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Intrathecal immunoreactivity in people with or without previous infectious mononucleosis

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ABSTRACT

Objectives: The risk of developing multiple sclerosis (MS) increases (OR: 3.1) after infectious mononucleosis (IM). However, the nature of this link is obscure. We tested the hypothesis that IM might incur long-term sequelae, including low-key inflammatory activity, with characteristics of an MS endophenotype (or presymptomatic trait), and that assays of MS-relevant cyto-/chemokines in the cerebrospinal fluid (CSF) post IM may show a trend in this direction.

Materials and methods: We selected seven CSF cytokines (IL-1b, IL-6, YKL-40, TNF-alpha) or chemokines (IL-8, CCL2, IP-10), representing pro-inflammatory factors previously associated with MS. We assayed the CSF levels of these seven cyto-/chemokines in healthy individuals with a median follow-up time of 10 years after serologically confirmed IM (post-IM group, n=22), and in healthy controls without a history of IM (n=19). A group of MS patients (n=23) were included as reference.

Results: The CSF levels of IP-10, YKL-40 and CCL-2 were higher in the post-IM group than in our IM unexposed controls (p = 0.021, 0.049, 0.028). Seven of seven cyto/chemokine assays showed a trend in the predicted direction (p of binomial ratio = 0.008). However, this trend was non-significant in a multivariate test (p = 0.22). A power analysis indicated that similar studies including a larger cohort would be numerically realistic.

Conclusions: These results do not reject the hypothesis that the established epidemiological association between IM and MS results from a stepwise inflammatory propagation from IM sequele to an MS endophenotype (or presymptomatic trait) in a proportion of IM patients, pending confirmation with adequate power.

1. INTRODUCTION

Multiple sclerosis (MS) is associated with complex immunopathological events, which evolve over years, or even decades, before onset. A useful way to characterize the presymptomatic period is to divide it into the first, latent stage, where genetic and environmental risk factors are assumed to interact, and a second, subsequent stage, when intrathecal inflammation and focal inflammatory lesions develop insidiously ¹. Furthermore, serum specimens stored in repositories, some from adolescence ², revealed

that individuals that subsequently developed MS had elevated anti-Epstein-Barr virus antibodies 1 (EBNA-1) and anti-HHV6A serum antibody titers years or decades before the clinical onset³. A logical early starting-point for prospective studies would be infectious mononucleosis (IM), a manifestation of the primary EBV infection, with an established increased risk of subsequent MS (OR 3.1)⁴. A previous report of IM with a virtually prospective design demonstrated three cross-reactive serum antibodies, which were associated with MS in a preceding study, >48 months after IM in twenty-eight patients. These antibodies were specific against EBV, a mycobacterial antigen and human interferon regulatory factor 5⁵. In three individuals of the post-IM group, the authors showed that stimulation with such peptides induced production of IFN-gamma (in T cells) and TNF-alpha and IL-6 (in monocytes), suggesting cellular immunity sequelae after IM⁶. However, a more relevant indication of the development of a relationship between IM and subsequent MS would be a similar immunological response in the intrathecal compartment, if mimicking an early MS response. We here perform a virtually prospective pilot study of the intrathecal innate immunology in 22 healthy volunteers a decade after serologically verified IM.

Objective

We examine whether the CSF level of seven selected MS-related cytokines (the “post-IM state”) distinguishes between healthy individuals with a history of serologically confirmed IM a decade earlier and healthy controls reporting no history of IM.

2. METHODS AND MATERIALS

2.1 Patients

We conducted a follow-up investigation on individuals that had contracted IM during the years 2003 to 2007. We searched the database of the Department of Virology of the Sahlgrenska University hospital for serum specimens with IgM anti-viral capsid antigen (VCA) titers $\geq 1:160$. We identified 331 samples from 316 individuals. With additional inclusion criteria, residency in the Gothenburg region and age 7 to 30 years at the time of sampling, the number of individuals was 129, to whom we sent letters to request participation in a follow-up investigation. Fifty-one individuals accepted to participate in our studies. They completed a health questionnaire with standardized questions concerning previous IM. We excluded three of these individuals, two with other autoimmune diseases (systemic lupus erythematosus [SLE] and

rheumatoid arthritis [RA]), and one infected with HIV, since they were likely to show an elevated immune response. We acquired new blood specimens for these 48 individuals for a virological follow-up study. From 23 of these we also obtained CSF specimens for the present study. We did not expect to find individuals with MS in the post-IM follow-up cohort, because a recent study on presymptomatic MS had queried the Swedish MS registry for records associated with MS that matched individuals in our Virology Department database, and they had exported all matching records. Nevertheless, in the present follow-up study, we retrieved data on one person with MS that had developed the disease after the call from the national registry. Therefore, we excluded that person from the present group of healthy individuals.

In the final analyses, we included CSF samples from 22 healthy individuals with previous IM (post-IM group), and all of these individuals confirmed a history of a typical IM episode in the questionnaire (Table 1). The median follow-up time was 10 years (range 9-13 years). We also included, through advertisements, 18 healthy controls (HC group) that completed a questionnaire and denied any previous IM infection, any autoimmune disorder, and any other serious disorder. For reference, we used samples from untreated individuals with MS (MS group). These samples were stored at -80°C after they were acquired in a previous study⁷.

2.2. Clinical data

The pre-specified study design included cell counts of the CSF samples; quantification of immunoglobulins, IgG and IgM, in the serum and CSF; quantification of albumin in the serum and CSF; quantification of the neurofilament light chain (NFL) levels in the CSF; and analyses of ten cytokines or chemokines in the CSF. The cyto/chemokines pre-selected for this study were: YKL-40 (Chitinase-3-like protein 1), IP-10 (CXCL10), CCL2 (MCP1), CXCL13 (MCP4), TNF-alpha, IL-1b, IL-6, IL-8, IL-23, and CXCL13 (Table S1). These cyto/chemokines are essentially pro-inflammatory (table S1). We did not add to the selection during the study. However, we had to cancel the analyses of three cytokines, MCP-4, CXCL13, and IL-23, because few or no samples harbored these cytokines above the lower level of quantification (LLOQ), probably due to low analytical sensitivity. Hence, we excluded them from further analyses. Thus, the final analyses included the remaining seven cyto/chemokines.

2.3 Laboratory methods

The CSF cell counts and the IgG and IgM quantifications were analyzed as described⁷.

We measured the CSF NFL concentration with an in-house ELISA, as previously described. The lower level of quantification (LLOQ) was 78 pg/mL; the average intra- and inter-assay coefficients of variation were 10%. The upper reference values were age-dependent, as reported ⁸. We measured CSF YKL-40 levels with solid-phase sandwich ELISA (R&D Systems, Minneapolis, MN, USA). The LLOQ was 2.32 pg/mL, and the average intra- and inter-assay coefficients of variation were 10% ⁹. We assayed CSF CXCL13 with ELISA (Human CXCL13/BLC/BCA-1 Immunoassay; R&D Systems Inc., Abingdon, UK), according to the manufacturer's instructions. The LLOQ was 7.8 pg/mL, and the average intra- and inter-assay coefficients of variation were <10% ¹⁰. We analyzed IP-10, CCL2, TNF-alpha, IL-1b, IL-6, IL-8, and IL-23, selecting these assays from the V-plex Neuroinflammation Panel (MesoScale Discovery, Rockville, MD, USA). The LLOQ for these assays were 1.37, 1.09, 0.69, 0.65, 0.63 and 0.59 respectively. Two samples had IL-1b measurements below the detection limit, and we used zero (the smallest detected value in the remaining samples) for the missing measurements. We did not adjust for CSF IgG values or signs of blood-brain-barrier lesion, as some cytokine levels (e.g. YKL-40 ¹¹) are higher in the CSF than in serum, and cytokine levels were independent of intrathecal IgG synthesis ^{12,13}.

IgM and IgG anti-VCA were originally analyzed 2003-2007 in serum specimens from acute IM cases by an in-house immunofluorescence method at the Department of Clinical Microbiology, Sahlgrenska University Hospital. We currently analyzed Anti-EBNA1 and anti-VCA in serum specimens from acute IM cases 2003-2007, post-IM cases, healthy controls and MS cases, using Architect ELISA.

For the follow-up investigation we collected 20 ml CSF from a lumbar puncture with the participant in the lateral position. We followed the protocols of an international standardization publication ¹⁴. Board-certified laboratory technicians in the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital performed the analyses. The technicians were blind to the clinical data.

2.4 Statistical methods

Because of the non-normal distribution, we compared concentrations of the seven biomarkers between groups with the Mann-Whitney test. We compared the post-IM and MS groups to the HC group, and considered P-values < 0.05 significant. We used a one-sided test for the pre-determined pro-inflammatory hypothesis of each biomarker. We accomplished these statistical analyses with SPSS version 22 and R 3.4.4. (R Core Team, 2018, www.R-project.org). As stated in the discussion, based on the literature we expected the reaction for CCL2 to be pro-inflammatory at this stage. To allow for multiple tests of the differences between two groups, we used a multivariate Hotelling's T² test. This approach had two

advantages: we avoided the problem of multiple testing and we could potentially detect group differences when differences were small between groups for each biomarker ¹⁵. Hotelling's T^2 test is known to be robust; it provided valid results for some biomarkers with values below the LLoQ ¹⁶. This test allowed us to compute scores for each patient, plotting the 7-dimensional biomarker data in a two-dimensional plot. To visualize differences between the three groups, we performed a principal component analysis and a linear discriminant analysis.

Finally, we calculated the sample sizes required to obtain 80% power with a univariate, Bonferroni corrected, Mann-Whitney test, at a 5% significance level. The result provided the number of individuals required in each group. Two markers required larger samples than the rest. Excluding these markers reduced the need of correction for mass significance and diminished the required numbers.

3. RESULTS

3.1 Cytokine levels.

The levels of YKL-40, IL-1b, IL-8, and TNF-alpha were significantly increased and the levels of CCL-2 significantly decreased in the MS group (Table 2). A multivariate test focusing on the seven selected cyto-/chemokines showed significantly higher levels in the MS group than in the HC group ($p=0.00001$). The levels of IP10, YKL-40 and CCL2 were higher in the post-IM group than in the HC group ($p = 0.021, 0.0495, 0.028$, Table 2, Figure 1). The average levels of the seven cyto-/chemokines showed a tendency towards higher values in the post-IM than in the HC group (binomial ratio 7/7, one-tailed $p = 0.008$, Figure 1). However, a multivariate test showed that the overall difference between the levels of the seven selected cyto-/chemokines in the post-IM vs. HC groups was not significant ($p=0.22$). A discriminant analysis and principal component analysis visually confirmed the non-significant result (Figure S1).

3.2 Basic CSF data

The level of NFL was significantly increased in the MS group (Figure 1). All individuals with MS ($n = 23$) had ≥ 2 intrathecally enriched oligoclonal immunoglobulin G (IgG) bands. No HC individual had CSF pleocytosis, ≥ 2 CSF-enriched oligoclonal IgG bands, or increased CSF albumin. No individuals in the post IM group ($n = 22$) had ≥ 2 oligoclonal IgG bands or increased CSF albumin. One individual in the post IM group had one intrathecally enriched igG band. One individual in the post-IM group had 70×10^6 mononuclear cells/liter.

However, that cell count was normal in a repeated lumbar puncture. IgG: immunoglobulin G; IM: infectious mononucleosis; MS: multiple sclerosis; HC: healthy controls; n.a.: Not applicable.

3.3 Anti-EBV antibodies

Re-analysis of 20 of 23 acute IM sera sampled 2003-7 revealed that one individual in the IM group had positive EBNA1 and 5 had borderline values, while the remainder were negative. All IM patients had clearly positive titers for IgM and IgG anti-VCA (Table S2). In the HC group two individuals had negative values for all tested EBV antibodies (IgM anti-VCA, IgG anti-VCA and IgG anti-EBNA1) indicating never having encountered the infection. We performed a re-analysis excluding these two individuals and the individual with positive IgG anti-EBNA1 in the original IM group. This modification of the study design only slightly changed the results of the cytokine analyses (data not shown).

3.4 Power analysis

We included these results in a power analysis, which showed that, to ensure sufficient statistical power for testing associations between the post-IM state and increased levels of the individual cytokines and chemokines tested here, between 25 and 115 specimens would be required. When we excluded IL-1b and IL-6 from the power analysis, between 20 and 75 specimens were required (Table 3).

4. DISCUSSION

This pilot study showed a trend towards higher levels of seven MS-associated cyto/chemokines in the CSF of healthy persons with serologically verified IM approximately 10 years ago, as compared to healthy persons without a history of IM (also valid for EBNA1+ persons without a history of IM). While seven of seven examined cyto-/chemokines had higher CSF levels in the post-IM group, with significant difference in three of these, a multifactorial analysis did not confirm a significant dependence on previous IM. This result did not reject our pre-stated hypothesis that there is – in addition to the established epidemiological association between IM and MS – a stepwise IM-MS-association at the cyto-/chemokine level. Rather than a fully developed pre-MS effect, we anticipated a non-obligatory intermediate effect similar to what has been termed an endophenotype. This is a necessary assumption, as there is a striking imbalance between the population prevalence of IM in the second decade (10-15%) and MS (reaching 2

pro mille in the fourth decade). The “endophenotype” concept defines an “at risk” population with genetics or laboratory findings suggestive of a specific disease. Individuals with the endophenotype may or may not develop the corresponding disease. Therefore, MS associated endophenotypes occur more frequently than manifest MS ¹⁷. Among potential “endophenotypes”, the MS trait, an MS-like oligoclonal intrathecal reaction in healthy people, was prevalent in approximately 20% of 1st degree relatives of individuals with MS and tentatively in 4% (2/50) of the young adult population. The MRI criteria for MS were met in 5% of asymptomatic 1st degree family members of individuals with MS and in 11% of 1st degree family members in multiplex MS families ¹⁸. A power analysis showed that a confirmatory study based on the trend we found in the present study would require a moderate increase in the number of post-IM CSF specimens in healthy people to show a significant increase in CSF cytokine concentrations a decade after IM.

The fundamental question behind the present study design is whether the nature of the epidemiological association between IM and MS (OR = 3.1) ⁴ is direct or reverse. Reverse causality would occur if a presymptomatic MS state, immunodeficient or abnormally immunoreactive, predisposed a person for the IM phenotype as a manifestation of the primary EBV infection. An early differentiated NK cell subset attenuates IM, and successive loss of this subset during the first decades of life might predispose an individual to IM during adolescence ¹⁹. Although NK cell levels in the serum, CSF, and bone marrow may be lower in individuals with MS than in controls ^{20,21}, these studies did not include specific NK cell subsets. Direct causality has support in prospective studies in college students, which revealed that two thirds of IM cases, but only one tenth of asymptomatic EBV infections, were attributable to oral sexual contact ²². The conclusion is that IM is an independent exogenous event, not readily attributable to individual predisposition.

We selected putative “pre-MS” cyto-/chemokine levels as outcome parameter based on 1) detectable in the CSF; 2) plausible involvement in early MS pathogenesis; 3) documented as early as possible in presymptomatic or in manifest MS; and 4) assays available in the laboratories of the Sahlgrenska University Hospital. The term “Involvement in early MS pathogenesis” is extremely wide. The potential number of relevant cytokines is large ²³; CC chemokines often behave synergistically, with partially common receptors, so our selection intends to be representative rather than MS specific. Three sources of approximative data are available: A) MS trait materials that show intrathecal oligoclonal immunoreactivity in first-degree relatives ⁷; these materials are probably biased towards genetic risk factors. B) subtle manifestations before the clinical MS onset, e.g. in the Radiologically Isolated Syndrome (RIS). C) results from pre-MS repositories or biobanks, until now limited to viral serology. That said, there

is a consensus, based on analogy from experimental autoimmune encephalomyelitis, that microglia and other immunocompetent central nervous system (CNS) cells actively produce several cytokines during the presymptomatic stage ²⁴.

Table S1 states the rationale behind each of the selected “pre-MS” cyto-/chemokines. Briefly, high CSF IL-8 in the Radiologically Isolated Syndrome (RIS) was associated with clinical progression, and YKL-40 predicted the conversion from RIS to CIS or to MS, in one of two studies ²⁴⁻²⁶. At that stage, also CSF oligoclonal IgG bands and kappa light chains predict the evolution to MS ^{26,27}. Microglia secrete a number of the selected cytokines or chemokines, and PET studies revealed extensive microglia activation in normal-appearing white matter in MS ²⁸. The behavior of CCL2 in MS is exceptional. Because immunohistochemistry revealed CCL2 in active MS lesions, we included CCL2 in the group of pro-inflammatory cytokines, although later investigators found a significant reduction in CSF CCL2 levels (and normal serum levels) in manifest MS, interpreted to be a result of cytokine consumption ²⁹. Two of the selected CSF cytokines, IP-10 and IL-23, are specifically associated with EBV infections ³⁰. Gamma interferon, produced during infections, induces IP-10 expression. IP-10 was consistently elevated in sera from patients with acute IM ³¹. IP-10 was also expressed in chronic viral infections ³². The authors correlated it with the treatment response in chronic hepatitis C infections ³³. Furthermore, the IP-10 level was elevated in both the sera and CSF from patients with relapsing- remitting and secondary progressive MS ³⁴. IP-10 levels predicted MS activity at different time points, and it was higher in patients that converted from CIS to MS than in those that did not convert ³⁵. We could not quite exclude the possibility that individuals that contracted IM might have had a constitutionally increased IP-10 drive. After obtaining negative results for IL-23, probably due to low analytical sensitivity, we did not pursue analysis of this cytokine in the present study, which was based on 7 of the originally selected cyto-/chemokines.

A more precise question would be whether IM *per se*, but not mild or asymptomatic EBV infection induces the MS endophenotype. The definition of IM in the 2003-2007 group was an IgM anti-VCA titer of 1:160 or more. The fact that physicians had requested this specific analysis, and that participating research persons later confirmed a history of symptom compatible with IM, further supports the serological IM diagnosis. Nevertheless, five of these patients had gray zone IgG titers against EBNA1, and one had a titer well above the cut-off. This makes the IM diagnosis less convincing, particularly in the latter case but still probable, as the proportion of IM patients with positive EBNA1 titers increase slowly during several weeks, even months, after the onset ³⁶. We approached the IM *per se* issue by excluding one individual with less convincing IM diagnosis in the original IM group and two individuals in the control group who had negative values for all tested EBV antibodies, indicating that they never encountered EBV. This

modification of the material only marginally changed the results. On the other hand, there is no way to determine if the presence of EBV seropositive individuals in the control group who had IM, and were unaware or oblivious of that, have attenuated the significance of our results.

A recent study used a similar post-IM design, i.e. imitating a prospective aspect from the primary EBV infection. A complex of cross-reactive antibodies against EBNA1, anti-mycobacterial and human antigens (myelin basic protein or anti-interferon regulatory factor 5) was documented by alignment analysis and competitive assays in MS patients ^{5,37}, and this antibody complex was subsequently found in twenty-eight patients >48 months post IM (with 40 healthy controls without a history of IM). In three of the post-IM group, the authors showed that stimulation with such peptides could induce production of IFN-gamma (in T cells) and TNF-alpha and IL-6 (in monocytes) ⁶. However, the authors confined this study to blood, and the strength of the present study is the achievement of follow-up with CSF analysis.

The non-significant trend towards higher CSF cytokine levels in the present study did not reject the hypothesis that IM may incur persisting alterations in pro-inflammatory cyto-/chemokine expression approaching an MS-related endophenotype. Future studies on the post-IM state with improved statistical power are warranted and might re-evaluate the selection of “pre-MS” cyto-/chemokines ²³, and include cellular immunity assays to probe the persistence of the post-IM state.

5. CONCLUSION

Long-term sequelae after IM provisionally demonstrated in serum ⁶, and here in the CSF, may contribute to the gradual induction of CNS inflammatory processes observed in the pre-symptomatic stages of MS, thereby explaining the established epidemiological relationship between IM and MS.

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STATEMENT OF ETHICS

All participants provided written consent. The Research Ethics Committee of Umeå Dnr 2017-484-32M approved the national MS registry study and all local extensions of the study.

DATA AVAILABILITY STATEMENT

Raw data from this study are available from the corresponding author on reasonable request.

REFERENCES

1. Giovannoni G. The neurodegenerative prodrome in multiple sclerosis. *Lancet Neurol.* 2017;16(6):413-414.
2. Levin LI, Munger KL, O'Reilly EJ, Falk KI, Ascherio A. Primary infection with the Epstein-Barr virus and risk of multiple sclerosis. *Ann Neurol.* 2010;67(6):824-830.
3. Sundstrom P, Juto P, Wadell G, et al. An altered immune response to Epstein-Barr virus in multiple sclerosis: a prospective study. *Neurology.* 2004;62(12):2277-2282.
4. Hawkes CH, Boniface D. Risk associated behavior in premorbid multiple sclerosis: A case-control study. *Mult Scler Relat Disord.* 2014;3(1):40-47.
5. Cossu D, Mameli G, Galleri G, et al. Human interferon regulatory factor 5 homologous epitopes of Epstein-Barr virus and Mycobacterium avium subsp. paratuberculosis induce a specific humoral and cellular immune response in multiple sclerosis patients. *Mult Scler.* 2015;21(8):984-995.
6. Mameli G, Madeddu G, Cossu D, et al. Immune response induced by Epstein-Barr virus and Mycobacterium avium subsp. paratuberculosis peptides in current and past infectious mononucleosis: a risk for multiple sclerosis? *Eur J Neurol.* 2016;23(1):140-147.

7. Haghighi S, Andersen O, Rosengren L, Bergstrom T, Wahlstrom J, Nilsson S. Incidence of CSF abnormalities in siblings of multiple sclerosis patients and unrelated controls. *J Neurol*. 2000;247(8):616-622.
8. Gaetani L, Hoglund K, Parnetti L, et al. A new enzyme-linked immunosorbent assay for neurofilament light in cerebrospinal fluid: analytical validation and clinical evaluation. *Alzheimers Res Ther*. 2018;10(1):8.
9. Hermansson L, Yilmaz A, Axelsson M, et al. Cerebrospinal fluid levels of glial marker YKL-40 strongly associated with axonal injury in HIV infection. *J Neuroinflammation*. 2019;16(1):16.
10. Novakova L, Axelsson M, Khademi M, et al. Cerebrospinal fluid biomarkers of inflammation and degeneration as measures of fingolimod efficacy in multiple sclerosis. *Mult Scler*. 2017;23(1):62-71.
11. Canto E, Tintore M, Villar LM, et al. Chitinase 3-like 1: prognostic biomarker in clinically isolated syndromes. *Brain*. 2015;138(Pt 4):918-931.
12. Maimone D, Gregory S, Arnason BG, Reder AT. Cytokine levels in the cerebrospinal fluid and serum of patients with multiple sclerosis. *J Neuroimmunol*. 1991;32(1):67-74.
13. Comabella M, Fernandez M, Martin R, et al. Cerebrospinal fluid chitinase 3-like 1 levels are associated with conversion to multiple sclerosis. *Brain*. 2010;133(Pt 4):1082-1093.
14. Teunissen CE, Tumani H, Engelborghs S, Mollenhauer B. Biobanking of CSF: international standardization to optimize biomarker development. *Clin Biochem*. 2014;47(4-5):288-292.
15. Farcomeni A. A review of modern multiple hypothesis testing, with particular attention to the false discovery proportion. *Stat Methods Med Res*. 2008;17(4):347-388.
16. Thulin M. Two-sample tests and one-way MANOVA for multivariate biomarker data with nondetects. *Stat Med*. 2016;35(20):3623-3644.
17. Ramagopalan SV, Dobson R, Meier UC, Giovannoni G. Multiple sclerosis: risk factors, prodromes, and potential causal pathways. *Lancet Neurol*. 2010;9(7):727-739.
18. De Stefano N, Cocco E, Lai M, et al. Imaging brain damage in first-degree relatives of sporadic and familial multiple sclerosis. *Ann Neurol*. 2006;59(4):634-639.
19. Chijioke O, Landtwing V, Munz C. NK Cell Influence on the Outcome of Primary Epstein-Barr Virus Infection. *Front Immunol*. 2016;7:323.
20. Svenningsson A, Andersen O, Hansson GK, Stemme S. Reduced frequency of memory CD8+ T lymphocytes in cerebrospinal fluid and blood of patients with multiple sclerosis. *Autoimmunity*. 1995;21(4):231-239.

21. Jons D, Kneider M, Fogelstrand L, Jeppsson A, Jacobsson S, Andersen O. Early hematopoiesis in multiple sclerosis patients. *J Neuroimmunol*. 2016;299:158-163.
22. Crawford DH, Swerdlow AJ, Higgins C, et al. Sexual history and Epstein-Barr virus infection. *J Infect Dis*. 2002;186(6):731-736.
23. Magliozzi R, Howell OW, Nicholas R, et al. Inflammatory intrathecal profiles and cortical damage in multiple sclerosis. *Ann Neurol*. 2018;83(4):739-755.
24. Rossi S, Motta C, Studer V, et al. Subclinical central inflammation is risk for RIS and CIS conversion to MS. *Mult Scler*. 2015;21(11):1443-1452.
25. Thouvenot E, Hinsinger G, Demattei C, et al. Cerebrospinal fluid chitinase-3-like protein 1 level is not an independent predictive factor for the risk of clinical conversion in radiologically isolated syndrome. *Mult Scler*. 2018:1352458518767043.
26. Matute-Blanch C, Villar LM, Alvarez-Cermeno JC, et al. Neurofilament light chain and oligoclonal bands are prognostic biomarkers in radiologically isolated syndrome. *Brain*. 2018;141(4):1085-1093.
27. Pawlitzki M, Sweeney-Reed CM, Bittner D, et al. CSF-Progranulin and Neurofilament Light Chain Levels in Patients With Radiologically Isolated Syndrome-Sign of Inflammation. *Front Neurol*. 2018;9:1075.
28. Zrzavy T, Hametner S, Wimmer I, Butovsky O, Weiner HL, Lassmann H. Loss of 'homeostatic' microglia and patterns of their activation in active multiple sclerosis. *Brain*. 2017;140(7):1900-1913.
29. Malmestrom C, Andersson BA, Haghighi S, Lycke J. IL-6 and CCL2 levels in CSF are associated with the clinical course of MS: implications for their possible immunopathogenic roles. *J Neuroimmunol*. 2006;175(1-2):176-182.
30. Broderick G, Katz BZ, Fernandes H, et al. Cytokine expression profiles of immune imbalance in post-mononucleosis chronic fatigue. *J Transl Med*. 2012;10:191.
31. Abbott RJ, Pachnio A, Pedroza-Pacheco I, et al. Asymptomatic Primary Infection with Epstein-Barr Virus: Observations on Young Adult Cases. *J Virol*. 2017;91(21).
32. Fortuna D, Hooper DC, Roberts AL, Harshyne LA, Nagurney M, Curtis MT. Potential role of CSF cytokine profiles in discriminating infectious from non-infectious CNS disorders. *PLoS One*. 2018;13(10):e0205501.
33. Lagging M, Romero AI, Westin J, et al. IP-10 predicts viral response and therapeutic outcome in difficult-to-treat patients with HCV genotype 1 infection. *Hepatology*. 2006;44(6):1617-1625.

34. Scarpini E, Galimberti D, Baron P, et al. IP-10 and MCP-1 levels in CSF and serum from multiple sclerosis patients with different clinical subtypes of the disease. *J Neurol Sci.* 2002;195(1):41-46.
35. Hakansson I, Tisell A, Cassel P, et al. Neurofilament levels, disease activity and brain volume during follow-up in multiple sclerosis. *J Neuroinflammation.* 2018;15(1):209.
36. Grimm JM, Schmeling DO, Dunmire SK, et al. Prospective studies of infectious mononucleosis in university students. *Clin Transl Immunology.* 2016;5(8):e94.
37. Mameli G, Cossu D, Cocco E, et al. Epstein-Barr virus and Mycobacterium avium subsp. paratuberculosis peptides are cross recognized by anti-myelin basic protein antibodies in multiple sclerosis patients. *J Neuroimmunol.* 2014;270(1-2):51-55.
38. Bonne-Barkay D, Wang G, Starkey A, Hamilton RL, Wiley CA. In vivo CHI3L1 (YKL-40) expression in astrocytes in acute and chronic neurological diseases. *J Neuroinflammation.* 2010;7:34.
39. Hu WT, Howell JC, Ozturk T, et al. CSF Cytokines in Aging, Multiple Sclerosis, and Dementia. *Front Immunol.* 2019;10:480.
40. Novakova L, Axelsson M, Khademi M, et al. Cerebrospinal fluid biomarkers as a measure of disease activity and treatment efficacy in relapsing-remitting multiple sclerosis. *J Neurochem.* 2017;141(2):296-304.
41. Gaudreault E, Fiola S, Olivier M, Gosselin J. Epstein-Barr virus induces MCP-1 secretion by human monocytes via TLR2. *J Virol.* 2007;81(15):8016-8024.
42. Ockinger J, Stridh P, Beyeen AD, et al. Genetic variants of CC chemokine genes in experimental autoimmune encephalomyelitis, multiple sclerosis and rheumatoid arthritis. *Genes Immun.* 2010;11(2):142-154.
43. Mendez-Enriquez E, Garcia-Zepeda EA. The multiple faces of CCL13 in immunity and inflammation. *Inflammopharmacology.* 2013;21(6):397-406.
44. Stein J, Xu Q, Jackson KC, et al. Intrathecal B Cells in MS Have Significantly Greater Lymphangiogenic Potential Compared to B Cells Derived From Non-MS Subjects. *Front Neurol.* 2018;9:554.
45. Ribeiro CM, Oliveira SR, Alfieri DF, et al. Tumor necrosis factor alpha (TNF-alpha) and its soluble receptors are associated with disability, disability progression and clinical forms of multiple sclerosis. *Inflamm Res.* 2019;68(12):1049-1059.
46. Rossi S, Motta C, Studer V, et al. Interleukin-1beta causes excitotoxic neurodegeneration and multiple sclerosis disease progression by activating the apoptotic protein p53. *Mol Neurodegener.* 2014;9:56.

- Accepted Article
47. Sisay S, Lopez-Lozano L, Mickunas M, et al. 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. *J Neuroimmunol*. 2017;306:40-45.
 48. Hunter CA. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol*. 2005;5(7):521-531.
 49. Shajarian M, Alsahebhosoul F, Etemadifar M, et al. IL-23 plasma level measurement in relapsing remitting multiple sclerosis (RRMS) patients compared to healthy subjects. *Immunol Invest*. 2015;44(1):36-44.
 50. Puthenparampil M, Federle L, Miente S, et al. BAFF Index and CXCL13 levels in the cerebrospinal fluid associate respectively with intrathecal IgG synthesis and cortical atrophy in multiple sclerosis at clinical onset. *J Neuroinflammation*. 2017;14(1):11.
 51. Khademi M, Kockum I, Andersson ML, et al. Cerebrospinal fluid CXCL13 in multiple sclerosis: a suggestive prognostic marker for the disease course. *Mult Scler*. 2011;17(3):335-343.

Table 1. Study participants

Disorder	N	F/M	Mean age, y (range)	P: F vs. M	P: Age IM vs. MS
Post IM	22	13/9	29 (22-40)	ref	Ref
MS	23 or 17*	17/6	35 (20-46)	ns	0.06
Healthy controls	18	13/5	26 (18-45)	0.85	0.08

*The numbers of MS serum samples were 23 for the neurochemical assays and 17 for the immunochemical assays. IM: infectious mononucleosis; MS: multiple sclerosis

Table 2. CSF concentrations of NFL and 7 selected immunological markers in post-IM, MS, and control samples

Cyto/chemokine	Post-IM median (IQR)	MS median (IQR)	HC median (IQR)	P*) Post-IM vs. HC	P*) MS vs. HC
NFL	262.0 (226.0- 360.0)	914.5 (649.3– 1642.5)	246.0 (205.0- 331.0)	0.193	<0.0001
YKL-40	72.0 (56.8- 83.2)	124.0 (99.7- 217.5)	63.4 (43.2- 72.9)	0.049	<0.0001
IL-1b	0.040 (0.020- 0.060)	0.050 (0.04-0.06)	0.030 (0.020- 0.040)	0.148	0.0003
IL-6	0.94 (0.73- 1.46)	0.94 (0.57- 1.70)	0.90 (0.52- 1.25)	0.185	0.170
IL-8	33.1 (28.5- 42.8)	43.7 (36.9- 52.1)	31.8 (26.0- 36.1)	0.103	0.0002
TNF-alpha	0.14 (0.09- 0.18)	0.20 (0.15- 0.30)	0.12 (0.08- 0.16)	0.266	0.0003
IP-10	246.5 (154.8- 335.3)	158.0 (56.0- 326.5)	176.0 (128.6- 227.4)	0.021	0.194
CCL2	296.5 (258.0- 371.5)	185.0 (28.0 –245.0)	264.0 (229.5- 287.8)	0.028	0.0014

*) One-sided tests uncorrected for multiple comparisons; NFL: neurofilament light chain; IM: infectious mononucleosis; MS: multiple sclerosis; HC: healthy controls; IQR: interquartile range.

Table 3. Power calculations for a future study; number of participants required

Cyto/chemokine	Required number, seven markers ^a	Required number, five markers ^b
YKL-40	48	45
IP10	25	20
CCL2	58	55
TNF-alpha	80	75
IL1b	95	-
IL6	115	-
IL8	80	70

^aAssuming that the same 7 biomarkers are used; ^bassuming that the two biomarkers that required the largest samples are excluded.

Figure 1. CSF NFL and selected cytokines/chemokines in control, post-IM, and MS groups. Significance was tested between the control (contr) and post-IM groups (short bar), and between the control and MS groups (long bars). NFL: neurofilament light chain; IM: infectious mononucleosis; MS: multiple sclerosis; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.