












# Interactions between High Seroreactivity to Human Herpes Virus 6A and Epstein–Barr Virus in MS Development: A Presymptomatic Case–Control Study

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Synergistic interactions between human herpesvirus 6A (HHV-6A) and Epstein–Barr virus (EBV) are hypothesized in the etiopathogenesis of multiple sclerosis (MS). This study investigated if HHV-6A and EBV seroreactivities interact regarding the risk of developing MS. Antibodies against viral antigens were analyzed in biobank samples from 670 individuals who later developed MS and matched controls. Additive interactions were analyzed. A significant interaction between HHV-6A and EBNA-1 seroreactivities was observed in study participants above the median age of 24.9 years (attributable proportion due to interaction = 0.45). This finding supports the hypothesis that HHV-6A and EBV infections interact in MS development.

ANN NEUROL 2024;96:302–305

Epstein–Barr virus (EBV) infection is an established risk factor for developing multiple sclerosis (MS).<sup>1</sup> The effect of EBV on MS risk is age-dependent; infection at a young age is associated with a protective effect and later infection with an increased risk of MS.<sup>2</sup> Repeated studies have reported EBV seropositivity in virtually all individuals with MS, indicating that the infection is a

prerequisite for the development of MS.<sup>1</sup> However, the seroprevalence of EBV in the general population is also high, suggesting that additional factors are important in the etiopathogenesis of MS. Recent studies indicate that human herpesvirus 6A (HHV-6A) infection is also associated with an increased risk of developing MS.<sup>2,3</sup> Latently EBV-infected B-cells appear susceptible to co-infection with HHV-6A, resulting in an increased expression of EBV antigens.<sup>4,5</sup> This suggests a mechanistic link between the viruses in MS aetiopathogenesis.<sup>6</sup> However, supporting data from prospective studies are lacking.

The objective of this study was to determine if levels of EBV- and HHV-6A antibodies interact regarding the risk of developing MS.

## Methods

We performed a nested case–control study by crosslinking MS registries with 6 Swedish biobanks, thereby identifying serum samples from cases who later developed relapsing–remitting MS. All samples were drawn before the clinical onset of MS and before the age of 40. For each case, 1 control was randomly selected and matched for biobank, sex, age at sampling, and birth date, as previously described.<sup>7</sup>

## Laboratory Procedures

A bead-based multiplex assay<sup>8</sup> was used to detect antibodies against all the human herpesviruses: herpes simplex virus type 1 (gG); herpes simplex virus type 2 (mgG), varicella zoster virus (VZV; gE, gI), EBV (EBNA-1, amino acids 385–420);<sup>9</sup> cytomegalovirus (CMV; pp28, pp52, pp150); HHV-6A (IE1A); HHV-6B (IE1B, 101 K),

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Additional supporting information can be found in the online version of this article.

Received Jan 30, 2024, and in revised form May 31, 2024. Accepted for publication May 31, 2024.

View this article online at [wileyonlinelibrary.com](https://www.wileyonlinelibrary.com). DOI: 10.1002/ana.27009.

HHV-7 (U14), and HHV-8 (LANA, K8, Orf65). Seroreactivities were dichotomized as high or low, using the median seroreactivity in controls (for EBNA-1) or the 75<sup>th</sup> percentile in controls (for the remaining antigens) as cutoffs, as in previous studies.<sup>3,10</sup>

### Statistical Methods

Additive interactions between high EBNA-1 and IE1A seroreactivity regarding the risk of developing MS were analyzed with conditional logistic regression, calculating odds ratios (OR) and attributable proportion due to interaction (AP). Comparative analyses were also performed for additive interactions between high seroreactivity against EBNA-1 and the remaining human herpes viruses. Since the association between EBV and MS risk depends on the age at infection,<sup>2</sup> analyses were stratified by median age (24.9 years). The exposure with the lowest risk of MS was used as the reference. Confidence intervals (CI) for AP were calculated as described by Hosmer and Lemeshow. The significance level was 0.05. The analyses were performed in SPSS, version 28.0, and R, version 4.3.

### Ethical Considerations

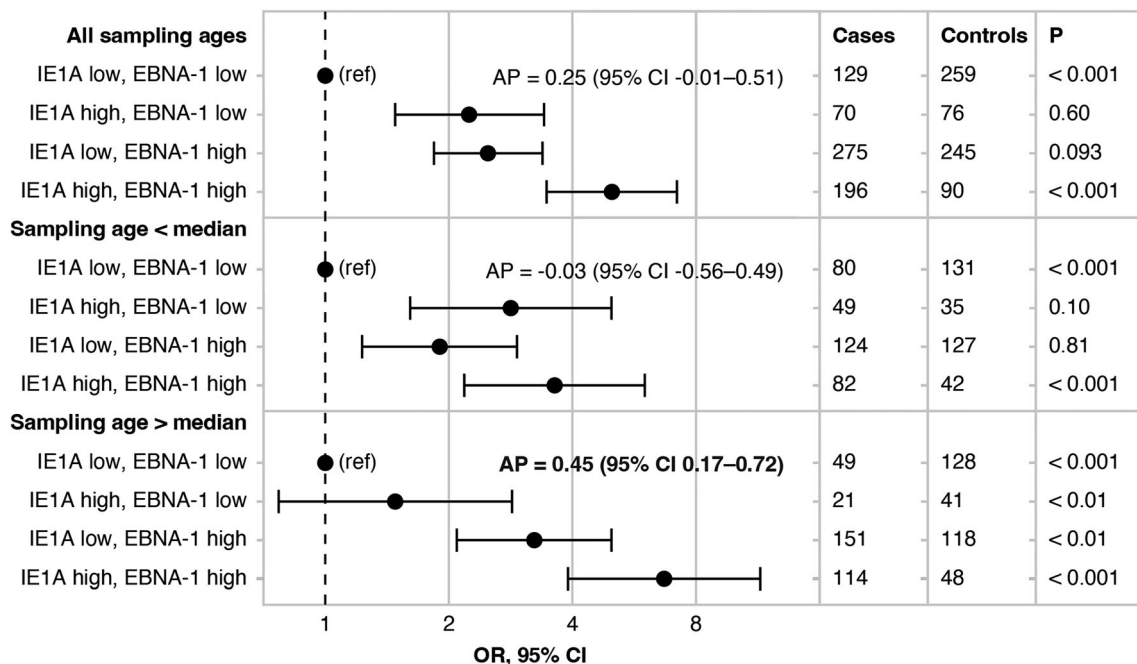
The regional ethical review board in Umeå, Department of Medical Research, approved this study (2011–

198-31 M with amendments). No written informed consent was required.

### Results

Samples from 670 cases and 670 matched controls were included. In controls, the median seroreactivity against EBNA-1 was 4,321 MFI and the 75<sup>th</sup> percentile of IE1A seroreactivity was 49.24 MFI (Figs S1 and S2, supplement). High EBNA-1 seroreactivity was observed in 471 cases and 335 controls ( $p < 0.001$ ), and high IE1A seroreactivity in 266 cases and 166 controls ( $p < 0.001$ ). High seroreactivity against both antigens was observed in 196 cases and 90 controls ( $p < 0.001$ ). The proportion with high IE1A and low EBNA-1 seroreactivity was similar in cases and controls (70 vs 76,  $p = 0.60$ ).

No significant interactions were observed in the whole sample or the younger subgroup (Fig). In the older subgroup, we observed a significant additive interaction between high EBNA-1 and high HHV-6A seroreactivity (AP = 0.45, 95% CI 0.17–0.72). The combination of the 2 risk factors was associated with a substantially increased risk of developing MS (OR = 6.7, 95% CI 3.9–11.5). Antagonistic interactions were observed for high seroreactivity against CMV antigens (pp28, pp52, pp150) and EBNA-1 (Fig S4, supplement). The antagonistic interaction between CMV and EBNA-1 seroreactivity was



**FIGURE:** Additive interactions between seroreactivities against EBV and HHV-6A in the risk of developing MS, calculated with conditional logistic regression. The odds ratios (OR) are presented with 95% confidence intervals (CI) on Log<sub>2</sub> scale. The subgroups are divided by median age (24.9 years). P values are calculated with Pearson's  $\chi^2$  test. AP, attributable proportion due to interaction; EBNA-1, Epstein-Barr virus nuclear antigen 1; EBNA-1 low/high, seroreactivity below/above median in controls; IE1A, truncated immediate-early protein 1 from human herpes virus 6A; IE1A low/high, seroreactivity below/above the 75<sup>th</sup> percentile in controls.

also significant on the multiplicative scale ( $p < 0.001$ ). In the older strata, an antagonistic interaction was also observed between high seroreactivity against VZV (gI) and EBNA-1 (Fig S3, supplement). No significant interactions were observed between high seroreactivity against EBNA-1 and the remaining human herpesviruses, including HHV-6B (Figs S3–S5, supplement).

## Discussion

In this study on samples collected before the clinical onset of MS, we report a synergistic effect between high seroreactivities against EBV and HHV-6A regarding the risk of developing MS later in life. The observed additive interaction – indicating biological interaction between the two infections – strengthens the hypothesis that a synergistic interplay between HHV-6A and EBV contributes to the development of MS.

A previous study, performed on samples collected after the clinical onset of MS, also reported interactions between EBV and HHV-6A antigens ( $AP = 0.24$ ).<sup>3</sup> Since HHV-6A is a neurotropic virus, residing in oligodendrocytes, the observed seroreactivity may be an epiphenomenon secondary to the disease, ie, affected by reverse causality. However, the current study was performed on presymptomatically collected samples, reducing this risk.

In the older half of the sample, we observed a strong interaction and a high risk of developing MS among those exposed to both high EBNA-1 and high IE1A seroreactivity. Since EBV infection in childhood is associated with a reduced MS risk,<sup>2</sup> we did not expect an interaction in that age strata. However, the presence of an interaction closer to MS onset is compatible with the involvement of both viruses—on a group level—in MS development.

The long prodromal phase of MS is a challenge when differentiating risk factors acting near the onset of the disease from factors secondary to the disease. The median time from sampling to MS onset in the older group was 6.5 years. Some cases in the older group may thus have developed a subclinical disease at the time of serum sampling. However, the interaction remained significant in samples collected more than 6.5 years before the clinical onset of MS:  $AP = 0.53$ , 95% CI 0.20–0.86. In addition, no significant synergistic interactions with EBNA-1 were observed for antibodies against the remaining human herpesviruses, which argues against a non-specific reaction secondary to inflammation.

The assay for the HHV-6A antibody IE1A has not been validated, since no reference sera are yet available. However, the assay has been tested for specificity in HHV-6B reference sera, indicating absence of cross-reactivity between HHV-6A and -6B.<sup>2,3</sup> Still, the association between

HHV-6A and MS remains tentative. Further studies are needed before we can draw any conclusions on the implications for individual patients.

Our findings are consistent with the hypothesis of viral interaction by reactivation.<sup>6</sup> Hypothetically, infection with EBV in adolescence or later could be sufficient to cause MS in individuals with genetic susceptibility or other risk factors. Conversely, MS risk following EBV infection in early childhood could depend on EBV reactivation by HHV-6A co-infection. While HHV-6A primarily infects T-cells, the virus has also been observed to infect B-cells if these are already latently infected with EBV.<sup>4,5</sup> Such co-infection leads to an increased expression of EBV antigens<sup>4,5</sup> and could be the hypothetical link between EBV infection in early childhood and later development of MS.<sup>6</sup>

In summary, we report a significant additive interaction between HHV-6A and EBV antibodies regarding the risk of later developing MS. This synergy supports the hypothesis of viral interaction in the development of MS.

## Acknowledgments

This study was supported by the Swedish Research Council, grant 2015-02419; the Visare Norr Fund, Northern County Councils' Regional Federation, grant 940405; the Research and Development Unit, Region Jämtland Härjedalen, grant JLL-967380; the Research Fund for Clinical Neuroscience at the University Hospital of Northern Sweden; Oskarfonden; and NEURO Sweden. PSt received funding from Margaretha af Ugglas stiftelse, the Swedish Neuro Foundation and the MS Research fund. TO received funding from the Swedish Research Council, The Swedish Brain Foundation and Margaretha af Ugglas stiftelse. IK received funding from Magareta af Ugglas stiftelse and The National MS Society, grant RFA-2104-37575. JH, PSt and IK were partially supported by Horizon 2020 MultipleMS, grant 733161.

## Author Contributions

P.Su., M.B., J.S., P.St., T.O., I.K., J.H., and V.G. contributed to the study's conception and design; all authors contributed to the acquisition and analysis of data; V.G., P.Su., M.B., J.S., and P.St. contributed to drafting the text and preparing the figures.

## Potential Conflicts of Interest

Nothing to report.

## Data Availability

Anonymized data are available from the corresponding author upon request.

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