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LEADING TOPIC

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Anti-EBNA1 IgG titre is not associated with fatigue in multiple sclerosis patients

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ABSTRACT

Introduction. Fatigue is the most frequent symptom in multiple sclerosis (MS), although it is still poorly understood due to its complexity and subjective nature. There is an urgent need to identify reliable biomarkers to improve disease prognosis and therapeutic strategies. Epstein-Barr virus (EBV) is the major environmental risk factor associated with MS aetiology, and trials with EBV-targeted T cell therapies have reduced fatigue severity in MS patients.

Aim of the study. We investigated whether the serum amount of immunoglobulin (Ig)G-specific for EBV antigens could be a suitable prognostic marker for the assessment of MS-related fatigue.

Material and methods. A total of 194 MS patients were enrolled. We quantified EBV nuclear antigen 1 (EBNA1) and EBV viral capsid antigen (VCA) immunoglobulin (Ig) G levels and B cell-activating factor of the tumour necrosis factor family (BAFF) concentration in the serum of patients with relapsing-remitting MS (RRMS) and chronic progressive MS (CPMS), and we analysed their correlation with aspects of fatigue and other clinical disease parameters.

Results. A complete EBV seropositivity could be detected in our cohort. After adjusting for confounding variables and covariates, neither EBNA1 nor VCA antibody titres were associated with levels of fatigue, sleepiness, depression, or with any of the clinical values such as expanded disability status scale, lesion count, annual relapse rate, or disease duration. However, patients with RRMS had significantly higher EBNA1 IgG titre than those with CPMS, whereas this was not the case under therapies targeting $CD20^+$ cells. BAFF levels in serum were inversely proportional to anti-EBNA1 IgG.

Conclusions and clinical implications. Our results show that EBNA1 IgG titre is not associated with the presence or level of fatigue. Whether the increased EBNA1 titre in RRMS plays a direct role in disease progression, or is only a consequence of excessive B cell activation, remains to be answered in future studies.

Key words: multiple sclerosis, RRMS, progressive MS, EBV, EBNA1, fatigue, BAFF

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS), and a distinction is drawn between the relapsing and the chronic progressive forms of the disease.

The progressive phase of MS is characterised by the slow expansion of pre-existing lesions and an exacerbated innate

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immune response in the CNS, with trapped immune cells behind the closed blood-brain barrier or the cerebrospinal fluid-brain barrier. Although the different disease courses are now understood as a continuum or spectrum of MS pathologies, this continuum is arbitrarily divided using different criteria to define each course. Diagnosing secondary chronic progressive MS (CPMS) is challenging since no standardised objective definition criteria or biomarkers are available [1].

Regardless of the disease course, B cells constitute a significant lymphocyte population in the CNS of MS patients being located in the active or chronic active lesions and, at later disease stages, high numbers of plasma cells are found in inactive lesions [2].

One of the major environmental risk factors associated with MS aetiology is an infection with the human Herpesviridae family member Epstein-Barr virus (EBV) [3]. EBV predominantly infects B cells and transforms them, leading to cellular activation, proliferation, and failure in viral regulation and clearance [4]. B cells play a pivotal role in driving new relapses in MS, as demonstrated by the high efficacy of anti-CD20-treatment [5, 6]. In addition, excessive B cell activation and other dysfunctions, such as abnormal B cell-activating factor of the tumour necrosis factor family (BAFF) expression, have been detected among MS patients [6, 7]. Although extensive literature supports the involvement of EBV infection in aberrant B cell biology and pathophysiology in MS [3, 8, 9], the causative and functional relations are less clear [10–15].

Fatigue is the most frequent and disabling MS symptom, with an estimated prevalence of up to 86% [16–18]. MS-caused fatigue is a multidimensional condition that includes physical, cognitive, and psychosocial aspects [19]. It commonly precedes the diagnosis of MS along with EBV infection [20, 21], suggesting that fatigue in MS might be related to a deficiency in controlling EBV-specific immune responses. Two recent small studies showed a reduction in fatigue severity in MS patients following EBV-specific T cell therapy [22, 23]. However, direct attribution of EBV status in MS-related fatigue has not been fully addressed.

In this study, we set out to investigate whether the serum antibody titres specific for EBV antigens might be suitable biomarkers for fatigue assessment in MS patients. We quantified EBV nuclear antigen 1 (EBNA1) and EBV viral capsid antigen (VCA) immunoglobulin (Ig) G levels and BAFF concentration in serum of patients with relapsing-remitting MS (RRMS) and CPMS, and we analysed their correlation with fatigue aspects and other clinical disease parameters.

Material and methods

Cohort

A total of 194 participants (age > 18 years) were recruited in the MS outpatient clinic at the University Medicine Essen, Germany during the period from June 2018 until April 2019. Patient selection was random and sequential. Both untreated and disease-modifying therapy (DMT) treated MS patients were included in the study. After receiving consent, all patients underwent a thorough clinical assessment and magnetic resonance imaging (MRI) examination. Study participants completed questionnaires in an assisted interview, and 7.5 mL of blood was collected into EDTA-coated Serum-Monovette (Sarstedt, Nümbrecht, Germany) by peripheral venepuncture. All serum samples were processed according to the standard operating procedures and stored at –20°C. The expanded disability status scale (EDSS) score and the annual relapse rate (ARR) for the last two and five years were determined. Based on the 2017 revised McDonald criteria, the cohort was divided into RRMS and CPMS groups, wherein the latter group included patients with primary progressive and secondary progressive MS (PPMS and SPMS, respectively).

Assessment of fatigue levels, sleepiness, and depression

The original Fatigue Impact Scale (FIS) by Fisk et al. in a German language adaptation was used to assess fatigue levels [24, 25]. Sleepiness was determined using the Epworth sleepiness scale (ESS), a robust and well-validated tool to distinguish sleepy from a normal condition with high retest reliability. For the evaluation of depression, the GRID-Hamilton Rating Scale for Depression (GRID-HAMD-21) in its German adaptation was used [26].

MRI lesion count

A total of 180 MRI FLAIR sequences were analysed for the presence of typical MS lesions by two independent raters. MRI lesions were classified into the following categories: (1) total lesion count; (2) concerning the location: supratentorial, infratentorial, and brainstem lesions; and (3) concerning the size: small (≥ 3–< 5 mm), medium (≥ 5–< 10 mm), and large (≥ 10 mm). Images were acquired on a 1.5 Tesla Avanto MRI scanner (Siemens Healthineers, Erlangen, Germany). The following MRI sequences were applied: T1 3D FSPGR tra (Slice Thickness 1 mm), T2 prop tra (Slice Thickness 3 mm/ /Spacing 0.3), 3D Sag T2 Cube FLAIR FS (Slice Thickness 2 mm), Sag DIR (Slice Thickness 1 mm), epi DWI tra (Slice Thickness 3 mm/Spacing 0.3), and T1 3D FSPGR tra + KM (Slice Thickness 1 mm).

Anti-EBV antibodies

IgG antibodies specific to EBNA1 and VCA were measured in patients' sera using automated chemiluminescent immunoassays LIAISON® EBNA IgG and VCA IgG assays (DiaSorin, Sallugia, Italy) with the LIAISON XL (DiaSorin) according to the manufacturer's instructions. Results are given in units (U)/mL. Values above the upper range (VCA IgG > 750 U/mL and EBNA1 IgG > 600 U/mL) were re-measured in a 1:20 dilution. EBNA1 IgG and VCA IgG levels < 20 U/ /mL were considered negative.

BAFF

BAFF concentration was determined in serum samples taken from 150 patients (138 in the RRMS group and 12 in the CPMS group) by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (R&D Systems Inc, Minneapolis, MN, USA) and according to the manufacturer's instructions. Minimum detection limit of human BAFF was 2.68 pg/mL.

Statistical analysis

Statistical analyses were performed using SPSS version 27. Normal distribution was tested with the Shapiro-Wilk test. Correlations were analysed with the Spearman's Rho test. The Mann-Whitney U test was used for comparisons of mean values. Multiple correlations and regression analyses were used to identify confounding factors associated with fatigue and test correlations between disease parameters.

Ethical statement

The ethics committee of the University Essen-Duisburg approved this study (18-8280-BO).

Results

Baseline characteristics of total cohort

In this observational study, 194 patients with MS were enrolled (we refer to the 194 as the total cohort). The mean age was 44.1 years, and 60% of participants were females (Tab. 1). The disease duration (DDY) ranged from 0.5 to 12.5 years, with a mean of 9.3 years. The median EDSS was 3.0. The median ARR over the last two and five years was 0.5 and 0.4 relapses, respectively. The mean lesion count determined by MRI was 27.0 ± 21.8 (n = 119 patients; Tab. 1).

Baseline characteristics of subgroups in matched cohort

To avoid bias in analyses that include clinical course variables, we matched the RRMS and CPMS subgroups for age and sex (matched cohort). The mean age was comparable between the groups, being 55.6 ± 1.3 years in the RRMS (n = 36) and 57.4 ± 1.8 years in the CPMS group (n = 37). The proportion of females was 58.3% in the RRMS group and 51.3% in the CPMS group (Tab. 2). The mean DDY in the RMS group was 10.5

Table 1. Demographic characteristics of total cohort of MS patients, serum parameters, disease-related parameters, and questionnaires

Demographics		Disease-related parameters	
No. of patients, n	194	Mean disease duration ± SD, y	9.3 ± 1.27
Mean age ± SD, y	44.1 ± 13.2	Median disease duration, y	6.0
Median age, y	45.0	Disease duration, y	0–12.5
Gender, female in %	60.0	Mean EDSS ± SD	3.1 ± 2.1
RRMS, n	150	Median EDSS	3.0
CPMS (SPMS + PPMS), n	37	Mean ARR (last 2 y) ± SD	0.74 ± 1.6
		Median ARR (last 2 y)	0.5
		Mean ARR (last 5 y) ± SD	0.65 ± 1.2
Serum parameters		Median ARR (last 5 y)	0.4
Mean EBNA1 ± SD, [U/mL]	26,389 ± 55,426	MRI mean lesion count	27.0 ± 21.8
Median EBNA1, [U/mL]	10,700	MRI median lesion count	21.0
Mean VCA ± SD, [U/mL]	8,300 ± 15,687		
Median VCA, [U/mL]	443	Questionnaires	
Mean BAFF ± SD, [pg/mL]	336.9 ± 169.0	Mean FIS ± SD	62.7+ 35.6
Median BAFF, [pg/mL]	292.2	Median FIS	65.5
		Mean ESS ± SD	8.3 ± 4.9
		Median ESS	8.00
		Mean GRID-HAMD-21 ± SD	10.4 ± 7.0
		Median GRID-HAMD-21	10.0

ARR — annual relapse rate; BAFF — B cell-activating factor of the tumour necrosis factor family; CPMS — chronic progressive MS; EBNA1 — Epstein-Barr virus nuclear antigen 1; EDSS — expanded disability status scale; ESS — Epworth sleepiness scale; FIS — fatigue impact scale; GRID-HAMD-21 — GRID-Hamilton rating scale for depression; MRI — magnetic resonance imaging; PPMS — primary progressive MS; RRMS — relapsing-remitting MS; SD — standard deviation; SPMS — secondary progressive MS; VCA — viral capsid antigen

Table 2. Demographic characteristics, serum parameters, disease-related parameters, and questionnaires in MS patients matched for age and sex, grouped according to clinical course

Demographics	RRMS	CPMS
No. of patients, n	36	37
Mean age ± SD, y	55.6 ± 1.3	57.4 ± 1.8
Median age, y	56.0	57.0
Gender, female in %	58.3	51.3
Disease-related parameters		
Mean disease duration ± SD, y	10.5 ± 9.0	10.7 ± 11.9
Median disease duration, y	11.0	6.5
Mean EDSS ± SD	3.3 ± 1.8	5.0 ± 1.3
Median EDSS	3.0	5.7
Mean ARR (last 2 y) ± SD	0.89 ± 1.3	0.13 ± 0.35
Median ARR (last 2 y)	0.5	0.0
Mean ARR (last 5 y) ± SD	0.84 ± 1.2	0.09 ± 0.2
Median ARR (last 5 y)	0.4	0.0
MRI mean lesion count	21.0 ± 19.5	27.1 ± 14.0
MRI median lesion count	24.0	26.0
Questionnaires		
Mean FIS ± SD	47.6 ± 29.9	57.4 ± 34.9
Median FIS	43.0	59.0
Mean ESS ± SD	4.5 ± 3.4	5.8 ± 3.1
Median ESS	5.0	7.0
Mean GRID-HAMD-21 ± SD	7.7 ± 5.6	9.7 ± 5.2
Median GRID-HAMD-21	7.0	9.0
Serum parameters		
Mean EBNA1 ± SD, [U/mL]	32.076 ± 41.320	9.371 ± 13.281
Median EBNA1, [U/mL]	20.020	518
Mean VCA ± SD, [U/mL]	11.102 ± 16.151	8.340 ± 18.116
Median VCA, [U/mL]	595	439
Mean BAFF ± SD, [pg/mL]	311.9 ± 176	387 ± 170
Median BAFF, [pg/mL]	257	338

ARR — annual relapse rate; BAFF — B cell-activating factor of the tumour necrosis factor family; CPMS — chronic progressive MS; EBNA1 — Epstein-Barr virus nuclear antigen 1; EDSS — expanded disability status scale; ESS — Epworth sleepiness scale; FIS — fatigue impact scale; GRID-HAMD-21 — GRID-Hamilton rating scale for depression; MRI — magnetic resonance imaging; RRMS — relapsing-remitting MS; SD — standard deviation; VCA — viral capsid antigen

± 9.0 versus 10.7 ± 11.9 years in the CPMS group. The EDSS mean value was 3.3 ± 1.8 in the RRMS group and 5.0 ± 1.3 in the CPMS group. The mean ARR value was 0.89 ± 1.3 (last two years) and 0.84 ± 1.2 (last five years) in the RRMS group, while the CPMS group had values of 0.13 ± 0.35 (last two years) and 0.09 ± 0.2 (last five years). The mean MRI lesion count was 21.0 ± 19.5 in the RRMS group and 27.1 ± 14.0 in the CPMS group (Tab. 2).

Association of fatigue with MS disease parameters

The mean FIS value of the total cohort was 62.7 ± 35.6, whereas the mean ESS was 8.3 ± 4.9, including 30.8% of the patients who had a state of excessive sleepiness, defined as ESS ≥ 11 (Tab. 1). The FIS score was markedly elevated in participants suffering from excessive sleepiness (87.7 ± 26.2) compared to those with ESS < 11 (51.19 ± 33.6, $p < 0.001$). When we analysed the effect of sleepiness on three FIS dimensions, logistic regression revealed its positive correlation with the impact of fatigue on cognitive functioning (B = 0.09, $p < 0.001$), but not on physical or psychosocial functioning (data not shown).

The mean GRID-HAMD-21 score in the total cohort was 10.4 ± 7.0 (Tab. 1). 81.2% showed no sign of depression or mild depression, 14.1% of patients were suffering from moderate depression, whereas 4.7% of individuals were severely depressed. Severely depressed patients (GRID-HAMD-21 ≥ 24) had considerably elevated FIS score (101.2 ± 31.6) compared to those with GRID-HAMD-21 < 24 (60.5 ± 34.8, $p < 0.01$). To avoid the confounding influence of high sleepiness and severe depression on the evaluation of fatigue, patients with ESS ≥ 11 (n = 47) and GRID-HAMD-21 ≥ 24 (n = 4) were not considered in further analyses. In the remaining cohort (n = 143), mean FIS score value (corrected FIS) was 50.3 ± 32.7 and positively correlated to sleepiness (Spearman's rho 0.554, $p < 0.001$, Fig. 1A) and depression (Spearman's rho 0.616, $p < 0.001$, Fig. 1A). Moreover, corrected FIS score significantly correlated with the EDSS ($r = 0.335$, $p < 0.001$; Fig. 1B). When stratifying FIS into the three subdomains, a significant correlation of EDSS with physical and psychosocial functioning ($r = 0.441$, $p < 0.01$, and $r = 0.362$, $p < 0.01$, respectively) could be found, but not with cognitive functioning ($r = 0.112$, ns; Fig. 1B). The other relevant MS disease parameters, such as DDY, AAR (over the last two or five years), and MRI lesion count (including total lesions, supra- and infratentorial lesions, brainstem lesions, and lesions categorised according to their size), showed no correlation to fatigue (Fig. 1B).

Next, we analysed the fatigue levels in the two subgroups of the matched cohort. Patients with CPMS showed statistically elevated fatigue levels over patients with RRMS (47.6 ± ± 29.9 vs. FIS CPMS: 57.4 ± 34.9, $p < 0.5$, Fig. 1C). We found a significantly higher fatigue impact on physical functioning in the CPMS group than in the RRMS group (RRMS 13.6 ± 9.7 vs. CPMS 19.5 ± 11.6, $p < 0.05$). In contrast, no differences were found for the psychosocial or cognitive dimensions between the two patient groups (Fig. 1C).

Of note, female participants had significantly higher levels of fatigue than males demonstrated by both total FIS (62.5 ± 3.2 in females vs. 53.4 ± 4.0 in males, $p < 0.01$) and by corrected FIS (56.4 ± 3.8 in females vs. 41.8 ± 4.1 in males, $p < 0.05$; not shown). Concerning age, we could not identify any statistically significant correlation with either total or corrected FIS value.

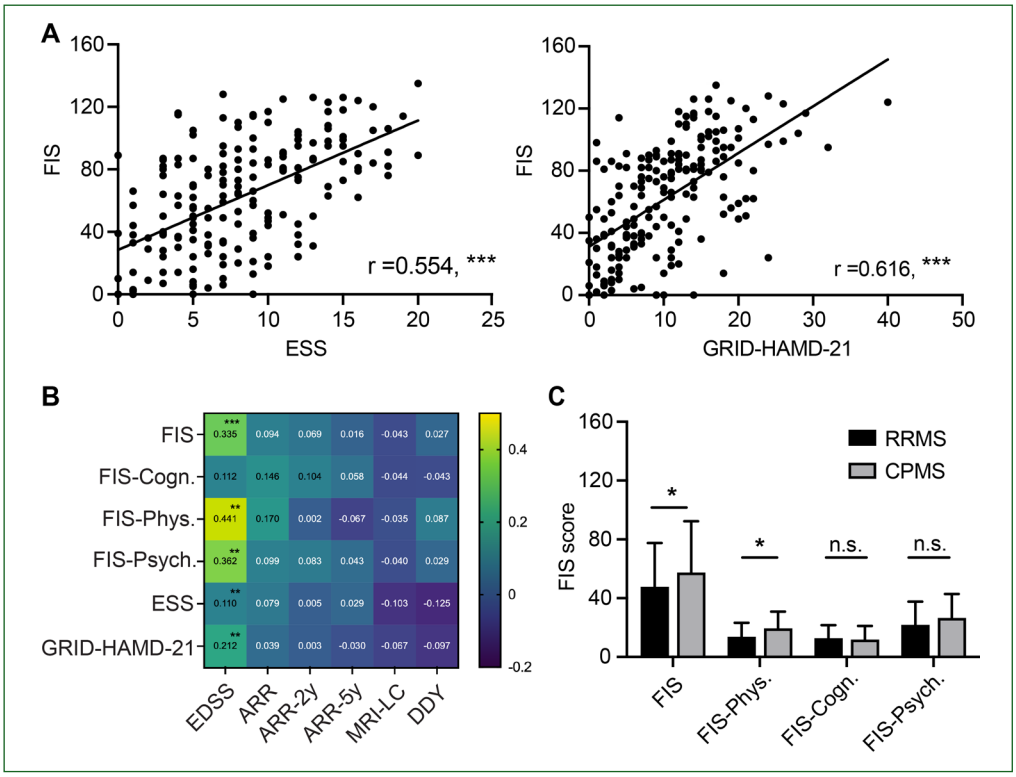

Figure 1. Fatigue is in positive correlation with sleepiness, depression, and disability in MS patients; **A.** Level of fatigue determined by fatigue impact scale (FIS) in correlation with level of sleepiness (ESS, r = 0.554, p < 0.001) and with severity of depressive symptoms (GRID--HAMD-21, r = 0.616, p < 0.001) in total cohort; **B.** Correlation heatmap (Spearman) of FIS, subdimensions of FIS (cognitive, physical, and psychosocial), ESS, and GRID-HAMD-21 to MS disease-related parameters: expanded disability status scale (EDSS), annual relapse rate (ARR), ARR over last two years (ARR-2y), ARR over last five years (ARR-5y), MRI lesion count (MRI-LC), and disease duration in years (DDY). Colours are coded for Spearman's correlation coefficient rho from –0.2 to 0.5. Corrected FIS values were used, excluding patients with high levels of sleepiness (ESS ≥ 11) and high levels of depression (GRID-HAMD-21 ≥ 24); **C.** Total fatigue and its subdimensions in sex- and age-matched subgroups differentiated according to clinical MS course — relapsing-remitting MS (RRMS) and chronic progressive MS (CPMS); Significant correlations are labelled with asterisks. * p < 0.05; ** p < 0.01; *** p < 0.001; n.s. — not significant

Association of sleepiness and depression with clinical disease activity

We next analysed the association of sleepiness and depression with the MS disease parameters in the total cohort. GRID-HAMD-21 positively correlated with EDSS, but there was no association with the mean ARR over two or five years, lesion count, or DDY (Fig. 1B). Sleepiness score (ESS) was not associated with any of these clinical parameters (Fig. 1B). In addition, we found no statistically significant difference in ESS or GRID-HAMD-21 scores between the RRMS and CPMS subgroups (Tab. 2).

Association of EBNA1- and VCA-specific antibodies with radiological and clinical markers of disease activity

In the total cohort (n = 194), serum levels of IgG antibodies specific to EBNA1 were detectable in 193 patients and showed a broad range, with a mean of 26,389 U/mL ± 55,426 and median value of 10,700 U/mL (Tab. 1). VCA-specific IgG antibodies were detected in 100% of patients with mean and median values of 8,300 U/mL ± 15,687 and 443, respectively (Tab. 1).

To determine whether MS-related fatigue was associated with the humoral immune response to EBV infection, we evaluated the correlation of EBV antigen-specific antibodies in serum with the corrected FIS score. Neither EBNA1 nor VCA antibody titres showed associations with fatigue, calculated as general FIS or stratified on cognitive, physical, or psychosocial dimensions (Figs. 2A, B). Likewise, there was no correlation of EBNA1 or VCA IgG amounts with sleepiness and depression levels, neither in the matched cohort nor when MS patients who were suffering from excessive daytime sleepiness (ESS ≥ 11) or severe depression (GRID-HAMD-21 ≥ 24) were analysed separately (Figs. 2B, C and not shown). Furthermore, EBNA1 and VCA IgG levels did not correlate with sex, age, or other clinical disease parameters analysed, such as EDSS, ARR over the last two or five years, MRI lesion count, and DDY (not shown). Nevertheless, when we analysed differences between the subgroups in the matched cohort corrected for age and

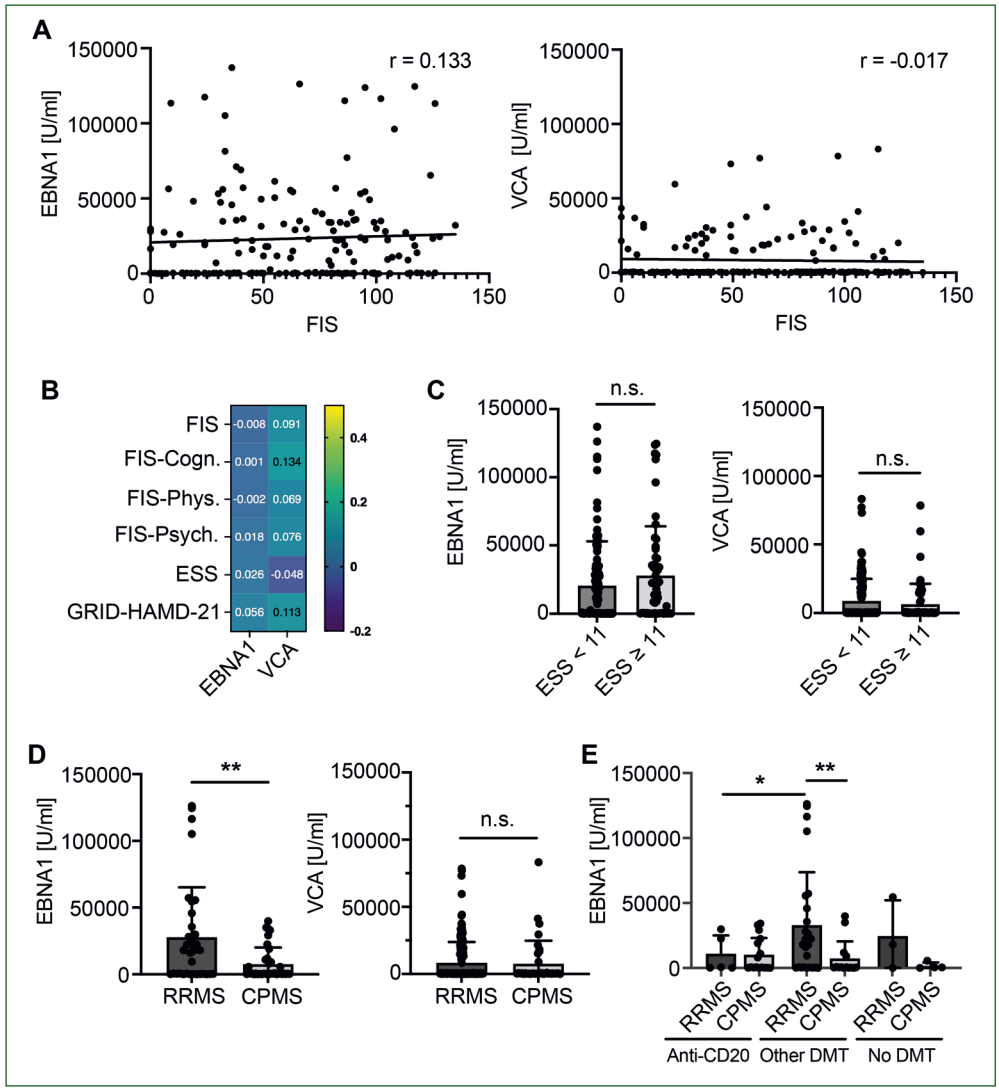

Figure 2. EBNA1 IgG titre is not associated with radiological and clinical parameters of MS activity, but it is elevated in patients with RRMS; **A.** Epstein-Barr virus nuclear antigen 1 (EBNA1)- and viral capsid antigen (VCA)-specific IgG antibody titre in correlation with total fatigue levels (FIS); **B.** Correlation heatmap of fatigue level and its subdimensions, sleepiness (ESS), and depression (GRID-HAMD-21) with EBNA1- and VCA-specific IgG titer. Colours are coded for Spearman's correlation coefficient rho from –0.2 to 0.5; **C.** EBNA1- and VCA-specific IgG titres in participants with excessive sleepiness (ESS ≥ 11) and those with ESS < 11; **D, E.** EBNA1- and VCA-specific IgG titer concentrations in total relapsing-remitting MS (RRMS) and chronic progressive MS (CPMS) subgroups (D) and divided according to treatment into 'anti-CD20' (rituximab and ocrelizumab), 'other DMT' and 'no DMT' (E). *p < 0.05, ** p < 0.01, *** p < 0.001; n.s. — not significant

sex, we found significantly higher EBNA1 IgG antibody levels in patients with RRMS (32,076 ± 41,320 U/mL) than in those with CPMS (9,371 ± 13,281 U/mL; p < 0.05, Fig. 2D). VCA IgG amount did not significantly differ between the RRMS and CPMS clinical subgroups (Fig. 2D).

Next, we checked whether the specific DMT or absence of DMT influences this result (Suppl. Tab.). Interestingly, under anti-CD20 modifying therapies (rituximab and ocrelizumab) and without DMT no differences in EBNA1-specific antibody titres were detected between the RRMS and CPMS subgroups. In contrast, patients treated with DMT other than anti-CD20 (other DMT group; Suppl. Tab.) had higher EBNA1 titres in the RRMS than in the CPMS group (Fig. 2E). Nevertheless, no significant correlation was detected between EBNA1 IgG levels and any of the clinical parameters in the RRMS subgroup (not shown).

BAFF levels in patients' sera in relation to therapy, disease parameters and clinical course

BAFF, a B cell survival and activating factor, has been implicated in the development of MS, and BAFF-producing EBV-infected B cells have been found in acute MS lesions and

ectopic B cell follicles [27]. Therefore, we analysed whether BAFF serum levels correlated with EBV antigen-specific antibody titres, fatigue, and other MS disease parameters. The mean concentration of BAFF in the serum samples of the total cohort was 336.9 ± 169.0 pg/mL (Tab. 1). We then analysed BAFF concentration regarding DMT type, since increased BAFF levels following treatment with rituximab have been reported [28]. In accordance with this, we found significantly elevated BAFF concentration in patients under therapies that selectively target CD20 compared to those treated with other DMTs or without DMT (Suppl. Fig. 1A). However, no significant correlation of BAFF values with corrected FIS, cognitive, physical, or psychosocial aspects of FIS, ESS, or GRID-HAMD-21 scores were found when we analysed either the total cohort (n = 150) or when the patients were grouped according to their therapy (Suppl. Fig. 1B). Furthermore, BAFF concentration in serum samples of the total cohort did not correlate with sex, age, serum EBNA1 IgG levels, serum VCA IgG levels, or any other clinical parameter of MS disease (EDSS, ARR over two or five years, MRI lesion count, and DDY; data not shown).

In contrast to EBNA1 IgG, BAFF serum levels did not differ between the RRMS and the CPMS clinical subgroups in the matched cohort (Tab. 2). However, the same pattern of higher BAFF levels under treatment with one of the anti-CD20 therapies than with other DMT or without DMT was also present when the RRMS and CPMS subgroups were analysed separately (Suppl. Fig. 1C, Suppl. Tab.).

Discussion

This prospective cohort study revealed significantly higher serum EBNA1 IgG antibody levels in RRMS patients than in those with CPMS, and DMT influenced these differences. Neither EBNA1 nor VCA IgG titres in the serum of MS patients correlated with fatigue, sleepiness, or depression. Likewise, levels of EBNA1 and VCA titres were not associated with sex, age, EDSS, ARR, lesion count, DDY, or serum BAFF levels. This was the case when we analysed both the total cohort of 194 patients and the age- and sex-matched subgroups divided according to the clinical course.

Fatigue is one of the most frequent symptoms in MS, affecting most patients, with more than half of them reporting it as one of the worst symptoms experienced [29]. Notwithstanding its high prevalence and pronounced impact on quality of life, fatigue remains poorly understood. MS-related fatigue is ascribed to multifactorial aetiologies, including immunological abnormalities and infection [30]. So far, the current assessment is based entirely on symptoms and self-rating scales, and there is no precise tool for differential diagnosis or viable biomarker. Due to the increased prevalence of several sleep disorders and depression related to fatigue, we adjusted our cohort for excessive daytime sleepiness, severe depression, sex, and age.

In line with previous studies [31–33], fatigue positively correlated with sleepiness, depression, and EDSS in our cohort. The physical dimension of fatigue appeared to be more severe in patients with CPMS than in those with RRMS, although this may in part be explained by differences in disability levels. ARR, DDY or MRI T2 lesion burden did not correlate with fatigue in our cohort, suggesting that these parameters do not reflect relevant aspects in the pathophysiology of MS-related fatigue.

EBV-infected autoreactive B cells have been implicated in MS pathology [34]. Given that reduction of fatigue severity occurs after EBV-targeted immunotherapy in MS patients [22, 23], we hypothesised that there could be a mechanistic relation between fatigue levels and humoral response against EBV. Nonetheless, in our study, neither EBNA1 nor VCA IgG titres in patients' sera were associated with MS-related fatigue. Moreover, in our cohort, these infection parameters did not correlate with sleepiness, depression, or levels of BAFF, a major survival and maturation factor for B cells that is elevated in MS and related to EBV immunity [35]. Even though fatigue has been reported in some patients receiving ocrelizumab [36], we did not detect changes in fatigue, sleepiness, or depression levels under the anti-CD20-targeted treatment in our cohort (not shown).

There is strong evidence for a link between EBV and MS. Virtually all patients with clinically isolated syndrome (CIS, i.e. a first symptomatic episode suggestive of MS) and early MS are seropositive for EBV antigens [37, 38]. Furthermore, abundant EBNA1 IgG levels and a history of symptomatic EBV infection (infectious mononucleosis) in HLA-DRB1*1501 carriers have been associated with an increased odds ratio for MS [39, 40]. EBV infection was recently shown to increase 32-fold the risk of MS, consistently preceding symptom onset and the detection of the neurofilament light chain in serum, one of the earliest markers of preclinical MS [41].

In line with these studies, a complete EBV seropositivity was also present in our total cohort of MS patients. Furthermore, in our matched subgroups with similar mean disease duration of over ten years, patients with RRMS had higher EBNA1 titres than those with CPMS, suggesting that EBV might contribute to MS clinical course. This finding is consistent with prior reports of higher serum EBNA1 IgG titres in RRMS than in progressive MS forms [42]. Of note, we observed no distinct pattern of a VCA-specific serological response between these clinical MS subgroups. This is in accordance with one previous study [42], but not with another, where higher VCA IgG values were measured in patients with progressive MS [43].

The pathobiological importance of antibodies specific for EBNA1 in MS is unclear. Several hypotheses based on EBV's role in the propagation of aberrant immune responses both in the periphery and the CNS have been proposed [3, 44]. A recent study demonstrated that the naïve EBNA1-restricted antibody could develop into a mature antibody that

is cross-reactive with CNS residing glial cells adhesion molecule, suggesting high-affinity molecular mimicry as one of the mechanistic links [9].

Interestingly, when we stratified groups according to the therapy, anti-EBNA1 IgG titre in RRMS patients being treated with rituximab or ocrelizumab decreased to the levels detected in the CPMS group. These therapies strongly deplete circulating B cells and a small subset of highly activated $CD20^+$ T cells [45], but not antibody-secreting plasmablasts and plasma cells. Inversely proportional to anti-EBNA1 IgG, BAFF levels significantly increased in MS patients after anti-CD20 therapies, although irrespective of the clinical course. Removal of circulating B cells probably changes the availability of growth and survival factors that were previously consumed by these cells.

Overall, these findings support the notion that dysfunction in controlling EBV infection may be related to sustaining B cell activation, possibly setting the stage for new relapses.

Several studies have reported the association of humoral response to EBV with radiological or clinical disease activity and the risk of CIS conversion to MS [11, 12, 14, 15, 42]. In contrast, other studies could not identify these links [10, 13]. In our cohort, we did not detect the association of EBNA1 IgG antibodies in serum with demographic, radiological, or clinical disease activity parameters, neither in the total RRMS group nor in patients with RRMS who received DMT other than anti-CD20 therapies. Reasons for the divergent findings across studies may include different types of immunomodulatory treatment, different clinical characteristics of the analysed cohorts, and/or discrepancies in applied statistical methods. Whether abundant EBNA1-specific antibodies in the serum of RRMS patients directly participate in disease progression, or are simply a consequence of altered B cell activation, remains to be answered.

Of note, patients were enrolled for this study before the global COVID-19 pandemic began, and we could not analyse whether infection with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) could potentially influence our results. Our previous study suggests that both MS diagnosis and the administration of highly effective DMTs do not increase the risk of severe COVID-19 course [46]. However, since COVID-19 and MS share some specific components of pathomechanisms [47], the re-evaluation of the clinical MS parameters related to fatigue and SARS-CoV-2 infection, especially in the context of 'long COVID-19 syndrome', remains to be addressed.

Among the strengths of our study are the comprehensive acquisition of clinical and MRI data and the standardised blood sample collection and storage. Moreover, inpatient participants with possibly more severe symptoms were not included in the study. Some of the study limitations are the relatively small cohort sample size, and the possible selection bias, although the exclusion of confounding variables and covariates should be acknowledged.

Clinical implications/future directions

Our study indicates that in MS patients, EBNA1 and VCA IgG levels in serum are not associated with fatigue, sleepiness, or depression. We found no correlations of these parameters with MRI or clinical markers of disease activity. However, the decreased humoral response to EBNA1 at later MS stages requires further attention, and might be an interesting prognostic biomarker for assessing progressive MS, particularly for secondary progressive MS.

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References

1. Adamczyk-Sowa M, Adamczyk B, Kułakowska A, et al. Secondary progressive multiple sclerosis - from neuropathology to definition and effective treatment. Neurol Neurochir Pol. 2020; 54(5): 384–398, doi: 10.5603/PJNNS.a2020.0082, indexed in Pubmed: 33058113.
2. Absinta M, Lassmann H, Trapp BD. Mechanisms underlying progression in multiple sclerosis. Curr Opin Neurol. 2020; 33(3): 277–285, doi: 10.1097/WCO.0000000000000818, indexed in Pubmed: 32324705.
3. Bar-Or A, Pender MP, Khanna R, et al. Epstein-Barr virus in multiple sclerosis: theory and emerging immunotherapies. Trends Mol Med. 2020; 26(3): 296–310, doi: 10.1016/j.molmed.2019.11.003, indexed in Pubmed: 31862243.
4. Mrozek-Gorska P, Buschle A, Pich D, et al. Epstein-Barr virus reprograms human B lymphocytes immediately in the prelatent phase of infection. Proc Natl Acad Sci U S A. 2019; 116(32): 16046–16055, doi: 10.1073/pnas.1901314116, indexed in Pubmed: 31341086.

5. Mulero P, Midaglia L, Montalban X. Ocrelizumab: a new milestone in multiple sclerosis therapy. Ther Adv Neurol Disord. 2018; 11: 1756286418773025, doi: 10.1177/1756286418773025, indexed in Pubmed: 29774057.
6. Myhr KM, Torkildsen Ø, Lossius A, et al. B cell depletion in the treatment of multiple sclerosis. Expert Opin Biol Ther. 2019; 19(3): 261–271, doi: 10.1080/14712598.2019.1568407, indexed in Pubmed: 30632834.
7. Steri M, Orrù V, Idda ML, et al. Overexpression of the cytokine BAFF and autoimmunity risk. N Engl J Med. 2017; 376(17): 1615–1626, doi: 10.1056/NEJMoa1610528, indexed in Pubmed: 28445677.
8. Burnard S, Lechner-Scott J, Scott RJ. EBV and MS: Major cause, minor contribution or red-herring? Mult Scler Relat Disord. 2017; 16: 24–30, doi: 10.1016/j.msard.2017.06.002, indexed in Pubmed: 28755681.
9. Lanz TV, Brewer RC, Ho PP, et al. Clonally expanded B cells in multiple sclerosis bind EBV EBNA1 and GlialCAM. Nature. 2022; 603(7900): 321–327, doi: 10.1038/s41586-022-04432-7, indexed in Pubmed: 35073561.
10. Gieß R, Pfuhl C, Behrens J, et al. Epstein-Barr virus antibodies in serum and DNA load in saliva are not associated with radiological or clinical disease activity in patients with early multiple sclerosis. PLOS ONE. 2017; 12(4): e0175279, doi: 10.1371/journal.pone.0175279.
11. Kvistad S, Myhr KM, Holmøy T, et al. Antibodies to Epstein-Barr virus and MRI disease activity in multiple sclerosis. Multiple Sclerosis Journal. 2014; 20(14): 1833–1840, doi: 10.1177/1352458514533843.
12. Lünemann JD, Tintoré M, Messmer B, et al. Elevated Epstein-Barr virus-encoded nuclear antigen-1 immune responses predict conversion to multiple sclerosis. Ann Neurol. 2010; 67(2): 159–169, doi: 10.1002/ana.21886, indexed in Pubmed: 20225269.
13. Munger KL, Fitzgerald KC, Freedman MS, et al. No association of multiple sclerosis activity and progression with EBV or tobacco use in BENEFIT. Neurology. 2015; 85(19): 1694–1701, doi: 10.1212/WNL.0000000000002099, indexed in Pubmed: 26453645.
14. Zivadinov R, Chin J, Horakova D, et al. Humoral responses to herpesviruses are associated with neurodegeneration after a demyelinating event: results from the multi-center set study. J Neuroimmunol. 2014; 273(1-2): 58–64, doi: 10.1016/j.jneuroim.2014.04.012, indexed in Pubmed: 24907902.
15. Zivadinov R, Zorzon M, Weinstock-Guttman B, et al. Epstein-Barr virus is associated with grey matter atrophy in multiple sclerosis. J Neurol Neurosurg Psychiatry. 2009; 80(6): 620–625, doi: 10.1136/jnnp.2008.154906, indexed in Pubmed: 19168469.
16. Broch L, Simonsen CS, Flemmen HØ, et al. High prevalence of fatigue in contemporary patients with multiple sclerosis. Mult Scler J Exp Transl Clin. 2021; 7(1), doi: 10.1177/2055217321999826, indexed in Pubmed: 33796331.
17. Manjaly ZM, Harrison NA, Critchley HD, et al. Pathophysiological and cognitive mechanisms of fatigue in multiple sclerosis. J Neurol Neurosurg Psychiatry. 2019; 90(6): 642–651, doi: 10.1136/jnnp-2018-320050, indexed in Pubmed: 30683707.
18. Weiland TJ, Jelinek GA, Marck CH, et al. Clinically significant fatigue: prevalence and associated factors in an international sample of adults with multiple sclerosis recruited via the internet. PLoS One. 2015; 10(2): e0115541, doi: 10.1371/journal.pone.0115541, indexed in Pubmed: 25692993.
19. Billones R, Liwang JK, Butler K, et al. Dissecting the fatigue experience: A scoping review of fatigue definitions, dimensions, and measures in non-oncologic medical conditions. Brain Behav Immun Health. 2021; 15: 100266, doi: 10.1016/j.bbih.2021.100266, indexed in Pubmed: 34589772.
20. Berger JR, Pocoski J, Preblick R, et al. Fatigue heralding multiple sclerosis. Mult Scler. 2013; 19(11): 1526–1532, doi: 10.1177/1352458513477924, indexed in Pubmed: 23439577.
21. Levin LI, Munger KL, O'Reilly EJ, et al. Primary infection with the Epstein-Barr virus and risk of multiple sclerosis. Ann Neurol. 2010; 67(6): 824–830, doi: 10.1002/ana.21978, indexed in Pubmed: 20517945.
22. Ioannides ZA, Csurhes PA, Douglas NL, et al. Sustained Clinical Improvement in a Subset of Patients With Progressive Multiple Sclerosis Treated With Epstein-Barr Virus-Specific T Cell Therapy. Front Neurol. 2021; 12: 652811, doi: 10.3389/fneur.2021.652811, indexed in Pubmed: 33790852.
23. Pender MP, Csurhes PA, Smith C, et al. Epstein-Barr virus-specific T cell therapy for progressive multiple sclerosis. JCI Insight. 2018; 3(22), doi: 10.1172/jci.insight.124714, indexed in Pubmed: 30429369.
24. Fisk JD, Ritvo PG, Ross L, et al. Measuring the functional impact of fatigue: initial validation of the fatigue impact scale. Clin Infect Dis. 1994; 18 Suppl 1: S79–S83, doi: 10.1093/clinids/18.supplement_1.s79, indexed in Pubmed: 8148458.
25. Häuser W, Almouhtasseb R, Muthny FA, et al. [Validation of a German Version of the Fatigue Impact Scale FIS-D]. Z Gastroenterol. 2003; 41(10): 973–982, doi: 10.1055/s-2003-42927, indexed in Pubmed: 14562194.
26. Tabuse H, Kalali A, Azuma H, et al. The new GRID Hamilton Rating Scale for Depression demonstrates excellent inter-rater reliability for inexperienced and experienced raters before and after training. Psychiatry Res. 2007; 153(1): 61–67, doi: 10.1016/j.psychres.2006.07.004, indexed in Pubmed: 17445908.
27. Serafini B, Severa M, Columba-Cabezas S, et al. Epstein-Barr virus latent infection and BAFF expression in B cells in the multiple sclerosis brain: implications for viral persistence and intrathecal B-cell activation. J Neuropathol Exp Neurol. 2010; 69(7): 677–693, doi: 10.1097/NEN.0b013e3181e332ec, indexed in Pubmed: 20535037.
28. Topping J, Dobson R, Lapin S, et al. The effects of intrathecal rituximab on biomarkers in multiple sclerosis. Mult Scler Relat Disord. 2016; 6: 49–53, doi: 10.1016/j.msard.2016.01.001, indexed in Pubmed: 27063622.
29. Flensner G, Ek AC, Landtblom AM, et al. Fatigue in relation to perceived health: people with multiple sclerosis compared with people in the general population. Scand J Caring Sci. 2008; 22(3): 391–400, doi: 10.1111/j.1471-6712.2007.00542.x, indexed in Pubmed: 18840223.
30. Braley TJ, Chervin RD. Fatigue in multiple sclerosis: mechanisms, evaluation, and treatment. Sleep. 2010; 33(8): 1061–1067, doi: 10.1093/sleep/33.8.1061, indexed in Pubmed: 20815187.
31. Braley TJ, Boudreau EA. Sleep Disorders in Multiple Sclerosis. Curr Neurol Neurosci Rep. 2016; 16(5): 50, doi: 10.1007/s11910-016-0649-2, indexed in Pubmed: 27068547.
32. Popp RFJ, Fierlbeck AK, Knüttel H, et al. Daytime sleepiness versus fatigue in patients with multiple sclerosis: A systematic review on the Epworth sleepiness scale as an assessment tool. Sleep Med Rev. 2017; 32: 95–108, doi: 10.1016/j.smrv.2016.03.004, indexed in Pubmed: 27107751.
33. Siegert RJ, Abernethy DA. Depression in multiple sclerosis: a review. J Neurol Neurosurg Psychiatry. 2005; 76(4): 469–475, doi: 10.1136/jnnp.2004.054635, indexed in Pubmed: 15774430.
34. Baker D, Marta M, Pryce G, et al. Memory B cells are major targets for effective immunotherapy in relapsing multiple sclerosis. EBioMedicine. 2017; 16: 41–50, doi: 10.1016/j.ebiom.2017.01.042, indexed in Pubmed: 28161400.

35. Mameli G, Cocco E, Frau J, et al. Serum BAFF levels, Methypredsinolone therapy, Epstein-Barr Virus and Mycobacterium avium subsp. paratuberculosis infection in Multiple Sclerosis patients. Scientific Reports. 2016; 6(1), doi: 10.1038/srep29268.
36. The Wearing-off Phenomenon of Ocrelizumab in Patients With Multiple Sclerosis [2000]. https://clinicaltrials.gov/ct2/show/record/NCT04478591 (16.02.2022).
37. Abrahamyan S, Eberspächer B, Hoshi MM, et al. German Competence Network Multiple Sclerosis (KKNMS), Other members of the KKNMS that acted as collaborators in this study. Complete Epstein-Barr virus seropositivity in a large cohort of patients with early multiple sclerosis. J Neurol Neurosurg Psychiatry. 2020; 91(7): 681–686, doi: 10.1136/jnnp-2020-322941, indexed in Pubmed: 32371533.
38. Dobson R, Kuhle J, Middeldorp J, et al. Epstein-Barr–negative MS: a true phenomenon? Neurol Neuroimmunol Neuroinflamm. 2017; 4(2): e318, doi: 10.1212/nxi.0000000000000318.
39. Jacobs BM, Giovannoni G, Cuzick J, et al. Systematic review and meta-analysis of the association between Epstein-Barr virus, multiple sclerosis and other risk factors. Mult Scler. 2020; 26(11): 1281–1297, doi: 10.1177/1352458520907901, indexed in Pubmed: 32202208.
40. Mescheriakova JY, van Nierop GP, van der Eijk AA, et al. EBNA-1 titer gradient in families with multiple sclerosis indicates a genetic contribution. Neurol Neuroimmunol Neuroinflamm. 2020; 7(6), doi: 10.1212/NXI.0000000000000872, indexed in Pubmed: 32796079.
41. Bjornevik K, Cortese M, Healy BC, et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. Science. 2022; 375(6578): 296–301, doi: 10.1126/science.abj8222, indexed in Pubmed: 35025605.
42. Zivadinov R, Cerza N, Hagemeier J, et al. Humoral response to EBV is associated with cortical atrophy and lesion burden in patients with MS. Neurol Neuroimmunol Neuroinflamm. 2016; 3(1): e190, doi: 10.1212/NXI.0000000000000190, indexed in Pubmed: 26770996.
43. Ramroodi N, Niazi AA, Sanadgol N, et al. Evaluation of reactive Epstein-Barr Virus (EBV) in Iranian patient with different subtypes of multiple sclerosis (MS). Braz J Infect Dis. 2013; 17(2): 156–163, doi: 10.1016/j.bjid.2012.09.008, indexed in Pubmed: 23465600.
44. Cencioni M, Mattoscio M, Magliozzi R, et al. B cells in multiple sclerosis — from targeted depletion to immune reconstitution therapies. Nature Reviews Neurology. 2021; 17(7): 399–414, doi: 10.1038/s41582-021-00498-5.
45. Gingele S, Jacobus TL, Konen FF, et al. Ocrelizumab depletes CD20+ t cells in multiple sclerosis patients. Cells. 2018; 8(1), doi: 10.3390/cells8010012, indexed in Pubmed: 30597851.
46. Möhn N, Konen FF, Pul R, et al. Experience in multiple sclerosis patients with COVID-19 and disease-modifying therapies: a review of 873 published cases. J Clin Med. 2020; 9(12), doi: 10.3390/jcm9124067, indexed in Pubmed: 33339436.
47. Adamczyk B, Morawiec N, Arendarczyk M, et al. Multiple sclerosis immunomodulatory therapies tested for effectiveness in COVID-19. Neurol Neurochir Pol. 2021; 55(4): 357–368, doi: 10.5603/PJNNS.a2021.0051, indexed in Pubmed: 34346052.