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Virus-specific antibody responses in multiple sclerosis patients treated with Ocrevus

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ABSTRACT

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system. B cell-depleting therapy is highly efficient in treating patients with relapsing-remitting MS (RRMS), although the mechanisms behind reducing disease progression with this type of therapy is unknown. Virus infections are associated with the onset of MS and antibodies to these have previously been suggested to supplement MS diagnostics. Based on this, we aimed to investigate the effect of Ocrevus (OCR) (B cell depletion therapy) on selected virus antibody levels.

Blood samples were collected from RRMS patients before ($n = 13$) and during OCR treatment ($n = 29$) and from healthy controls (HCs) ($n = 15$). Serum antibodies to virus antigens from Epstein-Barr virus (EBV), severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), Rubella virus, Measles virus, John Cunningham polyomavirus, Mumps virus, Merkel cell polyomavirus, Varicella zoster virus, Influenza A virus, Human herpes virus 6, and Cytomegalovirus were analyzed by enzyme-linked immunosorbent assay.

EBV nuclear antigen 1 (EBNA1) IgG levels were elevated in RRMS patients compared to HCs independent of OCR treatment. However, no significant difference in virus antibody levels was observed following OCR treatment. Only SARS-CoV-2 spike protein IgG levels were significantly reduced following OCR treatment. The effect of OCR treatment on antibody levels may correlate with the time of infection. Only EBV EBNA1 IgG levels were significantly elevated RRMS patients at baseline compared to HCs, supporting that EBV infection is involved in the development of MS and confirming the diagnostic value of EBNA1 IgG.

1. Introduction

Multiple sclerosis (MS) is a chronic disease associated with inflammation in the central nervous system (CNS), leading to demyelination and axonal loss [1,2]. The disease is mostly characterized by clinical attacks or relapses, which typically show a dissemination in time and space, one of the cornerstones of the revised McDonald criteria for diagnosis of MS [3,4]. MS usually occurs in young adults and is more frequent in women than in men with an increased incidence in the westernized countries [1,5]. Typical symptoms include fatigue, numbness, loss of balance, physical and cognitive disabilities [1,2].

Although the etiology of MS remains to be fully understood, Epstein-Barr virus (EBV) appears to be strongly associated with the development of MS [6–9]. EBV is a herpes DNA virus primarily infecting B cells, which induces asymptomatic infection in most people and can cause infectious mononucleosis in young adults [8,10]. Studies have described a 32-fold increase in the risk of developing MS after EBV infection [6], which is supported by recent findings describing that EBV infection associated with host genetic pre-dispositions increases the risk of developing MS up to 260-fold [9]. Even though a link has been determined between EBV and MS, the mechanism by which EBV contributes to the development of MS has not been identified, although EBV-infected B cell migration across the blood-brain barrier has been suggested to be a crucial step in the initiation and maintenance of inflammation in the CNS [7–9,11–13]. Furthermore, it has been hypothesized that molecular mimicry between EBV proteins and self-antigens contributes to the development of MS

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[14–16], although it remains to be determined whether the mechanisms associated with molecular mimicry resembles immunological cross-reactivity without necessarily functioning as a main contributor to the development of autoimmunity [17].

Besides a strong link between EBV and MS, other viruses occasionally have been proposed as potential triggering agents. These viruses among others include Human herpes virus (HHV) 6, Varicella-zoster virus (VZV), John Cunningham polyomavirus (JCV) and human endogenous retroviruses [18–22]. These viruses are ubiquitous and have a high prevalence in the adult population, moreover, they can establish lifelong infections and reactivate, which may be linked to MS relapses [20,23–25]. On the contrary Cytomegalovirus (CMV) has been reported to have a protective role in the development of the disease [26,27].

Independent of environmental triggering factors, MS was originally described as an autoimmune T cell-mediated disease [28]. However, studies have shown that B cells play a role in the disease pathogenesis as well [28]. This knowledge has been used in the development of highly effective disease-modifying therapies (DMT) targeting B cells, which have become available for MS treatment [29]. A majority of B cells express the surface molecule CD20, which may serve as a target for therapeutic monoclonal antibodies. CD20 is expressed by pre-B cells in the bone marrow, and naïve B cells and memory B cells in the germinal centers and lymphoid tissues [30]. Similarly, a small subset of CD3-positive T cells also express CD20 [31,32]. In contrast, most plasma blasts, antibody-producing plasma cells and hematopoietic stem cells do not express CD20 [30].

Ocrevus (OCR) was the first immunosuppressive humanized monoclonal antibody targeting CD20, which was approved for treatment of relapsing forms of MS and primary progressive (PP) MS [33,34]. Depletion of B cells from the circulation using DMTs such as OCR markedly reduces disease activity in MS patients, potentially by reducing the number of virus-infected B cells, although the mechanism by which B cell depletion reduces disease activity remains to be determined [13,35].

Based on this, the objective of this study was to investigate the effect of B cell-depleting OCR therapy on virus antibody responses in RRMS patients by screening serum samples from RRMS patients for antibody reactivity before and during OCR treatment.

2. Materials and methods

2.1. Reagents

NaCl was purchased from Unikem (Copenhagen, Denmark). Diethanolamine, Tween-20, Na_2CO_3, $NaHCO_3$, phenol red and $MgCl_2$ were from Merck (Darmstadt, Germany). Alkaline phosphatase (AP)-labelled goat anti-human IgG, Tris HCL and *p*-nitrophenylphosphate (*p*NPP) substrate tablets were from Sigma Aldrich (St. Louis, MO, USA). Polysorp microtiter plates were from Thermo Fisher Scientific (Roskilde, Denmark). EBV Epstein-Barr nuclear antigen (EBNA)1 was from Abcam (Cambridge, UK). CMV phosphoprotein (pp)52 was obtained from ProSpec-Tany TechnologyGene Ltd. (Rehovot, Israel). HHV 6 A polymerase processivity factor (p41) and EBV BamHI-A rightward frame (BARF)1 were from MyBioSource (San Diego, USA). VZV recombinant glycoprotein E (gE) was from Virogen (Boston, USA). Virus antigens influenza A (IAV) nucleoprotein (NuP), severe acute respiratory syndrome corona virus (SARS-CoV)2 spike (S) protein, Measles virus (MeV) NuP and Mumps virus (MuV) NuP were from Baltymas (Vilnius, Lithuania). Rubella virus (RuV) capsid protein (CaP), the Merkel cell polyomavirus (MCV) major capsid viral protein 1 (VP1) assembled into virus-like particles (VLPs) and JCV VP1 VLPs were produced In-house at the Life Sciences Center, Institute of Biotechnology, Vilnius, Lithuania, as previously described [36–38]. Further information about the tested virus proteins is found in Appendix 1.

2.2. Design, study population and procedure

RRMS patients ($n = 27$) and healthy controls (HCs) ($n = 15$) were recruited at the Department of Neurology, Rigshospitalet Glostrup between 2021 and 2022. HCs were negative for any known neurologic diseases and were gender- and age-matched to RRMS patients when possible. RRMS patient inclusion and exclusion criteria are listed in Table 1.

RRMS patients at baseline (-OCR) donated blood samples prior to their first OCR treatment, whereas the remaining samples were collected pre-infusion on the same day for RRMS patients receiving OCR treatment (+OCR). RRMS patients received OCR treatment according to the current standard guidelines for the Department of Neurology, Rigshospitalet Glostrup.

The collected blood samples were centrifuged at 2500*g* for 10 min whereafter serum was moved to new tubes and stored at −20 °C until further use.

2.3. Detection of virus antibodies in plasma by enzyme-linked immunosorbent assay

The presence of virus IgG in serum samples from RRMS patients and HCs was assessed by enzyme-linked immunosorbent assay (ELISA). Briefly, Polysorb microtiter plates were coated with virus protein (1 μg/mL) in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.5 % Phenol red, pH 9.6) overnight at 4 °C. Following coating, plates were blocked for 30 min in TTN (0.3 M NaCl, 20 mM Tris, 1 % Tween-20, pH 7.5). Next, patient sera diluted in TTN (1:100) were added to each well and incubated for 1 h, whereafter wells were washed 3 times in TTN and incubated for another hour with AP-labelled goat-anti-human IgG (1:5000). Bound antibodies were quantified by addition of *p*NPP (1 mg/mL) diluted in AP-substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8), whereafter absorbances were measured at 405 nm with background subtractions measured at 650 nm using a microtiter plate reader (Versamax, Molecular Devices, Sunnyvale, Ca, USA). Plates were washed between each step in TTN buffer (200 μL, 3 × 1 min).

For each microtiter plate a standard curve was included, which was composed of a two-fold serial dilution of a donor pool serum sample, starting from a dilution of 1:100. A RRMS pool ($n = 50$) and a donor pool ($n = 100$) were used as high positive and low positive controls, respectively. Samples were tested in duplicates (Appendix 2).

2.4. Determination of total IgG by competitive inhibition assay

Total IgG levels in serum samples of RRMS patients and HCs were assessed by a competitive inhibition assay. Briefly, human serum (1:100) preincubated with AP-conjugated goat-anti-human IgG (1:4000), diluted in TTN, for 1 h on a shaking table, whereafter the solution was transferred to a Polysorp microtiter plate precoated with human IgG (1 μg/mL) in carbonate buffer over night at 4 °C. The plates incubated for 1 h on a shaking table whereafter *p*NPP substrate tablets diluted in AP-substrate buffer were added to each well, whereafter absorbances were measured at 405 nm with background subtractions

Table 1
Inclusion and exclusion criteria.

Inclusion criteria	*Exclusion criteria*
1. Diagnosis of RRMS according to the revised McDonald criteria (2017) 2. Persons between 18 and 70 years	1. Persons under 18 and over 70 years 2. Persons with other neurological disease 3. Persons treated with cytostatic drugs or prednisolone >10 mg/day 4. Persons with malignant disease 5. Persons with anti-coagulant treatment (INR > 1.5) 6. Persons with thrombocytopenia (platelets $<100 \times 10^9$/L)

measured at 650 nm using a microtiter plate reader. Plates were washed between each step in TTN buffer (200 μL, 3 × 1 min).

For each plate a standard curve was included. This curve was composed of a ten-fold serial dilution of human IgG diluted in AP-conjugated Goat-anti human IgG (1:4000). Samples were tested in duplicates.

2.5. Ethics

The current study was conducted according to current guidelines and was approved by the Regional Scientific Committee of Copenhagen (No H-20012823, no. H-19036891). All individuals were notified and provided written informed consent.

2.6. Data analysis

MyAssays was used to create a 4-parameter logistic curve fit for calculation of IgG levels in serum (U/mL). Antibody levels being lower than the quantification limit of the respective assay were re-tested in a lower concentration or set to a background value, before being included in the statistical analysis. Antibody levels that were above the upper point of the standard curve were extrapolated using the 4-parameter polynomic function. For repeated measurements an average of virus IgG titers were used.

The intra-assay variation (% CV), standard deviation and standard error were calculated by MyAssays for each replicated sample. Only a variation <10 % for replicates were accepted for data analysis. Measurements of duplicates with a variation >10 % were repeated. The inter-assay variation of plates used for measuring serum and CSF titers was <15 % for all measurements. These data are available upon request.

Statistical analysis and visualization of results was performed using GraphPad Prism software (v 5.0, Graphpad, San Diego, CA, USA). Statistical analysis was performed by a nonparametric approach, where Mann Whitney *t*-tests were used for statistical analyses. A value of $p < 0.05$ was accepted as statistically significant, where * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$.

A correlation coefficient (r) measuring the strength between various variables was determined, where r was defined as follows: 0–0.25 = no correlation, 0.25–0.5 = weak positive correlation, 0.5–0.75 = moderate positive correlation, 0.75–1.0 = strong positive correlation.

3. Results

3.1. Population description

A total of 42 serum samples were collected from 27 RRMS patients. Twelve RRMS patients donated one serum sample, whereas the remaining 15 RRMS patients donated two samples and were followed for a period of 6 months or 12 months. Twenty-nine samples were collected from patients already in OCR treatment, whereas the remaining 13 samples were collected at baseline prior to OCR treatment. Three patients each donated 2 consecutive (6 months) blood samples while in OCR treatment. Patient characteristics are shown in Table 3.

3.2. Evaluation of baseline virus IgG levels in relapsing-remitting multiple sclerosis patients and healthy controls

Initially, baseline RRMS blood samples (n = 13) were tested for antibody reactivity to a panel of viruses by ELISA. Antibodies to two EBV antigens were determined, to account for that EBV cycles between a latent and a lytic cycle, where EBNA1 represents the latent cycle and BARF1 represents the lytic cycle.

The majority of RRMS serum samples were seropositive for antibodies to most virus antigens although not all samples from RRMS patients tested positive for SARS-CoV-2 S protein IgG, as some individuals neither had been vaccinated nor exposed to SARS-CoV-2 infection (Appendix 3) (Fig. 1). Significantly elevated EBV EBNA1 IgG levels were observed in RRMS patient samples compared to HCs ($p = 0.0382$), however for the remaining virus IgG levels, no difference in titers were observed between RRMS patient samples and HCs.

Table 3
Sample characteristics of included relapsing-remitting multiple sclerosis patients. * Twelve RRMS patients donated 2 samples, 1 prior to Ocrevus treatment and again after 6 or 12 months of treatment. ** Three patients donated 2 samples +3 years following initial Ocrevus treatment. *** Twelve patients donated 1 sample at baseline or after 0.5, 1–2 or + 3 years of Ocrevus treatment. Collectively, 42 samples obtained from 27 individuals were enrolled. For two patients their OCB status was missing and for three patients their IgG index was missing.

Characteristics	Number (%)
Gender	
Female	15 (56)
Male	12 (44)
Age (years)	
Medium age	38
Age range	18–66
IgG index	14/24 (58)
OCBs	25/25 (100)
Vitamin D (nmol/L)	79 (42–145)
BMI	25 (19–38)
RRMS patients donating both baseline and follow-up samples	(n = 12)*
Visit 1 (baseline)	12
Visit 2 (6 months after baseline)	4
Visit 2 (12 months after baseline)	8
RRMS patients donating 2 follow-up samples	(*n* = 3)**
RRMS patients donating one sample following OCR treatment	(n = 12)***
At baseline	1
0.5 years	1
1–2 years	2
+3 years	8

3.3. Changes in virus antibody levels upon Ocrevus treatment

Next, the effect of OCR treatment on antibody levels to virus antigens in RRMS samples was evaluated by ELISA, where serum samples were categorized into - OCR (*n* = 13) and + OCR groups (*n* = 26). For initial analyses three samples were excluded from the + OCR group, as three patients each contributed with two samples at close intervals (6 months) to this population.

When comparing virus IgG levels between RRMS baseline samples (-OCR) and RRMS patients in OCR treatment (+OCR) no statistically significant change in virus antibody levels was observed for most virus antigens (Fig. 2). Hence, similar antibody titers to virus antigens were roughly observed for HCs and RRMS patients independent of OCR treatment.

Only SARS-CoV-2 S protein IgG levels in RRMS serum samples were significantly reduced upon OCR treatment ($p = 0.0186$). Moreover, S protein IgG levels were significantly reduced in the +OCR group when compared to HCs ($p < 0.0001$), whereas no difference in antibody levels were observed between the –OCR and the HC group, as previously presented (Fig. 2) ($p = 0.2495$).

Although significantly elevated EBV EBNA1 IgG titers were observed in the –OCR group compared to HCs ($p = 0.0382$), no statistically significant difference (albeit a trend) was observed between the +OCR group and the HC group ($p = 0.0960$), or between the –OCR and + OCR groups ($p = 0.6875$).

To determine whether the effect of OCR treatment on all enrolled RRMS patients was representative for the directly comparable samples, baseline RRMS patient samples (-OCR) and follow-up samples from RRMS patients (+OCR) (*n* = 12) with two visits were examined (Fig. 3).

As seen, the effect of OCR treatment on the individual virus antibody expression levels varied (Fig. 3) and no general pattern could be identified. The majority of matched serum pairs experienced no distinct difference in antibody expression levels over time. For each virus

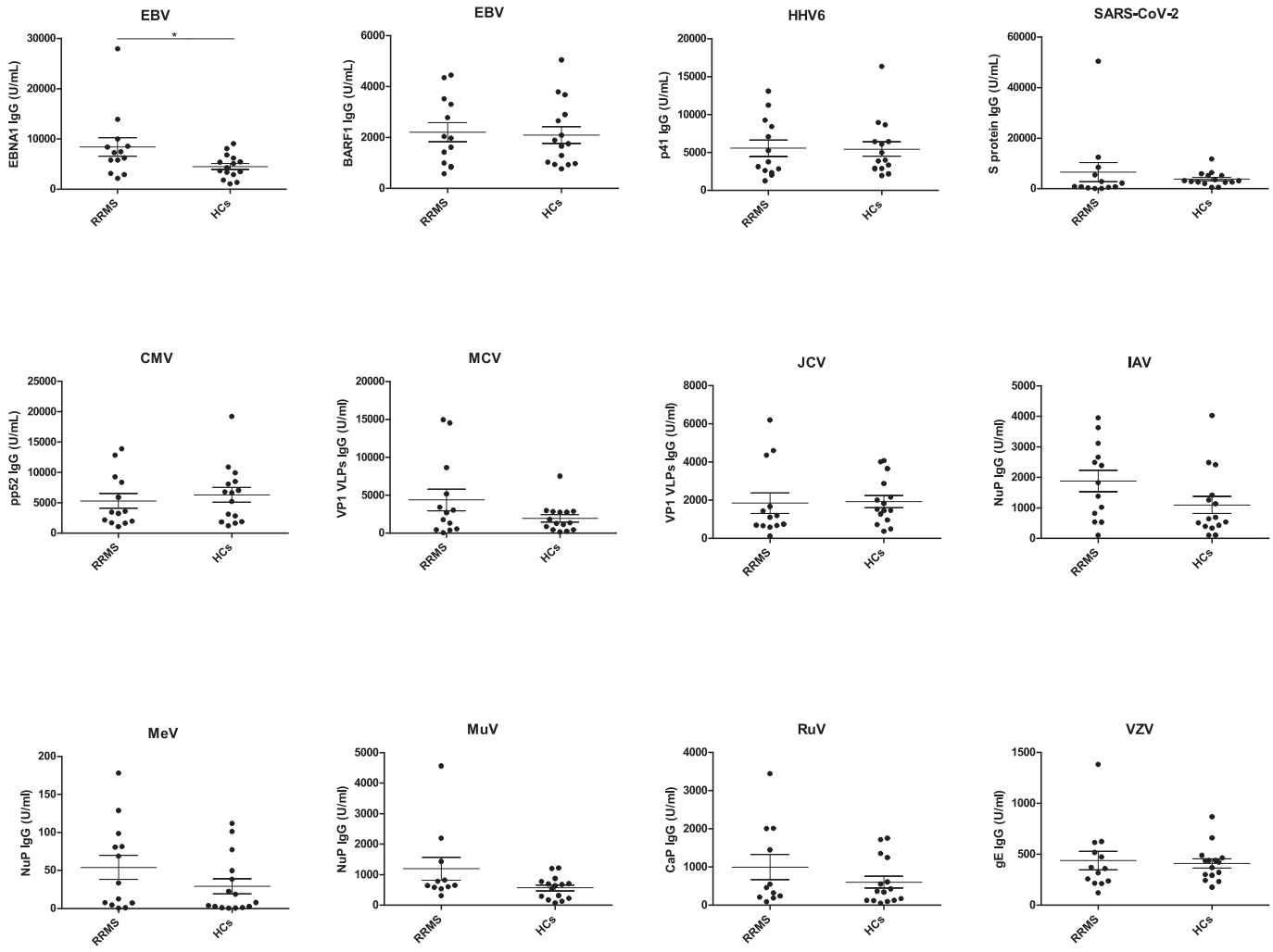

Fig. 1. Quantification of virus antibody titers in serum of relapsing-remitting patients (n = 13) collected at baseline and healthy controls (HCs) (n = 15). No significant difference in virus IgG levels in MS patients were detected compared to HCs. EBV EBNA1, $p = 0.0382$; EBV BARF1, $p = 0.9633$; HHV6 p41, $p = 0.8719$; SARS-CoV-2 S protein, $p = 0.2495$; CMV pp52, $p = 0.6450$; MCV VP1 VLPs, $p = 0.3109$; JCV VL1 VLPs, $p = 0.3569$; IAV NuP, $p = 0.0800$; MeV NuP, $p = 0.2310$; MuV NuP, $p = 0.1462$; RuV CaP, $p = 0.3502$; VZV gE, $p = 0.7822$. Error bars present mean with standard deviation. Statistical analyses were conducted using Mann Whitney t-tests, where a value of $p < 0.05$ was accepted as statistically significant, where * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$.

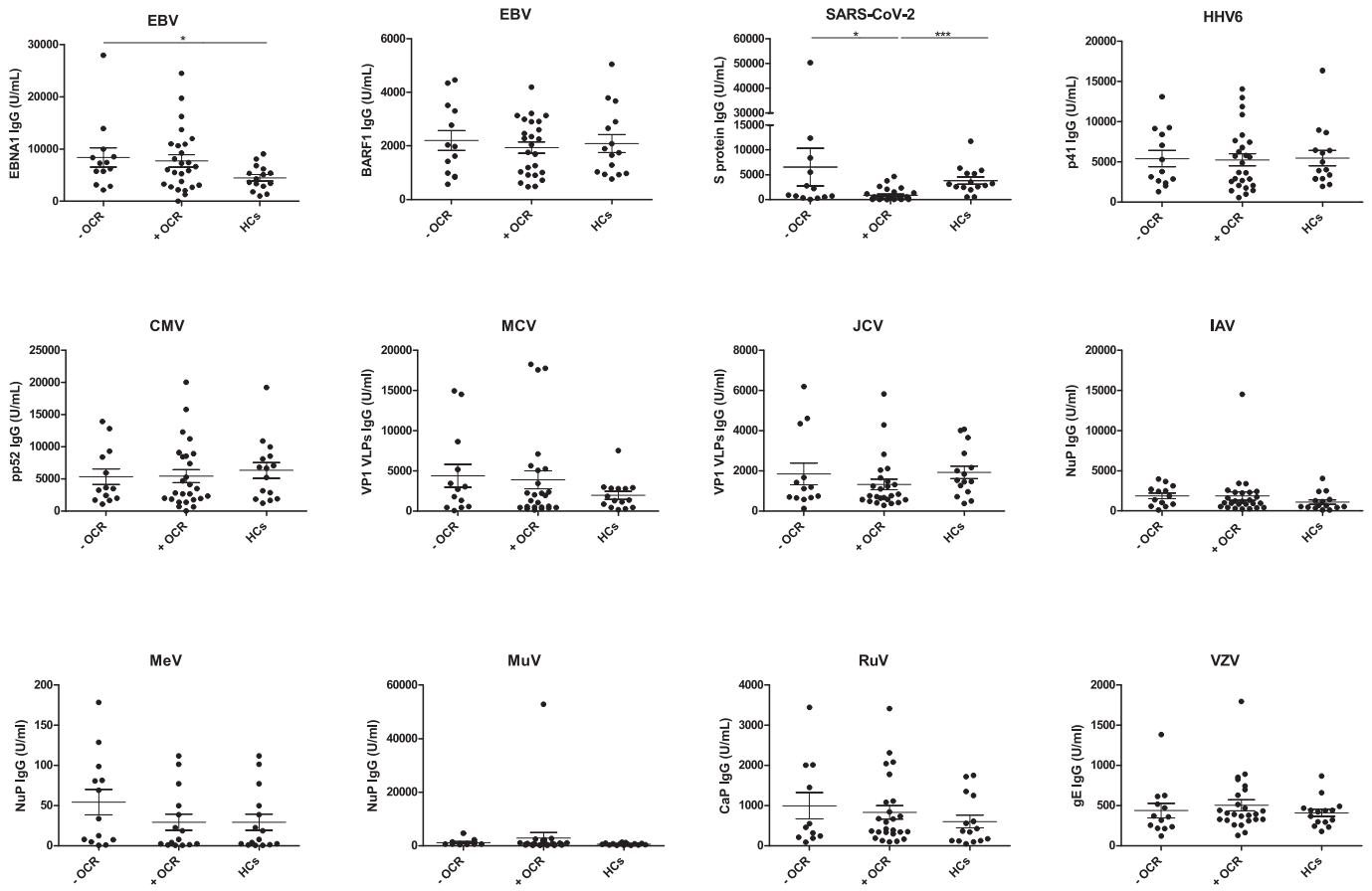

Fig. 2. Effect of Ocrevus treatment on virus antibody levels analyzed by enzyme-linked immunosorbent assay before (− OCR) and after Ocrevus (+ OCR) treatment compared to healthy controls (HCs) (n = 15). No statistically significant differences in virus IgG levels were observed between - OCR (n = 13) and + OCR groups (n = 26), besides from SARS-CoV-2 S protein IgG (p = 0.0186). EBV EBNA1 p = 0.6875; EBV BARF1 p = 0.6892; HHV6 p41 = 0.7777; CMV pp52 p = 0.8232; MCV VP1 VLPs p = 0.6444; JCV VP1 VLPs p = 0.4237; IAV NuP = 0.2639; MeV NuP p = 0.2310; MuV p = 0.3906; RuV CaP = 0.9179, VZV gE p = 0.3099. Error bars present mean with standard deviation. Statistical analyses were conducted using Mann Whitney t-tests, where a value of $p < 0.05$ was accepted as statistically significant, where * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$.

antigen tested, a majority of antibody levels remained within a range of −/+ 20 % variation compared to baseline concentrations, whereas a few antibody levels were observed to increase or decrease outside this range. Similarly, no statistically significant change in total IgG was found for the directly comparable RRMS samples ($p > 0.05$).

Finally, three RRMS patients donated two samples in the +OCR group within a short period (6 months). No difference was observed for virus IgG levels in blood samples from these patients as well, hence the virus antibody profiles for these RRMS patient samples followed the same trends as the directly comparable samples (Appendix 4).

3.4. Evaluation of total IgG levels

RRMS patient samples were evaluated with respect to total IgG levels and to OCR treatment and compared to HCs (Fig. 4).

No statistically significant change was observed between total IgG levels in RRMS baseline and HC samples ($p = 0.8262$). Moreover, neither gender nor age influenced total IgG at baseline when compared to HCs. OCR treatment was not observed to interfere with total IgG levels ($p = 0.8330$). Furthermore, neither gender nor age influenced total IgG in RRMS patients independent of treatment (appendix 5).

3.5. Correlation between clinical factors and Epstein-Barr virus antibodies

To analyze for possible virus infections associated to MS development, various virus antibody levels were compared to EBV EBNA1 and BARF1 IgG titers in RRMS patient baseline samples.

As presented in Table 4, a significant moderate positive correlation was determined between CMV pp52 IgG and EBV EBNA1 IgG ($r = 0.5758$, $p = 0.050$), while the remaining antigens presented with non-significant weak positive or weak negative correlations. Similarly, no correlation was observed between EBV EBNA1 IgG and BARF1 IgG ($r = 0.0454$, $p = 0.8829$).

Furthermore, correlations between EBV EBNA1 IgG and BARF1 IgG titers and clinical factors such as IgG index, D-vitamin and BMI were determined, however, no specific correlations were identified (Table 5).

4. Discussion

In this study, we evaluated the effect of B cell-depleting OCR therapy on virus antibody levels, and in particular EBV. We found that most antibody levels remained relatively stable after initiation of OCR therapy and persisted at this level at follow-up analysis at 6 or 12 months although with some exceptions, as SARS-CoV-2 S protein IgG levels were significantly reduced following OCR treatment. Similar studies have been conducted with varying results [13,39]. In some studies, a minor decrease in virus antibody levels was observed, whereas in other studies virus antibody levels were increased, depending on the specific virus antibody analyzed [13,39–42]. E.g., Rød et al. described that the humoral response to EBV and CMV in MS patients treated with OCR was reduced by approximately 15 % over a longer period [13]. These findings are in accordance with findings published by Zivadinov and Pham,

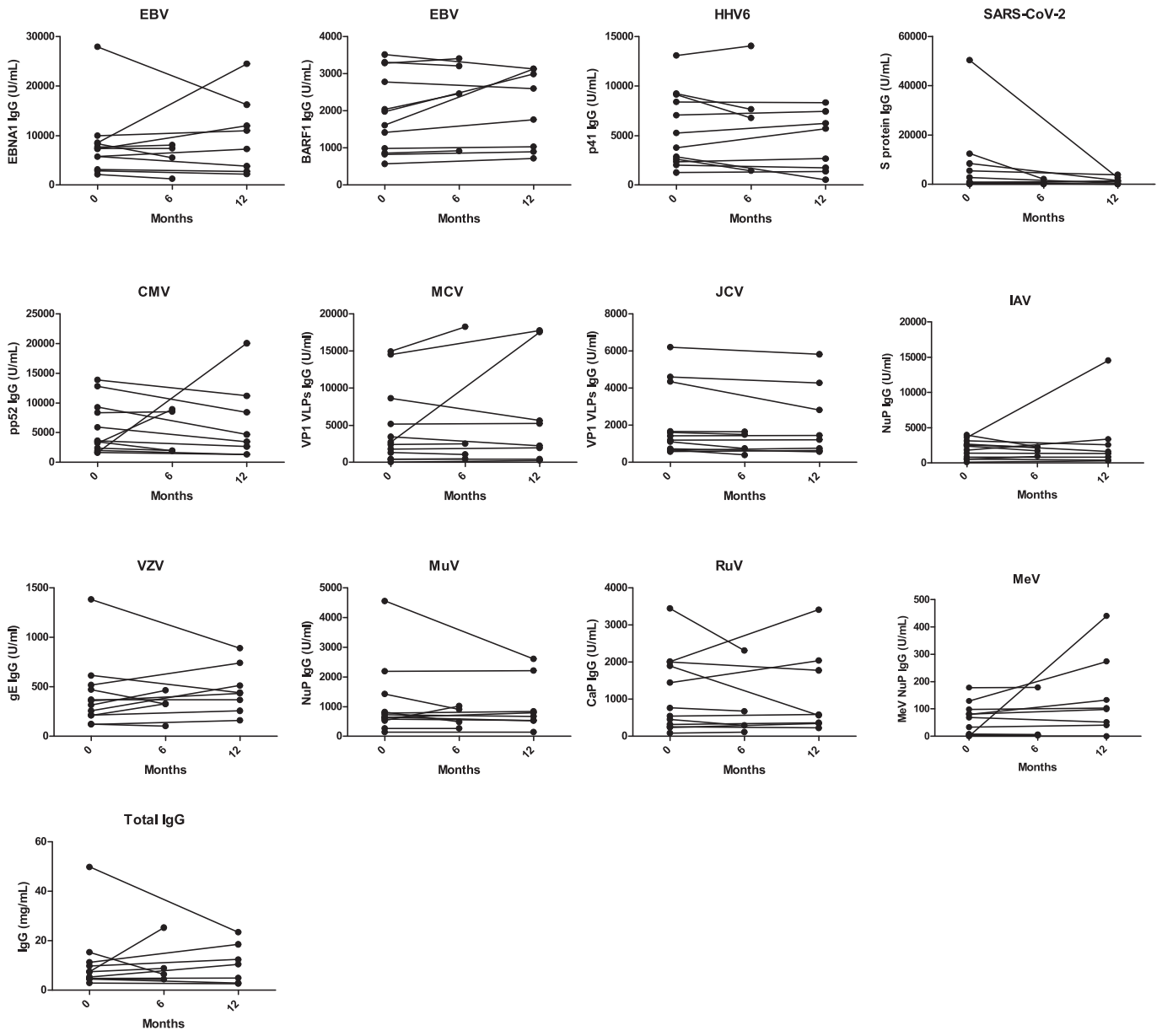

Fig. 3. Effect of Ocrevus treatment on virus antibody levels over time analyzed by enzyme-linked immunosorbent assay. No statistically significant difference in virus IgG levels was observed after 6 and 12 months of OCR therapy ($p > 0.05$ for all analyses comparing 0–6 months and 0–12 months). Error bars present mean with standard deviation. Statistical analyses were conducted using Mann Whitney t-tests, where a value of $p < 0.05$ was accepted as statistically significant, where * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$.

where significant decrease of EBV EBNA-1 IgG was evidenced as well [41,43]. On the contrary, Rød et al. described that EBV VCA IgG levels were increased by approximately 14 % three months after initiation of OCR treatment. Furthermore, Rød and colleagues observed a significant decrease in total IgG levels over time, whereas Pham and researchers could not confirm these findings [13,41]. Collectively, the effect of OCR treatment on the humoral virus response appear to vary from study to study. A trend indicates that virus antibody levels are reduced, although our findings could not confirm this conclusion.

The evidence relating EBV to MS disease development is compelling, although the precise role of EBV remains to be determined [6–9]. Based on this, the levels of EBV antibodies in serum and CSF of RRMS patients have received most attention. However, only EBV EBNA1 serum IgG levels were elevated in RRMS patients compared to HCs, confirming the diagnostic value of EBV EBNA1 IgG as previously reported [11,14,18]. Moreover, these findings indicate that EBV in RRMS patients primarily is found in a latent state, as lytic expression of EBV presumably would have resulted in increased EBV BARF1 IgG concentrations. In addition, no notable correlations were found between EBV EBNA1 IgG or EBV BARF1 IgG titers to the remaining virus antibodies, which is supported by earlier findings [11,12]. These findings may indicate that EBV may not solely be responsible for the ongoing pathology of MS. The key role of EBV may be early in the disease pathogenesis, e.g., at the initial trigger phase, where an ongoing immunopathology may also depend on (re-) activation of other neurotrophic viruses. In general, the role of EBV EBNA1 IgG remains to be determined. A persistently elevated EBV EBNA1 IgG level could in theory continue to have a continuous effect in neurodegeneration, or they could have no effect in MS disease in any stage of disease [17]. This remains to be elaborated. The former is in accordance with findings describing that molecular mimicry between EBNA1 and host proteins is essential for disease onset [15,16].

However, a moderate positive correlation was found between CMV

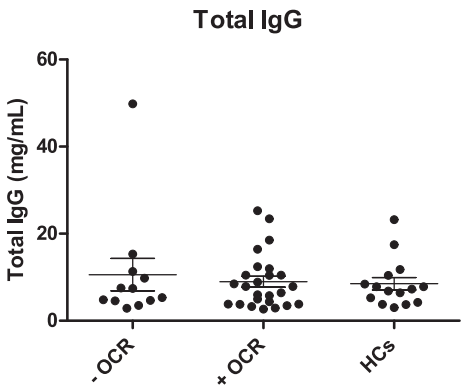

Fig. 4. Quantification of total IgG levels in Ocrevus treated-(-OCR, +OCR) relapsing-remitting multiple sclerosis patients and healthy controls (HCs) analyzed by enzyme-linked immunosorbent assay. Error bars present mean with standard deviation. Statistical analyses were conducted using Mann Whitney *t*-tests, where a value of $p < 0.05$ was accepted as statistically significant, where * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$.

Table 4
Correlation between antibodies to Epstein-Barr virus antigens EBNA1 and BARF1 and various viruses measured in baseline samples from relapsing-remitting multiple sclerosis patients (cohort 2).

	EBNA1		BARF1	
	R	*p*	*R*	*P*
SARS-CoV-2	0.037	0.909	−0.054	0.867
MuV	−0.177	0.604	−0.410	0.210
RuV	0.295	0.378	−0.001	0.998
VZV	−0.241	0.427	0.357	0.232
JCV	−0.254	0.402	−0.189	0.537
MCV	−0.162	0.598	−0.351	0.240
IAV	−0.331	0.267	−0.420	0.155
CMV	0.576	0.050	−0.168	0.601
MeV	0.272	0.3689	−0.228	0.456

Table 5
Correlation between Epstein-Barr virus antigens and clinical factors.

Antigen	BMI	D-vitamin	IgG index
EBV EBNA1	−0.1803	−0.1846	−0.2926
EBV BARF1	−0.1621	0.1550	−0.2908

pp52 IgG and EBV EBNA1 IgG, which is very interesting but difficult to interpret in relation to earlier findings, suggesting that CMV may have a protective role against MS development [27].

As observed, OCR treatment did not affect EBV EBNA1 virus antibody levels. These findings are in accordance with similar studies of treatment with Natalizumab as well as OCR, where small changes or no changes in antibody levels were observed [13,44]. In a recent study by Rød et al, results only showed minor differences in EBNA1 IgG levels after 6, 12 and 18 months of OCR treatment [13]. Similarly, CMV IgG levels in RRMS patients, who were CMV IgG positive at baseline did not change over a period of 10 months after initiation of B cell depletion therapy when compared to baseline concentrations, which is in accordance with our findings [13].

Common for viruses such as EBV, CMV and HHV6 is that these viruses typically infect the host early in life and persist in immune cells following infections [8,45–46]. To determine whether antibody levels to viruses recently presented to the immune system behaves in a similar way, antibody levels to SARS-CoV-2 S protein were determined. Interestingly, only SARS-CoV-2 S protein IgG levels were significantly decreased following OCR treatment. The effect of OCR treatment on antibody levels may correlate with the time of infection. Thus, antibody levels to virus infections obtained early in life such as EBV, HHV6 and CMV are not significantly influenced by OCR treatment, whereas antibody titers to the recent/current SARS-CoV-2 infection are significantly reduced in response to OCR treatment. This may be explained by that SARS-CoV-2 S protein-antibody-producing cells have not all migrated to the bone marrow as fully differentiated plasma cells. These results remain to be elaborated on.

SARS-CoV-2 S protein IgG results are interesting in relation to the effect of vaccines, as obtained results indicate that DMTs in RRMS treatment may reduce vaccine responses and increase the risk of SARS-CoV-2 infections. These findings are in accordance with that the durability of vaccinations is influenced by various factors, e.g., lymphocyte levels, age, and IgG levels [45,47].

A limitation of the present study is that not all MS patients donated samples at baseline and follow-up, which complicates interpretation of data. Moreover, the study population is relatively small, and the results should be repeated and extended using a larger cohort. Optimally, data on lymphocyte dynamics should also be collected.

Recent findings indicate that the HLA status may influence virus antibody levels, as Rød and colleagues reported that MS patients positive for HLA-DRB1*15:01 presented with elevated EBV EBNA1 IgG levels at baseline compared to HLA-DRB1:15:01-negative MS patients [13,48,49,50]. Based on this, one could argue that it may be necessary to match controls accordingly. However, as no significant difference was observed in EBV EBNA1 IgG levels in MS patients over time, the presented data may indicate that the effect of OCR is not influenced by the HLA status. This remains to be elaborated using a larger cohort.

Collectively, these findings indicate that OCR treatment does not interfere with antibody levels to past infections but may reduce antibody levels to recent infections or vaccinations. This should to be taken into account in relation to vaccination of MS patients. Specifically, our results on SARS-CoV-2 S protein antibodies, indicate that for some time after a recent infection and/or vaccination (SARS-CoV-2), CD20-positive antibody-producing cells persist in the immune system and are accessible to OCR treatment.

CRediT authorship contribution statement

Nadia Zivlaei: Methodology, Investigation, Formal analysis, Data curation. **Daut Can Asani:** Methodology, Investigation, Formal analysis, Data curation. **Nicole Hartwig Trier:** Writing – original draft, Visualization, Validation, Supervision, Software, Investigation, Formal analysis, Data curation. **Danguolė Žiogienė:** Resources. **Alma Gedvilaitė:** Resources. **Rasa Petraitytė Burneikienė:** Resources. **Evaldas Ciplys:** Resources. **Rimantas Slibinskas:** Resources. **Gunnar Houen:** Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Jette Lautrup Frederiksen:** Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

Jette Lautrup Battistini Frederiksen received funding from Roche Pharmaceuticals. Nadia Zivlaei, Daut Can Asani, Nicole Hartwig Trier, Danguolė Žiogienė, Alma Gedvilaitė, Rasa Petraitytė Burneikienė, Evaldas Ciplys, Rimantas Slibinskas and Gunnar Houen have nothing to disclose.

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Appendix 1

Appendix 1
Table S1. Antigen proteins applied in the current study.

Antigen	Sequence	Production host	Source	Reference, link
Epstein-Barr virus BamHI-A rightward frame (BARF)1	BARF1 full-length (221 a.a.) UniProtKB: P03228	E. coli	MyBioSource (San Diego, SD, USA) Cat # MBS1170055	MBS1170055 | Recombinant Epstein-Barr virus Secreted protein BARF1 (BARF1)
Epstein-Barr nuclear antigen (EBNA)1	EBNA1 full-length (Strain B95-8) (641 a.a.) UniProtKB: P03211	E. coli	Abcam (Cambridge, UK) Cat # 138345	Recombinant EBV Nuclear Antigen/EBNA1 protein (ab138345) | Abcam
Cytomegalovirus phosphoprotein (pp)52	Recombinant CMV pp52 fragment (a.a. 202-433). UniProtKB: P16790	E. coli	ProSpec-Tany TechnologyGene Ltd. (Rehovot, Israel). Cat # CMV-214	Cytomegalo Virus Pp52 (UL44) | CMV Pp52 Antigen | ProSpec
Human herpes virus 6A polymerase processivity factor (p41)	Full-length p41 (393 a.a.) Strain not specified by the manufacturer.	Yeast *Saccharomyces cerevisiae*	MyBiosource Cat # MBS1185516	MBS1185516 | Recombinant Human herpesvirus 6A DNA polymerase processivity factor
Varicella zoster virus recombinant glycoprotein E (gE)	Recombinant gE fragment (a.a. 48-135) Strain not specified by the manufacturer.	E. coli	Virogen (Boston, USA) Cat # 00200-V	VZV gE recombinant antigen | Varicella Zoster Virus & Antigens by ViroGen Corporation
Influenza A virus (IAV) nucleoprotein (NuP)	IAV strain A/New York/384/2005(H3N2) full-length NuP (498 a.a.). UniProtKB: Q3YQ35	Yeast *S. cerevisiae*	UAB Baltymas (Lithuania), prod. code #15-IAUlt-U2L	baltymas.lt/products/viral-nucleocapsid-proteins/influenza-a-virus/15-iault-u2l/
SARS-CoV-2 Spike (SCoV2-S) glycoprotein trimeric ectodomain	Expressed ectodomain included (a.a. 1-1208). UniProtKB: P0DTC2	Mammalian (hamster) CHO cells	UAB Baltymas (Lithuania), prod. code #20-S2S-TCg-G	baltymas.lt/products/viral-surface-proteins/spike-glycoprotein/20-s2s-tcg-g/
Measles virus (MeV) NuP	MeV strain Edmonston (Schwarz vaccine) full-length NuP (525 a.a.). GenBank: AAF85699.1	Yeast *S. cerevisiae*	UAB Baltymas (Lithuania), prod. code #12MeN-BSc-Gly	baltymas.lt/products/viral-nucleocapsid-proteins/measles-virus-recombinant-nucleoprotein-me-rn-expressed-in-yeast/12men-bsc-gly/
Mumps virus (MuV) NuP	MuV wild-type strain Glouc1/UK96 full-length NuP (549 a.a.). GenBank: AAG37826.1	Yeast *S. cerevisiae*	UAB Baltymas (Lithuania), prod. code #12MuNP-ASc-Gly	baltymas.lt/products/viral-nucleocapsid-proteins/mumps-virus/12munp-asc-gly/
Rubella virus (RuV) capsid protein (CaP)	RuV vaccine strain RA27/3 full-length CaP (300 a.a.). UniProtKB: P19725. Expressed CaP included a. a. 1-300 of the structural polyprotein.	Yeast *S. cerevisiae*	Institute of Biotechnology at Life Sciences Centre (LSC) of Vilnius University (VU)	[36]
Merkel cell polyomavirus (MCV) major capsid viral protein 1 (VP1)	MCV full-length VP1 (423 a.a.). UniProtKB: B6DVZ3	Yeast *S. cerevisiae*	Institute of Biotechnology at LSC of VU	[37]
John Cunningham polyomavirus (JCV) major capsid protein VP1	JCV full-length VP1 (354 a.a.). UniProtKB: P03089	Yeast *S. cerevisiae*	Institute of Biotechnology at LSC of VU	[38]

Appendix 2

A.1. Loading of 96-microtiter well plates for testing antibody levels towards different virus antigens in relapsing-remitting multiple sclerosis patients

For each ELISA experiment two plates were loaded. Loading of samples and standards was identical on both plates, however samples 21–39 were placed instead of 1–20 and 41 instead of 40 on plate 2. Additionally, healthy controls 8–15 were placed instead of 1–7.

Example of loaded ELISA plate well plate 1 with serum samples from MS patients and healthy controls:

Blank, non-coated wells with TTN buffer. Used as control for background noise.

Standards based on a pool from healthy controls. Standards were conducted from a 2-fold serial dilution starting at 1:100.

Samples from patients with MS.

1: High positive control, 2: Low positive control, 3: Negative control based on pool from blood donors, 4: TTN-buffer.

Healthy controls.

HC1 and 2 and placed on all plates used for inter- and intra-variation tests.

Raw data for ELISA standard.
Example of standard for coated EBNA1 EBV protein.
EBNA1 is coated 1 μg/mL in carbonate buffer.

Dilution (u/mL)	**Absorbance in duplicates ($A_{405–650}$)**
100	2.74/2.72
50	2.6/2.58
25	2.41/2.37
12.5	2.02/1.91
6.25	1.49/1.41
3.125	1/0.921
1.5625	0.725/0.653
0.78125	0.526/0.476

Appendix 3

Appendix 3
Severe acute respiratory syndrome coronavirus-2 infection and vaccination status in relapsing-remitting multiple sclerosis patients prior to Ocrelizumab treatment.

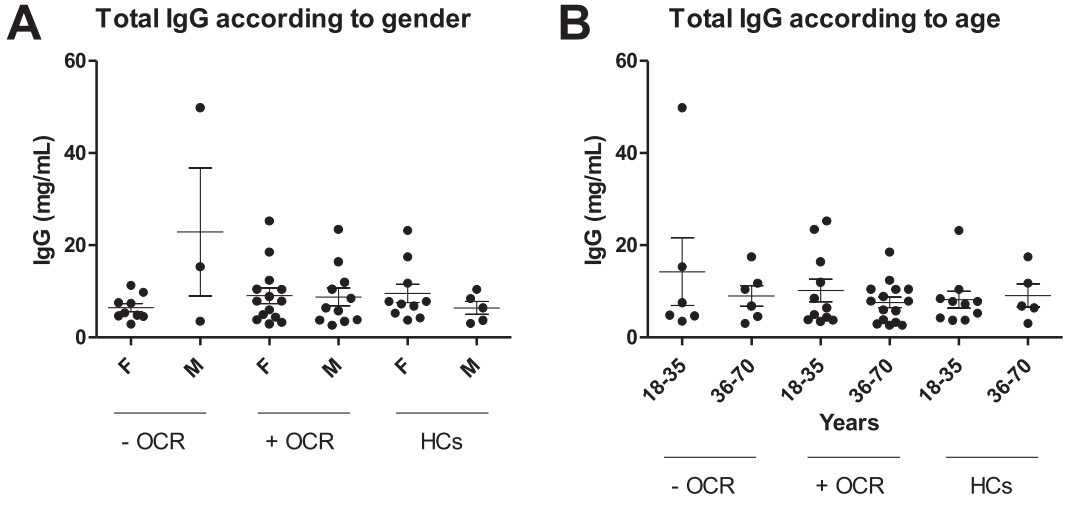
Baseline RRMS sample no	S protein IgG (U/mL)	Vaccinated	Infected
1	0.8	–	–
2	717	++	–
3	484	++	–
4	2773	++	–
5	845	++	+
6	8397	+++	–
7	50,373	+++	–
8	348	+++	–
9	5468	+++	–
10	12,400	++++	–
11	705	++++	–
12	2194	+++	–
13	211	–	–

Appendix 4

Appendix 4
Antibody virus titers in follow-up samples from three relapsing-remitting multiple sclerosis patients in Ocrevus treatment (+ 3 years).

Antigen	Patient 1		Patient 2		Patient 3	
	1. visit	*2. visit*	*1. visit*	*2. visit*	*1. visit*	*2. visit*
EBV EBNA1	9656	5250	7800	5	8460	5827
EBV BARF1	2900	18,644	1185	1039	613	648
CMV	3462	2796	2466	2	1835	19,140
HHV6	4242	3614	2845	2890	2403	2018
SARS-CoV-2	15	6	1	1	35	21
MCV	5064	3538	2208	1950	1277	1517
JCV	274	110	1311	923	832	494
IAV	1207	7094	2303	1395	3405	4832
VZV	406	297	258	254	390	576
MuV	285	200	285	202	1088	1089
MeV	1	1	22	17	250	14
RuV	730	548	163	114	343	295

Appendix 5. Quantification of total IgG levels in – OCR and + OCR-treated relapsing-remitting multiple sclerosis patients



Quantification of total IgG levels in – OCR and + OCR-treated RRMS patients. A. Quantification of total IgG levels according to gender in RRMS baseline samples and to HCs; (Female (F), $p = 0.3562$; Male (M) $p = 0.3929$). Total IgG according to gender following OCR treatment of RRMS patient (F $p = 0.3950$, M $p = 0.4363$). B. Quantification of total IgG levels according to age in RRMS baseline samples and to HCs (18–35 years $p = 1.000$, 36–70 years $p = 0.9266$). Comparison of total IgG levels according to age in RRMS baseline samples and RRMS samples in OCR treatment (18–35 years $p = 0.9599$, 36–70 $p = 0.5591$). Error bars present mean with standard deviation. Statistical analyses were conducted using Mann Whitney *t*-tests, where a value of $p < 0.05$ was accepted as statistically significant, where * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$.

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