Cytomegalovirus seropositivity is associated with reduced risk of multiple sclerosis—a presymptomatic case–control study

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Abstract

Background and purpose: Epstein–Barr virus (EBV) and human herpesvirus 6A (HHV-6A) are associated with increased risk of multiple sclerosis (MS). Conversely, infection with cytomegalovirus (CMV) has been suggested to reduce the risk of MS but supporting data from presymptomatic studies are lacking. Here, it was sought to increase the understanding of CMV in MS aetiology.

Methods: A nested case–control study was performed with presymptomatically collected blood samples identified through crosslinkage of MS registries and Swedish biobanks. Serological antibody response against CMV, EBV and HHV-6A was determined using a bead-based multiplex assay. Odds ratio (OR) with 95% confidence interval (CI) for CMV seropositivity as a risk factor for MS was calculated by conditional logistic regression and adjusted for EBV and HHV-6A seropositivity. Potential interactions on the additive scale were analysed by calculating the attributable proportion due to interaction (AP).

Results: Serum samples from 670 pairs of matched cases and controls were included. CMV seropositivity was associated with a reduced risk for MS (OR = 0.70, 95% CI 0.56–0.88, p = 0.003). Statistical interactions on the additive scale were observed between seronegativity for CMV and seropositivity against HHV-6A (AP 0.34, 95% CI 0.06–0.61).
**INTRODUCTION**

Multiple sclerosis (MS) is an autoimmune disease driven by inflammation against central nervous system antigens. The prevailing hypothesis is that environmental risk factors may trigger inflammatory processes resulting in demyelination in the central nervous system, which may be facilitated by certain genetic predispositions [1]. Epstein–Barr virus (EBV) is now firmly established as such a risk factor for MS [1]. Infection in early childhood is often asymptomatic and does not appear to increase the risk for MS, whilst symptomatic infection—‘infectious mononucleosis’—in adolescents and adults is strongly associated with increased MS risk [2]. EBV infection appears to be a prerequisite for MS in adults [3,4], and it was recently shown that the association of EBV seropositivity and MS risk is age-dependent; EBV seropositivity was associated with a decreased MS risk before 20 years of age and an increased risk after that age [5]. Human herpesvirus 6A (HHV-6A) has in recent years emerged as a risk factor for MS [6,7]. Contrary to EBV, HHV-6A appears to be associated with increased MS risk in all age groups [5]. Cytomegalovirus (CMV), another virus from the *Herpesviridae* family (denoted HHV-5), has been suggested to play a protective role in MS aetiology. A negative association of past CMV infection and risk of developing MS has been observed in serological studies on samples collected after MS diagnosis [8–12]. However, reverse causation may explain associations seen between exposures and diseases when samples are drawn after disease onset. Therefore, presymptomatic samples are preferred, but such previous studies on MS and CMV have been underpowered [13–16]. The aim of the present study was to increase the understanding of CMV in MS aetiology through a sufficiently powered study with serological analyses of presymptomatically collected serum samples.

**METHODS**

**Trial design and patients**

By crosslinkage of MS registries and microbiological biobanks, plasma or serum samples were identified and retrieved from individuals who later in life developed relapsing–remitting MS, as described elsewhere [17]. In short, the Swedish MS registry was crosslinked with five Swedish microbiological biobanks, containing remainders of sera from clinical analyses. An additional microbiological biobank was crosslinked with a local MS database at the University Hospital of Umeå, Sweden. All samples were donated before the age of 40 and prior to MS symptom onset. For each case, one control was randomly selected, matched for biobank, sex, date of blood sampling and date of birth (in order of priority).

**Laboratory procedures**

Serological responses to viruses were analysed using a bead-based multiplex assay, as previously described in detail [18]. Briefly, each bacterially expressed viral antigen was loaded onto a fluorescence-labelled glutathione-casein-coated bead set and presented to primary serum antibodies in a multiplex. Bound primary antibodies were detected using a biotinylated goat-α-human immunoglobulin G (IgG)/IgM/IgA secondary antibody and streptavidin-R-phycocerythrin as reporter dye. A Luminox 200 analyser was used to measure median fluorescence intensities (MFI) for each bead set, that is, antigen, thereby quantifying antibody levels. The following recombinantly expressed antigens were used to detect serum antibodies against CMV: pp28; pp52; and pp150 N-terminus (pp150-N) [19]. For EBV, the following antigens were used: EBV nuclear antigen 1 truncated, amino acids 325–641 (EBNA-1 trunc); EBV nuclear antigen 1 peptide, amino acids 385–420 (EBNA-1 pep); and viral capsid antigen p18 (VCA p18) [19]. For HHV-6A, truncated immediate-early protein 1 from HHV-6A (IE1A) was used [7]. Inter-batch controls and linear or modified logarithmic models were used to correct for batch-related variability.

Antigen serostatus for each virus was assessed in accordance with previously validated cut-offs [19]. In the validation study, the CMV serological assay was validated against two reference assays on two independent reference serum panels. Sensitivity and specificity in comparison to both reference assays was high and resulted in two sets of cut-offs. The differences in the cut-offs were most probably driven by the different serum panels and reference assays used [19]. The antibody responses against CMV are nearly dichotomous [19] and the published cut-offs are probably influenced by a small number of samples with intermediate seroreactivity. After assessing the antibody reactivity distribution in the present study, the higher set of cut-offs was selected: pp28, 200 MFI; pp52, 1101 MFI; and pp150N, 655 MFI. A sensitivity analysis was performed with the lower set of cut-offs: pp28, 73 MFI; pp52, 854 MFI; and
pp150N, 100 MFI. Overall seropositivity for CMV was defined as seropositivity against at least two CMV antigens, as suggested in the validation study [19].

For EBV, the following validated cut-offs were used: EBNA-1 trunc, 1800 MFI; EBNA-1 pep, 411 MFI; and VCA p18, 2526 MFI [19]. Seropositivity for EBV was defined as serological response exceeding cut-offs for one or more of the three antigens. A post hoc analysis was performed where EBV antigen EBNA-1 pep was used separately. EBNA-1 pep has been validated as a single antigen for detection of EBV antibodies and demonstrated high specificity (90.8%) and sensitivity (88.2%) [19].

The assay for HHV-6A has been partly validated for specificity by assessing seroconversion in acute and convalescent sera from children with exanthema subitum, a syndrome confirmed to be caused by HHV-6B [20], where seroconversion was seen for IE1B but not for IE1A [7]. However, no established gold standard reference assay is yet available for comparison studies. Therefore, seropositivity for HHV-6A was defined as IE1A over 50 MFI to maximize sensitivity and specificity, as described elsewhere [5]. The data on EBV and HHV-6A serostatus have been published previously [5,7]. A subset of the data on CMV has been used in a previous study as covariate in a regression analysis to adjust for possible confounders [7]. These data have been expanded for the current study and were used to assess a different hypothesis.

Statistical analyses

Analyses were performed on the entire cohort and stratified based on age at blood sampling: <20 years and 20–39 years of age. Matched pairs with participants on different sides of the age limits were assigned to the younger group, which contained fewer individuals. Odds ratio (OR) for CMV seropositivity as risk factor for MS was calculated using conditional logistic regression and adjusted for EBV and HHV-6A by including serostatus for all three viruses in the model. A sensitivity analysis was performed for samples drawn >8 years before symptom onset. A subgroup analysis was also performed, stratified by sex.

Interactions between CMV and HHV-6A or EBV serostatus were analysed on an additive scale using conditional logistic regression, calculating attributable proportion (AP) due to interaction [21,22]. Confidence intervals for AP were calculated as described by Hosmer and Lemeshow [21]. The post hoc analysis assessed interactions between serostatus for CMV and EBV antigen EBNA-1 pep with regard to MS risk. Conditional logistic regression analyses of all combinations of serostatus for the three viruses were performed in the older group. For all interaction analyses, the exposure group with the lowest MS risk was used as reference category. Interactions on the multiplicative scale were assessed by modelling the CMV–EBV and CMV–HHV-6A product terms in conditional logistic regression. The distribution of categorical variables was analysed using Pearson’s $\chi^2$ test or Fisher’s exact test, where appropriate.

Statistical analyses were performed in IBM SPSS version 26, SAS version 9.4 and R Studio version 1.3.

Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki and approved by the Regional Ethical Review Board in Umeå (2011-198-31M).

RESULTS

In total, 670 pairs of matched cases and controls were included. Median age at the time of blood sampling was 25 years and median time from sampling to symptom onset of MS was 8 years. The absolute mean differences for sampling date and sampling age between cases and controls were 6 days and 152 days, respectively.

Viral serostatus as risk factor for MS

Seropositivity against CMV was more common amongst females than males (56.8% vs. 43.5%; $p < 0.001$) and increased by age (Table 1). CMV seropositivity was significantly associated with a decreased risk of developing MS at age 20–39 (Figure 1) and in the total cohort (unadjusted OR = 0.71, 95% CI 0.57–0.90, $p = 0.003$). The result was similar after adjustments for EBV and HHV-6A serostatus (OR 0.70, 95% CI 0.56–0.88, $p = 0.003$). The sensitivity analysis using the lower set of cut-offs for CMV antigens yielded similar results (Figure S1). The negative association of CMV and MS risk remained in the sensitivity analysis of samples drawn >8 years before symptom onset (OR 0.66, 95% CI 0.48–0.93, $p = 0.016$, n = 664). The result was also similar in the female subgroup (unadjusted OR = 0.69, 95% CI 0.54–0.88, $p = 0.002$) but not statistically significant amongst men (unadjusted OR = 0.91, 95% CI 0.51–1.65, $p = 0.76$).

Epstein–Barr virus seropositivity was associated with an increased MS risk in the older group and a non-significant decreased MS risk in the younger group (Figure 1). HHV-6A seropositivity was consistently associated with an increased risk for MS in both age groups and in the total cohort (Figure 1).

Interactions of viral serostatus on MS risk

Compared to controls, a significantly higher proportion of cases were both seropositive for EBV and seronegative for CMV. This difference was observed at sampling age 20–39 and in the whole cohort (Table 2). The interaction analysis was complicated by the high proportion of EBV seropositive individuals. At age 20–39, the reference category of EBV seronegative and CMV seropositive individuals contained only one case and five controls (Table 2). Therefore, no interaction analysis could be performed for CMV and EBV serostatus.
In the post hoc analysis of CMV and EBNA-1 pep, a significant additive interaction was observed with regard to MS risk in the older stratum (Figure 2a).

As for CMV and HHV-6A serostatus, additive interactions with regard to MS risk were observed at sampling age 20–39 years and in the whole cohort (Figure 2b). The same trend was present in the younger group, although not statistically significant.

When all combinations of viral serostatus were analysed simultaneously, the highest MS risk was observed at age 20–39 for those seronegative for CMV and seropositive for both HHV-6A and EBNA-1 pep (OR = 16.4, 95% CI 5.7–47, p < 0.0001; Figure 3), although the confidence interval was very wide and some of the exposure groups were small.

No interactions were observed on the multiplicative scale between CMV and EBV or HHV-6A with regard to MS risk.

DISCUSSION

The key result of this study is the association between CMV seropositivity and a reduced risk of developing MS in a large presymptomatic cohort. In addition, additive interactions were observed with regard to MS risk between serostatus for CMV and the EBV antigen EBNA-1 pep, as well as for CMV and HHV-6A. Altogether, these findings indicate a role for CMV in MS aetiology.

The primary strength of this study is the large number of cases (n = 670) with samples collected before symptom onset. This study design contradicts previous concerns that the negative association of CMV and MS could be affected by reverse causation. A relatively large group of young cases were included, which allowed for analysis of risk factors for MS in two separate age groups. Cases and controls were matched on sex, age and date of sampling and the matching was kept intact through all statistical analyses. The method for assessment of CMV serostatus has recently been validated and demonstrated high sensitivity and specificity to detect antibodies against CMV [19]. The observed rates of CMV seropositivity, as well as the sex differences in this regard, are consistent with previous investigations of CMV seroprevalence [23,24].

Still, some limitations must be acknowledged. The mean time from sampling to MS symptom onset was 8 years. Even this latency might not be sufficient to ensure that samples are presymptomatic, considering the emerging evidence of a long prodromal phase of MS [25,26]. However, the negative association of CMV serostatus and MS risk remained in the sensitivity analysis of samples drawn more than 8 years before MS onset.

Sex stratified subgroup analyses were performed, but the low number of male cases (n = 108) limits the possibility of drawing conclusions from such assessments. Whether the association of CMV serostatus and reduced MS risk applies to men remains to be evaluated, but would require a larger sample.

Whilst the study was adequately powered for the regression analyses of antiviral serostatus and MS risk, the interaction analyses were
### TABLE 2 Comparison of viral serostatus between cases and controls

<table>
<thead>
<tr>
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<th>Case, n (%)</th>
<th>Controls, n (%)</th>
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<tr>
<td><strong>CMV and EBV</strong></td>
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<td><strong>All ages</strong></td>
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<tr>
<td>CMV+, EBV−</td>
<td>11 (1.6%)</td>
<td>10 (1.5%)</td>
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<td>CMV−, EBV−</td>
<td>30 (4.5%)</td>
<td>36 (5.4%)</td>
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<td>CMV+, EBV+</td>
<td>329 (49.1%)</td>
<td>382 (57.0%)</td>
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<td>CMV−, EBV+</td>
<td>300 (44.8%)</td>
<td>242 (36.1%)</td>
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<td>5 (3.5%)</td>
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<tr>
<td>CMV−, EBV−</td>
<td>22 (15.4%)</td>
<td>17 (11.9%)</td>
<td>0.39</td>
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<td>CMV+, EBV+</td>
<td>51 (35.7%)</td>
<td>61 (42.7%)</td>
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<td>CMV−, EBV+</td>
<td>60 (42.0%)</td>
<td>60 (42.0%)</td>
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<td><strong>Age 20–39</strong></td>
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<tr>
<td>CMV+, EBV−</td>
<td>1 (0.2%)</td>
<td>5 (0.9%)</td>
<td>0.22†</td>
</tr>
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<td>CMV−, EBV−</td>
<td>8 (1.5%)</td>
<td>19 (3.6%)</td>
<td>0.03</td>
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<td>CMV+, EBV+</td>
<td>278 (52.8%)</td>
<td>321 (60.9%)</td>
<td>0.007</td>
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<tr>
<td>CMV−, EBV+</td>
<td>240 (45.5%)</td>
<td>182 (34.5%)</td>
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<td><strong>CMV and EBNA-1 pep</strong></td>
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<td><strong>All ages</strong></td>
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<td>CMV+, EBNA−</td>
<td>27 (4.0%)</td>
<td>41 (6.1%)</td>
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<td>CMV−, EBNA−</td>
<td>43 (6.4%)</td>
<td>61 (9.1%)</td>
<td>0.07</td>
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<td>CMV+, EBNA+</td>
<td>313 (46.7%)</td>
<td>351 (52.4%)</td>
<td>0.04</td>
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<td>CMV−, EBNA+</td>
<td>287 (42.8%)</td>
<td>217 (32.4%)</td>
<td>&lt;0.0001</td>
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<td><strong>Age &lt;20</strong></td>
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<tr>
<td>CMV+, EBNA−</td>
<td>16 (11.2%)</td>
<td>9 (6.3%)</td>
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<td>CMV−, EBNA−</td>
<td>29 (20.3%)</td>
<td>25 (17.5%)</td>
<td>0.55</td>
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<td>CMV+, EBNA+</td>
<td>45 (31.5%)</td>
<td>57 (39.9%)</td>
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<tr>
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<td>53 (37.1%)</td>
<td>52 (36.4%)</td>
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<td><strong>Age 20–39</strong></td>
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<td>CMV+, EBNA−</td>
<td>11 (2.1%)</td>
<td>32 (6.1%)</td>
<td>0.001</td>
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<td>14 (2.7%)</td>
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<td>0.001</td>
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<td>234 (44.4%)</td>
<td>165 (31.3%)</td>
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<tr>
<td><strong>CMV and HHV-6A</strong></td>
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<tr>
<td><strong>All ages</strong></td>
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</tr>
<tr>
<td>CMV+, HHV-6A−</td>
<td>208 (31.0%)</td>
<td>289 (43.1%)</td>
<td>&lt;0.0001</td>
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<tr>
<td>CMV−, HHV-6A−</td>
<td>199 (29.7%)</td>
<td>215 (32.1%)</td>
<td>0.34</td>
</tr>
<tr>
<td>CMV+, HHV-6A+</td>
<td>132 (19.7%)</td>
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<td>CMV−, HHV-6A+</td>
<td>131 (19.6%)</td>
<td>63 (9.4%)</td>
<td>&lt;0.0001</td>
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<td><strong>Age &lt;20</strong></td>
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<td>CMV+, HHV-6A−</td>
<td>40 (28.0%)</td>
<td>51 (35.7%)</td>
<td>0.16</td>
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<tr>
<td>CMV−, HHV-6A−</td>
<td>56 (39.2%)</td>
<td>63 (44.1%)</td>
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<td>CMV+, HHV-6A+</td>
<td>21 (14.7%)</td>
<td>15 (10.5%)</td>
<td>0.29</td>
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<tr>
<td>CMV−, HHV-6A+</td>
<td>26 (18.2%)</td>
<td>14 (9.8%)</td>
<td>0.04</td>
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(Continues)
Figure 2 Additive interactions between serostatus for CMV, EBV antigen EBNA-1 pep and HHV-6A as risk factors for MS. AP, attributable proportion due to interaction; CI, confidence interval; CMV+, positive seroresponse for ≥2 CMV antigens (pp28, pp52, pp150N); EBNA+, positive seroresponse for EBV nuclear antigen 1 peptide; HHV-6A+, positive seroresponse for HHV-6A antigen (IE1A). Statistically significant results in bold.

Figure 3 Odds ratios for MS by different combinations of viral serostatus. Red box indicates exposure to risk factor (CMV seronegativity, HHV-6A seropositivity, EBNA-1 pep seropositivity). OR, odds ratio; CI, confidence interval; CMV–, negative seroresponse for ≥2 CMV antigens (pp28, pp52, pp150N); EBNA+, positive seroresponse for EBV nuclear antigen 1 peptide; HHV-6A+, positive seroresponse for HHV-6A antigen (IE1A). *p < 0.05, **p < 0.01, ***p < 0.001, significance for odds ratios [Colour figure can be viewed at wileyonlinelibrary.com]

which also reported a negative association of CMV and MS risk with OR = 0.73, 95% CI 0.58–0.92, p = 0.005 [10]. Both results are consistent with that of the present study, where the OR for CMV as a risk factor for MS was 0.70, 95% CI 0.56–0.88, p = 0.003.

To our knowledge, interactions on the additive scale between CMV and EBV or HHV-6A concerning MS risk have not been studied previously in presymptomatically collected samples. In a study on samples drawn after disease onset, containing 8742 cases and 7215 controls, no significant interaction between CMV and HHV-6A was found using a different measure of antibody response [7]. In the present study, validated cut-offs were used for each CMV antigen and a validated definition of CMV seropositivity [19].

Cytomegalovirus infects the majority of the adult population worldwide. About 60% of the adult population in developed countries and almost 100% in developing countries are seropositive for CMV [30]. After primary infection, CMV establishes a latent infection through several immune evasive mechanisms, affecting both innate and adaptive immune responses [30,31]. Immunocompetent hosts manage to control both the primary and the latent infection and rarely develop symptoms of the disease. In order to control the latent infection, a gradually increasing proportion of the immune system is committed to CMV. After years of latency, the immune response against CMV constitutes a major proportion of the immune system [30,32,33].

Whether this results in a reduced immunocompetence against other viruses remains unclear [32,34]. However, CMV seropositivity may reduce the immune response to EBV infection [35]. Against this background, it has been hypothesized that immune competition between CMV and EBV could be a possible mechanism behind the observed risk reduction for MS in CMV seropositive individuals [10]. The latent CMV infection is suggested to occupy a large proportion of the immune system, thereby reducing the adverse immune reaction against EBV that could lead to MS. Alternative mechanisms have also been suggested. For example, CMV infection promotes expansion of a subset of mature natural killer cells, which could modulate the control of EBV [36,37]. Interestingly, both the above hypotheses involve the immune response against EBV. Here, a significant additive interaction was observed between CMV and EBNA-1 pep with the combination of CMV seronegativity and EBNA-1 pep seropositivity inferring the highest risk for MS development. It is noteworthy that a single peptide is representing EBNA-1, and antibodies directed towards other fragments or the whole protein may differ in their effect on interaction. Still, this finding is consistent with the above hypotheses that CMV infection affects the immune response to EBV, either by immune competition or by expansion of mature natural killer cells. However, significant interactions between CMV and HHV-6A were also observed, suggesting that these hypotheses could be extended to also involve HHV-6A.

Cytomegalovirus infection can be acquired throughout the entire life span. During infancy, infection is often transferred through breastfeeding. Viral reactivation and viral shedding in breast milk
occur for over 90% of seropositive mothers, which leads to sub-
clinical infection of many infants [38]. Accumulating evidence indi-
cates that breastfeeding may reduce the risk for MS in the offspring
[39,40]. Considering the results from the present study, the trans-
mition of CMV through breast milk could contribute to the ob-
served risk reduction for MS in individuals that were breastfed.

When acquired during adolescence or later, CMV infection can
cause mononucleosis with symptoms almost indistinguishable from
those caused by EBV. Hypothetically, the interaction between CMV
and EBV-1 pep serostatus that was observed in the present study
could reflect a decreased risk for EBV induced infectious mononu-
cleosis in individuals already infected with CMV. Past CMV infection
would then add to the hypothesis that young individuals with im-
munity against EBV are protected against infectious mononucleosis,
which could be the key event that triggers the adverse immune re-
action causing MS.

A statistical interaction was also observed between CMV and
HHV-6A serostatus, with the combination of CMV seronegativity
and HHV-6A seropositivity inferring the highest risk for MS de-
velopment. Similar to the case for CMV and EBV, past CMV infection
could reduce the risk for an HHV-6A-related autoimmune response.
These two statistical interactions are suggestive of biological inter-
action and may relate to their evolutionary relationship. All three
viruses belong to the *Herpesviridae* family and are thus genetically
homologous. Still, the genetic distances between these three viruses
are considerable, and future research will tell whether the relation-
ship has a bearing on their interaction.

In conclusion, our results provide further evidence of the nega-
tive association between CMV serostatus and MS risk, as well as the
significance of HHV-6A and EBV in MS aetiology.

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CONFLICT OF INTEREST
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stitutional consultancy fees from Mabion S.A. AFH has received a
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have provided honoraria for advisory boards and/or lectures. None
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NBr, JB, NBe, AL, LAM, MG, MV, TB, OA, IK, TW and PSu report no
disclosures.

AUTHOR CONTRIBUTIONS
Viktor Grut: Formal analysis (lead); writing—original draft (lead);
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DATA AVAILABILITY STATEMENT
Anonymized data are available from the corresponding author upon
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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.