RESEARCH ARTICLE OPEN ACCESS

Disease-Modifying Treatments for Multiple Sclerosis Affect Measures of Cellular Immune Responses to EBNA-1 Peptides

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Neurol Neuroimmunol Neuroinflamm 2024;11:e200217. doi:10.1212/NXI.0000000000200217

Abstract

Background and Objectives

Epstein-Barr virus (EBV) has been strongly implicated in the pathogenesis of multiple sclerosis (MS). Despite this, there are no routinely used tests to measure cellular response to EBV. In this study, we analyzed the cellular response to EBV nuclear antigen-1 (EBNA-1) in people with MS (pwMS) using a whole blood assay.

Methods

This cross-sectional study took place in a dedicated MS clinic in a university hospital. We recruited healthy controls, people with epilepsy (PWE), and pwMS taking a range of disease-modifying treatments (DMTs) including natalizumab, anti-CD20 monoclonal antibodies (mAbs), dimethyl fumarate (DMF), and also treatment naïve. Whole blood samples were stimulated with commercially available PepTivator EBNA1 peptides and a control virus-cytomegalovirus (CMV) peptide. We recorded the cellular response to stimulation with both interferon gamma (IFN-γ) and interleukin-2 (IL-2). We also compared the cellular responses to EBNA1 with IgG responses to EBNA1, viral capsid antigen (VCA), and EBV viral load.

Results

We recruited 86 pwMS, with relapsing remitting MS, in this group, and we observed a higher level of cellular response recorded with IFN-γ (0.79 IU/mL ± 1.36) vs healthy controls (0.29 IU/mL ± 0.90, $p = 0.0048$) and PWE (0.17 IU/mL ± 0.33, $p = 0.0088$). Treatment with either anti-CD20 mAbs (0.28 IU/mL ± 0.57) or DMF (0.07 IU/mL ± 0.15) resulted in a cellular response equivalent to control levels or in PWE ($p = 0.26$). The results of recording IL-2 response were concordant with IFN-γ: with suppression also seen with anti-CD20 mAbs and DMF. By contrast, we did not record any differential effect of DMTs on the levels of IgG to either EBNA-1 or VCA. Nor did we observe differences in cellular response to cytomegalovirus between groups.

Discussion

This study demonstrates how testing and recording the cellular response to EBNA-1 in pwMS may be beneficial. EBNA-1 stimulation of whole blood samples produced higher levels of IFN-γ and IL-2 in pwMS compared with controls and PWE. In addition, we show a differential effect of currently available DMTs on this response. The functional assay deployed uses whole blood samples with minimal preprocessing suggesting that employment as a treatment response measure in clinical trials targeting EBV may be possible.

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Go to Neurology.org/NN for full disclosures. Funding information is provided at the end of the article.

The Article Processing Charge was funded by the authors.

Glossary

CMV = cytomegalovirus; **DMF** = dimethyl fumarate; **DMT** = disease-modifying treatment; **EBNA-1** = EBV nuclear antigen-1; **EBV** = Epstein-Barr virus; **EDSS** = Expanded Disability Status Scale; **IFN-γ** = interferon gamma; **mAbs** = monoclonal antibodies; **MS** = multiple sclerosis; **NTZ** = natalizumab; **OCR** = ocrelizumab; **OMB** = ofatumumab; **PBMCs** = peripheral blood mononuclear cells; **PWE** = people with epilepsy; **pwMS** = people with MS; **RLU** = relative luminescence units; **VCA** = viral capsid antigen.

Introduction

Multiple sclerosis (MS) is an autoimmune disorder that results in CNS demyelination and disability as a consequence.[1] Recent epidemiologic evidence has resulted in a paradigm shift, arising from the observation that Epstein-Barr virus (EBV) plays a critical role in the pathogenesis of MS.[2] Although the precise mechanistic process is not fully elucidated, 2 potential examples of molecular mimicry between EBV and key CNS proteins have been reported.[3,4] These include glial cell adhesion molecule and α crystallin B, both of which have epitope similarities to EBV nuclear antigen-1 (EBNA-1). A deeper understanding of this process and elucidation of the effects of currently available disease-modifying treatment (DMT) on EBV have been identified as a research priority in MS.[5]

Initial control of EBV infection is mediated primarily by $CD8^+$ T cells,[6] with an aberrant T-cell response reported in MS.[7-9] Furthermore, clinical disease activity is reflected by cellular response to EBV.[10-12] Based on these observations, a hypothesis has been developed that CNS migration of EBV-activated $CD8^+$ T cells has been implicated in pathogenesis of MS. This is supported by evidence of an increased number of $CD8^+$ T cells in the CSF of people with MS (pwMS) relative to controls.[13-15] However, similar CSF findings have been noted in other inflammatory neurologic disease and with higher $CD8^+$ T cells seen than in blood samples.[16]

Arising from this observation, some studies have evaluated the cellular effect of currently available DMTs on EBV. These include an increase in $CD8^+$ T cells with glatiramer acetate and natalizumab,[9,10,17] and conflicting results on the effect of anti-CD20 monoclonal antibodies with both a decrease and no effect reported.[9,18,19] Similarly, reports of no effect or a decrease in cellular response are available for interferon beta.[9,10] In addition to such conflicting data, there are currently no available results available on the effect of dimethyl fumarate (DMF) on the cellular response to EBV.

Given the association between cellular responses to EBV and disease activity in MS, improvements in our understanding of inflammatory cytokine response to important EBV peptides such as EBNA1 have clinical relevance. This information could be used as a metric as part of a personalized profile of inflammatory activity (to complement existing measures such as MRI and clinic-demographic features), with a view toward patient stratification and treatment selection.[20] Furthermore, current clinical trials in MS are now not solely focused on immunomodulation, and EBV-specific T-cell therapies are being tested.[21] Future therapies in development include vaccines or other means of diminishing the immune response to EBV.[22] An assay that can measure cellular response to EBV would be highly desirable in such a context.

A rate-limiting step in this regard has been the methodologies used to capture the T-cell cytokine response to EBV. EBNA1-specific T cells expressing interferon gamma (IFN-γ) and IL-2 have been demonstrated in peripheral blood of patients with MS.[23] Previous studies have used isolated peripheral blood mononuclear cells (PBMCs) with intracellular staining for cytokine responses on flow cytometry or EliSpot testing.[11,19] These methods require complex preanalytical processing and are not readily standardized; this poses significant challenges for deployment in routine clinical laboratories.

The aims of this study are threefold: (1) to develop a scalable assay using whole blood samples to measure cellular response to EBV in MS; (2) to compare the cellular responses recorded with IgG responses to EBNA1, viral capsid antigen (VCA), and EBV viral load; and (3) to determine the influence of currently available DMTs on the cellular response to EBNA-1 in MS taking and compare with controls.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

The study was approved by St James's Hospital/Tallaght University Hospital Joint Research Ethics Committee; Ref No 840. The study was performed in concordance with the Declaration of Helsinki (1964), and all participants provided informed written consent.

Participants

We recruited people with MS (pwMS) from the MS Unit in St James's Hospital, all of whom fulfilled the 2017 McDonald criteria for diagnosis.[24] As the focus of the study was on DMTs, we recruited pwMS who have a relapsing remitting disease course.[25] We also recruited people with epilepsy as a disease control group (i.e., a neurologic disease where EBV is not implicated) and a cohort of age-matched and sex-matched healthy controls. Demographic details were also recorded and the DMT being used for the MS cohort. For each participant with MS, the level of physical disability was recorded using the Expanded Disability Status Scale (EDSS) by an experienced clinician.

Table Demographics of Patient and Control Cohorts Included in the Study

Variable (range)	Total	Group		
		Healthy control	Epilepsy	Multiple sclerosis
% (n)	145	27.6 (40)	13.1 (19)	59.3 (86)
Age, y, mean (SD)	40.2 (13.0)	39.7 (13.0)	38.4 (18.1)	40.8 (11.8)
≤34, % (n)	40.7 (59)	45.0 (18)	57.9 (11)	34.9 (30)
≥35, % (n)	43.4 (63)	35.0 (14)	10.5 (2)	54.7 (47)
≥55, % (n)	15.9 (23)	20.0 (8)	31.6 (6)	10.5 (9)
Sex: female, % (n)	64.1 (93)	67.5 (27)	36.8 (7)	68.6 (59)
Disease duration, y, median (IQR)	5.5 (15.0)	N/A	10.0 (12.0)	4.0 (13.0)
EDSS: median (range)				1.5 (0–8)
EDSS <1, % (n)				14.5 (12)
EDSS ≥1, % (n)				74.7 (62)
EDSS ≥5, % (n)				9.6 (8)
Disease-modifying therapies				
Treatment naïve, % (n)				25.6 (22)
Natalizumab, % (n)				32.6 (28)
Ocrelizumab/ofatumumab, % (n)				12.8 (22)
Dimethyl fumarate, % (n)				16.3 (14)

Evidence of EBV Infection

Serum samples were tested for IgG to EBNA1, and IgG and IgM to VCA using the Alinity i analyzer with the EBV EBNA1 reagent kit or the EBV VCA IgG or IgM reagent kits according to manufacturer's instructions (Abbott GmBH, 65205 Wiesbaden, Germany). These assays are chemiluminescent microparticle immunoassays used for the qualitative detection of antibodies to EBV antigens. Results are calculated based on the ratio of sample (S) relative luminescence units (RLU) to the cutoff (Co) RLU for each sample and reported as S/Co units. Interpretive ranges for negative, grayzone, and positive S/Co results were provided by the manufacturer for each antibody and have been verified locally.

To evaluate for evidence of active EBV infection, plasma samples were subject to DNA extraction with a NUCLISENS easyMAG Instrument (BIOMERIEUX, SA, 69280 Marcy-I'Etoile, France) followed by testing for the EBV genome using real-time PCR quantification with the ARGENE EBV R-GENE Kit (BIOMERIEUX, SA, 69280 Marcy-I'Etoile, France) on an Applied Biosystems 7500 Fast Real-Time PCR instrument following manufacturer's instructions.

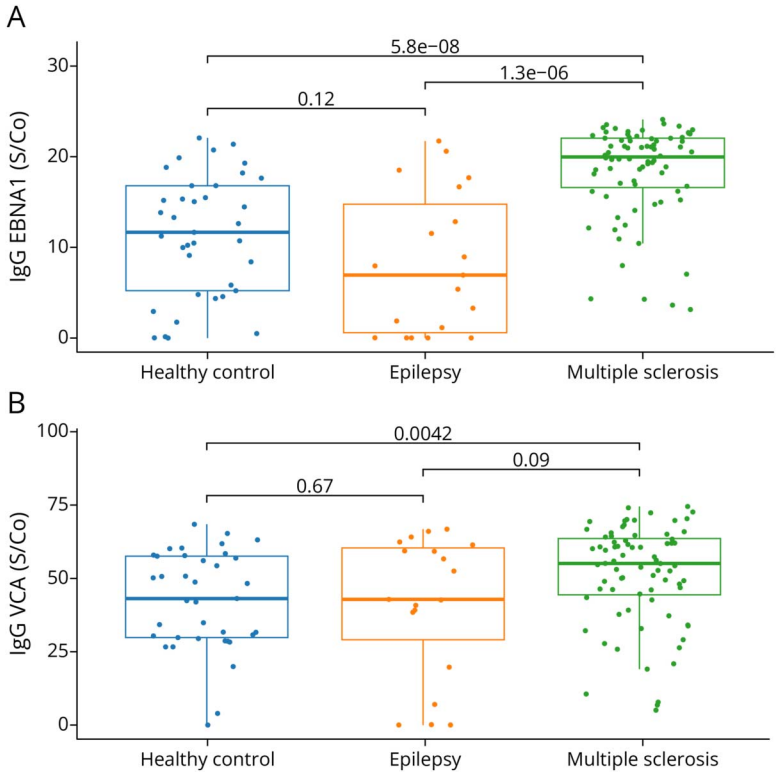
Laboratory Testing of the Cellular Response to EBNA-1

We collected whole blood samples from each participant using QuantiFERON Monitor (Qiagen, Germantown, MD) tubes containing lithium heparin anti-coagulant and a standardized culture medium. Serum and EDTA samples were collected at the same time point.

We stimulated each participant's whole blood samples overnight at 37 °C with PepTivator EBV EBNA1 premium grade 0.5 μg/mL or control virus (CMV pp65 premium grade) at 0.5 μg/mL peptides (Miltenyi Biotech B.V. & Co KG, Bergisch Gladbach, Germany). This commercially available peptide pool contains 15-mer proteins with 11 amino acid overlap providing coverage of EBNA-1 protein. An unstimulated control was included to measure background cytokine levels. A positive WB sample was stimulated with Cytostim (Miltenyi Biotech B.V. & Co KG), a reagent which cross links T-cell receptor to major histocompatibility receptors on antigen-presenting cells. Cytostim was used at 4 μL/mL to assess capacity of the participants' cells to produce cytokines.

All whole blood samples were incubated for 24 hours at 37 °C. Samples were centrifuged at 3,000 rcf for 15 minutes, and the supernatants were harvested and stored at −20 °C. Supernatants were tested for the presence of IFN-γ using a QuantiFERON Monitor ELISA assay (Qiagen Germantown). IL-2 levels (pg/mL) were measured using a Simple Plex cytokine panel on the ELLA platform (BioTechne 614 McKinley Pl NE, Minneapolis) following manufacturer's instructions. IFN-γ and IL-2 levels reported for EBNA1-specific and CMV-

Figure 1 Comparison of EBNA-1 IgG Levels in Healthy Controls and People With MS and Epilepsy



The EBNA-1 IgG levels recorded were significantly higher in the MS cohort compared with either epilepsy or controls. EBNA-1 = EBV nuclear antigen-1.

specific responses represent the measured level minus the unstimulated background level.

Statistical Analysis

First, to compare prior levels of EBV infection between groups, we used a *t*-test to evaluate for differences between: IgG responses to both EBNA1 and VCA, and also EBV PCR levels. In this analysis , we compared pwMS vs healthy controls and pwMS vs people with epilepsy (PWE), we used unpaired 2-sample Wilcoxon tests for these comparisons.

Second, to investigate the cellular response to EBNA-1, we compared both the IFN-γ and IL-2 levels between groups, i.e., MS vs controls and MS vs PWE using a Kruskal-Wallis test. To further interrogate the relationship between prior EBV infection and cellular response to EBNA-1, we conducted univariate correlation analysis between IFN-γ levels in response to EBNA-1 and IgG levels, as well as EBV PCR.

For analysis, we considered a p value of <0.05 to be statistically significant.

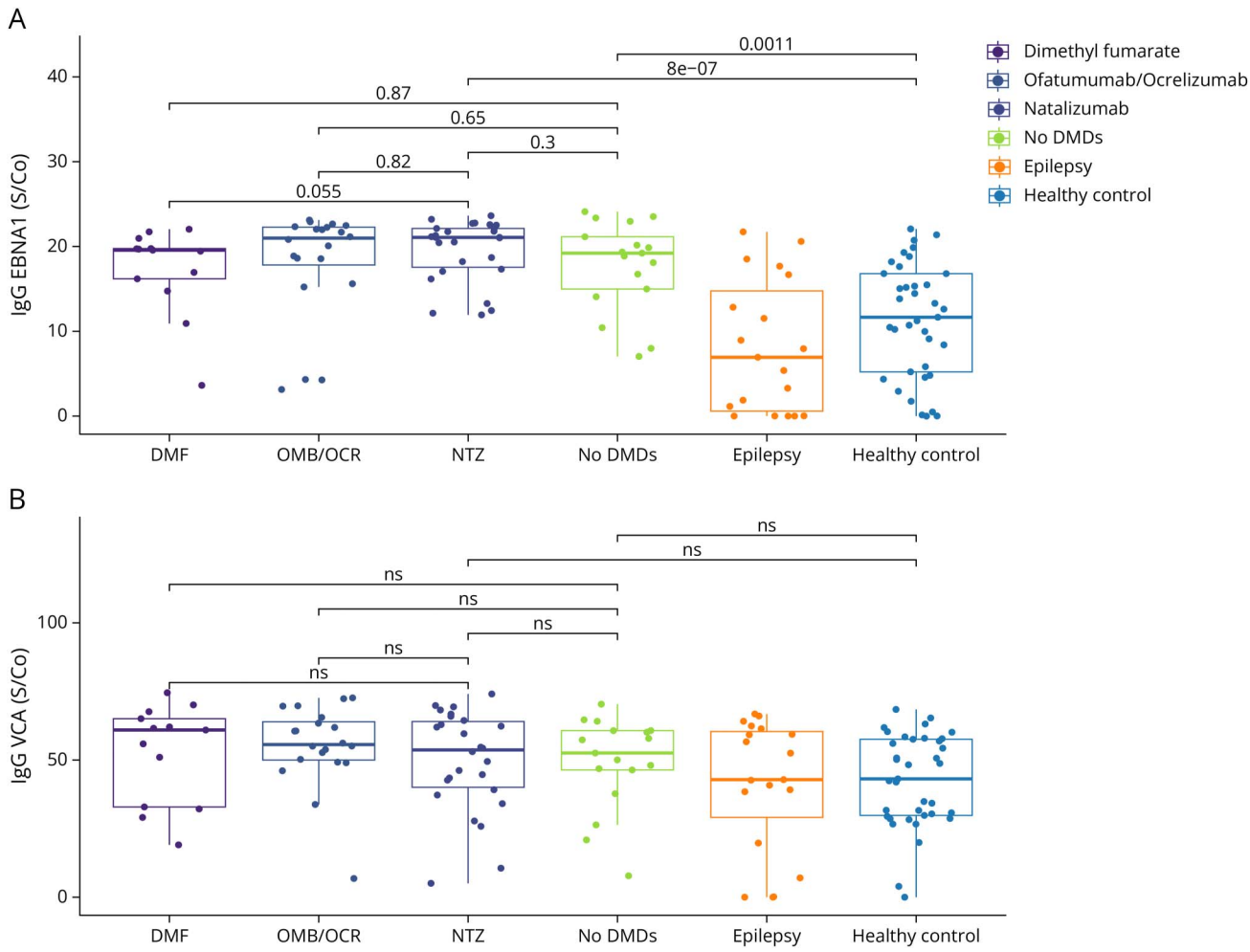
We conducted a receiver operating characteristic (ROC) analysis to evaluate the discriminatory capability of the cellular IFN-γ and IL-2 response to EBNA-1 in distinguishing between individuals with or without MS. The R package *caret* (version 6.0-94) was used to perform stratified partitioning of samples into equally sized training and testing groups (50% split ratio), ensuring that the representation of MS and non-MS cases remained balanced in both groups. An unadjusted logistic regression model was fitted using the training data set to perform binary classification of MS status as a function of IFN-γ response to EBNA-1. To assess the predictive performance of the model, class probabilities of the test data set were calculated using the fitted model. These predicted probabilities were then used to construct an ROC curve using the R package *pROC* (version 1.18.4).

Third, we then compared differences within the MS cohort to test the hypothesis that different DMTs would result in a change in the cellular response to EBV. We based these comparisons on the mechanism of action of the DMT and so considered anti-CD20 monoclonal antibodies (ofatumumab [OMB] and ocrelizumab [OCR]) together. Other groups included natalizumab (NTZ), DMF, and treatment naïve. We again used Kruskal-Wallis testing for comparisons.

To ascertain the relationship with physical disability, age, and disease duration, we performed a univariate Spearman rank correlation coefficient with IFN-γ and each of these variables.

Owing to the exploratory nature of the study, and as statistical tests were restricted solely to a priori hypotheses, adjustments for multiple comparisons were not applied.[26] Statistical analyses were performed using R Statistical Software (v4.1.2; R Core Team 2021).

Figure 2 Comparison of EBNA-1 IgG (A) and VCA IgG (B) Levels



(A) Comparison of EBNA-1 IgG levels, showing no difference in the EBNA-1 IgG levels recorded in MS cohort with different disease-modifying therapies used. (B) Similarly, the VCA IgG levels did not differ between groups using different disease-modifying drugs in MS. In both groups, all MS cohorts had higher VCA IgG levels compared with the epilepsy and healthy control cohorts. Statistical significance is indicated as follows: ns = $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$. EBNA-1 = EBV nuclear antigen-1; VCA = viral capsid antigen.

Data Availability

Anonymized data are available from research teams on reasonable request. These data will be available for other analyses. Transfer of data will require an access agreement between institutions.

Results

Study Participants

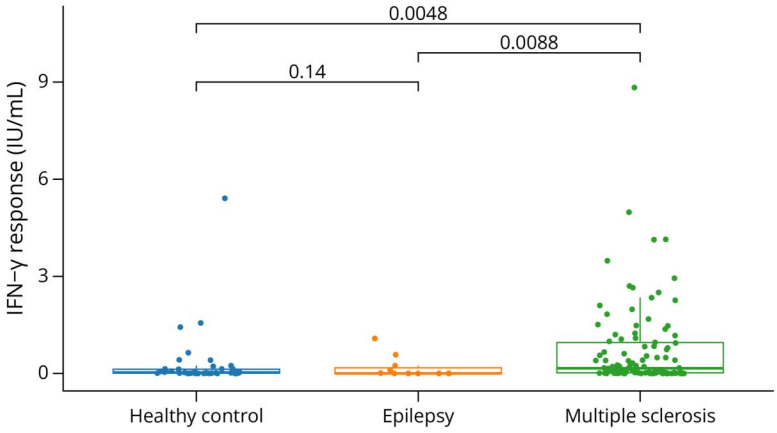
We studied the cellular response to EBNA-1 in 145 people. This groups consisted of 86 pwMS (59 female, 27 male), 19 PWE, and 40 controls. Within the MS group (n = 86), the mean age was 41.7 (± 12.6) and median EDSS score was 1.5 (range = 0–8), and median disease duration of 5.0 years (range = 0–29). All pwMS had RRMS (n = 86). The number of pwMS taking each DMT was as follows: NTZ, n = 28; anti-CD20 mAbs, n = 22; DMF, n = 14; and treatment naïve, n = 22. The demographics of each group are summarized in Table.

Differences in Prior EBV Infection and Relationship Between IgG Levels With DMTs

We observed a statistically significant difference in the mean IgG level to EBNA-1 in MS compared with both controls and PWE using an unpaired two-sample Wilcoxon tests. The mean IgG level was higher in pwMS vs controls (18.4 ± 5.0 vs 11.3 ± 6.7, p = 3.7e-08), and pwMS also had a higher IgG level against EBNA-1 than pwE (18.4 ± 5.0 vs 9.4 ± 7.9, p = 0.0003). By contrast, no differences were evidence in IgG levels against EBNA-1 between controls and PWE, highlighting the lack of a role for this virus in epilepsy pathogenesis in contrast to MS. Results of IgG levels against EBNA-1 and VCA are displayed in Figure 1 (Spearman rank correlation analysis between serum IgG levels against viral capsid antigen and EBNA-1 showing a positive correlation (r = 0.35, p = 4.2e-05) are displayed in eFigure 1).

Similarly, the mean IgG levels against VCA in MS (50.4 ± 18.8) were higher than healthy controls (42.4 ± 16.86, p =

Figure 3 Comparison of Cellular Response to EBNA-1 Between Cohorts



The results show a significantly higher level of interferon gamma between MS and controls and also with the epilepsy cohort. EBNA-1 = EBV nuclear antigen-1.

0.0098); a similar trend was seen in IgG responses to VCA between MS and PWE (37.8 ± 24.9). We did not observe evidence of PCR-confirmed active EBV infection in either the MS cohort or 2 comparator groups.

To evaluate the effects of DMTs on IgG responses, we compared the levels between groups and with the epilepsy and control cohort. With the MS cohort divided based on the DMT used, each subgroup had higher IgG levels against EBNA-1 and VCA compared with controls. However, there were no differences between groups indicating that IgG levels are not influenced by different mechanisms of action of DMTs in MS. Results are displayed in Figure 2.

Comparison of Cellular Response With EBNA-1 Between Groups

The mean IFN-γ level measured from cellular response to EBNA-1 was as follows in each group: pwMS = 0.79 IU/mL (± 1.36), PWE = 0.17 IU/mL (± 0.33), and healthy controls = 0.29 IU/mL (± 0.90). In the comparison between groups, the IFN-γ level was found to be higher in MS vs controls (p = 0.0048) and in MS vs PWE (p = 0.0088). However, no differences were seen in controls vs PWE.

Similarly, the IL-2 level was as follows in each group: pwMS = 17.07 pg/mL (± 28.03), pwE = 9.42 pg/mL (± 18.77), and healthy controls = 7.25 pg/mL (± 17.99). In common with IFN-γ levels, we observed higher IL-2 levels in MS vs controls (p = 0.0057) and in pwMS vs pwE (p = 0.014) (mean and SD in levels of interferon gamma (IU/mL) and interleukin 2 (pg/mL) in response to stimulation of whole blood samples with EBNA-1. eTable 1 and eFigure 2 present a significantly positive univariate correlation between IL-2 and interferon γ release as a response to EBNA-1). Again, no differences were seen in controls vs pwE. Results are displayed in Figure 3.

To demonstrate that these raised cellular cytokine responses were exclusive to EBV, we also repeated the experiment with cytomegalovirus and found no differences between MS, epilepsy, and healthy controls (eFigure 3 shows a comparison of interferon gamma cellular response with cytomegalovirus between groups. Comparison performed with the Kruskal-Wallis test with no significant differences seen and eFigure 4 shows a comparison of interferon gamma cellular response with CMV between groups with the MS cohort stratified by disease-modifying therapy. Again, no differences were seen between groups based on testing with Kruskal-Wallis analysis). This was in support of our hypothesis that the heightened cellular response in MS is specific to EBV and not a universal phenomenon.

Receiver Operator Curve Analysis

Based on the observation that the MS cohort had a higher level of cellular response to EBNA-1, we wished to explore the discriminatory capacity of this assay between MS and controls.

Based on this analysis, an optimal classification threshold of IFN-γ was identified as an ROC cutoff of 0.59 IU/mL and used to define a cutoff for classification using predicted probabilities (equating to approx. 0.64 IU/mL). The area under the ROC curve (AUC) was calculated to quantify the overall performance of the model in distinguishing MS from non-MS cases (AUC = 0.72).

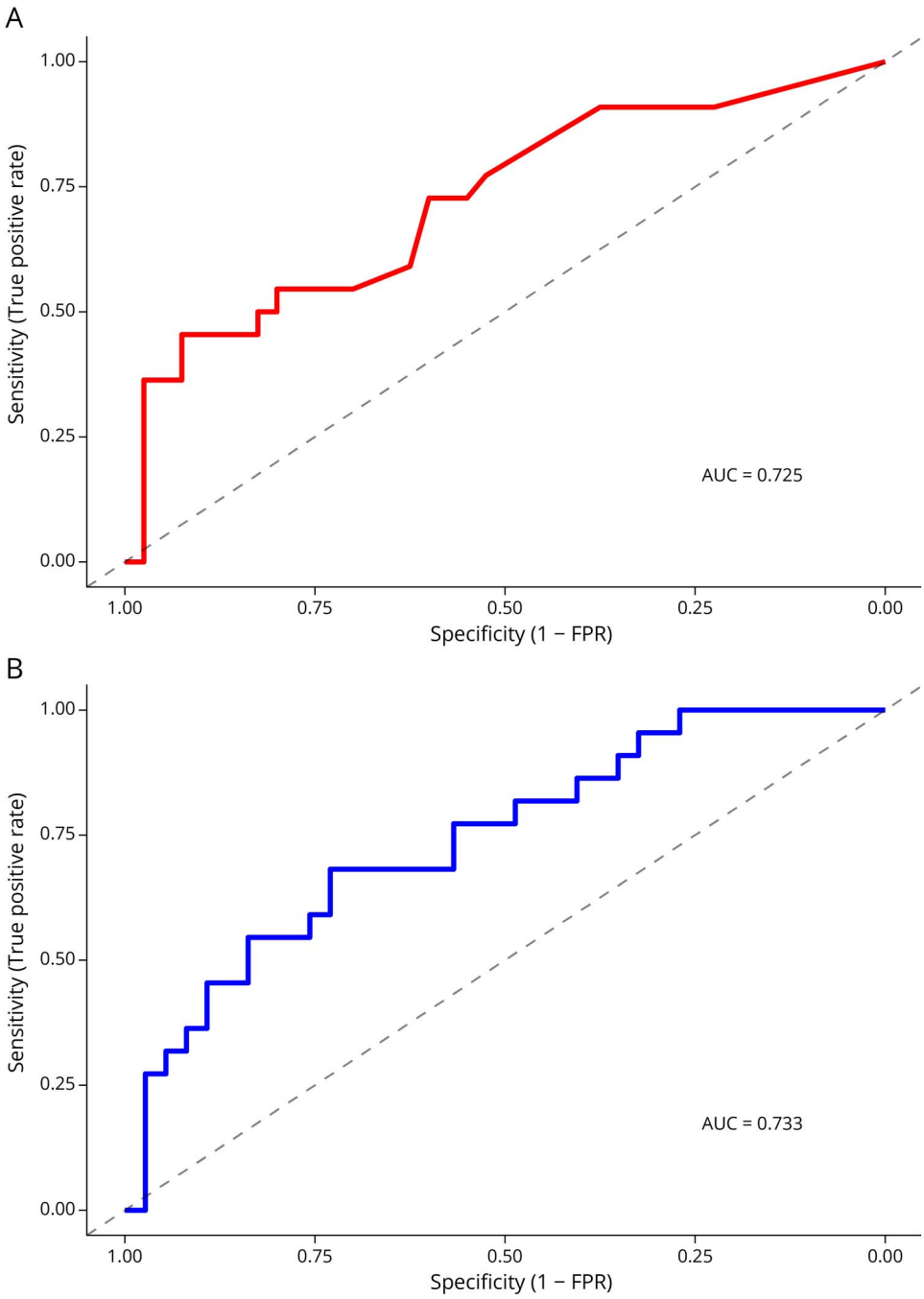
Again, in line with the results of IFN-γ, we conducted analysis with ROC to discriminate between MS and controls based on IL-2 response. In this analysis, an AUC of 0.73 was recorded.

Results are displayed in Figure 4 for ROC curves based on levels of both IFN-γ levels and also IL2.

Comparison of IFN-γ and IL-2 Levels With Different DMTs Between Group and Controls

The cellular response level measured with IFN-γ was as follows with each DMT: NTZ mean = 1.23 (± 1.71), OCR/OMB mean = 0.28 (± 0.57), and DMF mean = 0.07 (± 0.15). The IFN-γ level with NTZ treatment was higher than either

Figure 4 (A) Receiver Operator Curve to Discriminate Between MS and Controls Based on the Cellular Response to EBNA-1 Measured Using IFN-y and (B) ROC Curve Displayed Based on IL-2 Levels



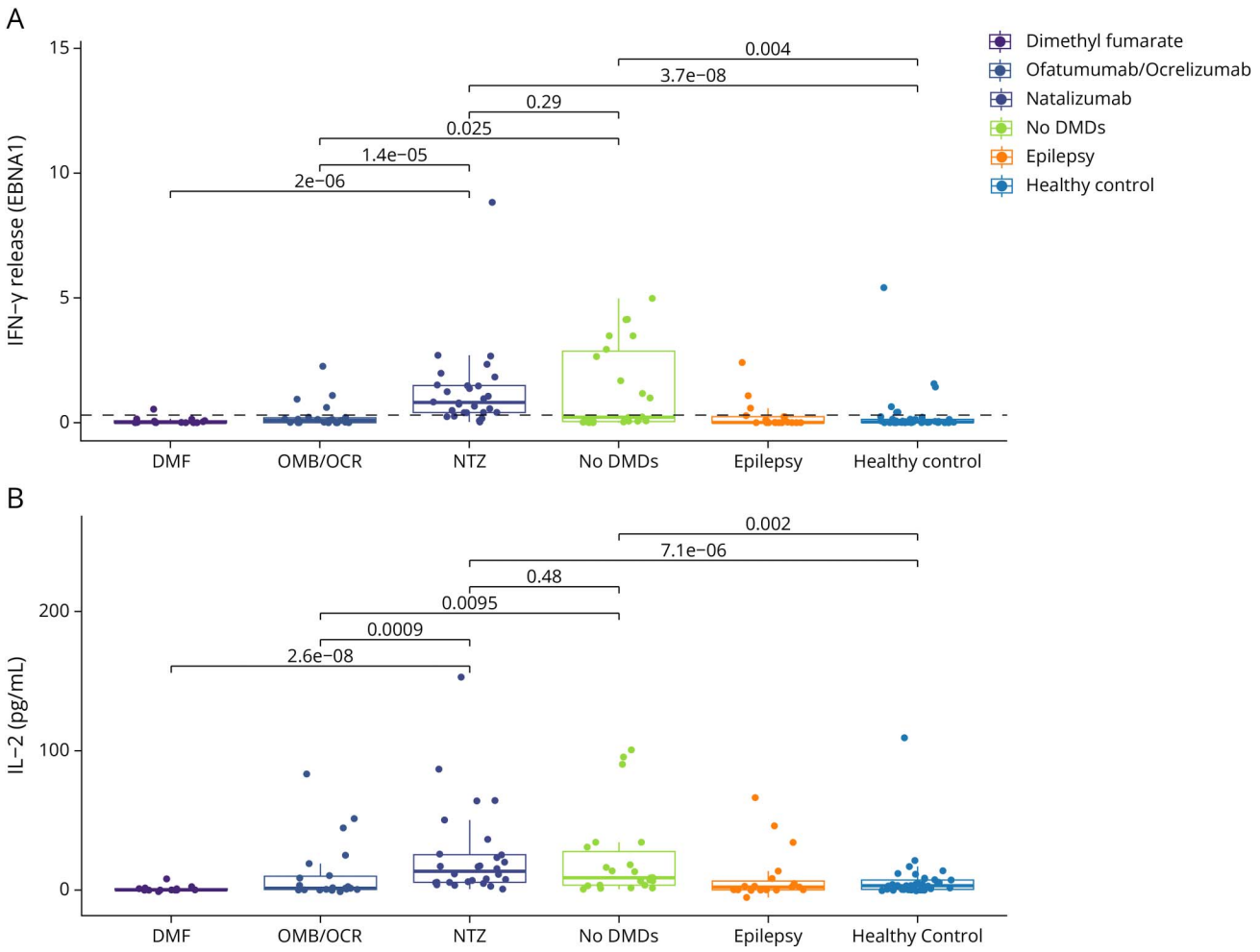
EBNA-1 = EBV nuclear antigen-1.

OCR/OMB (p = 4.9e-05) or DMF (p = 5.1e-06). In line with the high cellular response seen with NTZ treatment, pwMS who were treatment naïve had a mean IFN-γ level of 1.56 IU/mL (± 1.78), and this level was also higher than both controls (p = 0.0075) and PWE (p = 0.0055). Furthermore, this cellular response to EBNA-1 in patients receiving NTZ treatment was higher than controls (p = 1e-07) or PWE (p = 0.00013).

By contrast, no significant differences in cellular response were observed when comparing people receiving DMF or anti-CD20 antibodies with controls and PWE (Kruskal-Wallis, p = 0.26). Results are displayed in Figure 5.

In line with the results observed with IFN-γ, we recorded similar findings with IL-2 as follows: NTZ, mean = 24.97 pg/mL (± 33.06); OCR/OMB, mean = 11.59 pg/mL (± 21.61); and DMF, mean = 0.94 pg/mL (± 2.21). In line with the results seen with IFN-γ levels, the IL-2 levels with NTZ treatment were higher than either OCR/OMB (p = 0.0005) or DMF (p = 1.2e-07). Again, in line with the high cellular

Figure 5 Pairwise Comparison of Cellular Response to EBNA-1 Recorded Using IFN-γ Levels in (A) and IL-2 Levels in (B)



No differences were seen between the cellular response against EBNA-1 in controls and epilepsy or the MS groups taking dimethyl fumarate or anti-CD20 monoclonal antibodies. By contrast, significantly higher levels were recorded in the MS cohort on natalizumab and treatment naive groups relative to both healthy controls and epilepsy. EBNA-1 = EBV nuclear antigen-1.

response seen with NTZ treatment, pwMS who were treatment naïve had a higher mean IL-2 level than controls (p = 0.0057) but again did not differ from levels observed with NTZ treatment (p = 0.65).

These results differ from the measurement of IgG levels where no difference was seen in the levels recorded in MS cohorts taking different DMTs.

Relationship Between Cellular Response and IgG Levels Against EBNA-1 and VCA

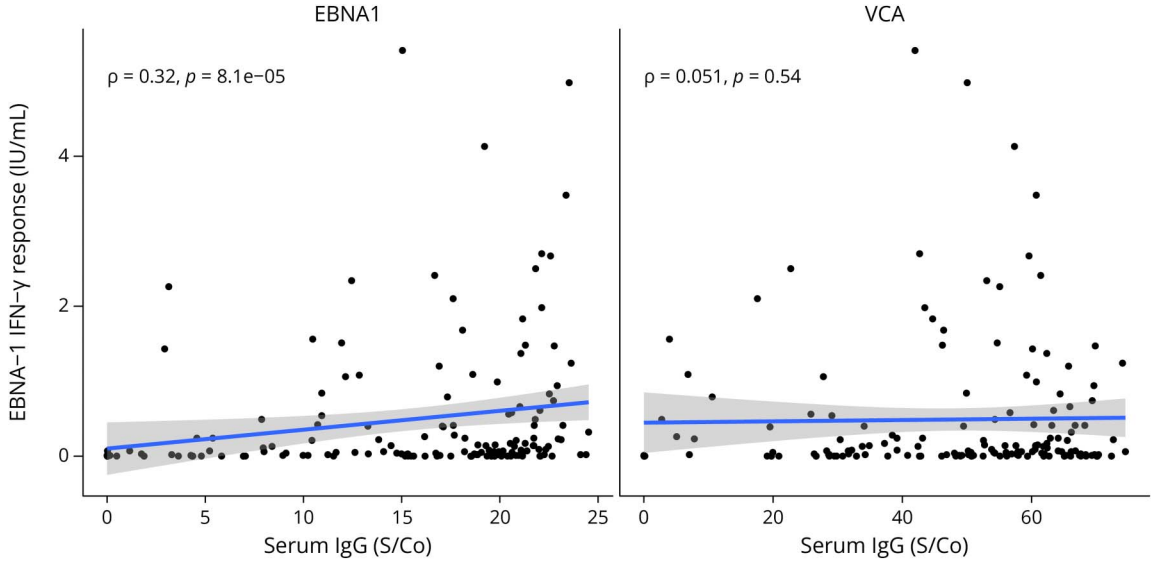
We conducted a Spearman correlation analysis to investigate the relationship between IFN-γ levels measured from cellular response to EBNA-1 and IgG levels against either EBNA-1 or VCA. Our analysis revealed a weak positive Spearman correlation between the IFN-γ response to EBNA-1 and the levels of IgG antibodies directed against EBNA-1 (r = 0.28, p = 0.0013). Results are displayed in Figure 6. Conversely, there was no correlation between IFN-γ response to EBNA-1 and antibodies against VCA (r = −0.013, p = 0.88). Finally, a positive Spearman correlation was found between both IgG measures together (r = 0.35, p = 4.2e-05).

The weak correlation between the cellular response to EBNA-1 and IgG levels indicates that the inflammatory cytokines measured as an immunologic response to EBNA-1 are not related solely to prior infection levels and seem to be an independent biological process seen in MS.

Associations Between Cellular Response to EBNA-1 With Physical Disability, Age, and Sex

No significant correlation was seen with either disease duration (r = 0.19, p = 0.079) or EDSS (r = 0.18, p = 0.13) (eFigure 5 shows univariate correlations between cellular response to EBNA-1 recorded using interferon gamma and age, disability, disease duration. No strong associations were seen between these variables, and eFigure 6 shows a linear regression model which regresses age and MS divided by DMT

Figure 6 Univariate Correlations Between Cellular Response and IgG Levels to EBNA-1 Where a Weak Positive Correlation Was Observed (r = 0.28, p = 0.0013) and No Significant Correlation Was Seen With IgG Levels Against VCA



EBNA-1 = EBV nuclear antigen-1; VCA = viral capsid antigen.

against the interferon gamma response to EBNA-1 and including healthy controls. These data show that age is not associated with the interferon gamma response to EBNA-1. Second, the MS status is only associated with an increase in interferon gamma in response to EBNA-1 when evaluating treatment naïve or natalizumab groups even when adjusting for age). An inverse correlation with age was seen in women with MS (r = −0.51, p = 0.013), but not in men (r = 0.081, p = 0.57).

Discussion

There are a number of novel findings in this study. First, we developed a novel, easily scalable, and translatable test of cellular response to the EBV latent antigen, EBNA-1. Second, we report for the first time the differential effect of a range of currently available DMTs in MS on the cellular response to EBNA-1. Third, we show the wide range of IFN-γ and IL-2 responses to EBNA-1 in a treatment naïve cohort.

The role of $CD8^+$ T cells has been reported to be of importance in establishing the outcome to infection with EBV.[6,27] A methodology, such as outlined in this study, which uses whole blood samples, could potentially enhance understanding of the effect of currently available DMTs on the cellular cell response to EBNA-1. Furthermore, this technique has the potential to be used in future clinical trials of treatments that directly target the virus or its immune response. The trade-off in using whole blood samples is that this approach does not allow identification of which specific cells are the source of the cytokines—but this may not be that relevant to potential clinical applications such as clinical trials of antivirals or vaccines directed against EBV in an MS population.

In this study, we show the use of an assay to provoke a cellular response which is similar to that used in routine clinical practice for the detection of latent tuberculosis infection.[28] However, in this study, we used a commercially available EBNA-1 reagent to stimulate whole blood samples that were taken and stored at room temperature. There are no complex preprocessing steps required before stimulation in the laboratory assay. This is in contrast to the technique of extracting PBMCs or running an elispot assay—requiring labor intensive analysis—which have been performed in previous research studies evaluating the cellular response to EBV in MS and have not resulted in widespread clinical translation.[18,19]

The low cellular responses in relation to OCR[19] and high cellular response with NTZ,[29] confirm the findings from previous reports, albeit with a different laboratory technique. Recent evidence has also demonstrated an effect of anti-CD20 antibody treatment in T-cell response, which may in part account for this observation.[30] In addition, B-cell depletion has been hypothesized to affect the latent EBV virus contained within the B-cell compartment.[31] The low cellular response to EBNA-1 with anti-CD20 antibodies did not result in a corresponding decrease in EBNA-1 IgG levels—as seen in a previous longitudinal study.[18] The differences may arise in part from study design considering the cross-sectional nature of this study and that CD20 is not expressed throughout the B-cell lineage resulting in differential levels of depletion.[31] By contrast, it has been shown that NTZ withdrawal can result in significant viral reactivation[32] and may result in alterations to serum levels of the virus.[5]

Our data show a lower cellular response to EBNA-1 in pwMS treated with DMF. The IFN-γ levels recorded did not differ from healthy controls or PWE in the context of this DMT. A previous report has suggested that the humoral response to EBV can be reduced by DMF.[33] However, our observation extends these findings through implication of a reduction in inflammatory cellular response to EBNA-1. This study was not designed to interrogate the exact mechanisms at play, but the metabolic effect of DMF may account for its effect on response to EBV. Recent evidence has shown that fumarate can play a role in mediating macrophage interferon production,[34] owing to the potential antiviral effects of interferon,[35] although will require confirmatory analysis.

At present, phenotyping of a person with newly diagnosis of MS involves recording clinical and radiologic abnormalities. The clinical[36] and MRI metrics may play a role in prognostication[37] but have not yet progressed to the point of routine use of precision medicine in MS.[20] A blood-based prognostic biomarker would be desirable at initial consultation, where an appropriate DMT is selected. Our results show a spectrum of cellular responses to EBNA-1 in an untreated cohort of pwMS. This cohort either had sampling before initiation of a DMT or had declined pharmacologic treatment. To develop the hypothesis, IFN-γ and IL-2 response to EBNA-1 may relate to overall disease activity and longitudinal follow-up would be required, which was not possible in this current cross-sectional study.

EBV is now being considered as a 'driver' of MS.[27] Arising from this hypothesis, techniques measuring immune response to EBV will be required in clinical trials. A recent clinical trial reported on the use of T-cell therapy targeting EBV.[21] However, outcome metrics included in this trial did not extend beyond the use of conventional clinical and MRI metrics that are used in DMT clinical trials. Our results show that measurement of IgG levels alone may not suffice in this regard because no effect on levels was observed with current DMTs, which is in contrast to the cellular response recorded with either IFN-γ or IL-2. With the treatment of EBV set as a potential treatment target in MS,[5] assays that can report on the immune response to the virus may be of assistance in this regard.

Limitations should be noted when considering the findings in this study. First, the cross-sectional nature of this study precluded observations with clinical outcomes for the newly diagnosed cohort. We plan to address this in a future study to understand the relationship between cellular response and response to DMTs. Second, as noted, our study uses a whole blood technique, we therefore cannot identify the cellular source of the cytokines measured. Despite this, measures of intra-assay and interassay variability provide encouragement that such assays could be developed to accreditation standards across a wide range of clinical blood science laboratories. Furthermore, our assay uses EBNA1 peptides rather than EBNA1 protein. The use of peptides will likely bypass antigen processing pathways, and further assessment using whole EBNA1 proteins might yield differential results and should be investigated further. Finally, this was a single-center study, with no independent validation cohort. To address this, we now plan to establish a collaborative study to confirm the findings in this study in another academic MS center.

In summary, we have measured the cellular response to EBNA-1 in MS, using a method that can be run with whole blood samples without significant preprocessing. We have shown the differential effect of a range of currently available disease-modifying drugs on this response and have also shown the potential for differing levels of response in an untreated cohort. With growing interest in EBV as a therapeutic target, methodologies will be required for clinical trials such as we present, to demonstrate an effect on the extent of the immune response to the virus and not solely IgG levels.

Study Funding

This publication has emanated from research supported in part by a research grant from Science Foundation Ireland (SFI) under Grant Number 21/RC/10294 and co-funded under the European Regional Development Fund and by FutureNeuro industry partners.

Disclosure

J. Dunne has filed British Patent Application No. 2301209.9 relating to the Whole Blood EBNA1 assay. All other authors report no disclosures relevant to the manuscript. Go to Neurology.org/NN for full disclosures.

Publication History

Received by *Neurology: Neuroimmunology & Neuroinflammation* October 27, 2023. Accepted in final form January 19, 2024. Submitted and externally peer reviewed. The handling editor was Deputy Editor Scott S. Zamvil, MD, PhD, FAAN.

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Jean Dunne, PhD	Department of Immunology, St James's Hospital, Dublin, Ireland	Major role in the acquisition of data; analysis or interpretation of data
Michael Savio	School of Medicine, Trinity College Dublin, Dublin, Ireland	Major role in the acquisition of data
Marianna Kalaszi, MD	MS Unit, Department of Neurology, St James's Hospital, Dublin, Ireland	Major role in the acquisition of data
Matt McElheron, BSc	Department of Medical Gerontology, School of Medicine, Trinity Translational Medicine Institute, Trinity College Dublin, Ireland	Analysis or interpretation of data

Appendix *(continued)*

Name	Location	Contribution
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Ammara Qureshi, MSc	Virology Laboratory, St James's Hospital, Dublin, Ireland	Major role in the acquisition of data
Brendan Crowley, MD	Virology Laboratory, St James's Hospital, Dublin, Ireland	Major role in the acquisition of data; analysis or interpretation of data
Niall Conlon, PhD	Department of Immunology, St James's Hospital, Dublin, Ireland; School of Medicine, Trinity College Dublin, Dublin, Ireland	Drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data
Hugh Kearney, PhD	MS Unit, Department of Neurology, St James's Hospital, Dublin; FutureNeuro SFI Research Centre, Academic Unit of Neurology, School of Medicine, Trinity College Dublin, Ireland	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data

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