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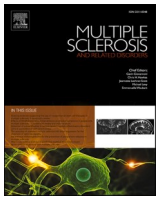

Original article

Markers of Epstein-Barr virus and Human Herpesvirus-6 infection and multiple sclerosis clinical progression

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ABSTRACT

Background: Infections with Epstein-Barr virus (EBV) and human herpesvirus-6 (HHV-6) have been implicated in multiple sclerosis (MS) onset but little work has studied their relationships in early disease.
Objective: Evaluate associations between markers of EBV and HHV-6 infection/reactivation and MS conversion, relapse and EDSS/MSSS amongst 205 CIS participants with EBV/HHV-6 data followed over 5 years.
Method: Baseline serological and viral load measures of EBV and HHV-6 exposure/reactivation were measured and infectious mononucleosis (IM) history recorded. Conversion to MS and relapses were assessed annually, and EDSS/MSSS measured at 5-year review. Determinants of MS conversion and relapse assessed by Cox regression, and disability progression by linear regression.
Results: IM history showed a strong positive trend with higher relapse risk (aHR=1.45,95%CI=0.97–2.16) but was not associated with MS conversion (aHR=0.92,95%CI=0.57–1.48). Anti-HHV-6 IgG titre>40 also showed strong positive trends with higher relapse (aHR=1.61,95%CI=0.99–2.63) and MS conversion risks (aHR=1.48,95%CI=0.89–2.46). Anti-HHV-6 IgG titre≥640 was significantly associated with higher MSSS (0.15 (95%CI=0.00,0.30) and also showed a strong positive trend with higher EDSS 0.10(95%CI=-0.02,0.21). HHV-6 DNA detection showed strong positive trends with 83%(95%CI=-6–357) and 77%(95%CI=-4–328) higher MS conversion and relapse risk. Anti-EBV-EA-D IgG titre was associated with a lower annualised disability progression by EDSS (p_{trend}=0.037) and also showed strong positive trend with higher MSSS (p_{trend}=0.053). No associations were seen for other serological or viral load markers.
Conclusion: Overall, our data provides evidence that higher HHV-6 IgG was associated with increased risk of MS conversion and relapse but of borderline significance, and greater annualised disability progression, while that for EBV was more limited.

1. Introduction

Multiple sclerosis (MS) is a complex autoimmune and neurodegenerative disorder of the central nervous system (CNS). The onset of MS has been ascribed to an interplay between environmental and genetic risk factors.(Mechelli et al., 2010; Ramagopalan et al., 2010) Amongst the environmental risk factors implicated in MS, a prominent evidence base exists for infection with human herpesviruses, especially Epstein-Barr virus (EBV). Recent meta-analyses support causal relationships between latent EBV infection and increased risk of developing MS, as at onset a higher proportion of MS patients than controls have seropositivity against Epstein-Barr nuclear antigen (EBNA IgG,

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OR=4.5, 95%CI=3.3–6.6),(Almohmeed et al., 2013) viral capsid antigen (VCA IgG, 95%CI=2.8–7.8) and a past history of infectious mononucleosis (IM, OR=2.17, 95%CI=1.97–2.39), but no association of early antigen (EA) IgG.(Almohmeed et al., 2013; Handel et al., 2010) This was more definitively demonstrated in the recent study by Bjornevik and colleagues using longitudinally collected data from US military service members, which conclusively showed that exposure to EBV was causally related to subsequent MS diagnosis Bjornevik, K., et al. (2022). However, markers of active or recent infection (EBV viral load or EA) have not been consistently associated with the development of MS. (Almohmeed et al., 2013; Lucas et al., 2011) Past infection with human herpesvirus-6 (HHV-6) may also be more prevalent in MS patients, although the evidence is less consistent than that seen with EBV, with some studies showing that HHV-6 IgG titres were higher in MS patients at onset than matched controls,(Virtanen et al., 2007) while others showed no significant differences.(Kuusisto et al., 2008; Xu et al., 2002)

In contrast to the literature regarding MS risk, there is less and inconsistent evidence on whether these viruses are involved in MS progression– including conversion from clinically isolated syndrome (CIS) to definite MS, and relapse and disability accumulation following MS diagnosis. Some studies suggested no associations between markers of EBV infection and conversion to MS or relapses(Lunemann et al., 2010; Munger et al., 2015; Simpson et al., 2012), while other studies have shown associations with greater MRI activity(Farrell et al., 2009; Jakimovski et al., 2020; Kvistad et al., 2014; Lünemann et al., 2010; Zivadinov et al., 2009) and disability progression.(Farrell et al., 2009; Horakova et al., 2013; Lunemann et al., 2010) Few studies have examined the association between markers of HHV-6 infection and MS disease course.(Ortega-Madueno et al., 2014; Simpson et al., 2012; Virtanen et al., 2014) Following 198 patients with established MS for over two years, our group previously demonstrated a robust dose-dependant positive association between HHV-6 IgG levels and subsequent hazard of relapse (p_{trend}=0.003)(Simpson et al., 2012), while another prospective study including 301 MS patients found that HHV-6 IgG and IgM titres peaked two weeks and one month prior to clinical relapse, respectively(Ortega-Madueno et al., 2014). Higher HHV-6 DNA load in cerebrospinal fluid (CSF) has been linked with a greater number of contrast-enhancing lesions on MRI(Virtanen et al., 2014), albeit in a small cross-sectional study of 37 MS patients.

Here, we examined the relationship between markers of EBV and HHV-6 infection and disease course (conversion to MS, relapse and change in disability) in early MS, using a multicentre longitudinal prospective cohort recruited soon after referral for a clinical episode of CNS demyelination.

2. Methods

2.1. Study design

As described elsewhere(Lucas et al., 2007), the Ausimmune Study was a multicentre case-control study which recruited individuals at their first clinical diagnosis of CNS demyelination (FCD) over the period 2003–6. Participating cases were aged 18 to 59 years old and resident in one of four study regions in the east of Australia. A subsequent longitudinal cohort study, the Ausimmune Longitudinal (AusLong) Study, has continued to follow the case participants of the Ausimmune Study annually with high retention (84.6% at five years) to over ten years following their initial participation in the Ausimmune Study, though the present analysis is constrained to up to the 5-year timepoint to focus on early disease progression.

Of the 282 cases initially considered to be eligible as having a FCD, three cases were subsequently diagnosed with a non-MS disorder (one neuromyelitis optica, one Susac's Syndrome, and one a pineal germinoma). Of the remaining 279 cases, review of all medical notes after 5 years resulted in 260 cases being considered to have a relapse-onset and 19 had progressive-onset disease; amongst the relapse-onset cases, 170 had their first demyelinating event (FDE) within the study recruitment period. At study entry (2003–7), 21% (58/279) patients used DMTs, solely of first-generation type, most using interferon-beta (51/58).

2.2. Data collection

Participants completed questionnaires prior to, and during, a face-to-face interview with a study nurse on entry to the Ausimmune Study. This included information on history of IM ('have you ever had glandular fever?') at baseline. Use (yes/no) of disease-modifying therapy (DMT) was reported at annual review. Blood was taken by venepuncture; aliquots of serum and whole blood were stored at −80 °C.

2.2.1. Measurement of EBV/HHV-6 variables

As previously described(Lucas et al., 2011), serological EBV and HHV-6 parameters were measured in blood taken at the initial interview for the Ausimmune Study in a subset of 205 cases, with 167 having their FDE during the recruitment period. A comparison of the data (sex, conversion to MS, progressive-onset, and age at FCD) between those patients who did and those who did not have the data of viral biomarkers showed that the two groups were very similar ($p>0.10$). Thus, this sample is likely representative of the entire AusLong cohort.

Quantitative IgG antibody titres to EBV viral capsid antigen (VCA) were measured by automated enzyme immunoassay (Star Corp, Stillwater, MN) and antibodies to EBNA complex and early antigen (diffuse and restricted, EA-D and EA-R, respectively) by immunofluorescence assay. EBV DNA load was quantified in whole blood using the EBNA-1 and BHRF-1 primers as described elsewhere.(Lay et al., 2010) Serum HHV-6 IgG titres were measured using indirect immunofluorescence assay (Panbio). EBNA and VCA IgG are indicators of previous EBV exposure, while EA-D and EA-R IgG and EBV viral load are indicators of EBV reactivation or recent infection, EBV-EAD and -EAR being released from cells after lytic replication and IgG specific thereto being detectable within 3–6 months of infection/reactivation but not thereafter(Luzuriaga and Sullivan, 2010). HHV-6 IgG was used as a marker of HHV-6 exposure, while HHV-6 DNA (yes or no) and HHV-6 IgM were used as markers of HHV-6 reactivation or new infection. Ig titres were assessed by serial dilution so were necessarily restricted to particular increments. These increments of Ig titres were consolidated appropriate to allow quantitative analysis.

2.2.2. Measurement of conversion to MS and relapse

Conversion to MS was defined primarily as the occurrence of a second clinical demyelinating event, thus satisfying the diagnostic requirements of dissemination in time and space, or a single clinical episode plus paraclinical evidence, as per the 2005 McDonald criteria (a minority of cases were diagnosed following neurologist-ordered MRI assessment ($n = 1$)), or were progressive from onset ($n = 19$).(Polman et al., 2005) Conversion to MS was reported at annual review or assessed by neurologist at face-to-face review, as well as derived from review of neurological records. Relapses were reported at annual review and defined according to the 2001 McDonald Criteria as the acute or subacute appearance or reappearance of a neurological abnormality (lasting at least 24 h) in the absence of other potential explanatory factors. (Polman et al., 2011)

2.2.3. Measurement of disability progression

Disability was assessed by EDSS at the 5-year review Kurtzke, J. F. (1983). From this, the MS Severity Score (MSSS) was estimated using functions described previously (Roxburgh et al., 2005). Annualised change in both were evaluated with the 5-year disability value divided by the duration in years since FDE.

2.3. Data analysis

Mean and standard deviation, median and interquartile range were

used as descriptive statistics. First, we examined the association between DMT use and viral indices at baseline. Ordinal logistic regression was used to assess the associations between baseline DMT (yes/no) and the dilution antibody titres of viral biomarkers. To test replication of the results of the association between baseline DMT and EBNA IgG levels, we also analysed data from MS cases from the Tasmanian MS case-control study (including 136 prevalent cases EBNA IgG titres were measured using an ELISA assay (Panbio, Brisbane, Australia)) and the Southern Tasmanian Multiple Sclerosis Longitudinal (MSL) Study (including 198 established MS cases, EBNA IgG titres were measured using an indirect immunofluorescence assay (IFA) (Panbio Inc., Columbia, MD, USA)).(Simpson et al., 2012; van der Mei et al., 2003)

The effect of predictors on time-to-conversion and time-to-relapse was estimated using Cox proportional hazards models. All analyses were adjusted for age, sex, study site; for time to relapse analysis, baseline use of DMT was also adjusted for. All covariates of interest satisfied the proportional hazards assumption. For conversion to MS analysis, the starting point was the date of FDE. We included only relapse-onset cases who had their FDE during the recruitment period (for better accuracy in determining the date of FDE), and who had data on viral parameters ($n = 194$) or information on IM history ($n = 274$). For the relapse analyses, Cox models were evaluated with shared frailties within each participant. In addition to stratum-specific estimates of measures of association, tests for trend were included for ordered polychotomous terms. This allows a single statistical test of the dose-dependency of the ordered polychotomous term expressed as a linear term.

Since only baseline DMTs were associated with viral biomarkers, we used baseline DMTs as the covariate in the primary multivariable model. However, longitudinal DMTs had a closer relationship with the disease course, so a sensitivity analysis with additional adjustment for longitudinal DMT use (yes/no) was performed. We also examined associations between viral biomarkers and disease course but results did not change materially when longitudinal DMTs was adjusted for, nor did adjustment for baseline smoking or serum vitamin D levels materially impact any associations. Thus, these results are not presented.

Predictors of annualised change in EDSS and MSSS were evaluated using linear regression, adjusted for whether participants were having a relapse at 5-year disability assessment. Because the annualised change in disability was highly skewed and for many cases the annualised change of EDSS was zero, a log-transformation of the original score plus one was applied to satisfy linear regression assumptions of minimal heteroskedasticity. All means and coefficients, however, were back-transformed and presented on the original scale of the annualised change in EDSS variable. We included only those who had been diagnosed as MS by the 5-year review (as the EDSS is designed for assessing MS-associated disability) and who had data on viral parameters ($n = 144$) and IM ($n = 183$).

3. Results

3.1. Participant characteristics

The characteristics of the total cohort ($n = 279$) and cases with an FDE during the recruitment period ($n = 170$) are shown in Table 1. The mean age was 39 years and 78% were female. Of the total cohort, 118 converted to MS and experienced 553 relapses. EBV and HHV-6 DNA were detected in only a small proportion of all cases (~15%).

3.2. Association between baseline covariates and viral markers

Though EBNA (p_{trend}=0.47), VCA (p_{trend}=0.92), or EBV-EA-D (p_{trend}=0.84) IgG titres were not associated with IM history, EBV-EA-R (p_{trend}=0.088) and EBV-EA-D ($p = 0.071$), and EBV viral load were higher ($p = 0.069$). Interestingly, HHV-6 IgG (p_{trend}=0.12) titre and HHV-6 viral load ($p = 0.084$) were higher in those reporting IM history, robust to adjustment for EBV-EA titres (data not shown). VCA IgG titres was positively correlated with EBNA IgG ($r = 0.35$, $p<0.001$) but measures of EBV reactivation (EBV-EAR/D, viral load) were not associated. EBV-EAR was positively correlated with VCA IgG ($r = 0.17$, $p = 0.053$), and EBV-EAR and EBV-EAD were positively correlated ($r = 0.34$, $p = 0.001$), while HHV-6 viral load was positively correlated with EBV-EAD ($r = 0.26$, $p = 0.004$) but not EBV-EAR ($r = 0.08$, $p = 0.26$). HHV-6 IgG was positively correlated with HHV-6 IgM ($r = 0.30$, $p = 0.007$) but was not associated with any measures of EBV nor with HHV-6 viral load, but interestingly, HHV-6 viral load was positively correlated with EBV-EAD IgG ($r = 0.26$, $p = 0.004$).

We found that those who used DMT before study entry were less likely to have higher levels of EBNA IgG (ordinal logistic regression OR 0.44, 95% CI: 0.23–0.84), this association robust to adjustment for age and sex (aOR 0.44, 95% CI: 0.23–0.84). We attempted to replicate these findings, but found no association in the cases of the Tasmanian case-control study ($p = 0.76$) or MSL study ($p = 0.73$) after adjustment for age and sex. Other EBV biomarkers (VCA IgG, EA-R IgG, EA-D IgG and EBV DNA) or HHV-6 (HHV-6 IgG, HHV-6 IgM and HHV-6 DNA) were not associated with baseline DMT use.

3.3. Association between serological & viral load parameters of EBVand HHV-6 and the hazard of conversion to MS

HHV-6 IgG titre above 40 was associated with an increased risk of conversion to MS (HR=1.68, 95%CI=1.03,2.76), though this attenuated on adjustment (aHR=1.48, 95%CI=0.89–2.46). No other markers of HHV-6 were associated with MS conversion risk, nor were any markers of EBV or IM history (Table 2).

3.4. Association between viral load & serological parameters of EBV and HHV-6 and the hazard of relapse

Positive IM history was associated with a 45% increased risk of relapse (95%CI=0.97, 2.16), albeit of borderline significance. At the same time, while no serological or viral load markers of EBV were associated with relapse risk, higher HHV-6 IgG titre (>40) was associated with 61% increased risk of relapse (95%CI=0.99–2.63), though of borderline significance. Neither HHV-6 IgM titre nor HHV-6 viral load were associated, however (Table 3).

Mutually adjusting IM history (aHR=1.43, 95%CI=0.88–2.34) and high HHV-6 IgG titre (aHR=1.52, 95%CI=0.90–2.56) found both persisted, albeit with some attenuation, suggesting each largely operates independently.

3.5. Association between immune response to EBV/HHV-6 and annualised disability progression

EBV-EA-D IgG titre was associated with lower annualised disability progression by both EDSS (p_{trend}=0.037, Table 4) and a trend for MSSS (p_{trend}=0.053), such that those with the highest titre had 0.13 (95% CI=−0.28,0.01) and 0.16 (95%CI=−0.34,0.02) lower annualised progression by each, respectively, compared to those with the lowest titre, albeit not reaching significance. No other markers of EBV exposure were associated with annualised disability progression, nor was IM history. Interestingly, those uncertain of their IM history had a lower annualised progression by EDSS (aβ=−0.12, 95%CI=−0.23,−0.01) and MSSS (aβ=−0.15, 95%CI=−0.28,−0.01) compared to those who did not have an IM history. For HHV-6, those with the highest anti-HHV-6 IgG titres (640 or higher) had higher annualised disability progression by EDSS (aβ=0.10, $p = 0.095$) and MSSS (aβ=0.15, $p = 0.046$), compared to those with the lowest titres, though there was no dose-dependency with increasing titres.

Table 1
Demographic and clinical characteristics of the cohort.

	Total cohort n(%)			Cases with an FDE during the recruitment period n (%)		
	All persons	Persons with 5-year follow-up	Cases with viral biomarkers	All persons	Persons with 5-year follow-up	Cases with viral biomarkers
Total	279	236	179	170	145	110
Female	214 (76.7%)	184 (78.0%)	140 (78.2%)	132 (77.7%)	114 (78.6%)	88 (80.0%)
Study site						
Queensland	91 (32.6%)	68 (28.8%)	57 (31.8%)	49 (28.8%)	38 (26.2%)	30 (27.3%)
NSW	39 (14.0%)	36 (15.3%)	23 (12.9%)	26 (15.3%)	24 (16.6%)	15 (13.6%)
Victoria	69 (24.7%)	61 (25.9%)	41 (22.9%)	33 (19.4%)	29 (20.0%)	20 (18.2%)
Tasmania	80 (28.7%)	71 (30.1%)	58 (32.4%)	62 (36.5%)	54 (37.2%)	45 (40.9%)
MS onset type						
Relapse-onset	260 (93.2%)	223 (94.5%)	169 (94.4%)	170 (100%)	145 (100%)	110 (100%)
Progressive onset	19 (6.8%)	13 (5.5%)	10 (5.6%)	0	0	0
DMT used at baseline	58 (20.8%)	49 (20.8%)	39 (21.8%)	33 (19.4%)	31 (21.4%)	23 (20.9%)
Conversion to MS during study	217 (77.8%)	189 (80.1%)	147 (82.1%)	110 (64.7%)	100 (69.0%)	78 (70.9%)
History of glandular fever/infectious mononucleosis?						
No	181 (66.1%)	153 (65.7%)	121 (68.4%)	111 (66.5%)	94 (65.7%)	74 (68.5%)
Yes	74 (27.0%)	62 (26.6%)	44 (24.9%)	45 (27.0%)	38 (26.6%)	26 (24.1%)
Not sure	19 (6.9%)	18 (7.7%)	12 (6.8%)	11 (6.6%)	11 (7.7%)	8 (7.4%)
(Missing)	(5 (1.8%))	(3 (1.3%))	(2 (1.1%))	(3 (1.8%))	(2 (1.4%))	(2 (1.8%))
	Mean (SD; Range)					
Age at study entry, years	38.7 (9.8; 18–58)	38.7 (9.6; 18–58)	38.9 (9.7; 18–58)	37.6 (9.6; 18–58)	37.7 (9.6; 18–58)	37.9 (9.6; 18–58)
MS duration from onset to 5-year review, years	6.6 (2.3; 4.8–21.2)	6.6 (2.3; 4.8–21.2)	6.6 (2.3; 4.8–21.2)	5.8 (0.6; 4.8–7.8)	5.8 (0.6; 4.8–7.8)	5.7 (0.6; 4.8–7.8)
Number of relapses during study	2.0 (2.6; 0–21)	2.0 (2.6; 0–21)	2.0 (2.7; 0–21)	1.7 (2.4; 0–17)	1.7 (2.4; 0–17)	1.6 (2.5; 0–17)
	Median (IQR)					
EDSS at 5-year study review	1.5 (1.0–2.5)	1.5 (1.0–2.5)	1.5 (1.0–2.5)	1.5 (1.0–2.0)	1.5 (1.0–2.0)	1.5 (0.5–2.0)
Annualised change in EDSS, baseline to 5-year review	0.3 (0.2–0.4)	0.3 (0.2–0.4)	0.3 (0.2–0.4)	0.3 (0.2–0.4)	0.2 (0.1–0.4)	0.2 (0.1–0.4)
Annualised change in MSSS, baseline to 5-year review	0.4 (0.1–0.7)	0.4 (0.2–0.7)	0.4 (0.2–0.7)	0.4 (0.2–0.7)	0.4 (0.2–0.7)	0.4 (0.2–0.7)
EBNA IgG titre						
≤40	44 (21.5%)	39 (21.8%)	21 (16.7%)	18 (16.4%)		
160	89 (43.4%)	77 (43.0%)	62 (49.2%)	54 (49.1%)		
≥640	72 (35.1%)	63 (35.2%)	43 (34.1%)	38 (34.6%)		
(Missing)	(74 (26.5%))	(57 (24.2%))	(44 (25.9%))	(35 (24.1%))		
EBV-VCA IgG titre						
≤160	39 (19.0%)	31 (17.3%)	20 (15.9%)	15 (13.6%)		
640	110 (53.7%)	101 (56.4%)	68 (54.0%)	62 (56.4%)		
≥1280	56 (27.3%)	47 (26.3%)	38 (30.2%)	33 (30.0%)		
(Missing)	(74 (26.5%))	(57 (24.2%))	(44 (25.9%))	(35 (24.1%))		
EBV-EA-R IgG titre						
≤10	69 (33.7%)	62 (34.6%)	39 (31.0%)	35 (31.8%)		
40	66 (32.2%)	58 (32.4%)	41 (32.5%)	36 (32.7%)		
≥160	70 (34.2%)	59 (33.0%)	46 (36.5%)	39 (35.5%)		
(Missing)	(74 (26.5%))	(57 (24.2%))	(44 (25.95))	(35 (24.1%))		
EBV-EA-D IgG titre						
0	45 (22.0%)	42 (23.5%)	20 (15.9%)	18 (16.4%)		
10	54 (26.3%)	48 (26.8%)	36 (28.6%)	31 (28.2%)		
40	81 (39.5%)	69 (38.6%)	53 (42.1%)	46 (41.8%)		
160–640	25 (12.2%)	20 (11.2%)	17 (13.5%)	15 (13.6%)		
(Missing)	(74 (26.5%))	(57 (24.2%))	(44 (25.9%))	(35 (24.1%))		
EBV DNA detection						
Negative	180 (87.8%)	156 (87.2%)	109 (86.5%)	94 (85.5%)		
Positive	25 (12.2%)	23 (12.9%)	17 (13.5%)	16 (14.6%)		
(Missing)	(74 (26.5%))	(57 (24.2%))	(44 (25.9%))	(35 (24.1%))		
HHV-6 IgG titre						
≤40	65 (31.9%)	60 (33.7%)	39 (31.0%)	35 (31.8%)		
160	95 (46.6%)	81 (45.5%)	54 (42.9%)	48 (43.6%)		
≥640	44 (21.6%)	37 (20.8%)	33 (26.2%)	27 (24.6%)		
(Missing)	(75 (26.9%))	(58 (24.6%))	(44 (25.9%))	(0 (0%))		
HHV-6 IgM titre						
0	193 (95.1%)	169 (95.5%)	118 (93.7%)	104 (94.6%)		
20	7 (3.5%)	6 (3.4%)	6 (4.8%)	5 (4.6%)		
40	3 (1.5%)	2 (1.1%)	2 (1.6%)	1 (0.9%)		
(Missing)	(76 (27.2%))	(2 (1.1%))	(44 (25.9%))	(0 (0%))		
HHV-6 DNA detection						
Negative	177 (86.3%)	152 (84.9%)	112 (88.9%)	96 (87.3%)		
Positive	28 (13.7%)	27 (15.1%)	14 (11.1%)	14 (12.7%)		
(Missing)	(74 (26.5%))	(0 (0%))	(44 (25.9%))	(0 (0%))		

Abbreviations: EBV = Epstein-Barr Virus; EBNA = EBV Nuclear Antigen; EA = EBV Early Antigen; VCA = EBV Viral Capsid Antigen; EDSS = Expanded Disability Status Scale; HHV6 = Human Herpesvirus 6; MSSS = MS Severity Score; NSW = New South Wales.

Table 2
Associations of baseline levels of viral markers and the hazard of conversion to MS in cases with an FDE during the recruitment period.

	Conversions/ person-years (annual rate)	Univariable analysis HR (95% CI)	Multivariable analysis aHR (95% CI)
History of infectious mononucleosis?			
No	70/293.54 (0.24)	1.00 [Reference]	1.00 [Reference]
Yes	29/123.53 (0.23)	0.99 (0.65, 1.52)	0.92 (0.57, 1.48)
Not sure	8/26.29 (0.30)	1.33 (0.61, 2.93)	1.59 (0.75, 3.37)
EBNA IgG titre			
≤40	15/49.37 (0.30)	1.00 [Reference]	1.00 [Reference]
160	40/171.77 (0.23)	0.75 (0.39, 1.45)	0.66 (0.32, 1.34)
≥640	29/118.19 (0.25)	0.78 (0.40, 1.52)	0.77 (0.39, 1.53)
Trend:		*p = 0.72*	*p = 0.98*
EBV-VCA IgG titre			
≤160	15/40.76 (0.37)	1.00 [Reference]	1.00 [Reference]
640	43/201.32 (0.21)	0.62 (0.34, 1.15)	0.55 (0.29, 1.02)
≥1280	26/97.25 (0.27)	0.74 (0.38, 1.42)	0.53 (0.27, 1.04)
Trend:		*p = 0.69*	*p = 0.14*
EBV-EA-R IgG titre			
≤10	26/116.04 (0.22)	1.00 [Reference]	1.00 [Reference]
40	26/109.17 (0.24)	1.05 (0.61, 1.82)	1.03 (0.58, 1.82)
≥160	32/114.12 (0.28)	1.20 (0.73, 1.98)	1.08 (0.65, 1.80)
Trend:		*p = 0.41*	*p = 0.73*
EBV-EA-D IgG titre			
0	10/62.23 (0.16)	1.00 [Reference]	1.00 [Reference]
10	27/83.37 (0.32)	1.78 (0.80, 3.99)	1.74 (0.74, 4.07)
40	35/158.77 (0.22)	1.31 (0.60, 2.86)	1.28 (0.55, 2.96)
160–640	12/34.96 (0.34)	1.93 (0.76, 4.93)	1.91 (0.74, 4.92)
Trend:		*p = 0.38*	*p = 0.37*
EBV DNA detection			
Negative	73/292.22 (0.25)	1.00 [Reference]	1.00 [Reference]
Positive	11/47.11 (0.23)	0.95 (0.51, 1.78)	0.98 (0.56, 1.71)
		p = 0.88	*p = 0.94*
HHV-6 IgG titre			
≤40	21/132.86 (0.16)	1.00 [Reference]	1.00 [Reference]
160	38/118.46 (0.32)	**1.80 (1.04, 3.11)**	1.53 (0.85, 2.77)
≥640	24/87.69 (0.27)	1.53 (0.88, 2.65)	1.42 (0.82, 2.47)
Trend:		*p = 0.39*	*p = 0.40*
≤40	21/132.86 (0.16)	1.00 [Reference]	1.00 [Reference]
>40	62/206.15 (0.30)	**1.68 (1.03, 2.76)**	1.48 (0.89, 2.46)
		p = 0.039	*p = 0.13*
HHV-6 IgM titre			
0	79/307.40 (0.26)	1.00 [Reference]	1.00 [Reference]
20	3/27.44 (0.11)	0.49 (0.19, 1.25)	**0.42 (0.19, 0.94)**
40	1/4.16 (0.24)	0.81 (0.13, 4.98)	0.61 (0.13, 2.81)
Trend:		*p = 0.28*	*p = 0.11*
HHV-6 DNA detection			
Negative	71/310.72 (0.23)	1.00 [Reference]	1.00 [Reference]
Positive	12/28.28 (0.42)	1.83 (0.94, 3.57)	1.54 (0.73, 3.26)
		p = 0.077	*p = 0.26*

Adjusted for age, sex, and study site.
Figures in boldface denote statistical significance ($p<0.05$).
Abbreviations: HR, hazard ratio; aHR, adjusted hazard ratio; EBV = Epstein-Bar virus; EBNA = EBV nuclear antigen; VCA = viral capsid antigen; EA-*R* = early antigen restricted; EA-*D* = early antigen diffuse; HHV-6 = human herpesvirus-6.

4. Discussion

Using one of the most comprehensive prospective studies of MS cases in the early stage of disease, we evaluated the roles of EBV and HHV-6 infection in disease course of early MS, including conversion to MS, relapse and disability progression. We found that a history of IM was associated with a near-significant 45% higher risk of relapse, though no serological markers of EBV exposure or reactivation were associated. Higher HHV-6 IgG above 40 units was associated with 68% higher risk of MS conversion and 41% higher risk of relapse, though neither was significant on adjustment. The associations of IM history and HHV-6 IgG with relapse risk seemed largely statistically independent. For disability progression, EBV-EA-D showed a significant inverse association, with those at the highest titres having 0.13 and 0.16 points lower annualised EDSS and MSSS progression, respectively, compared to those with the lowest titres, albeit not reaching statistical significance. At the same time, anti-HHV-6 IgG showed a significant positive association with both disability progression measures, those of titre 640 or higher having 0.10 and 0.15 greater annual disability progression compared to those with the lowest titres. However, no other markers of EBV or HHV-6 predicted disability progression, nor was IM history associated.

That higher HHV-6 IgG was associated with a greater risk of both MS conversion and relapse is in line with our previous finding that higher HHV-6 IgG was associated with greater relapse risk in established MS (Simpson et al., 2012). It is notable that whereas in our previous study the HHV-6 association with relapse was quite dose-dependant, that seen here exhibited a more threshold association. The internal consistency between the related but distinct outcomes of MS conversion and relapse is suggestive of a true association and may suggest that serological evidence of HHV-6 exposure at any level may be a risk factor for these outcomes. Our finding here of a positive association of anti-HHV-6 IgG with annualised disability progression conflicts with our prior findings (Simpson et al., 2012), where we found no significant association with disability progression by either EDSS or MSSS. This may reflect differences in cohort, however, particularly this being at the early phase of MS whereas that prior work was in an established MS cohort. In keeping with our previous work in the same established MS cohort(Simpson et al., 2014), we found no association of HHV-6 IgM with either MS conversion or relapse risk, nor with disability progression.

On the other hand, the inconsistent associations seen for IM history, being associated with relapse risk but not at all associated with MS conversion, gives pause. None of the other EBV markers were associated with relapse risk or conversion to MS, and EBV-EA-D IgG titres, surprisingly, were associated with less rather than more disability progression. It raises the question whether these are possibly spurious results. No association between EBV infection and change of EDSS was observed in the BENEFIT study (β=0.02, $p = 0.80$)(Munger et al., 2015),

Table 3
Associations of baseline levels of viral markers and the hazard of relapse.

	Relapses/person-years (annual rate)	Univariable analysis HR (95% CI)	Multivariable analysis aHR (95% CI)
History of infectious mononucleosis?			
No	154/591.7 (0.26)	1.00 [Reference]	1.00 [Reference]
Yes	92/246.6 (0.37)	1.44 (0.95, 2.19)	1.45 (0.97, 2.16)
Not sure	10/65.4 (0.15)	0.60 (0.25, 1.43)	0.68 (0.30, 1.56)
EBNA IgG titre			
≤40	34/115.7 (0.29)	1.00 [Reference]	1.00 [Reference]
160	98/332.2 (0.30)	0.97 (0.53, 1.75)	1.03 (0.57, 1.85)
≥640	53/234.7 (0.23)	0.72 (0.38, 1.37)	0.85 (0.46, 1.60)
Trend:		*p = 0.21*	*p = 0.45*
EBV-VCA IgG titre			
≤160	39/104.7 (0.37)	1.00 [Reference]	1.00 [Reference]
640	77/375.3 (0.21)	0.56 (0.32, 1.01)	0.65 (0.37, 1.16)
≥1280	69/202.5 (0.34)	0.91 (0.49, 1.68)	0.90 (0.49, 1.67)
Trend:		*p = 0.68*	*p = 0.97*
EBV-EA-R IgG titre			
≤10	54/217.3 (0.25)	1.00 [Reference]	1.00 [Reference]
40	51/220.8 (0.23)	0.93 (0.54, 1.62)	0.91 (0.54, 1.54)
≥160	80/244.5 (0.33)	1.29 (0.77, 2.15)	1.26 (0.77, 2.08)
Trend:		*p = 0.22*	*p = 0.23*
EBV-EA-D IgG titre			
0	31/105.2 (0.29)	1.00 [Reference]	1.00 [Reference]
10	43/194.3 (0.22)	0.73 (0.37, 1.43)	0.85 (0.44, 1.62)
40	79/294.0 (0.27)	0.87 (0.47, 1.62)	1.02 (0.56, 1.85)
160–640	32/89.1 (0.36)	1.19 (0.55, 2.54)	1.47 (0.70, 3.10)
Trend:		*p = 0.29*	*p = 0.13*
EBV DNA detection			
Negative	164/585.3 (0.28)	1.00 [Reference]	1.00 [Reference]
Positive	21/97.19 (0.22)	0.75 (0.40, 1.45) *p = 0.40*	0.88 (0.46, 1.67) *p = 0.69*
HHV-6 IgG titre			
≤40	43/207.0 (0.21)	1.00 [Reference]	1.00 [Reference]
160	92/295.2 (0.31)	1.49 (0.89, 2.51)	1.68 (1.00, 2.82)
≥640	48/176.8 (0.27)	1.27 (0.71, 2.29)	1.48 (0.82, 2.68)
Trend:		*p = 0.79*	*p = 0.54*
≤40	43/207.0 (0.21)	1.00 [Reference]	1.00 [Reference]
>40	140/472.0 (0.30)	1.41 (0.87, 2.29) *p = 0.16*	1.61 (0.99, 2.63) *p = 0.057*
HHV-6 IgM titre			
0	178/639.5 (0.28)	1.00 [Reference]	1.00 [Reference]
20	3/31.5 (0.10)	0.36 (0.09, 1.39)	0.40 (0.11, 1.50)
40	2/8.0 (0.25)	0.83 (0.13, 5.43)	0.90 (0.15, 5.36)
Trend:		*p = 0.30*	*p = 0.35*
HHV-6 DNA detection			
Negative	151/603.7 (0.25)	1.00 [Reference]	1.00 [Reference]
Positive	34/78.8 (0.43)	1.77 (0.96, 3.28) *p = 0.067*	1.62 (0.90, 2.92) *p = 0.11*

Adjusted for age, sex, study site, and baseline disease modifying therapy use.
Figures in boldface denote statistical significance ($p<0.05$).
Abbreviations: HR, hazard ratio; aHR, adjusted hazard ratio; EBV = Epstein-Bar virus; EBNA = EBV nuclear antigen; VCA = viral capsid antigen; EA-*R* = early antigen restricted; EA-*D* = early antigen diffuse; HHV-6 = human herpesvirus-6.

while Farrell et al. found that EBNA-1 IgG levels were associated with increased EDSS ($r = 0.3$, $p = 0.004$) over 5 years(Farrell et al., 2009). Of interest, HHV-6 IgG titre was higher in amongst those with IM, robust to adjustment for EBV-EA titres, suggesting that prior suspected glandular fever infections may have been due to HHV-6 rather than EBV. It is also possible that common factors activate both viruses, as described previously(Ponsonby et al., 2005). That we fail to show an association between serological measures of EBV exposure and IM history is in contrast to previous literature. Measures of EBV exposure (EBNA, VCA IgG) and EBV reactivation (EBV-EA IgG, viral load) positively correlated in the expected fashions, whereas HHV-6 IgG did not correlate with EBV measures. While it is unexpected that none of the measures of EBV exposure or reactivation were associated with IM history of MS outcomes, the consistency of relationships or lack thereof between these separate commercial assays would argue against a fault in these measures. It is possible that there is a systematic misreporting in IM history between those with EBV or HHV-6 infection histories but this cannot be ascertained from these data. The greater confidence in associations would be placed on objective serological measures over self-reported recalled infection history, particularly for a childhood/adolescence illness of vague and nonspecific presentation like glandular fever. In that case, the finding of an association of HHV-6 but not EBV serological measures aligns with our previous findings in other studies(Simpson et al., 2011).

We observed that those who used DMTs at baseline were less likely to have lower levels of EBNA IgG, which was an interesting finding suggesting that immune response to EBV could be down-regulated through DMTs as suggested elsewhere(Ciotti et al., 2020). However, we could not replicate this association among prevalent cases in the Tasmanian case-control and MSL studies. One possible explanation is that these participants in the baseline Ausimmune Study were incident cases with FCD, while participants in the other two studies were prevalent cases that were diagnosed as MS and in many cases had been on DMTs for many years. Two other studies also found that EBNA levels did not fluctuate materially when DMTs were used; however, sample size in these studies were small (both studies: $n = 20$), all were prevalent MS cases (disease duration 10+ years) and forms of DMTs were not identical.(Castellazzi et al., 2015; Raffel et al., 2014)

Strengths of our study include the use of a prospective longitudinal study design following cases with a first diagnosis of CNS demyelination. We had a high retention rate at 5-year follow-up (84.6%), and a high rate (76%) of conversion to MS amongst those with follow-up data. A limitation was that levels of EBV/HHV-6 and viral load were only measured at baseline, so the association between longitudinal variation in immune response to HHV-6/EBV and MS disease course could not be measured. As previous studies have suggested that EBV/HHV-6 IgG levels are comparatively stable over time,(Braun et al., 1997; Henle et al., 1987) our capacity to detect associations was only limited for

Table 4
Associations of baseline levels of viral markers and annualised change in EDSS & MSSS amongst those who converted to MS at 5-year review.

		Annualised change in EDSS		Annualised change in MSSS	
	n (%)	Model 1[a] aβ (95% CI)	Model 2[b] aβ (95% CI)	Model 1[a] aβ (95% CI)	Model 2[b] aβ (95% CI)
History of infectious mononucleosis?					
No	152 (66.4%)	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Yes	59 (25.8%)	0.01 (−0.08, 0.10)	0.01 (−0.07, 0.10)	0.01 (−0.11, 0.12)	0.01 (−0.10, 0.12)
Not sure	18 (7.9%)	−0.10 (−0.22, 0.01)	**−0.12 (−0.23, −0.01)**	−0.14 (−0.28, 0.00)	**−0.15 (−0.28, −0.01)**
EBNA IgG titre					
≤40	37 (21.0%)	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
160	77 (43.8%)	−0.00 (−0.11, 0.11)	0.02 (−0.10, 0.13)	−0.05 (−0.20, 0.10)	0.00 (−0.14, 0.15)
≥640	62 (35.2%)	−0.01 (−0.12, 0.10)	−0.01 (−0.12, 0.11)	−0.09 (−0.24, 0.07)	−0.04 (−0.19, 0.10)
Trend:		*p = 0.79*	*p = 0.72*	*p = 0.31*	*p = 0.43*
EBV-VCA IgG titre					
≤160	30 (17.1%)	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
640	99 (56.3%)	0.05 (−0.06, 0.15)	0.06 (−0.04, 0.17)	−0.04 (−0.19, 0.11)	0.00 (−0.15, 0.15)
≥1280	47 (26.7%)	0.03 (−0.09, 0.14)	−0.00 (−0.12, 0.12)	0.01 (−0.16, 0.18)	−0.00 (−0.17, 0.17)
Trend:		*p = 0.80*	*p = 0.85*	*p = 0.72*	*p = 0.99*
EBV-EA-R IgG titre					
≤10	61 (34.7%)	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
40	57 (32.4%)	0.01 (−0.09, 0.11)	0.01 (−0.09, 0.12)	−0.06 (−0.19, 0.07)	−0.05 (−0.18, 0.08)
≥160	58 (33.0%)	−0.02 (−0.12, 0.08)	−0.02 (−0.12, 0.08)	−0.04 (−0.17, 0.09)	−0.03 (−0.16, 0.11)
Trend:		*p = 0.62*	*p = 0.57*	*p = 0.76*	*p = 0.85*
EBV-EA-D IgG titre					
0	42 (23.9%)	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
10	46 (26.1%)	0.02 (−0.10, 0.15)	0.03 (−0.10, 0.15)	−0.01 (−0.17, 0.16)	0.01 (−0.15, 0.17)
40	68 (38.6%)	−0.07 (−0.18, 0.04)	−0.08 (−0.19, 0.04)	−0.10 (−0.25, 0.04)	−0.08 (−0.22, 0.07)
160–640	20 (11.4%)	−0.12 (−0.25, 0.02)	−0.13 (−0.28, 0.01)	**−0.19 (−0.36, −0.02)**	−0.16 (−0.34, 0.02)
Trend:		***p = 0.043***	***p = 0.037***	***p = 0.025***	*p = 0.053*
EBV DNA detection					
Negative	54 (87.5%)	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Positive	22 (12.5%)	0.02 (−0.11, 0.15)	0.01 (−0.13, 0.14)	−0.01 (−0.17, 0.15)	−0.03 (−0.19, 0.12)
		p = 0.73	*p = 0.93*	*p = 0.90*	*p = 0.69*
HHV-6 IgG titre					
≤40	59 (33.7%)	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
160	80 (45.7%)	−0.01 (−0.10, 0.08)	0.00 (−0.09, 0.09)	0.04 (−0.08, 0.15)	0.02 (−0.09, 0.13)
≥640	36 (20.6%)	0.07 (−0.05, 0.19)	0.10 (−0.03, 0.22)	0.14 (−0.02, 0.29)	**0.16 (0.00, 0.33)**
Trend:		*p = 0.17*	*p = 0.089*	*p = 0.083*	***p = 0.031***
≤160	139 (79.4%)	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
≥640	36 (20.6%)	0.08 (−0.03, 0.18)	0.10 (−0.02, 0.21)	0.11 (−0.03, 0.26)	**0.15 (0.00, 0.30)**
		p = 0.16	*p = 0.095*	*p = 0.12*	***p = 0.046***
HHV-6 IgM titre					
0	166 (95.4%)	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
20	6 (3.5%)	−0.12 (−0.33, 0.09)	−0.12 (−0.33, 0.09)	−0.11 (−0.34, 0.12)	−0.12 (−0.35, 0.11)
40	2 (1.2%)	0.47 (−0.10, 1.05)	0.43 (−0.14, 1.01)	0.93 (−0.18, 2.04)	0.72 (−0.27, 1.72)
Trend:		*p = 0.23*	*p = 0.29*	*p = 0.24*	*p = 0.38*
HHV-6 DNA detection					
Negative	150 (85.2%)	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]	0.00 [Reference]
Positive	26 (14.8%)	−0.05 (−0.15, 0.05)	−0.06 (−0.17, 0.04)	−0.01 (−0.15, 0.14)	−0.05 (−0.19, 0.09)
		p = 0.30	*p = 0.25*	*p = 0.94*	*p = 0.49*

a: adjusted for relapse or not in the 5-year review; b: further adjusted for sex, age, relapse number, study site and baseline disease modifying therapy.
Figures in boldface denote statistical significance ($p<0.05$).
Abbreviations: EA-*D* = early antigen diffuse; EA-*R* = early antigen restricted; EBV = Epstein-Bar virus; EBNA = EBV nuclear antigen; EDSS = Expanded Disability Status Scale; MSSS = MS Severity Score; VCA = viral capsid antigen; HHV-6 = human herpesvirus-6.

active viral parameters. We cannot exclude the possibility that some results were produced by chance, so replication in other similar cohorts is necessary. Also, our measure of anti-EBV-EBNA Ig titre is limited to measuring EBNA as a complex, rather than individual EBNA-1, −2, and −3 components, precluding more detailed assessment, and may account for the failure to show associations between EBNA Ig titre and outcomes. Similarly, our measure of anti-HHV-6 IgG was not able to distinguish individual HHV-6-A and -B strains, which is a limitation that precludes assessment of individual relationships.

Overall, our data provides evidence that higher HHV-6 IgG was associated with increased risk of MS conversion and relapse but of borderline significance, and greater annualised disability progression. Also, HHV-6 viral load detection showed positive trends with MS conversion and relapse risk, though no association with disability progression was seen. A history of IM was associated with an increased hazard of relapse but not conversion to MS, while EBV-EA-D IgG were inversely associated with clinical outcomes. This is in line with other evidence showing a lack of consistent associations between markers of EBV/HHV-6 infection and MS disease course. Thus, epidemiological studies provide some evidence that herpesvirus infection and host immune are involved in MS disease course, but the evidence is not consistent for mechanism.

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Supplementary materials

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