Received: 26 February 2023 | Accepted: 7 June 2023

DOI: 10.1111/ene.15919

european journal of neurology
the official journal of the european academy of neurology

ORIGINAL ARTICLE

Risk of a first clinical diagnosis of central nervous system demyelination in relation to human herpesviruses in the context of Epstein–Barr virus

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Funding information
Multiple Sclerosis Australia; National Health and Medical Research Council; National Multiple Sclerosis Society

Abstract

Background and purpose: Epstein–Barr virus (EBV) is implicated in multiple sclerosis (MS) risk; evidence for other herpesviruses is inconsistent. Here, we test blood markers of infection with human herpesvirus 6 (HHV-6), varicella zoster virus (VZV), and cytomegalovirus (CMV) as risk factors for a first clinical diagnosis of central nervous system demyelination (FCD) in the context of markers of EBV infection.

Methods: In the Ausimmune case–control study, cases had an FCD, and population controls were matched on age, sex, and study region. We quantified HHV-6- and VZV-DNA load in whole blood and HHV-6, VZV, and CMV antibodies in serum. Conditional logistic regression tested associations with FCD risk, adjusting for Epstein–Barr nuclear antigen (EBNA) IgG, EBV-DNA load, and other covariates.

Results: In 204 FCD cases and 215 matched controls, only HHV-6-DNA load (positive vs. negative) was associated with FCD risk (adjusted odds ratio = 2.20, 95% confidence interval = 1.08–4.46, $p = 0.03$). Only EBNA IgG and HHV-6-DNA positivity were retained in a predictive model of FCD risk; the combination had a stronger association than either alone. CMV-specific IgG concentration modified the association between an MS risk-related human leucocyte antigen gene and FCD risk. Six cases and one control had very high HHV-6-DNA load ($>1.0 \times 10^6$ copies/mL).

Conclusions: HHV-6-DNA positivity and high load (possibly due to inherited HHV-6 chromosomal integration) were associated with increased FCD risk, particularly in association with markers of EBV infection. With growing interest in prevention/management of MS through EBV-related pathways, there should be additional consideration of the role of HHV-6 infection.

KEYWORDS
case–control, human herpesviruses, multiple sclerosis, viral infections

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INTRODUCTION

Past infection with Epstein–Barr virus (EBV), a member of the Herpesviridae family, is a well-documented risk factor for the development of multiple sclerosis (MS) [1, 2], with accumulating causal evidence. The importance of other viruses, particularly other human herpesviruses, is less clear, although their neurotropic behaviour and neuronal latency would fit with an effect on MS onset and relapse [3]. Furthermore, the interplay between EBV and other herpesviruses has not been examined.

Past infection with human herpesvirus 6 (HHV-6) has been repeatedly investigated as a risk factor for MS, with inconsistent findings [3–7]. There are two distinct variants; HHV-6A is more neurotropic, whereas HHV-6B causes the common infant infection roseola infantum. As with other herpesviruses, HHV-6 can persist in the host in latent form, reactivating at times of immunosuppression. Occasionally, the virus integrates into the chromosomes of host germline cells; every nucleated cell in offspring carries a copy of the virus genome. This "inherited chromosomally integrated HHV-6" [8] is uncommon, with a prevalence of ~1% (with various reports of 0.2%–2.9%), and is identified by persistently high viral DNA loads of $>1.0\times10^6$ copies/mL [8].

Primary infection with varicella zoster virus (VZV) causes chickenpox, and reactivation of the virus, herpes zoster (shingles). The evidence for an association between VZV infection and MS has been largely based on history of chickenpox and/or shingles, showing increased [9], reduced [10], or no association [11]. Cytomegalovirus (CMV) infection may be asymptomatic or mild, and can present as infectious mononucleosis. Past infection with CMV may be associated with reduced risk of MS [12–14] in at least some populations [15].

Of these herpesviruses, only VZV infection has characteristic skin lesions to provide confidence in a positive history of past infection. However, the presence of virus-specific serum antibodies provides evidence of recent or past infection, whereas the presence of viral DNA in whole blood indicates current/recent or persistent infection. Here, we test the association between blood markers of infection with multiple human herpesviruses and the risk of a first clinical diagnosis of central nervous system (CNS) demyelination (FCD) as a precursor to MS diagnosis.

METHODS

The Ausimmune Study was a multicentre case–control study to identify environmental risk factors for the development of MS [16]. The study recruited participants in four regions in eastern Australia (Brisbane city and surrounds, Newcastle city and surrounds, Geelong and the Western District of Victoria, and Tasmania) from 1 November 2003 to 31 December 2006. Cases (aged 18–59 years) had an incident FCD, including a classic clinically isolated syndrome (CIS; $n=216$), a first diagnosis of primary progressive MS (PPMS; $n=18$), and those with a prior undiagnosed, probable CIS ($n=48$). Cases were notified to the study by medical specialists; study neurologists confirmed the diagnosis based on patient-reported symptoms and clinical evidence (according to the 2001 McDonald criteria) [17]. Controls without evidence of CNS demyelination were randomly selected from the Australian Electoral Roll, matched to cases (1:1 to 4:1 matching ratio, depending on study region) on age (within 2 years), sex, and study region. Participation rates in the Ausimmune Study were 91% for cases and 60% for controls [18].

This viral biomarker substudy was undertaken while recruitment for the Ausimmune Study was ongoing; funding allowed for assays on 432 samples. Eligibility for inclusion rested on appropriate biological samples being available for cases and at least one matched control. There were no specific exclusion criteria. We first selected those with a classic CIS only; the remainder had an FCD but evidence of a prior, undiagnosed, probable CIS or a first diagnosis of PPMS.

We collected a broad range of self-reported sociodemographic and environmental exposure data [16], including education level and smoking history [18].

Venous blood was stored as serum and EDTA whole blood in 1-mL aliquots at −80°C until analysis. Vitamin D status was assessed as the serum 25-hydroxyvitamin D (25[OH]D) concentration [18] (using a liquid chromatography dual mass spectrometry assay), and DNA was genotyped for a range of MS-related genes, using proxy single nucleotide polymorphisms (SNPs) [19].

Viral DNA was extracted from EDTA whole blood and measured for viral load using quantitative real-time polymerase chain reaction (reported as copies/mL). For HHV-6, the *U67* gene was targeted, as it is highly conserved, contains one copy per genome, and was the target region routinely used in our diagnostic laboratory. Primers spanned a 108-bp section of the viral genome to detect both HHV-6A and HHV-6B variants [20]. Additional funding 1 year after the initial viral assays allowed genotyping of HHV-6-DNA [20]; DNA was re-extracted from archived samples and reamplified using a second set of primers spanning a larger segment of the *U67* gene. This served as a check to confirm previously positive samples as well as generating a longer region of freshly amplified DNA for sequencing. For VZV, a 244-bp section of the VZV *IE* gene was targeted [21].

Serum antibody levels were measured using immunofluorescence, reported as dilutions/titres (HHV-6-specific IgG and IgM) or optical density (CMV-specific IgG, VZV-specific IgG).

Epstein–Barr nuclear antigen (EBNA) IgG and EBV-DNA load were also measured, as previously described [19]. All assays were completed at the Clinical Virology Laboratory, Institute of Clinical Pathology & Medical Research, Westmead Hospital, Sydney, Australia; HHV-6-DNA quantification was replicated at the virology laboratory at Queensland Berghofer Institute for Medical Research, Brisbane, Australia, with good agreement.

Data analysis

Data management

DNA load was highly skewed for all tested viruses, with no detectable viral DNA in nearly 50% of samples. We thus modelled HHV-6-DNA

load as dichotomous (positive/negative) and as an ordinal categorical (negative/low/high) variable. VZV-DNA load and HHV-6-specific IgM were modelled as dichotomous variables only (due to the small number of nonnegative values when separated by case vs. control status). HHV-6-specific IgG (and EBNA IgG) was modelled as dichotomous (negative/positive), three ordinal categories (≤40, 160, >160), and as a continuous variable (log base 2 of the reciprocal of the dilution measured as titres) [19]. In the log base 2 transformation, the regression coefficient estimates the natural logarithm of the odds ratio (OR) associated with a twofold difference. CMV-specific IgG levels were highly skewed with no suitable transformation and were considered as dichotomous (low/high) and in categories defined by the quartiles of the control distribution. VZV-specific IgG was approximately normally distributed and modelled as dichotomous, quartiles of the control distribution, or a continuous (nontransformed) variable.

Statistical analysis

We used counts and proportions for categorical data and mean and SD or median and interquartile range (IQR) for continuous data (for normally or nonnormally distributed data, respectively) to describe the viral parameters and participant characteristics.

We used Spearman correlation to examine the correlation between the viral markers separately in the case and control groups, adjusting for multiple comparisons (Bonferroni).

To identify potential confounders for inclusion in multivariable analyses, we considered directed acyclic graphs based on prior literature where that exists, as well as data adaptive approaches, including univariate analyses. We thus assessed whether factors were antecedents of exposure, mediators, or disease consequences before considering their potential confounding role [22]. Univariate analyses used chi-squared test, *t*-test, analysis of variance, and Kruskal–Wallis test (to test the association between viral markers and other covariates within the control group only), and conditional logistic regression to test associations with case versus control status. We did not adjust for multiple comparisons in these exploratory univariate analyses, as, although this reduces the risk of false positives, it increases the risk of false negatives [23].

We used multivariable conditional logistic regression to test the association of viral markers with case versus control status, adjusting for potential confounders. Separate models were run in relation to each viral parameter and for any two viral parameters together (positive vs. negative for each). We used backward stepwise regression including all of the viral markers and those for EBV infection, to test the best (viral) predictors of case (vs. control) status.

We tested for multiplicative interaction between the different markers of viral infection, and those markers in relation to human leucocyte antigen (HLA) SNPs, testing the significance of the relevant product term when added to the model. We tested additive interaction using the Stata "ic" module [24] to estimate the relative excess risk due to the interaction, attributable proportion, and Synergy Index.

We report adjusted ORs (aORs) and 95% confidence intervals (CIs). Rather than emphasizing an arbitrary significance threshold, we present *p*-values as continuous estimates of the compatibility of the results with null hypotheses [25]. Missing data on covariates were replaced with an indicator, to retain in the analysis all participants with available exposure and outcome data. All analyses were undertaken using Stata (v17, StataCorp).

Standard protocol approvals, registrations, and patient consents

The Ausimmune Study was approved by nine regional Human Research Ethics Committees (led by the Human Research Ethics Committee of the Australian National University). All participants gave written informed consent prior to participation.

RESULTS

This viral substudy was based on data from 204 cases ($n=164$ with a classic CIS, $n=29$ with FCD and a probable prior undiagnosed CIS, $n=11$ with a first diagnosis of PPMS) and 215 matched controls (see Table 1). HHV-6 genotyping was undertaken on HHV-6-DNA-positive samples; most of these samples had HHV-6-DNA loads at the assay's limit of detection (2.0×10^2 copies/mL); thus, not all could be reamplified. Of the 24 (of 45) samples that could be genotyped, one case (of 15 cases and nine controls) was HHV-6A (U1102-like), with all other samples HHV-6B (HST-like). Of the 25 participants positive for VZV-DNA load, 24 had a wild-type genotype; one (control) participant carried VZV with a Eur C genotype and had a very high VZV-DNA load (2.7×10^8 copies/mL).

Seven participants had HHV-6-DNA loads $>1.0\times10^6$ copies/mL. All were female; six were cases (with four of these being from Brisbane). The one control was from Tasmania, had a low 25(OH)D level of 34 nmol/L, and was the only control with an HHV-6-DNA load of $>2.0\times10^2$ copies/mL.

Table S1 shows that, although there appeared to be several significant correlations between viral markers, and differences in the magnitude of those correlations between cases and controls, after adjustment for multiple comparisons only the correlation between HHV-6-specific IgM and HHV-6-specific IgG, in both cases and controls, was statistically significant.

In univariate analyses, only HHV-6-DNA load (apart from EBNA IgG), modelled as either a dichotomous or ordinal categorical variable, showed evidence of a significant association with case (vs. control) status (Table S2).

We next tested associations between viral markers and other covariates, including MS risk-related SNPs, in controls only (Table S3). HHV-6-specific IgG (log2) increased significantly with increasing education level. The median CMV-specific IgG concentration was

TABLE 1 Characteristics of participants in this study, focusing on markers of past viral infection.

Characteristic	Cases with FCD	Controls
Sex, % (*n*)		
Male	22.1 (45)	21.4 (46)
Female	77.9 (159)	78.6 (169)
Age, years, mean (SD)	39.0 (9.8)	39.9 (10.0)
Education category, % (*n*)		
Year 10 or less	24.1 (49)	32.2 (69)
Year 12 or TAFE	49.8 (101)	42.5 (91)
University	26.1 (53)	25.2 (54)
Total years smoking, median (IQR)	5.4 (0–18.6)	3.3 (0–16.2)
Serum 25(OH)D level, nmol/L, mean (SD)[a]	76.8 (31.2)	82.1 (31.9)
rs9271366 (*HLA-DRB1*1501*), % (*n*)[b]		
A:A	43.2 (82)	69.0 (129)
Any G	56.8 (108)	31.0 (58)
rs6904029 (HLA class 1 region), % (*n*)[c]		
Any A	37.7 (72)	54.8 (102)
G:G	62.3 (119)	45.2 (84)
rs2523393 (linked to *HLA-F-AS1*), % (*n*)[d]		
G:G	11.5 (22)	22.8 (43)
Any A	88.5 (170)	77.3 (146)
EBV-DNA positivity, % (*n*)		
Negative	87.8 (179)	88.8 (191)
Positive	12.3 (25)	11.2 (24)
EBNA antibody titre, % (*n*)		
≤40	21.6 (44)	41.9 (90)
160	43.1 (88)	38.1 (82)
≥640	35.3 (72)	20.0 (43)
HHV-6-DNA load, copies/mL, % (*n*)		
0 [not detected]	85.8 (175)	92.6 (199)
200	9.8 (20)	7.0 (15)
400	1.5 (3)	0.0
>1,000,000	2.9 (6)	0.5 (1)
HHV-6-specific IgM titre, % (*n*)[e]		
0	95.0 (190)	94.1 (191)
20	3.5 (7)	3.0 (6)
40	1.5 (3)	3.0 (6)
HHV-6-specific IgG titre, % (*n*)[e]		
≤40	31.9 (65)	34.4 (74)
160	46.1 (94)	44.7 (96)
≥2500	22.1 (45)	20.9 (45)
CMV-specific IgG [OD], % (*n*)		
0	29.4 (60)	24.2 (52)
<1 [but not zero]	16.7 (34)	16.3 (35)
1–10	22.6 (46)	20.9 (45)
>10	31.4 (64)	38.6 (83)
VZV-DNA load, copies/mL, % (*n*)		
0 [not detected]	95.6 (195)	92.6 (199)
200–1000		2.3 (5)
1200–8200	2.0 (4)	2.8 (6)
≥10,200	2.5 (5)	2.3 (5)
VZV-specific IgG [OD], % (*n*)		
<0.5	6.4 (13)	10.2 (22)
0.5 to <1.0	36.8 (75)	36.3 (78)
1.0 to <1.5	52.5 (107)	48.4 (104)
1.5–2.0	4.4 (9)	5.1 (11)

Note: Category boundaries that are discontinuous (e.g., VZV-DNA load) reflect no values between those category boundaries.

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CMV, cytomegalovirus; EBNA, Epstein–Barr nuclear antigen; EBV, Epstein–Barr virus; FCD, first clinical diagnosis of central nervous system demyelination; HHV, human herpesvirus; HLA, human leucocyte antigen; IQR, interquartile range; OD, optical density; TAFE, technical and further education; VZV, varicella zoster virus.

[a]Deseasonalized, control date of blood collection moved to that of case.

[b]Correlated with *HLA-DRB1*1501* [26]; G is associated with increased disease risk in the Ausimmune dataset.

[c]Correlated with *rs2394160* located approximately 15 kb centromeric of the *HLA-F* gene in the class 1 region and a risk factor for multiple sclerosis [26]; the A allele is associated with reduced disease risk in the Ausimmune dataset.

[d]See Cree et al. [27]; the A allele is significantly associated with increased disease risk in the Ausimmune dataset.

[e]Antibody titres are reported as the reciprocal of the last dilution in which the antibodies could be detected; only specific discrete values are possible.

higher in women compared to men (females: 8.23, IQR = 0.10–12.64; males: 0.24, IQR = 0–8.05, $p < 0.001$), and the median CMV-specific IgG concentration was significantly lower in participants homozygous for the G allele of *rs2523393*, an SNP in the HLA-B region. The median CMV-specific IgG concentration increased with age, but this was not statistically significant.

In multivariable conditional logistic models, adjusting for educational category, 25(OH)D concentration, smoking, EBNA IgG (log2), and EBV-DNA load (positive vs. negative), only HHV-6-DNA load showed evidence of an association with case versus control status (Table 2).

We further tested, in multivariable models: (i) the effect on the coefficient for EBNA IgG (log2) of adding other viral markers into the model; and (ii) the effect of being positive for (or having higher levels of) two viral markers, compared to negative for (low levels of) both and either, on case versus controls status (Table S4). Addition of any other viral marker to the multivariable model testing the association with EBNA IgG (log2) on case versus control status did not change the coefficient for EBNA IgG (variation from aORs = 1.26–1.27). Some combinations of viral markers could not be modelled

TABLE 2 Results of multivariable conditional logistic models of the association between viral markers and risk of FCD.

Variable	aOR (95% CI), *p*	aOR (95% CI), *p*[a]
HHV-6 DNA		
Negative[b]	1.00	1.00
Positive	2.20 (1.08–4.46), $p=0.03$	2.29 (1.10–4.74), $p=0.03$
HHV-6-DNA load		
0 [not detected][b]	1.00	1.00
Low [200 or 400 copies/mL]	1.82 (0.85–3.93), $p=0.13$	1.94 (0.88–4.29), $p=0.10$
High [$>10^6$ copies/mL]	6.13 (0.73–51.64), $p=0.10$	5.56 (0.66–47.07), $p=0.12$
p for trend	0.02	
HHV-6-specific IgM		
Negative[b]	1.00	1.00
Positive	0.92 (0.36–2.35), $p=0.87$	0.96 (0.37–2.50), $p=0.93$
HHV-6-specific IgG [log2]	1.03 (0.95–1.13), $p=0.47$	1.03 (0.94–1.12), $p=0.51$
CMV-specific IgG		
Quartile 1 [0][b]	1.00	1.00
Quartile 2 [0.01–6.47]	0.88 (0.49–1.57), $p=0.66$	0.94 (0.52–1.70), $p=0.84$
Quartile 3 [6.68–12.02]	0.86 (0.48–1.51), $p=0.59$	0.89 (0.50–1.59), $p=0.69$
Quartile 4 [12.03–19.52]	0.62 (0.33–1.16), $p=0.13$	0.64 (0.34–1.21), $p=0.17$
p for trend	0.16	
VZV-DNA detection		
Negative[b]	1.00	1.00
Positive	0.34 (0.09–1.33), $p=0.12$	0.37 (0.09–1.42), $p=0.15$
VZV-specific IgG	1.54 (0.76–3.13), $p=0.23$	1.66 (0.80–3.43), $p=0.17$

Note: Models are adjusted for education category, total years smoking, deseasonalized 25-hydroxyvitamin D, Epstein–Barr virus DNA (positive vs. negative), and Epstein–Barr nuclear antigen antibody titre (log2).

Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; CMV, cytomegalovirus; HHV, human herpesvirus; VZV, varicella zoster virus.

[a]Additional adjustment for *HLA-DR15*.

[b]Reference.

due to the small number of cases or controls with nonzero values. Despite suggestive findings of enhancement or reduction of FCD risk with some combinations of viral markers, for example, a marked increase in FCD risk for the combination of higher EBNA IgG and HHV-6-DNA positivity compared to low EBNA IgG and/or absence of HHV-6-DNA, there were no statistically significant additive or multiplicative interactions.

In the predictive model incorporating all of the viral markers (and adjusting for covariates as in the multivariable models), only EBNA IgG titre (log2; $p<0.001$) and HHV-6-DNA load (positive/negative; aOR = 2.38, 95% CI = 1.16–4.89, $p=0.02$) were retained in the model.

We found no significant additive or multiplicative interactions between HHV-6-related viral markers or VZV-DNA positivity and any of the measured HLA SNPs. However, there was a significant multiplicative interaction between higher CMV-specific IgG levels and *rs2523393* (a SNP in the HLA-B region) genotype ($p=0.02$; see Table 3). Having higher CMV-specific IgG (>1 vs. ≤1) was associated with a reduction in the increased risk of FCD associated with carrying the A allele of *rs2523393*.

DISCUSSION

In this case–control study of cases with an FCD and age-, sex-, and region-matched controls, in contrast to EBNA IgG levels, we found no evidence to support an adverse or protective association between IgG levels for HHV-6, VZV, or CMV and disease risk. Positive, or higher, HHV-6-DNA load was associated with increased FCD risk. In a predictive model including all viral markers, only higher EBNA IgG and HHV-6-DNA positivity were retained as significant predictors of FCD risk. There were suggestive findings that co-occurrence of different viruses could be important in higher FCD risk, for example, the combination of higher EBNA IgG and HHV-6-DNA positivity. We also found that higher CMV-IgG modified the risk associations of an MS-risk-related HLA SNP.

Our findings highlight the possible importance of HHV-6 as a risk factor for the onset of MS, particularly in the uncommon subgroup who have very high HHV-6-DNA levels. In a recent review of HHV-6 infection and MS risk, of the six studies focusing on MS onset, there was a positive association with HHV-6 seropositivity in four studies,

TABLE 3 Significant multiplicative interactions between viral markers and multiple sclerosis risk-related HLA single nucleotide polymorphisms.

rs2523393 (HLA-B region, linked to *HLA-F-AS1*)	CMV-specific IgG	
	Low (<1.0)	High (≥1.0)
G:G	aOR = 1.00	aOR = 2.95, 95% CI = 0.86–10.19, $p = 0.09$
G:A or A:A	aOR = 6.06, 95% CI = 2.22–16.58, $p < 0.001$	aOR = 3.48, 95% CI = 1.30–9.30, $p = 0.01$

Abbreviation: CMV, cytomegalovirus; HLA, human leucocyte antigen; aOR, adjusted odds ratio; CI, confidence interval.

and higher prevalence of positive HHV-6-DNA in cases compared to controls in two studies [28]. The authors did not discuss possible publication bias (nonpublication of null findings), but noted the non-uniformity of thresholds for seropositivity as a major issue in comparing studies. Additional evidence linking HHV-6 to MS comes from pathological and experimental studies. Intrathecal HHV-6-specific IgG and oligoclonal bands have been found in people with MS (reviewed in Voumvourakis et al. [28]). In marmosets predisposed to experimental autoimmune encephalomyelitis (EAE), intranasal inoculation of HHV-6 virus resulted in significantly accelerated EAE compared with control animals, with HHV-6 concentrated in brain lesions [29]. Hogestyn and colleagues suggest that HHV-6 may inhibit the migration of oligodendrocyte precursor cells and myelin repair, increasing vulnerability to MS onset [3].

All but one of the samples that could be genotyped for HHV-6 carried HHV-6B; one case sample carried the HHV-6A variant. This is not surprising, as HHV-6B is the cause of the common childhood illness roseola infantum. Nevertheless, although the numbers are small, that one case (but no controls) carried HHV-6A is consistent with HHV-6A being more neurotropic. A large recent study ($n = 8742$ people with MS, $n = 7215$ controls) tested the serological response to HHV-6A (IgG to the immediate–early 1 protein, IE1A) separately from that to HHV-6B (IE1B). Comparing the highest to the lowest quartile, the IE1A antibody was positively associated with MS (OR = 1.55, $p = 9 \times 10^{-22}$), whereas the IE1B antibody was negatively associated with MS (OR = 0.74, $p = 6 \times 10^{-11}$) [30]. Null serology findings in the current study may be at least partly explained by the failure to distinguish between HHV-6A and HHV-6B variants.

The prevalence of very high HHV-6-DNA load (potentially chromosomal integration) in the entire control group was 0.5% (1/215) and within the control group from Tasmania was 1.5%, that is, in keeping with the population prevalence described in previous studies [8]. Within case participants, the overall prevalence was 2.9% (6/204), and within the Brisbane-only cases was 5.8% (4/69). If these high DNA loads are the result of chromosomal integration, we speculate that this could be an additional risk factor for MS that is apparent within an otherwise low risk population.

The increased risk associated with higher EBNA IgG persisted after adjustment for any of the viral markers in this study but was possibly enhanced by the presence of HHV-6-DNA. There is compelling evidence that EBV infection is a risk factor/necessary cause of MS [31], most recently coming from a nested case–control study within a large US military cohort study [1]. Among the proposed mechanisms for the link between EBV infection/serology and MS is the "dual virus hypothesis" [32], whereby HHV-6A activates EBV latent in B cells in the CNS [33] either directly or via activation of a human endogenous retrovirus [32, 33], to induce CNS inflammation and demyelination. Measuring HHV-6 serology and DNA load (in blood or other tissues, e.g., fingernails) along with a host gene would be a powerful method to test independent or additive effects of HHV-6 and investigate HHV-6 chromosomal integration.

Our findings are consistent with some, but not all, past studies of CMV and MS [33]. For example, in a large nested case–control study in Sweden ($n = 670$ matched pairs of cases and controls), there was a 30% reduction of odds of having MS associated with CMV seropositivity [14]. However, in a case–control study in the USA, a protective association of CMV seropositivity was apparent only in Hispanics, and not in Whites or Blacks [15]. In the US military cohort study, there was no difference in MS risk in relation to CMV seroconversion over the course of the follow-up period, although MS risk was lower in those who were CMV seropositive (compared to seronegative) at baseline [1].

A recent study reported that a greater proportion of people with MS, compared to the general population, reported a past history of chickenpox and/or herpes zoster [34]. Our previous analysis of self-reported chickenpox in the Ausimmune Study found no association with FCD risk [11]. In a French study, clinically observed chickenpox was associated with lower risk of MS onset during childhood [10]. A recent review suggests geographic heterogeneity in the association, with more than fourfold higher odds of VZV-specific IgG seropositivity in MS patients than controls in Asian countries, but no significant association across studies from European countries [35].

Strengths

A major strength of this study was the relatively large sample size, compared to previous studies examining viral DNA loads and risk of MS [28]. Cases had had a first diagnosis of CNS demyelination; during recruitment for the Ausimmune Study, therapeutic options for CIS in Australia were largely limited to steroids at the time of the acute event, with subsidized access to disease-modifying therapies requiring a definitive diagnosis of MS, at the time defined by a second clinical event. In this substudy, 25% (50/204) of cases had received steroids and 6% (12/204) interferon-beta, the latter having had a second event by the time of the blood draw. At the 5-year follow-up of Ausimmune Study cases, 69% of the classic CIS-only group had converted to MS [36], consistent with older studies of conversion from mixed-site CIS to MS (prior to modern disease-modifying therapies) [37]. We had concurrent EBV IgG and DNA

load and thus were able to adjust for these (and other potential confounders) in our analyses. In addition, concurrent genotype data for several MS-associated HLA SNPs allowed exploration of possible interactions. Our study had a broad geographic range, from low latitude/low risk (Brisbane) to relatively higher latitude/higher risk locations (e.g., Tasmania) [17], potentially providing a broader range of risk exposures than may occur in a single-area study.

Limitations

Our serological assays did not distinguish between HHV-6A and HHV-6B. Methods to assay HHV-6 type-specific serology were not available when this work was undertaken. In addition, we were unable to genotype all of the HHV-6-DNA-positive samples due to the low HHV-6-DNA loads. With only a single measure of HHV-6-DNA for each participant, we were unable to confirm that samples with high HHV-6-DNA loads represented chromosomal integration, and thus the role of this as a risk factor for FCD. Quantification of HHV-6-DNA load (rather than detected/not detected), particularly at FCD, is essential to further assess this risk. Previous studies have noted the low prevalence of HHV-6-DNA and VZV-DNA positivity in blood from people with MS and healthy controls [38], such that very large sample sizes are required to analyse these as risk factors for MS. Only 12 control and 10 case participants had measurable HHV-6-specific IgM, limiting our ability to assess the immune response to recent HHV-6 infection or reactivation. Covariate data were largely based on self-report, possibly resulting in some recall bias, and we cannot rule out some level of selection bias in the Ausimmune Study [16]. Postdiagnostic sample collection incurs a risk of reverse causation, possibly related to initial disease management.

Conclusions

Overall, our findings add to the body of evidence on the possible role of human herpesviruses in the onset of MS. We found that, of a number of markers of infection with herpesviruses, only EBNA IgG and the presence of HHV-6-DNA were retained in a predictive model for FCD risk. The increased risk associated with higher EBNA IgG was independent of any of the other measured markers of viral infection, but possibly enhanced by HHV-6-DNA positivity. Large studies undertaking quantitative measurement of HHV-6-DNA, with repeated sampling over time to determine chromosomal integration, are required to further investigate the links between HHV-6 infection and risk of MS.

AUTHOR CONTRIBUTIONS

Robyn M. Lucas: Conceptualization; methodology; investigation; formal analysis; data curation; supervision; funding acquisition; project administration; writing—original draft; writing—review and editing. **Meav-Lang J. Lay:** Methodology; data curation; investigation; formal analysis; writing—review and editing. **James Grant:** Formal analysis; writing—review and editing; writing—original draft. **Nicolas Cherbuin:** Formal analysis; supervision; writing—review and editing. **Cheryl S. Toi:** Methodology; data curation; investigation; formal analysis; writing—review and editing. **Keith Dear:** Conceptualization; methodology; data curation; formal analysis; supervision; funding acquisition; writing—review and editing. **Bruce V. Taylor:** Conceptualization; supervision; funding acquisition; writing—review and editing. **Dominic E. Dwyer:** Conceptualization; methodology; funding acquisition; investigation; writing—review and editing. **Anne-Louise Ponsonby:** Conceptualization; methodology; data curation; investigation; supervision; funding acquisition; project administration; writing—review and editing.

ACKNOWLEDGEMENTS

We would like to acknowledge and thank the physicians who notified case participants of the Ausimmune Study, the local research officers/nurses, and the Ausimmune Study project personnel. We thank Prof. S. Burrows and Ms. J. Burrows for duplication of the HHV-6-DNA viral load assays. The Ausimmune Investigator Group includes Dr. Caron Chapman, Barwon Health, Geelong, Victoria, Australia; Prof. Alan Coulthard, Royal Brisbane and Women's Hospital and the University of Queensland, Brisbane, Queensland, Australia; Prof. Keith Dear, School of Public Health, University of Adelaide, South Australia, Australia; Prof. Terry Dwyer, Murdoch Childrens Research Institute, University of Melbourne, Melbourne, Victoria, Australia; Prof. Trevor Kilpatrick, Centre for Neuroscience, University of Melbourne, Melbourne, Australia; Prof. Robyn Lucas, National Centre for Epidemiology and Population Health, Australian National University, Canberra, Australian Capital Territory, Australia; Prof. Tony McMichael (deceased), National Centre for Epidemiology and Population Health, Australian National University, Canberra, Australian Capital Territory, Australia; Prof. Anne-Louise Ponsonby, Florey Institute of Neuroscience and Mental Health, University of Melbourne, Melbourne, Victoria, Australia; Prof. Bruce Taylor, Menzies Institute for Medical Research, University of Tasmania, Hobart, Tasmania, Australia; A./Prof. Patricia Valery, QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia; Prof. Ingrid van der Mei, Menzies Institute for Medical Research, University of Tasmania, Hobart, Tasmania, Australia; and Dr. David Williams, Hunter Health, Newcastle, New South Wales, Australia. Open access publishing facilitated by Australian National University, as part of the Wiley - Australian National University agreement via the Council of Australian University Librarians.

FUNDING INFORMATION

The Ausimmune Study was supported by the National Multiple Sclerosis Society of the USA, the National Health and Medical Research Council of Australia, the ANZ William Buckland Foundation, and Multiple Sclerosis Research Australia.

CONFLICT OF INTEREST STATEMENT

K.D. received research support from Multiple Sclerosis Research Australia and the National Health and Medical Research Council of Australia. R.M.L. received research support from Multiple Sclerosis Research Australia, the Royal Australasian College of Physicians, and the National Health and Medical Research Council of Australia. A.-L.P. receives research support from Multiple Sclerosis Research Australia and the National Health and Medical Research Council of Australia. B.V.T. has received funding for travel and speaker honoraria from Bayer Schering Pharma, CSL Australia, Novartis, and Biogen Australia. He has served on advisory boards for Biogen, Novartis, and Merck. He serves as associate editor for the *Journal of Neurology Neurosurgery and Psychiatry* and *BMJ Open Neurology*, and receives/has received research support from the National Health and Medical Research Council of Australia, the Australian Medical Research Future Fund, MS Australia, the Health Research Council of New Zealand, the National MS Society of USA, and the MS Society of Tasmania. None of the other authors has any conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study may be available on request from the corresponding author subject to an agreement for use. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Lucas RM, Lay M-LJ, Grant J, et al. Risk of a first clinical diagnosis of central nervous system demyelination in relation to human herpesviruses in the context of Epstein–Barr virus. *Eur J Neurol*. 2023;00:1-9. doi:10.1111/ene.15919

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