

**Untreated relapsing remitting multiple sclerosis patients show antibody production against Latent Epstein Barr Virus (EBV) antigens mainly in the periphery and innate immune IL-8 responses preferentially in the CNS**

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## Abstract

**Background:** Multiple sclerosis (MS) is an inflammatory and neurodegenerative disorder of the central nervous system (CNS). Reliable biomarkers are urgently needed for its diagnosis and management, and as clues to its pathogenesis, in which EBV is implicated. **Objective:** To measure IgG antibodies against EBV nuclear antigen-1 (EBNA-1) and innate inflammation status in paired serum and cerebrospinal fluid (CSF) samples from untreated relapsing-remitting MS (RRMS) patients. **Materials and Methods:** Anti-EBNA-1 IgG titers and IL-8, IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$  and IL-12p70 cytokine levels were measured in 20 untreated RRMS-patients and 17 healthy controls. **Results:** We found higher serum anti-EBNA-1 IgG and IL-8 levels in RRMS-patients than in healthy controls. Interestingly, levels of IL-8 – relative to total protein – were much higher in the CSF, whereas the anti-EBNA-1 antibodies were significantly higher in the sera. More detailed analysis showed that anti-EBNA-1 antibodies relative to total IgG were higher in the serum in the majority of RRMS patients compared to CSF. Levels of anti-EBNA-1 IgG and IL-8 showed a strong correlation between serum and CSF. **Conclusion:** These findings in newly diagnosed RRMS-patients imply anti-EBNA-1 antibody production mainly in the periphery and innate immune responses preferentially in the CNS. Both their potential as disease biomarkers and their implications for the pathogenesis of MS warrant further investigation.

**Keywords:** relapsing-remitting multiple sclerosis, serum, cerebrospinal fluid, Epstein-Barr virus, innate immune response, IL-8 cytokine, anti - EBNA-1 antibodies

## List of Abbreviations

MS: multiple sclerosis; CNS: central nervous system; RRMS: relapsing remitting MS; EBV: Epstein-Barr virus; EBNA-1: Epstein-Barr nuclear antigen 1; CSF: cerebrospinal fluid; IL: Interleukin.

## 1.) Introduction

MS is characterized by inflammatory plaques of demyelination in the MS brain and spinal cord. The drivers of neuroinflammation and neurodegeneration in MS are subjects of intensive research, and so is the identification of potential risk factors and biomarkers.(1)

Late Epstein-Barr virus (EBV) infection is one established risk factor for MS. EBV is a  $\gamma$ -herpesvirus with a population prevalence of >90% in adults in western countries.(2) It infects predominantly B-lymphocytes and persists in rare memory B-cells in the periphery. A history of overt glandular fever (symptomatic infectious mononucleosis), especially in the late teens or in adulthood, confers a more than twofold higher MS-risk.(3) Conversely, the risk is extremely low in rare individuals who are seronegative for EBV(4, 5), and increases sharply within a few months of seroconversion.(4) Furthermore, a prospective study implicated latent EBV-infection; elevated antibody levels against the entire EB viral nuclear antigen complex (EBNA), and EBNA-1, one of its best characterised proteins – which are expressed during latent EBV-infection – correlated with higher MS-risks respectively.(6) Anti-EBNA-1 IgG antibodies may also warn of conversion from clinically isolated syndrome to clinically definite MS, as they correlate with CSF oligoclonal bands, the best predictors known currently.(7) Indeed, the oligoclonal bands include anti-EBV antibodies in some patients.(8) While these findings implicate EBV early in MS pathogenesis, elevated serum anti-EBNA-1 antibodies also associate with MRI activity in established disease.(9, 10) Several groups have checked for EBV-infected cells in MS-brains with opposing findings.(11) Our own study found latently EBV-infected cells in areas of innate immune activation in the presence of B-cells in active, perivascular white matter lesions, but also in stroke and brain lymphoma cases. We proposed that innate immune activation may be perpetuated by non-coding EBV-RNAs, which, via ligation to the innate pattern recognition receptor Toll like receptor (TLR)-3, trigger the production of the innate cytokine interferon- $\alpha$  and an inflammatory milieu in active MS-lesions.(12)

1 Pro-inflammatory cytokines may be valuable biomarkers for disease activity in MS. Indeed,  
2 during active disease, there are increased levels of several that promote cellular immunity  
3 against intracellular infections (e.g., IL-1 $\beta$ , IFN- $\gamma$ , TNF- $\alpha$ , IL-12, IL-6, IL-8), possibly also  
4 implicating them in MS pathology, where IL-17 and Th17 responses were also found to be  
5 detrimental.(13, 14) The effects of pro-inflammatory cytokines include increases in  
6 demyelination, in endothelial permeability affecting the blood-brain barrier, and in toxicity to  
7 neurons and oligodendrocytes.(15)

8 As IL-8 is an important mediator of innate immunity and acute phase responses, its role in  
9 inflammation is of great interest. Also known as neutrophil and macrophage chemotactic and  
10 activation factor, it regulates their adhesion to endothelial cells and migration across the  
11 vascular wall.(16),(17) IL-8 is production intrathecally by many different cell types, including  
12 brain endothelial cells(18), astrocytes(19) and microglia.(20)

13 We had previously reported elevated IL-8 levels in MS patient sera.(21) Furthermore, we  
14 showed that its rodent homolog CXCL1, when administered peripherally to Wistar rats,  
15 inhibited spontaneous activity in open field and burrowing behavior.(21) Whether similar  
16 sickness behavior and immune dysregulation may also play a role in MS and psychiatric  
17 diseases, where fatigue is a disabling co-morbidity, warrants further investigation.

18 In this study we compared anti-EBNA-1 IgG and pro-inflammatory cytokine levels in  
19 RRMS patients and controls. Analysis of central and peripheral biomarker profiles in paired  
20 serum and CSF samples from untreated RRMS patients may provide insights into their  
21 potential as disease biomarkers and shed light on the possible role of dysregulation of anti-  
22 EBV immune responses and inflammatory pathways in the pathophysiology of MS.

## 2.) Materials and methods

**Patient cohort, ethics and consent:** Newly diagnosed and untreated RRMS patients with short disease duration (n=20 Caucasians; age-range: 18-61 years; F:M ratio: 4:1; disease duration < 2 months) and controls were enrolled from the San Carlos Clinical Hospital, Madrid, Spain (CEIC Hospital Clinico San Carlos: 13/193-E) and from the Blizzard Institute, UK (REC: 05/Q1606/76) following local ethical approval and written informed consent. Paired serum and CSF RRMS samples and control serum samples (n=17 Caucasians, age-range: 20-60 years, F:M ratio: 1.4:1) were collected and aliquots immediately stored at  $-80^{\circ}\text{C}$  until further analysis.

Measurement of cytokines IL-1 $\beta$ , IL6, IL-8, IL-10, TNF- $\alpha$  and IL-12p70 levels was performed in serum and CSF samples (stored at  $-80^{\circ}\text{C}$ ) using the BD<sup>TM</sup> Cytometric Bead Array (CBA) (BD Bioscience, Oxford, UK) according to the manufacturer's recommendations. Briefly, cytokine capture beads were added to the samples before addition of PE-labeled detection reagent and flow analysis on a FACS Canto II flow cytometer. The calibration range was between 0 - 5000 pg/ml. The lower detection limits were:- 7.2 pg/ml for IL-1 $\beta$ : 2.5 pg/ml for IL-6: 3.6 pg/ml for IL-8: 3.3 pg/ml for IL-10: 3.7 pg/ml for TNF- $\alpha$ : 1.9 pg/ml for IL-12p70.

Anti-EBNA-1 IgG antibody levels were measured by enzyme-linked immunosorbent assay (ETI-EBNA-G, DiaSorin, Saluggia, Italy) according to the manufacturer's recommendation. Briefly, diluted serum (1:100) and CSF samples (1:10) were used. Horseradish peroxidase (HRP) was added followed by a chromogen substrate to start the reaction. The reaction was then stopped and absorbance measured at 450/630 nm and 405/630 nm using a standard spectrophotometer (Multidetection reader Biotek Instrument, Vermont, USA). The results were expressed as arbitrary units (AU)/ml. The calibration range was between 0 and 200 AU/ml.

**Statistical analysis:** Statistical analysis was performed using SSPS 15.0 (IBM, Armonk, New York, USA). We used non-parametric Mann-Whitney tests for comparing patients with controls, Wilcoxon signed rank tests for paired serum and CSF samples, and Spearman's rank correlation coefficient. Differences were considered significant if  $p < 0.05$ .

### 3.) Results

#### 3.1.) Anti-EBNA-1 IgG antibody levels are significantly higher in serum from untreated RRMS-patients than controls

Consistently with their well established status as risk factors for MS, levels of IgG antibodies against EBNA-1 were significantly higher in sera from RRMS-patients than controls (Figure 1a,  $P = 0.002$ ). Notably, antibody levels were low in many more of the controls' than the patients' sera, as has been reported previously.(22)

#### 3.2.) Untreated RRMS-patients have significantly higher anti-EBNA-1 IgG antibody levels in serum than CSF

Next, we checked for differences in humoral immune responses to latent EBV-infection between the periphery and the CNS in RRMS-patients. Levels of anti-EBNA-1 IgG antibodies were significantly higher in serum than CSF in these untreated RRMS-patients (Figure 1b,  $P < 0.0001$ ), even when expressed relative to total protein concentrations (Figure 1c,  $P < 0.0001$ ).

When expressed relative to total IgG, we found that the majority of MS patients had higher levels in serum than CSF, however, two MS patients had very high levels in CSF (Figure 1d).

We calculated the ratio between CSF/serum quotients for anti-EBNA-1 IgG ( $Q_{EBNA-1}$ ) and total IgG ( $Q_{IgG}$ ) to determine the antibody index ( $AI = Q_{EBNA-1} / Q_{IgG}$ ) for each RRMS patients as shown in table 1. As EBNA-1 antibodies and total immunoglobulin are of the same immunoglobulin class and have the same molecular size, they must have the same barrier

permeability. Consequently, the ratio between EBNA-1 CSF/serum quotient (Q EBNA-1) and Q IgG should equal 1.0 in normal conditions and increase in cases of additional synthesis in the brain. We found an elevated antibody index (AI > 1.5) in three patients, which may indicate specific synthesis of EBNA-1 IgG in the brain.(23)

### **3.3.) IL-8 cytokine levels are significantly higher in sera from untreated RRMS patients than controls**

To gain insight into the inflammatory signature in MS, we measured levels of IL-1 $\beta$ , IL-6, IL-8, IL-10, TNF- $\alpha$  and IL-12p70 by CBA. RRMS-patients showed significantly elevated, though variable, serum IL-8 levels, which were uniformly low in the controls (Figure 2a,  $P = 0.0016$ ). The other cytokines were below the level of detection in serum.

### **3.4.) Significantly altered levels of IL-8 in RRMS-patients' CSF**

These findings prompted us to compare IL-8 levels in the periphery and CSF in RRMS-patients. Absolute IL-8 levels were slightly lower in CSF (Figure 2b,  $P = 0.019$ ). However, relative to total protein concentrations, which is around 100-fold lower in CSF (0.15-0.6 mg/ml) vs serum, they were ~50-fold higher than in the paired sera (Figure 2c,  $P < 0.0001$ ). Interestingly, we also detected IL-6 in 45% of these CSF samples (2.4 pg/ml-14.9 pg/ml, data not shown), but not in matched serum samples, as previously described.(24 )

### **3.5.) Significant correlations of levels of IL-8 and of anti-EBNA-1 antibodies in serum and CSF**

Finally, we tested whether levels of IL-8 and anti-EBNA-1 antibodies in the periphery might serve as indicators of those in the CSF. We found strong CSF $\leftrightarrow$ serum correlations between:-

(i) absolute anti-EBNA-1 IgG antibody levels (Figure 3a,  $P = 0.0032$ ); (ii) relative anti-EBNA-1 levels (Figure 3c,  $P = 0.0006$ ); IL-8 levels, whether (iii) absolute (Figure 3b,  $P = 0.0081$ ) or (iv) relative to total protein (Figure 3d,  $P = 0.044$ ). However, we did not find significant correlations between absolute levels of IL-8 and anti-EBNA-1 IgG antibodies in either sera or CSF, however, we found a significant inverse correlation in CSF between anti-EBNA-1 antibody levels and IL-8 levels relative to total protein (Figure 3e,  $P = 0.0095$ ).

#### 4.) Discussion

Novel evidence of dysregulated immune responses in MS may provide insights into the underlying immunopathology. We found significantly higher anti-EBNA-1 IgG antibody and IL-8 levels in untreated RRMS-patients than controls. Moreover, whereas the antibody levels were higher in the periphery in the majority of RRMS patients, IL-8 and IL-6 were selectively elevated in the CSF, suggesting inflammatory responses localized to the CNS in RRMS. The bias towards IL-8 and IL-6, rather than other cytokines, particularly implicates the innate immune system. Furthermore, the strong serum↔CSF correlations we find for both anti-EBNA-1 IgG antibodies and IL-8 levels indicate that serum measurements may mirror the latent EBV- and inflammatory status in the CNS in RRMS-patients and support the less invasive blood test over lumbar puncture for clinically useful information.

Our study confirms previous findings of elevated anti-EBNA-1 IgG antibody levels in RRMS patients rather than controls.(22) The latency program of EBV is complicated and the functions of its EBNA-complex are not fully understood. EBNA-1 is thought to be essential for the maintenance of the episomal state of EBV in infected cells and binds to the origin of replication.



EBNA-1 is expressed in latently EBV-infected B-lymphocytes that persist for life in healthy virus carriers, and is the only viral protein regularly detected in all malignancies associated with EBV. Hence it is an important target for cytotoxic CD8<sup>+</sup> and CD4<sup>+</sup> T-cell recognition.(25, 26) Interestingly, CD4<sup>+</sup> T cells against EBNA-1 predominate in MS and respond to myelin as well, suggesting antigenic mimicry. Similarly, anti-EBNA-1 antibodies show cross-reactivity to several self-proteins in the brain.(27) The contribution of EBV and anti-EBV CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses in triggering such mimicry-based autoimmune pathogenesis in MS remains to be defined. Interestingly, a recent study described an intrathecal specific synthesis of IgG against myelin basic protein and EBNA1<sub>400-413</sub> in MS patients as indicated by the antibody index and suggested that the presence of antibodies against MBP and its EBV homologous in CSF during relapses suggests a possible role of the pathogens in enhancing inflammation.(28) Whether the hypothesized inadequate control of persistent EBV-infection leads to higher numbers of latently EBV-infected B-cells requires further investigation. So do the exact pathogenic role of EBV in MS and the cellular immune responses against it.(29-31) The lower EBNA-1 antibody levels we noted in CSF of most RRMS patients, even relative to whole protein content and total IgG, argue in favor of leakage via a focally disrupted blood-brain barrier (BBB). They also argue against substantial intrathecal synthesis, as does previous evidence,(32) though that may occur in an exceptional minority of MS-patients as part of a humoral polyreactivity driven by chronic brain inflammation, which may be supported by our findings of an increased antibody index in 3 RRMS patients.(33) However, our study is limited by the fact that we were not able to correct for individual albumin quotient of a single patient as albumin measurements were not available. Aberrant local EBV regulation may enable EBV-infected cells to secrete EBERs across the BBB, triggering inflammation and increased IL-8/IL-6 production in the CSF.(12)

1 Another important issue is the regulation of anti-EBNA-1 antibody levels. Previously, we  
2 reported that they correlated with EBV load in healthy individuals and were not influenced by  
3 suboptimal vitamin-D levels.(34) However, they were down-modulated by supra-physiological  
4 doses of vitamin-D in MS patients.(35) Interestingly, a recent genome-wide investigation into  
5 the role of host genetics found that amino acid variation in HLA class II proteins is a major  
6 determinant of humoral responses to EBV and found that amino acid haplotypes present in the  
7 classical alleles HLA-DRB1\*15:01 or HLADRB1\*16:01 were associated with higher anti-  
8 EBV IgG levels and influenza A seropositivity.(36)

9  
10 The role of EBV-infection in the CNS in MS is still a matter of debate.(11) The observation  
11 of latently EBV-infected cells in MS-lesions warrants further study, as do other persistent or  
12 endogenous viruses. Recent findings showed that anti-human herpesvirus 6A/B IgG levels  
13 correlated with relapse and progression in MS, and may act as informative biomarkers for  
14 disease activity and responses to disease-modifying treatments.(37) Furthermore, elevated  
15 antibody levels against the envelope protein of MS-associated retroviral element (MSRV), a  
16 member of the Human Endogenous Retrovirus family “W” (HERV-W) and a relation between  
17 EBV infection and elevated HERV-W/K activity has been suggested and may be relevant in  
18 the pathogenesis of MS. (38),(39),(40),(41) This envelope protein triggered the production of  
19 IL-8 and IL-6 in a human brain endothelial line via the ligation of TLR-4, a pattern recognition  
20 receptor of the innate immune system also expressed on endothelial cells.(42) The ensuing  
21 inflammatory response affected the BBB permeability and facilitated the adhesion and  
22 transmigration of activated T-cells.(42)

23 Our present results confirm the elevated IL-8 levels in RRMS sera that we reported  
24 previously.(21) Recent studies also showed significantly higher IL-8 levels in CSF from MS  
25 patients than controls,(43) which we could not test here, as we did not have access to CSF from

1 non-neurological diseases. Another limitation of our study is the inability to answer whether  
2 IL-8 is being produced intrathecally or entered passively. IL-8 is smaller than albumin it may  
3 enter the CNS more easily, which may lead to a higher concentration relative to total protein  
4 in CSF than serum. Interestingly, higher CSF IL-8 levels were also found in RRMS-patients  
5 with more active disease, higher relapse rates post-diagnosis and shorter first inter-attack  
6 intervals.(44) Furthermore, elevated IL-8 levels were associated with clinical progression in  
7 subjects with radiologically isolated syndrome or CIS patients who converted to MS.(44) Their  
8 inverse correlation with anti-EBNA-1 IgG antibodies may be explained by biases towards  
9 recent or long-standing responses. Indeed elevated IL-8/IL-6 levels within the CSF may point  
10 towards local inflammation, fuelled by a nearby trigger. The aberrant anti-EBNA-1 levels in  
11 contrast may be due to inadequate systemic control of EBV infection.

12 Interestingly, we found IL-6 in 45% of CSFs from MS-patients but not in paired sera, as  
13 previously described.(24) In a previous study, CSF IL-6 was elevated only in Systemic Lupus  
14 Erythematosus (SLE) patients with CNS involvement and not in its absence, nor in patients  
15 with non-inflammatory neurologic diseases.(45) Further studies are necessary to clarify the  
16 role of IL-6 in the CNS and the precise consequences of intrathecal immune activation.

17 Whether dysregulation of inflammatory pathways, which contributes to behavioral changes  
18 and sickness behavior in mice, may also play a role in psychiatric diseases like schizophrenia  
19 is far from understood, but our recent findings of an inflammatory signature with elevated IL-  
20 8 levels in schizophrenic patients warrants further studies into the role of inflammation in  
21 schizophrenia.(46) It is already well established that depression is a major side-effect of therapy  
22 with type I IFNs.(47)

23  
24 The clinical and pathophysiological complexity and heterogeneity in MS have so far made it  
25 difficult to establish satisfactory biomarkers. Obviously, the most meaningful evidence must

1 reside in the CNS parenchyma, which is only accessible *post-mortem* (with inevitable delays),  
2 and very rarely in active disease/ after short durations. Therefore, most studies focus on the  
3 CSF as the best available *ex vivo* sample of CNS interstitial fluid – and thus of biomarkers –  
4 though the invasiveness of lumbar puncture narrows its potential. The correlations between  
5 serum and CSF IL-8 and anti-EBNA-1 antibody levels may indicate that, when measured in  
6 combination in serum, they may prove to be valuable biomarkers of events in the CNS.

## 8 **5.) Conclusion**

9 The correlations we show between serum and CSF levels both of EBNA-1 antibodies and of  
10 IL-8 in RRMS suggest that peripheral readings are good indicators of inflammation in the CNS.  
11 Furthermore, since both levels were higher in the serum of MS patients than controls, our  
12 findings imply that elevated EBNA-1 antibodies – and thus EBV latency – are characteristic  
13 features in the periphery, whereas elevated IL-8 and IL-6 levels may be useful biomarkers of  
14 intrathecal inflammation in RRMS. Such insights into its underlying mechanisms are urgently  
15 needed to improve patient stratification, monitoring of disease activity and understanding of  
16 MS pathophysiology.

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## Figure legends

**Fig. 1 Anti-EBNA-1 IgG antibody status in untreated RRMS-patients.** Anti-EBNA-1 IgG antibody levels were measured in untreated MS-patients (n = 20) and controls (HC) (n = 17) by ELISA. **(a)** Significantly higher anti-EBNA-1 IgG antibody titers in the serum of MS-patients than controls ( $P = 0.002$ ). The boxplots show the median (MS: 234; HC: 106.2) and the 25<sup>th</sup> percentiles (MS: 136.6; HC: 6.75) and 75<sup>th</sup> percentiles (MS: 271.2; HC: 189.7). The whiskers of the graph show the largest and smallest values. The median values for each group are indicated by a horizontal bar and the significance of the differences between groups is shown. **(b)** Significantly higher absolute anti-EBNA-1 IgG antibody levels in serum than CSF in MS-patients ( $P = 0.0001$ ). The boxplots show the median (MS: 234.4; HC: 0.272) and the 25<sup>th</sup> percentiles (MS: 136.6; HC: 0.155) and 75<sup>th</sup> percentiles (MS: 271.2 ; HC: 0.58). **(c)** Significantly higher relative anti-EBNA-1 IgG antibody levels in serum than CSF in MS-patients (n = 14; MS/serum: 3.04 +/- 0.44; MS/CSF: 1.31 +/- 0.37;  $P = 0.0057$ ) showing ratio of EBNA-1 concentration to total protein concentration calculated and expressed as [AU anti-EBNA-1/mg total protein]. **(d)** Ratio of anti-EBNA-1 concentration to total IgG concentration calculated and expressed as {AU anti-EBNA-1/ total IgG (mg/ml)}. The mean values for serum and CSF groups are indicated by a horizontal bar and the difference between serum and CSF groups is shown.

**Fig. 2 IL-8 status in untreated RRMS-patients.** IL-8 levels were measured in untreated RRMS-patients (n = 20) and controls (n = 17) by multiplexed fluorescent bead-based immunoassay. **(a)** Significantly higher IL-8 levels in the serum of MS-patients than controls ( $P = 0.0016$ ). The boxplots show the median (MS: 22.90; HC: 6.37) and the 25<sup>th</sup> percentiles (MS: 9.71; HC: 5.18) and 75<sup>th</sup> percentiles (MS: 99.37; HC: 11.64). **(b)** Slightly higher absolute

IL-8 levels in serum than CSF in matched samples from MS-patients (median MS: 22.9; HC: 17.50;  $P = 0.019$ ). (c) Significantly higher relative IL-8 levels in CSF than serum in MS-patients ( $n = 15$ ; MS/serum:  $1.24 \pm 0.51$ ; MS/CSF:  $49.7 \pm 6.02$ ;  $P < 0.0001$ ) showing ratio of IL-8 concentration to total protein concentration calculated and expressed as [pg IL-8/mg total serum protein]. The mean values for serum and CSF groups are indicated by a horizontal bar and the significant difference between serum and CSF groups is shown.

**Fig. 3 Correlation of serum and CSF levels of IL-8 and anti-EBNA-1 IgG antibodies in RRMS patients.** Significant correlations were found between:- (a) absolute anti-EBNA-1 IgG levels in CSF and serum (Spearman  $r = 0.625$ ,  $P = 0.003$ ); (b) serum and CSF absolute IL-8 levels (Spearman  $r = 0.574$ ,  $P = 0.008$ ); (c) relative anti-EBNA-1 IgG titers in serum and CSF (Spearman  $r = 0.7525$ ,  $P = 0.0006$ ), expressed as in Fig 1c; (d) relative IL-8 levels in serum and CSF (Spearman  $r = 0.4558$ ,  $P = 0.044$ ), expressed as in Fig 2c. (e) Significant inverse correlation was found in CSF between relative anti-EBNA-1 IgG levels and IL-8 levels (Spearman  $r = -0.6645$ ;  $P = 0.0095$ )

**Table 1:**

The table shows  $Q_{EBNA-1}$  and  $Q_{IgG}$  of individual RRMS patients ( $n=19$ ) and their calculated antibody index (AI) without  $Q_{LIM}$  correction.