Epstein-Barr virus (EBV)-specific antibodies in Multiple Sclerosis patients, including targeted investigation of a candidate EBV nuclear antigen-1 (EBNA-1) B cell epitope.

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Introduction

Multiple Sclerosis (MS) is a very complex disease, with different genetic and environmental factors contributing to its risk. In particular, infection with Epstein-Barr Virus (EBV) has been shown to increase MS risk. This risk is moderately associated with anti-viral capsid antigen (VCA) antibodies, but very strongly associated for anti-EBV nuclear antigen-1 (EBNA-1) antibodies. Focus on EBNA-1 has uncovered that antibody reactivity against a C-terminal part the of the protein (amino acid (aa) positions 385-420) is a stronger risk marker than antibodies against the whole EBNA-1 protein. Recently, in a small study in twins (n=12), a putative B cell epitope within this region (aa 401-411) has been identified as a target for antibodies that are specifically enriched in MS patients compared with healthy controls.

It is also known that post-translational modification (citrullination) can occur in MS lesions, and in particular in association with MS auto-antigens. Post-translational modification has also been described in other auto-immune diseases, such as rheumatoid arthritis, where citrullination of antibody targets occurs and utilizing these modified targets did increase the specificity of screening.

Purpose

To determine whether antibodies against the B cell epitope may be specifically associated with MS cases, and whether antibodies directed against a citrullinated peptide sequence may further increase specificity, reflecting the pro-inflammatory environment. Additionally, investigating sequence variation in the B cell epitope in MS cases could further explain antibody specificity.

To compare these outcomes with results of a commercially-available anti-EBNA-1 ELISA assay and including also results from an anti-VCA ELISA could gain additional information for risk assessment.

Methods

A total of 440 MS patients in the Perth (Western Australia) Demyelinating Disease Database (PDDD) was included. The control cohort was established from the population of Busselton, Western Australia, with 196 participants. Serum samples were collected and stored at -80^{o}C until testing.

A commercial ELISA assay (DiaSorin) was used to determine the levels of antibodies against EBNA-1 and VCA of Epstein-Barr Virus (Fig. 1).

EBNA-1 sequences covering the B cell epitope were obtained using a novel semi-nested PCR approach using expand high fidelity Taq-polymerase (Roche).
An in-house ELISA was developed, specific to the B cell epitope, based on the results of the MS EBNA-1 sequencing. Two peptides were synthesized:

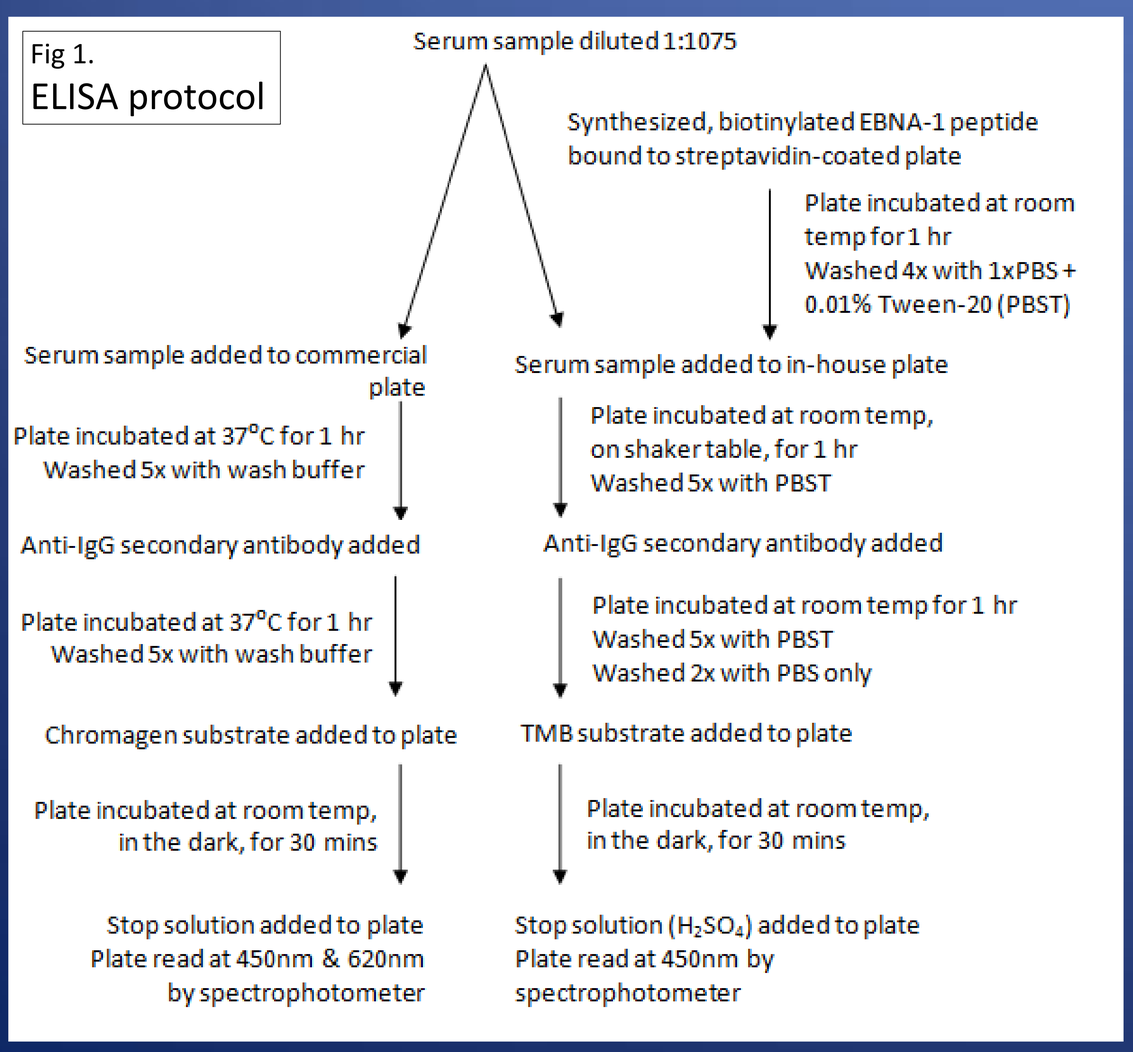
Uncitrullinated PPPGRRFFHPVGEAD

Citrullinated PPPG(Cit)(Cit)FFHPVGEAD

Both peptides were biotinylated at the N-terminus to enable binding to the streptavidin coated plates. Peptide sequences were confirmed by Proteomics Node, Perth, Australia. The ELISA protocol is summarized in Fig 1.

Statistical analysis was performed using IBM SPSS Statistics (version 19) and TIBCO Spotfire S^{+} (version 8.2). Proportion of positive samples were compared using Pearson's Chi-squared test, and medians of MS and control groups were compared using the Kruskal-Wallis test. A logistic regression was performed to observe predictive value of MS versus controls using combined EBNA-1 and VCA optical density (OD) readings obtained in the commercial ELISA. For this regression analysis, one outlier was excluded from the MS cohort.

Fig 1.
ELISA protocol



Results

31 MS samples have been successfully sequenced covering the B cell epitope. Conservation of the target epitope (GRRPFFHPVGE) was high (n=28). Three samples showed variation within the epitope (position 9:A/V, position 10:D).
The commercial anti-EBNA-1 assay detected 97% anti-EBNA-1 positivity for MS samples and 76% for control samples ($p<0.005$; Fig. 2). In the commercial anti-VCA ELISA, positivity was shown in 99% of MS samples and 91% of controls ($p<0.005$; Fig 2). With regard to the novel in-house B-cell epitope-targeted assay, MS serum samples showed 97% positivity (native peptide) and 83% positivity (citrullinated peptide). Rates of positive responses in control samples were 75% and 43%, respectively ($p<0.005$) (Table 1).
Inter-assay variability was low, with 4.4-9.1% for EBNA-1, 16.4-19.5% for VCA, 21.4-25.4% for the uncitrullinated peptide and 20.9-28.1% for the citrullinated peptide.

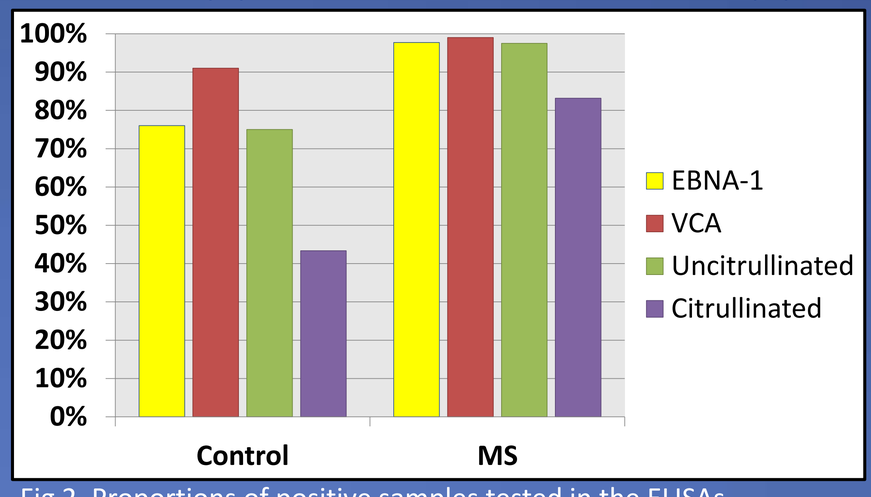


Fig 2. Proportions of positive samples tested in the ELISAs.

Table 1.	MS	Control
n	440	196
Commercial EBNA-1	430 (97%)	149 (76%)
In-house UNC	429 (97%)	147 (75%)
In-house CIT	366 (83%)	85 (43%)

Table 1. Comparing positive samples identified across EBNA-1 focused assays.

When comparing median log(OD) values between groups across the four assays (Fig. 3), the group median log(OD) was higher in MS patients than controls (EBNA-1: 4.5 times higher; $p<10^{-15}$; VCA: 2.1 times higher; $p<10^{-15}$; Uncitrullinated: 6.4 times higher; $p<10^{-15}$; Citrullinated: 11.5 times higher; $p<10^{-15}$).

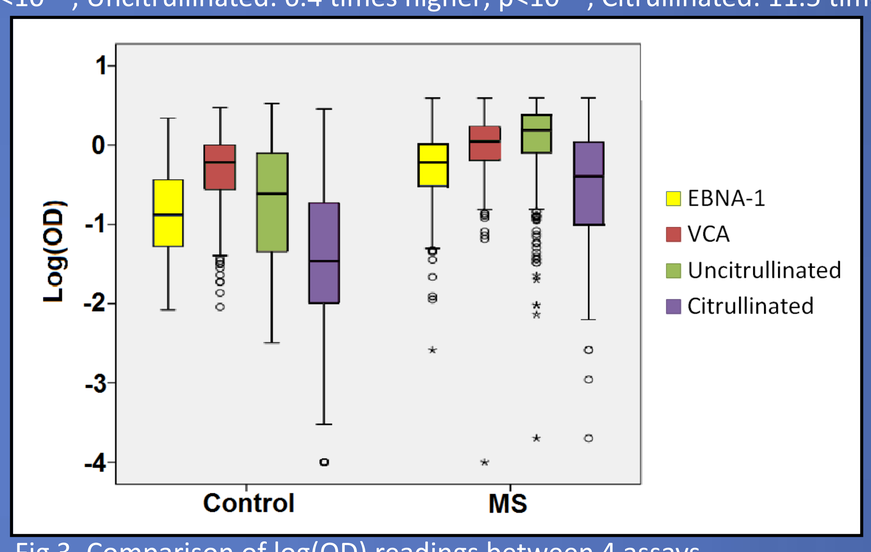


Fig 3. Comparison of log(OD) readings between 4 assays.

VCA and EBNA-1 are independently associated with risk after adjusting for each other in joint logistic regression (VCA $p=8.5x10^{-8}$, EBNA $p<10^{-15}$).
Joint logistic regression of EBNA-1 plus VCA increased the prediction for MS risk. A logistic score based on the cut-off of 0 would predict MS to be positive for 402 out of 439 MS samples with 91 false positives in the control group (Table 3).

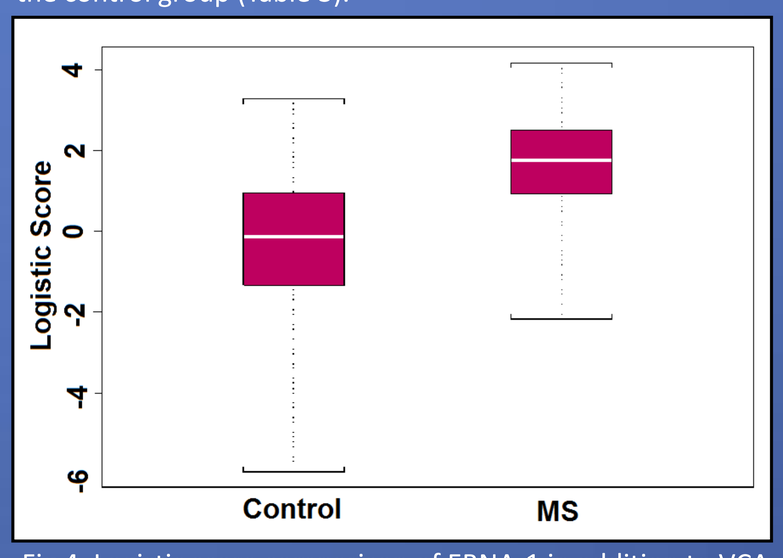


Fig 4. Logistic score comparison of EBNA-1 in addition to VCA.

Table 2.	MS	Control
FALSE	116	149
TRUE	323	48
Odds Ratio	8.64	

Table 2. Logistic score comparison of EBNA-1 and VCA; Score >1

Table 3.	MS	Control
FALSE	37	106
TRUE	402	91
Odds Ratio	12.7	

Table 3. Logistic score comparison of EBNA-1 and VCA; Score >0

Conclusions

High conservation of the target epitope among MS samples suggest minimal variation of the epitope in patients.
As expected, results show nearly universally–positive anti-EBNA-1 IgG responses in MS cases, with significantly increased log(OD) readings in MS patients. This was also observed for the commercial anti-VCA assay.
Targeted analysis of antibodies directed against the B-cell epitope only, also shows significant differences in positivity with higher reactivity found in MS cases in concordance with the commercial assay targeting the full EBNA-1 protein.
Antibodies against the citrullinated form of the B cell epitope showed less positive samples for both groups, but the MS group maintained to have significantly more positive samples and higher median log(OD) readings. This novel finding suggests citrullination may be a modification involved in some MS cases and has to be investigated in more detail in context of genetic risk factors (Human Leukocyte Antigen DRB*15), disease progression, relapse-remitting patterns and other demographic/clinical information. Lastly, prediction of MS risk utilising the combined anti-EBNA-1 and anti-VCA data strengthened the prediction compared to the individual risk contribution.

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