Current and past Epstein-Barr virus infection in risk of initial CNS demyelination

ABSTRACT

Objectives: To assess risk of a first clinical diagnosis of CNS demyelination (FCD) in relation to measures of Epstein-Barr virus (EBV) infection within the context of other known risk factors.

Methods: This was a multicenter incident case-control study. FCD cases (n = 282) aged 18-59 years and controls (n = 558, matched on age, sex, and region) were recruited from 4 Australian centers between November 1, 2003, and December 31, 2006. A nested study (n = 215 cases, n = 216 controls) included measurement of whole blood quantitative EBV DNA load and serum EBV-specific antibodies. Conditional logistic regression was used to analyze case-control differences.

Results: There were no significant case-control differences in the proportion with detectable EBV DNA (55.8% vs 50.5%, respectively, p = 0.28), or in quantitative EBV DNA load (p = 0.33). Consistent with previous work, higher anti-EBV-specific immunoglobulin G (IgG) titers and a history of infectious mononucleosis were associated with increased FCD risk and there was an additive interaction with HLA-DRB1*1501 status. We found additional interactions between high anti-EBNA IgG titer and SNPs in HLA-A (adjusted odds ratios [AOR] = 19.84 [95% confidence interval [CI] 5.95 to 66.21] for both factors compared to neither) and CTLA-4 genes (AOR = 0.31 [95% CI 0.13 to 0.76] for neither factor compared to both). EBV DNA load was lower at higher serum 25-hydroxyvitamin D concentrations in controls (r = −0.17, p = 0.01). An adverse effect of higher EBV DNA load on FCD risk was increased with higher 25-hydroxyvitamin D concentration (p[interaction] = 0.02).

Conclusion: Past infection with EBV, but not current EBV DNA load in whole blood, is significantly associated with increased FCD risk. These associations appear to be modified by immune-related gene variants. Neurology 2011;77:371-379

GLOSSARY

AOR = adjusted odds ratio; CI = confidence interval; EBNA = Epstein-Barr virus nuclear antigen; EBV = Epstein-Barr virus; FCD = first clinical diagnosis of CNS demyelination; FDE = first demyelinating event; HLA = human leukocyte antigen; IgG = immunoglobulin G; IM = infectious mononucleosis; IQR = interquartile range; MS = multiple sclerosis; OR = odds ratio; PBMC = peripheral blood mononuclear cell; PPMS = primary progressive multiple sclerosis; SNP = single nucleotide polymorphism; VCA = viral capsid antigen.

A strong and consistent finding in multiple sclerosis (MS) epidemiology is the association with evidence of past infection with Epstein-Barr virus (EBV). A history of infectious mononucleosis (IM), considered to be a manifestation of late (post-childhood) EBV infection, is more common in people with MS.1,2 Higher levels of EBV-specific antibodies, particularly to the Epstein-Barr virus nuclear antigen (EBNA), are also consistently associated with increased MS risk,3 with evidence of a prospective dose-response relationship.3,4

Biologically plausible mechanisms to explain this association include cross-reactivity between EBV and myelin proteins,5 EBV-induced activation of superantigens or other viruses,6

From the National Centre for Epidemiology and Population Health (R.M.L., K.D., A.J.M.), The Australian National University, Canberra; Murdoch Childrens Research Institute (A.-L.P., T.D.), Melbourne; Queensland Institute of Medical Research (P.V., J.M.B., S.R.B.), Brisbane; The University of Queensland and Royal Brisbane and Women’s Hospital (M.P.P., A.C.), Brisbane; Barwon Health (C.C.), Geelong; Virology Department (D.E.D., B.V.T.), The University of Melbourne, Melbourne; Menzies Research Institute Tasmania (B.V.T., I.A.F.v.d.M.), Hobart; and John Hunter Hospital (D.W.), Newcastle, Australia.

Study funding: Supported by the National Multiple Sclerosis Society of the United States of America, the National Health and Medical Research Council of Australia, the ANZ William Buckland Foundation, and Multiple Sclerosis Research Australia.

Disclosure: Author disclosures are provided at the end of the article.
EBV infection of autoreactive B cells,7 or defective CD8+ T-cell control of EBV-infected B lymphocytes.8 Elevated levels of EBV DNA in the peripheral blood, close to the time of diagnosis, might be expected but have been inconsistently observed.9–11 Previously described genetic12 and environmental13–15 risk factors for MS may operate, at least in part, by influencing the host response to infections such as EBV. Finally, the commonly observed latitudinal gradient in MS may be the result of variation in EBV infection,5 but this has not yet been formally assessed in individual level studies.

Here we examine EBV DNA load, EBV-specific antibody (immunoglobulin G [IgG]) titers, and history of IM in a large population-based study in relation to onset of CNS demyelination. We also examine the interplay (confounding or interaction) between these markers of EBV infection and genetic and environmental factors implicated in MS risk.

METHODS The Ausimmune Study is described in detail elsewhere.16 Briefly, this was a multicenter case-control study, recruiting individuals (aged 18–59 years) in 4 regions in eastern Australia. Cases had an incident first clinical diagnosis of CNS demyelination (FCD), including those presenting with a classic first demyelinating event (FDE, n = 216), a first diagnosis of primary progressive MS (PPMS, n = 18) and those in whom, in retrospect, there had been a previous, undiagnosed, probable FDE (n = 48). Controls were matched to cases (2:1) on age (within 2 years), sex, and study region. We collected self-report data on history of IM (“have you ever had glandular fever?”) and age at infection, smoking, exposure to infant siblings, and sun exposure over the lifetime. Silicone skin casts were used to objectively measure cumulative past sun exposure.17 Vitamin D status was assessed as the serum 25-hydroxyvitamin D [25(OH) D] concentration,18 and DNA was genotyped for a range of MS-related genes, using proxy single nucleotide polymorphisms (SNPs).12,18

In a nested EBV study (n = 215 cases, n = 216 controls), we measured whole blood quantitative EBV DNA load using 3 separate primers targeting EBNA-1, BHRF-1, and BWRF-1 gene regions, in 2 different laboratories. EBV DNA load quantification using the first 2 primers is described elsewhere.19 For the BWRF-1 gene region, DNA was extracted from EDTA whole blood using the Qiagen Blood mini kit (Qiagen, Hilden, Germany). One microgram of DNA was subjected to real-time quantitative PCR as previously described.20 The Namalwa cell line (containing 2 EBV copies/cell) was used in the standard curve to determine copy numbers21 (expressed as EBV copies/μg of DNA). Quantification was performed on EDTA whole blood rather than peripheral blood mononuclear cells (PBMC) to capture EBV sourced from plasma (representing active virus production) as well as PBMC (representing latent infection). Quantitative IgG antibody titers to EBV viral capsid antigen (VCA) were measured by automated enzyme immunoassay (Star Corp, Stillwater, MN)22 and antibodies to EBNA complex and early antigen (diffuse and restricted, EA-D and EA-R, respectively) by immunofluorescence assay.23 SNP genotyping was performed using the SNPlines method (KBIosciences, Hoddesdon Herts, UK).

Statistical analysis. Of 311 eligible cases, 282 (91%) agreed to participate. Of 1,118 controls initially selected, 937 were successfully contacted (84%) and 558 participated in the study (60% of those contacted). Results here are based on 276 cases and 543 matched controls (see table e-1 on the Neurology® Web site at www.neurology.org) with data on history of IM, including those in the EBV nested study (FCD cases, n = 215, matched controls, n = 216; serology unavailable for 9 cases).

EBV DNA load was highly skewed, with no detectable EBV DNA in nearly 50% of samples. We thus examined this factor as both a dichotomous (present/absent) and a continuous variable. EBV-specific antibody titers were positively skewed and were transformed to an approximately normal distribution using the base 2 logarithm of the reciprocal of the dilution of the titers as a continuous variable. In the log base 2 transformation, the regression coefficient estimates the logarithm of the odds ratio (OR) associated with a 2-fold difference. We used Spearman correlation to examine the correlation between different measures of EBV infection and logistic regression (IM as outcome), general linear models (antibody titers), and nonparametric tests (EBV DNA load). We examined associations between these measures and other factors, e.g., age and sex. Conditional logistic regression was used to examine FCD risk in relation to markers of EBV infection, testing other known MS risk factors as confounders or effect modifiers, the latter by adding the relevant product term to the model and assessing the resultant reduction in deviance using the likelihood ratio test.24 We also estimated the additive effect of having multiple risk factors. Adjusted ORs (AOR), including adjustment for educational level, smoking, and vitamin D status, and 95% confidence intervals (95% CI), are presented. All analyses were undertaken using Stata (version 9.2; StataCorp LP, College Station, TX). Participants with missing data on factors of interest were excluded from those specific analyses.

Standard protocol approvals, registrations, and patient consents. The Ausimmune Study was approved by 9 regional Human Research Ethics Committees. All participants gave written informed consent.

RESULTS EBV infection measures among control participants. EBV DNA load measured using the BWRF-1 gene region (but not EBNA-1 or BHRF-1) was correlated with anti-VCA and anti-EA-R IgG titers (anti-VCA r = 0.24, p < 0.001; anti-EA-R r = 0.24, p < 0.001) but not anti-EBNA (r = 0.08, p = 0.25) or anti-EA-D (r = 0.08, p = 0.25) titers.

A positive history of IM was associated with higher anti-EBV-specific IgG titers (log 2): anti-EBNA, OR = 1.12 (95% CI 0.93 to 1.35); anti-VCA, OR = 1.23 (95% CI 0.98 to 1.56); anti-EA-D, OR = 1.18 (95% CI 0.97 to 1.44); anti-EA-R, OR = 1.14 (95% CI 0.94 to 1.37). The highest mean anti-EBNA titers were for those reporting IM at 6–10 years of age, but the difference was statistically significant only in comparison to the 11–15 years age group (p = 0.02). EBV
DNA load was not associated with history of IM for any of the 3 gene regions examined.

Table 1 shows variation in the markers of EBV infection among controls. Additionally, EBV DNA load and anti-EBV specific IgG titers did not differ significantly by current or early childhood (0 to 5 years) latitude of residence, and there was no difference in EBV-specific IgG titers between Australian-born and overseas-born participants or according to age at immigration (data not shown). However, median EBV DNA load was higher in overseas-born controls than Australian-born (median [interquartile range (IQR)]: 0.25 [0–1.1]; 0 [0–0.65], respectively, \( p = 0.05 \)), with the greatest difference for those arriving in Australia as adults. Adjustment for sex and current age did not alter these findings.

Markers of EBV infection and FCD risk. Current EBV DNA load. The proportion with detectable EBV DNA was not significantly different for cases compared to matched controls (table 2, results shown only for the BWRF-1 gene region) and there was no increased

<table>
<thead>
<tr>
<th>Age at interview, y</th>
<th>BWRF-1 DNA positive, n (%)</th>
<th>BWRF-1 DNA load, median (IQR)(^a)</th>
<th>Anti-EBNA IgG titer, mean (SD)</th>
<th>Anti-VCA IgG titer, mean (SD)</th>
<th>History of IM, y, n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18–29</td>
<td>21 (50)</td>
<td>0.60 (0.20–1.70)</td>
<td>74.6 (4.0)</td>
<td>349.0 (4.9)</td>
<td>22 (22.9)</td>
</tr>
<tr>
<td>30–39</td>
<td>36 (53.7)</td>
<td>0.55 (0.30–1.50)</td>
<td>87.0 (4.7)</td>
<td>474.2 (3.9)</td>
<td>37 (19.6)</td>
</tr>
<tr>
<td>40–49</td>
<td>33 (47.8)</td>
<td>1.40 (0.3–3.4)</td>
<td>94.1 (4.2)</td>
<td>619.5 (2.7)</td>
<td>24 (14.5)</td>
</tr>
<tr>
<td>50+</td>
<td>19 (50.0)</td>
<td>0.7 (0.4–1.0)</td>
<td>115.2 (5.0)</td>
<td>477.8 (4.8)</td>
<td>6 (6.5)</td>
</tr>
<tr>
<td>( p(\text{trend}) )</td>
<td>0.80</td>
<td>0.99</td>
<td>0.20</td>
<td>0.15</td>
<td>0.002</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sex</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>25 (49.0)</td>
<td>0.70 (0.30–3.20)</td>
<td>55.7 (4.8)</td>
<td>292.3 (4.6)</td>
<td>17 (14.2)</td>
</tr>
<tr>
<td>Female</td>
<td>84 (50.9)</td>
<td>0.75 (0.30–1.85)</td>
<td>103.9 (4.3)</td>
<td>557.1 (3.6)</td>
<td>72 (17.0)</td>
</tr>
<tr>
<td>( p )</td>
<td>0.81</td>
<td>0.97</td>
<td>0.01</td>
<td>0.004</td>
<td>0.45</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Education, y</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>( \leq 10 )</td>
<td>31 (46.3)</td>
<td>0.80 (0.40–1.80)</td>
<td>90.2 (4.7)</td>
<td>492.8 (3.6)</td>
<td>15 (8.5)</td>
</tr>
<tr>
<td>12/TAFE</td>
<td>49 (51.6)</td>
<td>0.60 (0.20–2.70)</td>
<td>94.4 (4.0)</td>
<td>530.1 (3.5)</td>
<td>43 (19.0)</td>
</tr>
<tr>
<td>University</td>
<td>28 (52.8)</td>
<td>0.65 (0.45–1.65)</td>
<td>81.0 (4.6)</td>
<td>398.0 (4.9)</td>
<td>31 (22.3)</td>
</tr>
<tr>
<td>( p(\text{trend}) )</td>
<td>0.46</td>
<td>0.90</td>
<td>0.72</td>
<td>0.43</td>
<td>0.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HLA-DR 15</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A:A</td>
<td>65 (50.0)</td>
<td>0.80 (0.30–2.90)</td>
<td>88.6 (4.6)</td>
<td>521.8 (3.7)</td>
<td>62 (20.1)</td>
</tr>
<tr>
<td>G:A</td>
<td>26 (53.1)</td>
<td>0.80 (0.30–1.50)</td>
<td>96.4 (3.8)</td>
<td>477.3 (3.9)</td>
<td>14 (12.4)</td>
</tr>
<tr>
<td>G:G</td>
<td>3 (42.9)</td>
<td>0.40 (0.40–0.50)</td>
<td>97.5 (4.6)</td>
<td>780.2 (2.6)</td>
<td>3 (20.0)</td>
</tr>
<tr>
<td>( p )</td>
<td>0.97</td>
<td>0.62</td>
<td>0.72</td>
<td>0.87</td>
<td>0.15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HLA-A</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A:A</td>
<td>14 (50.0)</td>
<td>0.25 (0.10–1.50)</td>
<td>74.5 (4.8)</td>
<td>335.7 (5.6)</td>
<td>9 (18.0)</td>
</tr>
<tr>
<td>G:A</td>
<td>29 (39.7)</td>
<td>0.60 (0.30–1.10)</td>
<td>81.5 (3.8)</td>
<td>486.0 (3.5)</td>
<td>34 (19.4)</td>
</tr>
<tr>
<td>G:G</td>
<td>52 (61.2)</td>
<td>1.00 (0.40–3.40)</td>
<td>118.7 (4.5)</td>
<td>650.5 (3.3)</td>
<td>37 (17.2)</td>
</tr>
<tr>
<td>( p )</td>
<td>0.07</td>
<td>0.02</td>
<td>0.09</td>
<td>0.02</td>
<td>0.42</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serum 25(OH)D(^d)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tertile 1</td>
<td>42 (54.6)</td>
<td>1.15 (0.4–3.3)</td>
<td>79.3 (3.9)</td>
<td>499.0 (3.9)</td>
<td>25 (14.8)</td>
</tr>
<tr>
<td>Tertile 2</td>
<td>33 (52.4)</td>
<td>0.6 (0.3–1.7)</td>
<td>115.6 (4.2)</td>
<td>538.0 (3.6)</td>
<td>22 (12.9)</td>
</tr>
<tr>
<td>Tertile 3</td>
<td>33 (45.2)</td>
<td>0.5 (0.3–1.0)</td>
<td>85.4 (5.2)</td>
<td>456.8 (4.0)</td>
<td>35 (20.7)</td>
</tr>
<tr>
<td>( p )</td>
<td>0.26</td>
<td>0.06</td>
<td>0.76</td>
<td>0.69</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Abbreviations: EBNA = Epstein-Barr virus nuclear antigen; EBV = Epstein-Barr virus; HLA = human leukocyte antigen; IgG = immunoglobulin G; IM = infectious mononucleosis; IQR = interquartile range; TAFE = technical and further education college; VCA = viral capsid antigen.

\(^a\) EBV DNA load: positive samples only (copies/μg DNA).

\(^b\) Geometric mean (SD) of inverse of dilution.

\(^c\) Statistical significance is defined as \( p < 0.05 \).

\(^d\) Tertiles of unadjusted 25(OH)D concentration (1 \( < 68.5 \); 2 \( 68.5, \leq 93.6 \); 3 \( > 93.6 \) nmol/L).
risk of being a FCD case across the full range of EBV DNA load (0–73.5 copies/μg DNA) (AOR = 1.03 [95% CI 0.97 to 1.08], per copy/μg DNA, p = 0.33).

**Markers of past EBV infection.** Cases had higher titers of anti-VCA, anti-EBNA, and anti-EA-R IgG, but not anti-EA-D IgG (table 2). There was no significant variation in anti-EBV-specific IgG titers across the different types of FDE presentation, i.e., optic neuritis, spinal cord syndrome, brainstem/cerebellar syndrome, in relation to the length of time from the first demyelinating event to the interview/blood draw, or whether or not cases had had steroid treatment. A doubling of the anti-EBNA IgG titer was associated with a 28% increase in the odds of being an FCD case (AOR = 1.28 [95% CI 1.15 to 1.44]), with a similar effect size when only the classic FDE group was considered (AOR = 1.25 [95% CI 1.10 to 1.41]). In a model containing all 4 EBV-specific antibodies, only anti-EBNA IgG retained an independent effect (OR = 1.28 [95% CI 1.13 to 1.46]). A history of past IM was associated with an increased risk of being an FCD case (AOR = 1.90 [95% CI 1.28 to 2.81], p = 0.001) or FDE (AOR = 1.63 [95% CI 1.05 to 2.54], p = 0.03) case and risk did not vary by the reported age at IM (p = 0.90). Additional adjustment for EBV DNA load or for EBNA IgG titer did not substantially alter the association between markers of past EBV infection and FCD risk.

**FCD risk and EBV infection in the presence of a second risk factor. Environmental exposures.** Among controls, higher 25(OH)D concentrations were correlated with lower BWRF-1 DNA load (r = −0.17, p = 0.01). There was a significant multiplicative interaction (p[interaction] = 0.02) between 25(OH)D levels and EBV DNA load for FCD risk (both variables continuous, adjusted analysis). Categorizing both factors [25(OH)D low <80 nmol/L, high ≥80 nmol/L; EBV DNA load negative, low positive <1, high ≥1 copy/μg DNA], there was a twofold increased risk of being a FCD case for the combination of higher 25(OH)D concentration and high viral load (table 3). There was no multiplicative interaction between 25(OH)D concentration and anti-EBNA IgG titer on FCD risk (p = 0.41).

Among controls, there was no association between leisure-time sun exposure for 5-year age brackets from 6 to 25 years and history of IM (excluding from successive analyses those who had had IM at an earlier age). In case-control analyses, sun exposure and EBV infection measures were independently associated with FCD risk with no evidence of interaction (e.g., difference in effect of anti-EBNA titers on FCD risk by leisure-time sun exposure, p = 0.31). The findings obtained substituting in the silicone cast score as the sun exposure measure gave similar results.

A greater number of days of exposure to infant siblings was associated with a nonsignificant reduced FCD risk (AOR = 0.87 [95% CI 0.68 to 1.12]) per
1,000 days of exposure] that was independent of, and was not modified by, history of IM. For anti-EBNA titers, the results were very similar. Smoking history did not appear to be a major confounder or an effect modifier of the association between EBV infection and FCD risk in this study.

**Genetic factors.** HLA-DRB1*1501 status (HLA-DR15, proxy SNP rs9271366) was associated with a marked increase in FCD risk in unadjusted analyses (OR = 3.09 [95% CI 2.17 to 4.39]). For the association between IM and FCD risk, adjustment for HLA-DR15 status did not change the magnitude of effect (AOR = 1.79, vs AOR = 1.73 [95% CI 1.08 to 2.75]) in those with data on both factors. There was a borderline multiplicative interaction (p = 0.07): having both factors was associated with a marked increase in risk of being a FCD case (neither factor AOR = 1.00; IM+ but HLA-DR15− AOR = 1.15 [95% CI 0.60 to 2.21]; IM− but HLA-DR15+, AOR = 2.48 [95% CI 1.52 to 4.03]; IM+ and HLA-DR15+, AOR = 7.44 [95% CI 3.51 to 15.79]).

In the parallel analysis using anti-EBNA titer, both HLA-DR15 (AOR = 3.12 [95% CI 1.88 to 5.16]) and anti-EBNA titer (AOR = 1.27 [95% CI 1.12 to 1.43]) were independently associated with FCD risk when included in the same model (HLA-DR15: AOR = 3.47 [95% CI 1.99 to 6.06]; anti-EBNA: AOR = 1.31 [95% CI 1.14 to 1.51]). Here there was no significant multiplicative interaction (p = 0.50), but a marked increase in risk when both factors were present (figure).

There was increased FCD risk in association with the G allele of a SNP in the HLA-A region (rs6904029) (OR = 3.62 [95% CI 1.69 to 7.78]) and a marked increase in FCD risk with increasing anti-EBNA titer in association with this allele (figure).

Although there was no association between SNPs in the CTLA-4 gene and FCD risk in crude analyses (e.g., rs11571316: OR = 0.86 [95% CI 0.60 to 1.21]), this SNP was an effect modifier for the association between anti-EBNA titer and FCD risk (p(interaction) = 0.04). Here the adverse effect of high anti-EBNA titer (>320) was more marked in the absence, compared to the presence, of the A-allele (table 4). The odds of being a FCD case were markedly reduced when the anti-EBNA titer was low and the A allele was present.

**Latitudinal variation in EBV infection and the latitudinal FCD incidence gradient.** Among controls, EBV DNA load did not vary by latitude for any of the 3 gene regions examined and there was no association between latitude and anti-EBNA, anti-EA-D, or anti-EA-R IgG titers. After adjustment for age and sex, anti-VCA IgG titers tended to decrease with increasing latitude (β = −0.04 [95% CI −0.08 to 0.004], p = 0.08 change in anti-VCA [log2] titer per higher degree of latitude). The odds of a positive history of IM also decreased with increasing latitude (OR = 0.96 [95% CI 0.92 to 1.00]), with the magnitude of the estimate unaffected by adjustment for age, sex, educational level, or number of years smoking. This represents a 35% decrease in the odds for IM from the lowest latitude to the highest latitude region. There was no latitudinal variation in the age of reporting IM (p = 0.44) or in the associations between markers of EBV infection and FCD risk.
DISCUSSION No significant difference was observed in the whole blood EBV DNA load of cases with a first clinical diagnosis of CNS demyelination compared to control participants. In contrast, FCD cases were more likely than age- and sex-matched controls to report a history of IM and had higher anti-EBNA IgG titers, possibly reflecting not just past EBV infection, but impaired immune control of that infection. Of interest, the host constitution in relation to 3 gene variants, HLA-DR15, HLA-A, and CTLA4, altered the association between higher anti EBNA IgG titers and FCD risk. Neither EBV DNA load nor markers of past EBV infection accounted for the latitudinal gradient in FCD incidence previously observed in the Ausimmune Study.

Strengths of this multicenter study include recruitment of incident cases from study regions spanning a wide latitude range, the examination of a broad spectrum of environmental factors, and the measurement of quantitative EBV DNA load and titers for 4 EBV-specific antibodies. The focus was on early disease, with similar findings for the full FCD group, the classic FDE group, the FDE group with interview closest to the first event, and for those who had, and had not, received methylprednisolone treatment. One limitation was that data on EBV-specific antibodies and EBV DNA load were available for only a subset of participants, so that some analyses are limited by sample size. The EBV DNA load assay was sensitive and showed an expected correlation with antibodies produced relatively early in the host response to EBV infection. Nevertheless, undertaking measurements on whole blood does not permit evaluation of the relative contribution of EBV load in plasma (related to reactivation and virus production) compared to PBMCs (a marker of latent infection load).

Late infection with EBV (manifested as IM) could itself directly increase MS risk or be a marker of more hygienic conditions in early life. The hygiene hypothesis posits that lower infection exposure in early life results in a less targeted immune response to common infections, a generally more inflammatory immune milieu, and an increased risk of autoimmune diseases. Here, however, we found no evidence that the association between FCD and IM was altered by the reported age of IM. Higher infant sibling exposure was not associated with lower anti-EBNA IgG titer and did not modify the association between EBV indices and FCD risk.

Our findings are also not consistent with a strong role for acute EBV infection/reactivation being the trigger of the immediate demyelinating event. Even in FDE cases where the time from first event to blood collection was short (<3 months), we found no significant increase in EBV DNA load, as has been reported for some other Th-1 autoimmune diseases. Further, FCD risk associated with the host antibody response to acute infection/reactivation (anti-VCA and anti-EA-D IgG) did not persist after adjustment for anti-EBNA antibodies, and the latter appeared to be the most important serologic index.

Although several commentators have hypothesized that MS is caused by an interaction whereby low vitamin D status heightens the risk of EBV infection and subsequent autoimmune responses, we found no evidence of any interaction between either sun exposure or vitamin D status and history of IM or higher anti-EBNA titers. These factors appeared to be independent risk factors for onset of CNS demyelination in this study.

Higher 25(OH)D concentrations were associated with lower EBV DNA load in controls—this is consistent with the posited beneficial effect of vitamin D on viral and intracellular infections. However, the combination of having higher 25(OH)D concentrations and higher levels of EBV DNA in blood was associated with increased FCD risk. One explanation is that an unmeasured factor that allows reactivation of EBV even in a high vitamin D environment, e.g., defective CD8+ T cell function, is, in itself, a FCD disease determinant. Further studies are needed to confirm and further explore this result.

Human leukocyte antigen (HLA) class I molecules complex with viral peptides on virus-infected cells to allow recognition by CD8+ T cells responsible for controlling EBV infection. Thus, the observed interaction between markers of EBV infection and SNPs in the HLA class I region is plausible. A previous study has shown an increased frequency of CD8+ T cells recognizing EBV-derived peptides in association with HLA-A2 and B7 alleles in MS cases but not controls, although this was not confirmed in another study. As has been previously reported, it appears to be particularly adverse for people who are genetically susceptible to have higher levels of anti-EBNA IgG.
We have additionally shown that the presence/absence of the A allele of a SNP in the \textit{CTLA4} gene modifies the association between anti-EBNA titer and FCD risk. This has not been previously described in MS, but has been observed in systemic lupus erythematosus,\textsuperscript{37} another EBV-associated autoimmune disease.

These findings from this large, population-based study of first onset of CNS demyelination are consistent with other studies showing an association between markers of past EBV infection and MS risk. They do not provide strong support that whole blood EBV DNA load is of major importance at the onset of clinical disease, and we found no interaction with other risk factors including smoking, vitamin D status, or past sun exposure. We have confirmed previous findings of an additive interaction between HLA class II and EBV-specific antibody levels, and have additionally shown further interactions between SNPs in the HLA class I region and \textit{CTLA4}.

\textbf{AUTHOR CONTRIBUTIONS}

Dr. Lucas coordinated the collection of data, undertook data cleaning and analysis, and prepared the manuscript. Dr. Ponsonby was involved in the study design, data collection, analysis, and preparation of the manuscript. Dr. Dear was involved in the study design and analysis and provided comment on the manuscript. Dr. Valery was involved in data collection and provided comment on the manuscript. Dr. Pender was involved in the study design and data collection and provided comment on the manuscript. Dr. J. Burrows, Dr. S. Burrows, Dr. D. Dwyer, and M.-L. Lay undertook viral laboratory analyses and provided comment on the manuscript. Dr. Chapman was involved in the study design and data collection and provided comment on the manuscript. Dr. Coulthard was involved in data collection and provided comment on the manuscript. Dr. D. Dwyer, Dr. Kilpatrick, Dr. McMichael, Dr. Taylor, Dr. van der Mei, and Dr. Williams were involved in the study design and data collection and provided comment on the manuscript.

\textbf{ACKNOWLEDGMENT}

The authors thank the physicians who notified case participants to the Ausimmune Study: Joanne Anderson, FRANZCO, Coastal Eye Centre Queensland; Michael Bailey, FRANZCO, The Mount Gambier Eye Centre South Australia; Peter Batchelor, PhD, Barwon Health, Victoria; Jeffrey Blackie, FRACP, John Hunter Hospital, Newcastle, New South Wales; Richard Bourke, FRACGP, General Practice, Tasmania; Richard Boyle, FRACP, Princess Alexander Hospital, Brisbane, Queensland; John Cameron, MD, Princess Alexandra Hospital, Brisbane, Queensland; Ross Carne, MD, Deakin University, Victoria; Chris Charnley, FRACP, Southwest HealthCare, Warrnambool, Victoria; Ben Clark, FRANZCO, Geelong Hospital, Victoria; Steven Collins, MD, St Vincent’s Hospital, Melbourne; Diana Conrad, FRANZCO, Wesley Medical Centre, Auchentflower, Queensland; Michael Cororonos, FRACS, Private Practice, Brisbane, Queensland; Nicholas Downie, FRANZCO, Launceston General Hospital, Tasmania; Michael Dreyer, MD, Royal Hobart Hospital, Tasmania; Mervyn Eade, MD, Royal Brisbane and Women’s Hospital, Queensland; David Floyd, FRACP, John Hunter Hospital, Newcastle, New South Wales; Peter Gates, FRACP, Barwon Health, Geelong Hospital, Victoria; Kerryn Green, FRACP, University of Queensland, Queensland; Erwin Groeneveld, FRANZCO, Princess Alexander Hospital, Brisbane, Queensland; Mark Guigais, MBBS (Hons), Private Practice, Tasmania; John Harrison, FRANZCO, Royal Brisbane and Women’s Hospital and Princess Alexander Hospital, Brisbane, Queensland; Michael Haybittel, FRANZCO, North West Regional Hospital, Tasmania; Robert Henderson, FRACP, Royal Brisbane and Women’s Hospital, Queensland; John Henschaw, MMed, University of Tasmania, Tasmania; Keirh Ho, MBBS, Ballarat Medical Centre, Victoria; Eugene Hollenbach, MBBS, Private Practice, Newcastle, New South Wales; James Hurley, MD, University of Melbourne, Victoria; Dean Jones, FRACP, Royal Hobart Hospital, Tasmania; Michael Katekar, MBBS, John Hunter Hospital, Newcastle, New South Wales; Anthony Kemp, FRACP, Ballarat Health Services, Victoria; Mark King, FRACP, Geelon Private Hospital, Victoria; George Kiroff, FRACS, The Geelong Hospital, Victoria; Brett Knight, FRACP, Ballarat Health Services, Victoria; Thomas Kraemer, FRACP, The Geelong Hospital, Victoria; Cecile Lander, FRACP, Royal Brisbane and Women’s Hospital, Queensland; Jeanette Lechner-Scott, FRACP, John Hunter Hospital, Newcastle, New South Wales; Patrick Lockie, FRACS, St John of God Hospital, Geelong, Victoria; Andre Loisel, FRACGP, Hunter New England Health, Newcastle, New South Wales; Paul McCartney, FRANZCO, Royal Hobart Hospital, Tasmania; Pam McCombe, PhD, University of Queensland, Queensland; Mark McGree, FRANZCO, McCullough Medical Centre, Queensland; David McKnight, FRANZCO, Ballarat Base Hospital, Victoria; Dan MacLaughlin, PhD, Royal Brisbane and Women’s Hospital, Queensland; Ian Murrell, FRANZCO, The Eye Hospital, Launceston, Tasmania; Sarish Nagarajah, MBBS, St John of God Hospital, Geelong, Victoria; Robert Newton, MBBS, Bayside Medical Centre, Hobart, Tasmania; Rob Nightingale, FRACP, Calvary Hospital, Victoria; Terence O’Brien, MD, University of Melbourne, Victoria; John O’Sullivan, MD, Royal Brisbane and Women’s Hospital, Queensland; Gregory Outteridge, FRANZCO, Hunter Valley Private Hospital, Newcastle, New South Wales; Anthony Pane, FRANZCO, Queensland Eye Institute, Queensland; Mark Parsons, FRACP, Hunter Medical Research Institute, Newcastle, New South Wales; Melinda Pascoe, FRACP, Private Practice, Brisbane, Queensland; David Prentice, PhD, FRACP, St Vincent’s Hospital, Melbourne, Victoria; Richard Ralph, FRACGP, Cascade Road Medical Centre, Hobart, Tasmania; Stephen Read, FRACP, Royal Brisbane and Women’s Hospital, Queensland; Alison Reid, FRACP, Private Practice, Brisbane, Queensland; John Richardson, FRACP, Ballarat Health Services, Victoria; Jane Routley, FRANZCO, The Geelong Hospital, Victoria; Timothy Ruddell, FRANZCO, Private Practice, Newcastle, New South Wales; Noel Saines, FRACP, Wesley Medical Centre, Auchentflower, Queensland; Stan Siejka (dec), MBBS, Launceston General Hospital, Tasmania; Peter Silburn, PhD, FRACP, University of Queensland, Queensland; Christopher Staples, FRACP, Mater Health Services, Brisbane, Queensland; Alice Ann Sullivan, FRACP, Royal Brisbane and Women’s Hospital, Brisbane, Queensland; Paul Talman, FRACP, Barwon Health, Geelong Hospital, Victoria; Don Todman, FRACP, University of Queensland, Queensland; Nitin Verma, FRANZCO, Hobart Eye Surgeons, Tasmania; Brendan Votey, FRANZCO, University of Tasmania, Tasmania; Michael Waldie, FRANZCO, Queensland Eye Hospital, Queensland; Michael Weetch, FRACP, Private Practice, Bendigo, Victoria; Rodney Westmore, FRANZCO, Launceston General Hospital, Tasmania; Andrew Wong, FRACP, Princess Alexander Hospital, Brisbane, Queensland. Notifying physicians received no payment for case notification to the study. The authors also thank the paid research personnel, including the local research officers, for input: Susan Agland, BN, Hunter New England Health, Newcastle, New South Wales; Barbara Alexander, BN, Queensland Institute for Medical Research, Queensland; Marcia Davis, MD, Queensland Institute for Medical Research, Queensland; Zoe Dunlop, BN, Barwon Health, Geelong Hospital, Victoria; Rosalie Scott, BN, Royal Brisbane and Women’s Hospital, Queensland; Marie Steele, RN, Royal Brisbane and Women’s Hospital, Queensland; Catherine Turner, MPH/TM, Menzies Research Institute, Tasmania; Brenda Wood, RN, Menzies Research Institute, Tasmania; and the Ausimmune Study project officers during the course of the study: Jane Gresham, MA (Int Law), National Centre for Epidemiology and Population Health, The Australian National University, Canberra; Australian Capital Territory; Camilla Jurvick, BSc(Hons), National Centre for Epidemiology and Population Health, The Australian National University, Canberra; Australian Capital Territory; Helen Rodgers, RN, National Centre for Epidemiology and Population Health, The Australian National University, Canberra; Australian Capital Territory. The authors also thank Associate Professor Juleen Cavanagh, The Australian National University, Canberra, for assistance with DNA collection and A/Prof. Cavanagh and Dr.
Jim Stankovich, Menzies Research Institute Tasmania, University of Tasmania, Tasmania, for the genotyping facets of the Ausimmune Study.

DISCLOSURE
Dr. Lucas receives research support from Multiple Sclerosis Research Australia, The Royal Australasian College of Physicians, and the National Health and Medical Research Council of Australia. Dr. Ponsonby receives research support from Multiple Sclerosis Research Australia and the National Health and Medical Research Council of Australia. Dr. Dear receives research support from Multiple Sclerosis Research Australia and the National Health and Medical Research Council of Australia. Dr. van der Mei receives research support from MS Research Australia and the National Health and Medical Research Council of Australia. Dr. Lindsey receives research support from the National Health and Medical Research Council of Australia. Dr. Valery receives research support from Multiple Sclerosis Australia and the National Health and Medical Research Council of Australia. Dr. Simon receives funding for travel and research support from Multiple Sclerosis Research Australia and the National Health and Medical Research Council of Australia. Dr. Williams receives research support from Multiple Sclerosis Research Australia and the National Health and Medical Research Council of Australia. Dr. Williams reports no disclosures. Dr. S. Burrows serves on the editorial board of the Open Autoimmunity Journal and receives research support from Multiple Sclerosis Research Australia and the National Health and Medical Research Council of Australia. Dr. Chapman reports no disclosures. Dr. Coulthard serves as an Associate Editor of the Journal of Medical Imaging and Radiation Oncology. Dr. D. Dwyer has served on scientific advisory boards for Tibotec Therapeutics, Merck & Co., Inc., and CSL Behring; and has received research support from Merck & Co., Inc., CSL Behring, GlaxoSmithKline, and the National Health and Medical Research Council of Australia. Dr. T. Dwyer has received research support from the National Health and Medical Research Council of Australia. Dr. Kilpatrick has served on scientific advisory boards for GlaxoSmithKline, Neurosciences Victoria, and the Victorian Neurotrauma Initiative; has received funding for travel from Bayer Schering Pharma and Merck Serono; served on the editorial board of Therapeutic Advances in Neurological Disorders; is listed as an inventor on patents re: HIV test kit method for detecting anti-HIV-I antibodies in saliva; A method of modulating cell survival and reagents useful for same; Methods for the treatment and prophylaxis of demyelinating disease; and Method of treatment in the field of inflammatory neurodegeneration; and receives research support from Bayer Schering Pharma, Biogen Idec, the Australian Research Council, the National Health and Medical Research Council of Australia, MS Research Australia, and the National Multiple Sclerosis Society. M.-L. Lay reports no disclosures. Dr. McMichael serves on the editorial board of The Open Autoimmunity Journal and receives research support from Multiple Sclerosis Research Australia and the National Health and Medical Research Council of Australia. Dr. Pender serves on the editorial board of The Open Autoimmunity Journal and receives research support from Multiple Sclerosis Research Australia and the National Health and Medical Research Council of Australia. Dr. McMichael serves on the editorial board of The Open Autoimmunity Journal and receives research support from Multiple Sclerosis Research Australia and the National Health and Medical Research Council of Australia. Dr. Pender serves on the editorial board of The Open Autoimmunity Journal and receives research support from Multiple Sclerosis Research Australia and the National Health and Medical Research Council of Australia. Dr. Van der Mei receives research support from MS Research Australia and the National Health and Medical Research Council of Australia. Dr. Williams reports no disclosures. Dr. Williams reports no disclosures.

Received January 15, 2011. Accepted in final form April 5, 2011.

REFERENCES


