and HC were collected. MS patients were recruited from the following Hospitals in Spain: Hospital General Universitario Gregorio Marañón, Hospital Universitario de Getafe, Hospital Universitario de Torrejón, Hospital Universitario Quirónsalud Madrid, and Hospital Universitario Ramón y Cajal. HC were recruited from volunteered blood donors of Hospital Clínico San Carlos.

MS patients met the following inclusion criteria: (1) older than 18 years, (2) a diagnosis of PPMS, RRMS or SPMS according to 2010 or 2017 revised McDonald diagnostic criteria as appropriate, (3) gave the informed consent. Exclusion criteria for MS patients: (1) pregnant women, (2) MS patients with other concomitant neurological or autoimmune pathologies, (3) corticosteroid treatment in the last 3 months previous to the sample collection. Inclusion criteria for HC: (1) older than 18 years, (2) gave the informed consent. Exclusion criteria for HC: (1) pregnant women, (2) HC having any relationship of consanguinity with MS patients or patients with other autoimmune diseases.

Ethics statement

This study was conducted in accordance with the Declaration of Helsinki, and it was approved by the local Ethic Committee of the Hospital Clínico San Carlos (Comité Ético de Investigación Clínica del Hospital Clínico San Carlos).The informed consents were collected from all the subjects recruited in this study.

Data and sample collection

Data collection includes the following variables: (1) demographical variables: sex and age; (2) clinical variables at the sample collection: age at the disease onset, disease duration, EDSS score, MSSS (MS severity score), number of relapses since the disease onset, and in the last two years; (3) radiological variables at the sample collection: number of T2 and gadolinium-enhanced lesions at magnetic resonance imaging (MRI). In Table 1 is summarized the data collected from each cohort.

A serum sample was obtained by venipuncture from each patient included in the study. Samples were collected in a dry tube (BD vacutainer) for serum isolation. Aliquots were cryopreserved and eventually stored at -80 °C until use.

Antiviral serologies by automated ELISA

The following antibody titers were assessed using commercial tests: anti-EBNA-1 (Epstein-Barr nuclear antigen 1) and anti-VCA (Viral Capsid Antigen) IgG (Trinity Biotech, USA), both from EBV, anti-CMV IgG (Vircell, USA), and anti-HHV-6 A/B IgG and IgM (Vidia, Ltd., Czech Republic). The analysis was performed in an automated ELISA processing system (DS2, Dynex Technologies, USA). Results were reported in artificial units (AU), calculated by multiplying the index value by 10 (where the index value is the sample absorbance divided by the cut-off value)[9]. Each sample was tested in duplicate. Values below 9 AU were considered negative, while samples with values between 9 and 11 AU were reanalyzed.

Quantification of sNfL and sGFAP

Serum NfL (sNfL) and serum SGFAP (sGFAP) levels were measured using the Simoa NFLight™ Advantage Kit and the Simoa™ GFAP Discovery Kit, respectively (Quanterix, Billerica, MA, USA), on an SR-X instrument (Quanterix, Billerica, MA, USA), following the manufacturer's instructions. sNfL values were expressed as Z-scores, which adjust raw concentrations for age and body mass index[10]. Z-scores were calculated using the Serum Neurofilament Light Chain Reference App.

	RRMS (*n* = 280)	SPMS (*n* = 78)	PPMS (*n* = 166)	HC (*n* = 163)
Sex (male/female)	63/217	24/74	83/83	41/122
Age at sampling (years, m ± SD)	37.6 ± 9.0	45.5 ± 9.5	51.0 ± 13.8	38.6 ± 9.4
Age at MS onset (years, m ± SD)	31.2 ± 8.7	29.4 ± 10.7	40.8 ± 11.3	-
Disease duration (months, md (P25-P75))	54.5 (7.3-123.8)	180.0 (115.0-252.5)	94.6 (52.0-157.7)	-
EDSS score at sampling (md (P25-P75))	1.0 (0.0–2.0)	6.0 (5.0-6.5)	5.0 (3.5–6.5)	-
Relapses two years earlier (md (P25-P75))	1 (0–2)	1 (0–2)	0 (0–0)	-
Gadolinium-enhancing lesions (md (P25-P75))	0 (0–2)	0 (0–1)	0 (0–1)	-
T2 lesions at baseline (md (P25-P75))	23 (9–38)	43 (24–61)	25 (10–45)	-
Previous treatments: Naïve METs* HETs**	 174 90 16	 13 35 30	 133 9 24	 - - -

Table 1. Demographic and clinical characteristics of the multiple sclerosis (MS) patients and healthy controls (HC) included in the study. Continuous non-parametric variables are expressed as median (25th, 75th percentile) [md (P25-P75)] whereas parametric ones as mean ± standard deviation (m ± SD). * Moderate efficacy therapies (METs): beta-interferon, glatiramer acetate, dimethyl fumarate and teriflunomide; ** high-efficacy treatments (HETs): natalizumab (8-RRMS, 23-SPMS, 4-PPMS), fingolimod (8-RRMS, 7-SPMS, 17-PPMS) and azathioprine (3-PPMS).

Statistical analysis

Categorical variables were expressed as percentages, normal numerical variables as mean ± standard deviation, and non-normal as median (25th, 75th percentile). The association between/among categorical variables was analyzed using the Chi-square $\chi 2$ test, or Fisher's exact test when the value of the expected count less than 5 is more than 20%. For the quantitative variables, the means will be compared using the test Student's t-test or analysis of variance (ANOVA, for comparisons of more than two groups) or the Mann-Whitney U test, or the Kruskall Wallis test (to compare the medians of several groups) in case the quantitative variables did not fit a normal distribution. The parametric Pearson coefficient or the nonparametric Spearman coefficient will be applied to evaluate the correlation between two continuous quantitative variables. Subjects with missing data were omitted from the corresponding analyses. P-values < 0.05 were referred to as statistically significant in the text. When necessary, the Bonferroni adjustment will be carried out. Linear Discriminant Analysis (LDA) was applied to continuous viral predictive markers to identify linear functions that best discriminate the MS-clinical cohorts. Predictive accuracy was assessed using leave-one-out cross-validation. Analyses were performed using SPSS v28 (IBM, USA), R Studio v4.5.0 and Prism version 8.0 (GraphPad Prism, San Diego, CA, USA).

Results

Human herpesviruses prevalences and titers in the different MS clinical forms and in healthy controls

When we compared the prevalences and viral titers of the human herpesviruses included in the study, we found that EBNA-1 IgG titers were lower in PPMS (21.4 AU) group than in both RRMS (24.2 AU) ($p = 0.0004$) and SPMS (24.4 AU) ($p = 0.00003$) groups; although they were not different from those of the HC group (20.8 AU) ($p = 0.323$) (Table 2). Regarding EBV VCA IgG titers, they were higher in PPMS (49,9 AU) patients than in HC (44.0 AU) ($p = 0.00004$). On the contrary, CMV IgG titers were significantly higher in the PPMS group (30.1 AU) than in RRMS group (15.8 AU) ($p = 0.000002$) and SPMS group (24.5 AU) ($p = 0.030$); similarly, prevalence of detectable CMV IgG was higher in the PPMS group (73.4%) than in RRMS group (55.9%) ($p = 0.0006$; O.R.=2,2). Finally HHV-6 A/B IgM titers were higher in RRMS patients than in SPMS patients ($p = 0.002$). Results are shown in Table 2.

Human herpesviruses prevalences and titers and demographic variables

After stratifying by sex, we observed statistically significant associations in both males and females that were consistent with those found in the overall population (Supplementary Table 1). When we compared males and females in each one of the cohorts, we did not find differences for EBV. For HHV-6 A/B, only the prevalence of IgG antibodies was higher in female HC than in males ($p = 0.008$; O.R.=4.6). Regarding CMV prevalences and titers, they were higher in female than in males in all the cohorts, preserving statistical signification after Bonferroni correction in PPMS and RRMS cohorts (results are shown in Supplementary Table 1).

Regarding age, HHV-6 A/B IgG was inversely correlated and CMV IgG directly correlated in PPMS patients, EBV VCA IgG was directly correlated in the RRMS cohort (Supplementary Fig. 1). HHV-6 A/B IgG also inversely correlated with age in HC. When we analyzed separately those subjects that were above or below the median value age of the whole population (43 years), we found that below the median value only EBV VCA IgG

	PPMS	RRMS	SPMS	HC	PP vs. RR	PP vs. SP	PP vs. HC	RR vs. SP	RR vs. HC	SP vs. HC	
EBNA-1 IgG	21.4 AU	24.2 AU	24.4 AU	20.8 AU	0.0004	0.00003	*n*.s.	*n*.s.	*n*.s.	*n*.s.	*p* value*
	149/156 (95.5%)	227/243 (93.4%)	72/76 (94.7%)	137/156 (87.8%)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	p value** O.R. (95% CI)***
EBV VCA IgG	49.9 AU	47.6 AU	50.5 AU	44.0 AU	n.s.	n.s.	0.00004	n.s.	0.00003	0.00004	p value*
	151/158 (95.6%)	252/257 (98.1%)	74/75 (98.7%)	153/158 (96.8%)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	p value** O.R. (95% CI)***
CMV IgG	30.0 AU	16.0 AU	24.5 AU	25.8 AU	0.000002	n.s.	n.s.	n.s.	0.002	n.s.	p value*
	116/158 (73.4%)	118/211 (55.9%)	49/72 (68.1%)	101/132 (76.5%)	0.0006 2.2 (1.4–3.4)	n.s.	n.s.	n.s.	0.0001 2.6 (1.6–4.2)	n.s.	p value** O.R. (95% CI)***
HHV-6 IgG	25.7 AU	30.5 AU	26.9 AU	32.5 AU	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	p value*
	124/139 (89.2%)	218/246 (88.6%)	64/74 (86.5%)	142/156 (91.0%)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	p value** O.R. (95% CI)***
HHV-6 IgM	3.8 AU	5.0 AU	3.6 AU	5.2 AU	n.s.	n.s.	n.s.	0.002	n.s.	n.s.	p value*
	24/147 (16.3%)	39/252 (15.5%)	3/74 (4.1%)	30/151 (19.9%)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	p value** O.R. (95% CI)***

Table 2. Titers and prevalences of the herpesviruses included in the study and their comparisons among the MS cohorts and healthy controls. AU: artificial units (median values); those doubtful values, between 9–11 AU, where excluded. n.s.: not significant. Only significant values after Bonferroni correction are shown ($p < 0.0083$). * p values were calculated from Mann–Whitney U-test. ** p values were calculated from Chi-square test/ Fisher's exact test. *** Odds Ratios with the 95% Confidence Intervals.

correlated with age in the RRMS and HC cohorts. When we only considered those subjects above the median value of age, both HHV-6 A/B IgG and EBV VCA IgG inversely correlated with age in the PPMS group (Results are in Supplementary Fig. 1). In Fig. 1.A, we compared the herpesvirus titers between the different cohorts at different decades of age. In Fig. 1.B we show the herpesvirus prevalences for each one of the cohorts in the same decades of age.

Viral profile in PPMS, RRMS, and SPMS cohorts by the linear discriminant analysis (LDA) model

LDA was applied to evaluate the effectiveness of the viral immune response in discriminating between cohorts (Fig. 2). The model identifies two significant discriminant functions (DFs) equal to the number of cohorts (PPMS, RRMS, and SPMS) minus one. The predictor variables included IgG responses against CMV, HHV-6 A/B, EBNA-1, and EBV VCA, and IgM against HHV-6 A/B. DF1 was significant (Wilks' $\Lambda = 0.851$, $p < 0.0001$) and accounted for 82.5% of the between-group variance (Fig. 2). DF1 was mainly characterized by the anti-CMV IgG response (structure coefficient = -0.62) and the anti-EBNA-1 IgG response (0.61). DF2 also contributed significantly (Wilks' $\Lambda = 0.972$, $p = 0.019$), explaining 17.5% of the residual discriminatory variance. DF2 was predominantly defined by anti-HHV6 IgM response (-0.76), followed by the anti-VCA IgG response (0.24). The model showed moderate classification performance, with a correct classification rate of 49.8% (cross-validated 41.1%). Results are shown in Fig. 2.

Human herpesviruses prevalences and titers in relation with sNfL and sGFAP values

A positive correlation between CMV titers and sGFAP levels above the median was observed in PPMS and SPMS patients, whereas a negative correlation between CMV titers and sNfL levels below 10 pg/ml was observed in healthy controls (HC). Regarding EBV, EBNA-1 was positively correlated with sNfL and sNfL z-Score in HC, but mainly with lower values of sNfL and sNfL z-Score. Finally, HHV-6 A/B IgG was negatively correlated with sGFAP in PPMS patients ($r = -0.304$) and HHV-6 A/B IgM with sGFAP in RRMS patients ($r = -0.161$). Results are shown in Table 3.

Then, patients were stratified in three groups: NLGL (low sNfL [< 10 pg/ml] and sGFAP [< median value]), NH (high sNfL [> 10 pg/ml] at different levels of sGFAP), and NLGH (low sNfL [< 10 pg/ml] and high sGFAP [> median value]) (Fig. 3). In SPMS patients, the NLGH group showed higher CMV IgG titers ($p = 0.01$) and a higher CMV seroprevalence ($p = 0.03$; O.R.= 18.0) compared with the NLGL group. CMV IgG titers were also higher in SPMS patients of the NLGH group than in the NH group ($p = 0.02$). Regarding HHV-6 A/B IgG titers, they were statistical significant lower in RRMS patients belonging NLGH group than in NLGL ($p = 0.005$) and NH ($p = 0.03$) groups. Regarding HHV-6 A/B IgM prevalence, it was higher in PPMS patients belonging to the NH group than in the NLGL group ($p = 0.04$; O.R.=12.7). Finally, no difference was found for EBV (EBNA-1 or VCA) in any of the cohorts after stratification by sNFL and sGFAP (results are shown in Fig. 3).

Discussion

In this study, we investigated the potential relationship between antiviral immune responses and markers of neuroaxonal and glial damage across different clinical forms of MS. This approach aimed to clarify whether specific antiviral antibody profiles are linked to distinct pathogenic mechanisms underlying MS subtypes.

As we can see in Fig. 2, DF1 shows that CMV IgG has a negative correlation with EBNA-1 IgG. CMV IgG would be clearly associated with PPMS, EBV would be more related with RRMS/SPMS high titers (Fig. 1). In

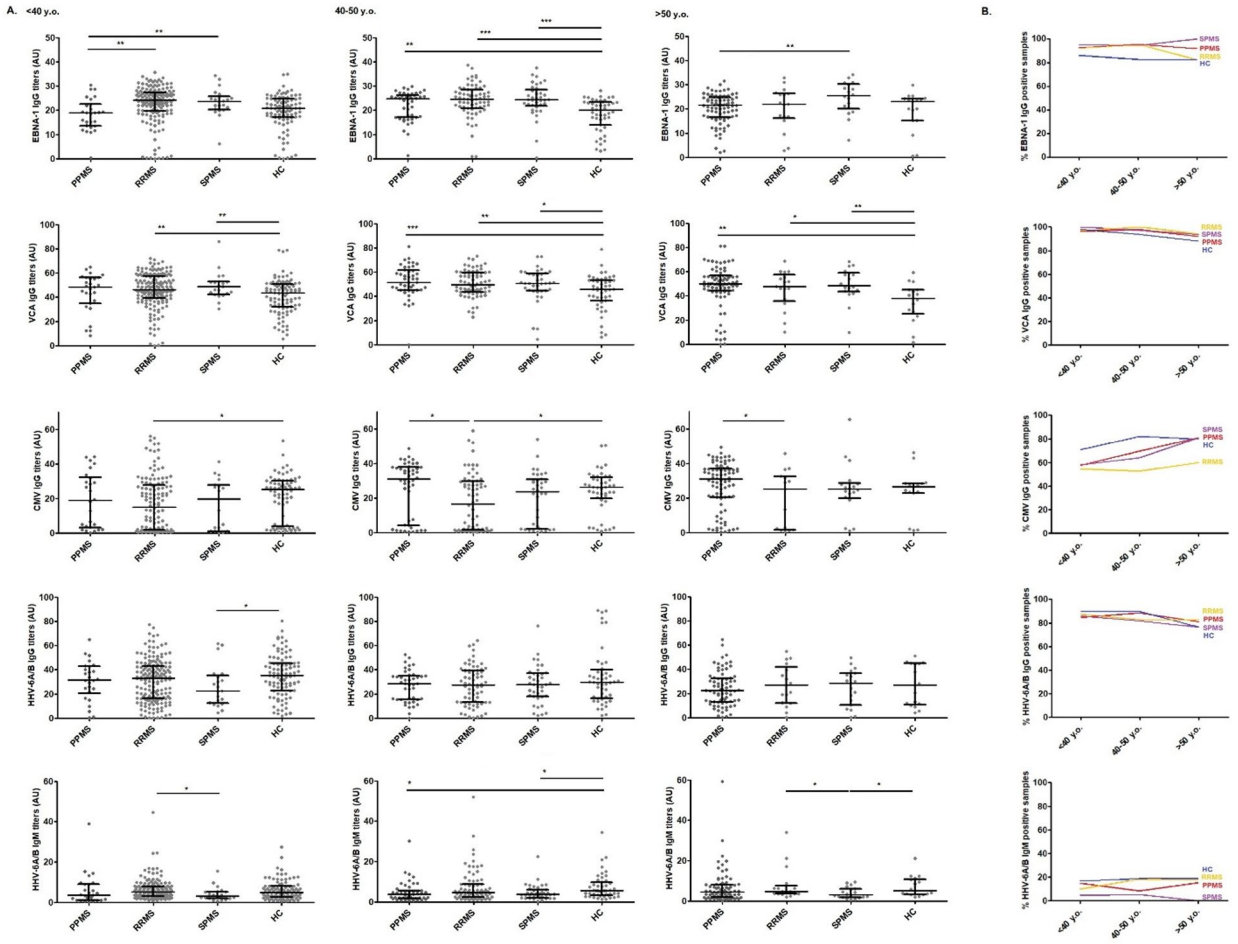
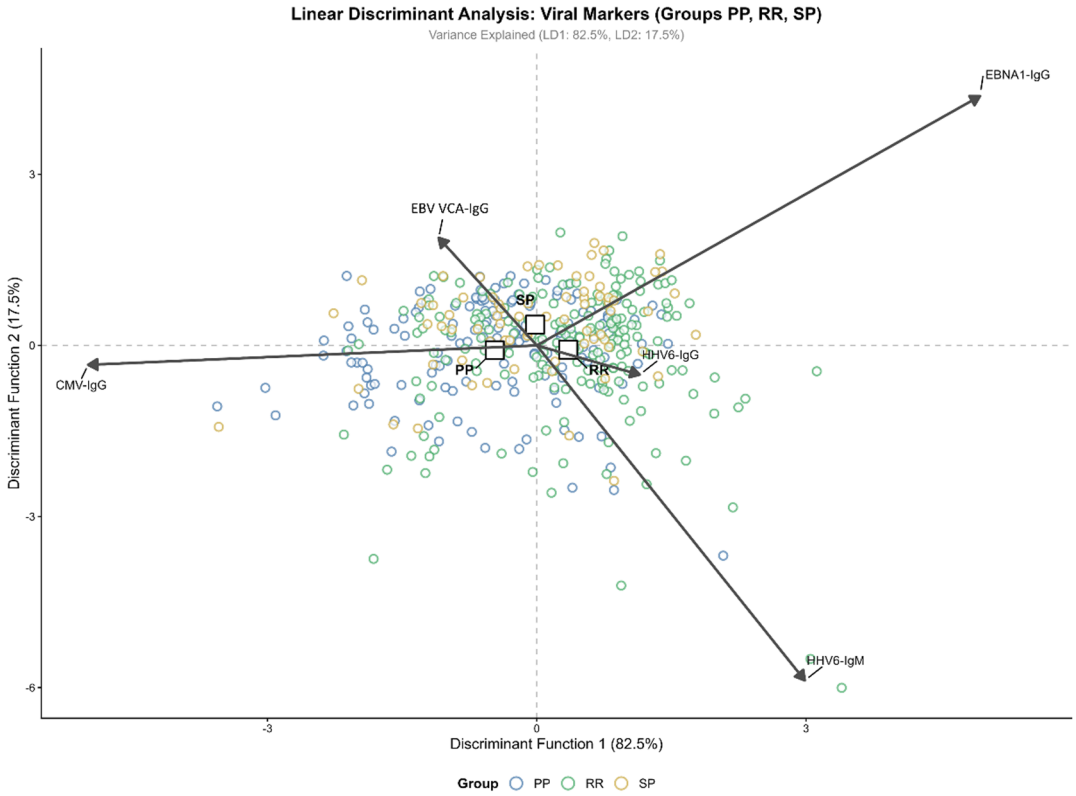


Fig. 1. **A**. Comparison of the herpesvirus antibody titers between the different cohorts at different decades of age: <40 years (PPMS: $n = 27$; RRMS: $n = 172$; SPMS: $n = 21$; HC: $n = 96$), between 40 and 50 years (PPMS: $n = 47$; RRMS: $n = 77$; SPMS: $n = 38$; HC: $n = 48$) and > 50 years (PPMS: $n = 84$; RRMS: $n = 17$; SPMS: $n = 19$; HC: $n = 17$). Median and interquartile range (P25 and P75) are shown. * ($p < 0.05$); ** ($p < 0.005$); *** ($p < 0.0005$). **B**. Evolution of the herpesvirus antibody prevalences in each one of the cohorts along different decades of age (< 40 years, between 40 and 50 years and with > 50 years).

addition, DF2 indicates that EBV VCA IgG appears to be more strongly associated with SPMS, but not with PPMS, whereas HHV-6 A/B IgG—and particularly HHV-6 A/B IgM—are associated with RRMS.

Given that EBV is considered one of the main triggers of MS[3], and considering the negative correlation observed in DF1 between CMV IgG and EBNA-1 IgG (Fig. 2), our findings support the hypothesis that CMV infection may be negatively associated with the risk of developing RRMS, suggesting a possible protective effect, as previously proposed by other authors[11]. Besides, CMV IgG prevalence and titers in RRMS were statistically significant lower than in HC. Similarly, a previous report found similar results[12]. High anti-CMV titers reduced the risk of MS symptoms in a study perform with 273 patients with radiologically isolated syndrome (RIS)[13]. We described that CMV IgG titers showed a strong negative correlation with high levels of sNfL (> 10 pg/ml) ($r = -0.794$) in the HC cohort, showing that CMV infection could be associated with a lower level of inflammation. Taken all these results together, CMV infection seems to be contributing to reduce the risk of developing MS. However, as we can see in Table 2, CMV showed higher prevalence and titers in the progressive forms of MS than in patients with RRMS, confirming also recent previous results[14]. Besides, CMV is positively correlated with higher levels (those above the median value) of sGFAP, a biomarker of neurodegeneration, in PPMS ($r = 0.420$) and in SPMS ($r = 0.321$). Also, in SPMS patients CMV prevalence and titers were significantly higher in those with low levels of sNfL and high levels of sGFAP. Then, we can conclude that while CMV infection seems to be a protective factor for RRMS, it would be associated with the progressive forms of MS and the neurodegeneration processes related to them, mainly in PPMS. Are these results contradictory? We think that the answer is no. CMV infection seems to help to control EBV infection. Previous results have shown that immune competition between CMV and EBV could be a possible mechanism behind the observed risk reduction for RRMS in CMV seropositive individuals[15]. Alternatively, CMV infection promotes expansion of a subset of mature natural killer (NK) cells, which could modulate the control of EBV[16,17]. The efficacy of the NKG2C + NK cell responses, a key cell population in the control of EBV infection, was significantly associated with previous infection with CMV[18].



Variance Explained	Wilk's/Lambda	P-Value	Variable	Structure of Coefficient (DF)[1]
DF1				
82.5%	0.851	< 0.0001	CMV-IgG †	-0.620
			EBNA1-IgG †	0.611
			HHV6-IgG †	0.154
			HHV6-IgM	0.373
			EBV VCA-IgG	-0.134
DF2				
17.5%	0.972	0.019	CMV-IgG	-0.067
			EBNA1-IgG	0.603
			HHV6-IgG	-0.153
			HHV6-IgM †	-0.758
			EBV VCA-IgG †	0.239
Cross-Validate Classify Accuracy: **PP** (45.8%); **RR** (51.3%); **SP** (38.4%); **Overall** (41.1%)				

1 Structure coefficients indicate the correlation between the variable and the latent discriminant function (DF). Statistical significance $p < 0.05$.
† Largest absolute correlation between each variable and DF1 and DF2.

Fig. 2. A discriminant analysis biplot with vectors representing EBNA-1 IgG, EBV VCA IgG, CMV IgG, HHV-6 A/B IgG and HHV-6 A/B IgM. Points close together are similar observations, and the angles between variable arrows indicate their correlation (acute for positive, right for 0, obtuse for negative).

But apart from the specific effect of CMV infection on EBV infection, chronic CMV infection influence the immune landscape impacting both, the composition and functionality of immune cell populations, inducing shifts in the inflammatory profile[19,20], supporting our finding about the negative correlation between CMV IgG titers and sNfL levels. Therefore, CMV infection could help to a better response to EBV infection and to a lower inflammatory profile reducing the risk of developing RRMS. But if the MS patient is already infected by CMV, the mechanisms related to CMV infection could act as a catalyst for neurodegenerative processes. This dual

	Cohorts*	rSpearman	Cohorts	rSpearman	Cohorts	rSpearman
sNfL	PPMS	n.s.	PPMS < 10 pg/ml	n.s.	PPMS > 10 pg/ml	n.s.
	RRMS	n.s.	RRMS < 10 pg/ml	n.s.	RRMS > 10 pg/ml	n.s.
	SPMS	n.s.	SPMS < 10 pg/ml	n.s.	SPMS > 10 pg/ml	n.s.
	HC	EBNA-1 IgG. $r = 0.192$	HC < 10 pg/ml	EBNA-1 IgG. $r = 0.255$	HC > 10 pg/ml	CMV IgG. $r = -0.794$
sNfL z-Score**	PPMS	n.s.	PPMS < 0	n.s.	PPMS > 0	n.s.
	RRMS	n.s.	RRMS < 0	n.s.	RRMS > 0	n.s.
	SPMS	n.s.	SPMS < 0	n.s.	SPMS > 0	n.s.
	HC	EBNA-1 IgG. $r = 0.163$	HC < 0	EBNA-1 IgG. $r = 0.221$	HC > 0	n.s.
sGFAP	PPMS	HHV-6 IgG. $r = -0.304$	PPMS< median	n.s.	PPMS> median	CMV IgG. $r = 0.420$
	RRMS	HHV-6 IgM. $r = -0.161$	RRMS< median	n.s.	RRMS> median	n.s.
	SPMS	n.s.	SPMS< median	n.s.	SPMS> median	CMV IgG. $r = 0.321$
	HC	n.s.	HC< median	n.s.	HC> median	n.s.

Table 3. Analysis of the correlation of herpesvirus antibody titers with the sNfL and SGFAP levels in each one of the cohorts of MS patients and healthy controls. Only significant correlations ($p < 0.05$) are shown. * On the left, we show the significant correlations between each herpesvirus analyzed and sNfL levels, sNfL z-scores, and sGFAP levels in each cohort (the entire population of each cohort was included). In the middle, we show the significant correlations between each herpesvirus analyzed and sNfL levels, sNfL z-scores, and sGFAP levels in each cohort (only patients with sNfL < 10 pg/ml, sNfL z-score < 0, and sGFAP values below the median were included). On the right, we show the significant correlations between each herpesvirus analyzed and sNfL levels, sNfL z-scores, and sGFAP levels in each cohort (only patients with sNfL > 10 pg/ml, sNfL z-score > 0, and sGFAP values above the median were included). Correlations were assessed by using the Spearman's rank correlation coefficient (r). * sNfL z-score adjust raw concentrations of sNfL for age and body mass index as it has been previously described[10](Serum Neurofilament Light Chain Reference App.: https://shiny.dkfbasel.ch/baselnflreference/).

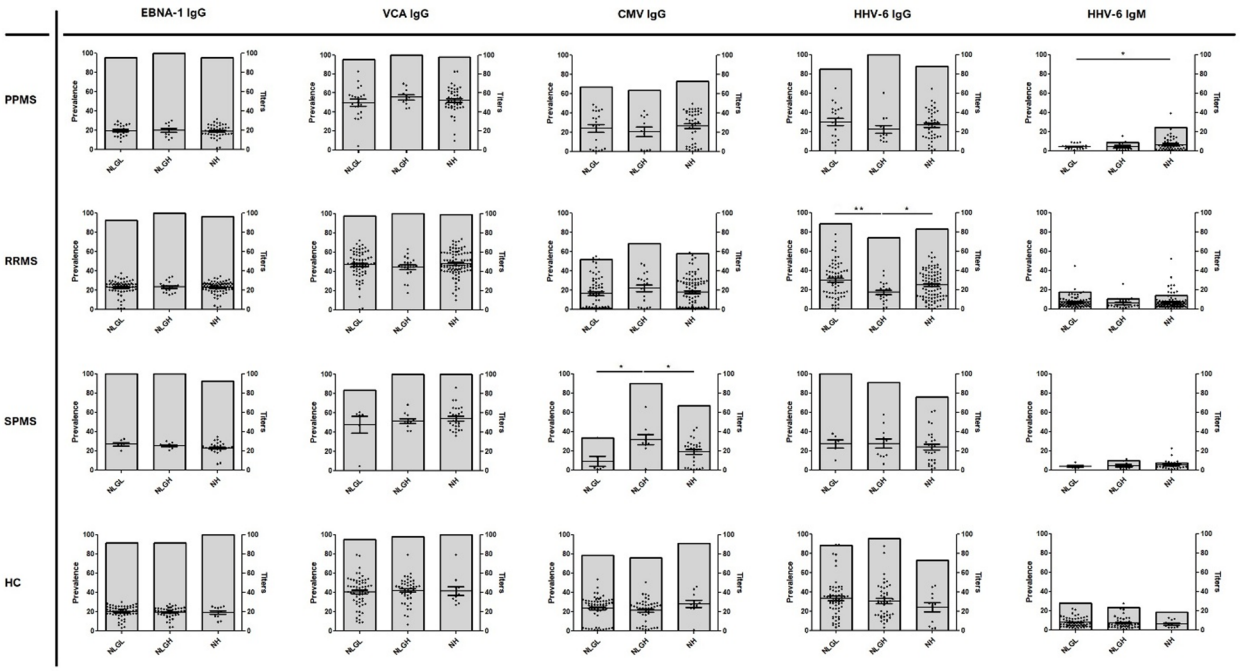


Fig. 3. EBNA-1, VCA, CMV and HHV-6 A/B IgG and IgM prevalences and titers in PPMS, RRMS, SPMS and HC cohorts after stratification by sNfL and sGFAP levels: NLGL (low sNfL [< 10 pg/ml] and low sGFAP [< median value]), NLGH (low sNfL [< 10 pg/ml] and high sGFAP [> median value]), and NH (high sNfL [> 10 pg/ml] at different levels of sGFAP). Height of the grey columns show the viral prevalences. Median and interquartile range (P25 and P75) for viral titers are also shown. * ($p < 0.05$); ** ($p < 0.005$); *** ($p < 0.0005$).

role of CMV infection in MS could help to explain the controversy surrounding this virus in the pathogenesis of MS in recent years. Could we speculate that an anti-CMV therapy in patients with progressive forms of MS might help reduce neurodegeneration in these individuals? SGFAP levels could be used to monitor the potential efficacy of this therapy.

In relation to EBV, EBNA-1 IgG and EBV VCA IgG prevalences and titers were higher in RRMS patients than in HC, confirming previous results[21]. However, although the prevalences were similar in the three cohorts of MS patients, the titers of EBNA-1 IgG were significantly lower in PPMS patients than in RRMS and SPMS patients. The literature is heterogeneous whether EBV, and overall EBNA-1, associates with the different clinical courses of MS in a different way. While some papers report higher EBNA-1 in relapsing forms compared with PPMS[22–24], others show elevated EBNA-1 in progressive subgroups or no clear difference between phenotypes[14,25]. In a brain tissue study, it was described an enrichment of EBV markers within lesions in progressive MS (both PPMS and SPMS) along with altered glial, astrocyte and neuronal interactions[26]. Therefore, while seems to be clear the association of EBV with the triggering of the disease, there does not appear to be a consensus regarding the possible role of the virus in the different clinical forms of the disease. When analyzing the association of EBV with sNfL and sGFAP as markers of neuroinflammatory and neurodegenerative processes, we found no significant associations in any MS cohort, even after stratification by sNfL and sGFAP levels (Fig. 3) for either EBNA-1 or EBV VCA. sNfL levels in individuals who were EBV-negative at baseline and went on to develop MS were similar to those of non-MS controls before and around the time of EBV infection but increased after EBV infection[3]. However, other recent study did not find a clear association between EBV and sNfL[27]. Taken together, these data confirm a strong association between EBV infection and RRMS but do not support a differential role of EBV across clinical subtypes. While it seems to be a temporal model in which EBV seroconversion seems to be followed by a latent period of growing axonal injury (reflected by rising sNfL) and eventual clinical MS, the contribution of EBV to neurodegenerative processes in progressive disease stages seems to be less clear and should be deeply studied.

Finally, HHV-6, especially HHV-6 A, has been increasingly investigated as a potential co-factor in the pathogenesis and clinical heterogeneity of MS[28]. Epidemiological and serological studies show that HHV-6 A seropositivity is more common in individuals who later develop MS compared with matched controls. HHV-6 A seropositivity was found in 40% of future MS cases versus 25% of controls, and was significantly associated with elevated sNfL levels, even years prior to clinical onset[29]. Although direct studies of HHV-6 A/B across all MS courses remain limited, some evidence points to HHV-6 A/B (reactivation or high serological response) being more frequent in relapse phases of RRMS than in SPMS or PPMS[30]. That suggests HHV-6 A/B may be more associated with the inflammatory relapsing component, characteristic of RRMS, and potentially with the transition to progression (SPMS), as we can see with a significant reduction of the prevalence and titers of HHV-6 A/B IgM in SPMS patients, and the negative correlation between HHV-6 A/B IgG and IgM with sGFAP. Whether HHV-6 A/B plays a role in PPMS is less clear. Given the pathophysiological differences, RRMS being characterized by focal inflammatory demyelination and SPMS or PPMS by neurodegeneration and smouldering inflammation, HHV-6 A/B may be more related to the inflammatory/relapsing axis rather than the purely degenerative one, when its role in the disease would be reduced as the disease evolves to more progressive forms and the astrocytic or glial processes become more dominant.

In conclusion, our results indicate that CMV may play a dual role in MS. While CMV appears to confer a protective effect against RRMS, this virus stands out as the main viral contributor to neurodegenerative processes, with particular importance in progressive MS forms, mainly in PPMS. Conversely, EBV and HHV-6 A/B seem to act primarily as risk factors in RRMS, but the relevance of HHV-6 A/B diminishes as neurodegeneration advances; thus, its role in SPMS and PPMS would be less clear.

Data availability

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

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Author contributions

MIDM, SR and RAL made the statistical analysis, discussed and interpreted findings, revised the manuscript critically. CRG, IOM, AAG, GPV and MAGM prepared the samples and processed the samples. LMV, LCF, NV, YA, ICP, MLMG, JMGD and RA provided unique reagents, discussed and interpreted findings, revised the manuscript critically. RAL contributed to the design of the study, guided the progress of the study and wrote the manuscript. All authors read and approved the final manuscript and have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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Declarations

Competing interests

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Additional information

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