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Relationship between cerebrospinal fluid biomarkers of inflammation and tissue damage in primary progressive multiple sclerosis

Jacob Talbot, MD¹; Helene Højsgaard Chow, PhD¹; Mie Mahler, MD¹; Sophie Buhelt, PhD¹; Rikke Holm HansenPhD¹; Henrik Lundell, PhD²; Tua Vinther-Jensen, PhD³,⁴; Marie N. N. Hellem, MD⁵; Jørgen E. Nielsen, PhD³; Hartwig Roman Siebner, DMSc²,⁴,⁵; Marina R. von Essen, PhD¹; Finn Sellebjerg, DMSc¹

¹Danish Multiple Sclerosis Center, Department of Neurology, Copenhagen University Hospital - Rigshospitalet, Glostrup, Denmark
²Danish Research Centre for Magnetic Resonance, Centre for Functional and Diagnostic Imaging and Research, Copenhagen University Hospital Amager and Hvidovre, Copenhagen, Denmark
³The Neurogenetics Clinic, Danish Dementia Research Center, Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark
⁴Department of Neurology, Copenhagen University Hospital Bispebjerg and Frederiksberg, Copenhagen, Denmark
⁵Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

Corresponding author:
Jacob Talbot, MD, PhD
Danish Multiple Sclerosis Center
Valdemar Hansens Vej 17, 5, 7
2600 Glostrup
Denmark
e-mail: jacob.lando.talbot@regionh.dk
phone: (+45) 3863 3205
Abstract

Background and Objectives

It is unclear to what extent intrathecal inflammation contributes to the pathogenesis in primary progressive multiple sclerosis (PPMS). We conducted an exploratory study to investigate the degree of intrathecal inflammation and its association with biomarkers of disease activity and severity in patients with PPMS.

Methods

We included patients with PPMS who participated in a randomized controlled trial conducted at the Danish Multiple Sclerosis Center, patients with relapsing-remitting multiple sclerosis (RRMS) and healthy controls. We analyzed concentrations of a panel of cytokines in CSF using electrochemiluminescence assays. We then explored the relationship between cytokines found in increased CSF concentrations in patients with PPMS (compared with healthy controls) with CSF concentrations of neurofilament light chain (NFL) and myelin basic protein (MBP), IgG-index, and magnetic resonance imaging (MRI) metrics (volume, magnetization transfer ratio and diffusion tensor imaging) from lesions, normal-appearing white matter, and cortical grey matter.

Results

We included 59 patients with PPMS, 40 patients with RRMS, and 21 healthy controls. In patients with PPMS, CSF concentrations of CC chemokine ligand 3 (CCL-3), CXC chemokine ligand 8 (CXCL-8), CXCL-10, interleukin (IL)-10, IL-15, and vascular endothelial growth factor (VEGF)-A were increased compared with healthy controls and comparable with CSF concentrations in patients with RRMS. In addition, patients with PPMS had increased CSF concentrations of IL-12p40, IL-17A, tumor necrosis factor (TNF)-α, and lymphotoxin (LT)-α compared with healthy controls, but concentrations of these cytokines were even higher in patients with RRMS. For the remaining seven cytokines (CCL22, interferon-γ, IL-5, IL-7, IL-16, IL-22, IL-27), we found no difference between patients with PPMS and healthy controls. CSF concentrations of NFL and MBP correlated weakly with concentrations of IL-15, while the remaining proinflammatory cytokines were not associated with CSF concentrations of NFL or MBP. The IgG-index correlated with four cytokines (IL-10, IL-12p40, TNF-α, and LT-α). We did not observe any significant associations between MRI metrics and CSF biomarkers of inflammation.

Discussion

In this exploratory study, we found few and weak associations between intrathecal inflammation and the extent of neuroaxonal damage and demyelination, and no associations between intrathecal inflammation and MRI metrics, in patients with PPMS. Our findings suggest that, for patients with PPMS, these measures of intrathecal inflammation are not associated with the extent of neuroaxonal injury, demyelination, and disease severity, and these processes may therefore have less relevance in PPMS than in relapsing forms of MS.
Introduction

Multiple sclerosis (MS) is an immune-mediated, demyelinating disease of the central nervous system (CNS) in which 10-15% of patients experience a slowly progressive course from onset, termed primary progressive MS (PPMS). The disease course in MS can be described using the terms disease activity (relapses, new or enlarging or Gd-enhancing [GEL] magnetic resonance imaging (MRI) lesions) and progression (gradual disability worsening not explained by superimposed relapses). Many disease-modifying treatments (DMTs), specifically targeting immune cells, inhibit relapse and focal MRI activity. The efficacy of DMTs in patients with progressive MS is lower and only B cell-depleting therapy has shown some efficacy in slowing disease progression in patients with PPMS. It is still unresolved why the therapeutic benefits of most DMTs on relapses and MRI activity do not translate into an effect on disease progression in PPMS. Intrathecally compartmentalized inflammation suggested as a primary mediator of disease progression in progressive MS may offer part of the explanation. Additionally, it has been proposed that inflammation subsides along with the progressive phase of the disease, and that the mechanisms responsible for propagating progression are independent from inflammatory activity in PPMS. However, the burden of inflammation seems evident especially in patients with secondary progressive MS (SPMS) in whom increased intrathecal concentrations of proinflammatory cytokines were related to worse disease course and extensive cortical inflammatory activity. However, few studies have assessed the degree of inflammation and the relationship with disease activity and progression in PPMS, although the processes applicable in SPMS seem relevant also in PPMS as cerebrospinal fluid (CSF) concentrations of biomarkers of inflammation are comparable in PPMS and SPMS.

We recently completed a randomized, placebo-controlled trial of dimethyl fumarate (DMF) treatment in PPMS where CSF concentrations of biomarkers of neuroaxonal damage and demyelination as well as selected biomarkers of inflammation were used as outcome measures. In this study, we further investigate the extent of inflammation in PPMS compared with untreated patients with early RRMS and healthy controls (HCs). We also characterize the relationship between intrathecal inflammation and disease activity and severity assessed with MRI and CSF biomarkers of demyelination and neuroaxonal damage.

Methods

Subjects and CSF collection

We enrolled 59 patients with PPMS. All patients with PPMS were screened for inclusion in a randomized, placebo-controlled trial conducted at the Danish Multiple Sclerosis Center, Copenhagen University Hospital – Rigshospitalet from Dec 2016 to Jan 2019. Primary inclusion criteria were age between 18 and 65 years, no immunomodulatory or immunosuppressive therapy within six months before inclusion, and no steroid treatment within three months before inclusion. The study was approved by the Capital Region Ethics Committee, Denmark (protocol H-16047666, Clinicaltrials.gov number: NCT02959658), and informed consent was obtained from all patients before enrolment. In addition, we collected CSF from 40 untreated patients with RRMS.
and 21 HCs according to the same procedure as described below. Patients with RRMS were untreated and recruited from the Danish Multiple Sclerosis Center between Feb 2016 and Mar 2021 (ethics committee protocol number H-17005703). All patients with RRMS were diagnosed according to the 2017 McDonald criteria. The HCs were huntingtin gene expansion negative family members of Huntington disease gene expansion carriers from the Neurogenetics Clinic, Copenhagen University Hospital - Rigshospitalet (ethics committee protocol number H2-2011-085 and H-17002606).

We collected 10 to 12 ml of CSF of which three ml was sent for routine diagnostic analysis of cell count, immunoglobulin-G (IgG)-index, albumin quotient, and oligoclonal bands. The remaining CSF was separated from cells by centrifugation and stored at -80°C until analysis.

CSF measurements

We measured a panel of cytokines and chemokines in CSF using electrochemiluminescence (ECL) assays (V-PLEX human cytokine 22-PLEX kit, Meso Scale Diagnostics, Rockville, MD, USA) according to manufacturer’s instructions. The following cytokines and chemokines were measured: vascular endothelial growth factor A (VEGF-A), tumor necrosis factor-α (TNF-α), lymphotoxin-α (LT-α), interferon (IFN)-γ, CC chemokine ligand (CCL)-3, CCL-20, CCL-22, CCL-26, CXC chemokine ligand (CXCL)-8, CXCL-10, interferon-γ (IFN-γ). The maximum lower limit of detection (LLoD_max) across all plates measured for each analyte was chosen as cut-off for detection (see supplementary figure e1), and we excluded cytokines with a median CV above 20% and samples from plates with an irregular standard curve compared with other plates. To account for large variations in concentrations within the lower curve area, we assigned the LLoD_max value to all samples in which concentrations fell below this limit. We used ELISA to measure CSF concentrations of neurofilament light chain (NFL) (Uman Diagnostics, Umeå, Sweden) and myelin basic protein (MBP) (R&D Systems, MN, USA) according to manufacturer’s instructions.) in patients with PPMS.

MRI

We performed MRI on all patients with PPMS at screening and after 48 weeks. Patients were scanned on the same 3T Verio MRI scanner (Siemens Healthcare, Erlangen, Germany) with a 32-channel head coil. T1-weighted (T1W), T2-weighted (T2W), and fluid-attenuated inverse recovery (FLAIR) 3D sequences with 1 mm³ isotropic resolution were acquired with structural imaging. In addition, diffusion tensor imaging (DTI) sequences and data for magnetization transfer ratio (MTR) were acquired. For the present study we analyzed the relationship between CSF biomarkers and lesion volume, MTR in lesions, normal-appearing white matter (NAWM) volume, fractional anisotropy (FA) and mean diffusivity (MD) in NAWM, scaled cortical gray matter (CGM) volume, and MTR in scaled CGM. Finally, we performed longitudinal analysis of new lesions and enlarging lesions developing over 48 weeks. Details regarding the MRI data acquisition and analysis has previously been described in detail.
Statistics

Data are presented as mean (SD) and median [Q1, Q3] as appropriate. We compared CSF cytokine concentrations across groups by applying analysis of variance (ANOVA) tests on linear models adjusted for age and sex to assess overall group differences. We used a multiple testing-corrected (Bonferroni) significance value of 0.05. Post-hoc, we conducted age and sex-adjusted linear models with log-transformed concentrations as outcomes to evaluate pairwise differences between the three groups. We performed model control for all generalized linear models. In post-hoc analysis we considered a p-value below 0.05 as statistically significant. Cytokines with increased CSF concentrations in patients with PPMS compared with HCs were analyzed for associations using Spearman rank correlation analysis. For all correlation analyses, a two-sided significance level of q<0.05 was considered significant (false discovery rate [FDR] corrected). All analyses and illustrations were conducted with RStudio v 1.2.5 (extension packages tidyverse, ggendro, factoextra, and patchwork).13–17
Results

Increased cytokine and chemokine concentrations in patients with PPMS

Baseline comparisons revealed differences in sex, age, disease duration, expanded disability status scale (EDSS) and CSF cell count between groups (Table 1). We performed Bonferroni-corrected analysis of variance on linear models adjusted for sex and age to compare concentrations of cytokines between the three groups. We found group differences for the following cytokines: CCL-3 (p<0.001), CCL-22 (p<0.001), CXCL-8 (p<0.001), CXCL-10 (p=0.002), IFN-γ (p<0.001), IL-7 (p=0.035), IL-10 (p<0.001), IL-12p40 (p<0.001), IL-15 (p=0.003), IL-17 (p<0.001), IL-27 (p=0.048), LT-α (p<0.001), TNF-α (p<0.001), and VEGF-A (p=0.006). We excluded five out of 22 cytokines (CCL-20, CCL-26, IL-21, IL-23, IL-31) from further analysis due to poor sensitivity (eTable 1 in the supplement).

We found considerable age differences between groups, and some of the investigated cytokines correlated with age (eTable 2 in the supplement), and we therefore conducted post-hoc test based on a linear model adjusted for age and sex to assess differences between groups for each cytokine (figure 1). We observed increased concentrations of the following cytokines in patients with PPMS compared with HCs (mean fold increase in patients with PPMS patients vs. HCs [95% CI], p-value): CCL-3 (1.97 [1.64 – 2.35], p<0.001), CXCL-8 (1.60 [1.34 – 1.91], p<0.001), CXCL-10 (1.76 [1.10 – 2.82], p<0.001), IL-10 (2.47 [1.68 – 3.62], p<0.001), IL-12p40 (2.11 [1.46 – 3.06], p<0.001), IL-15 (1.31 [1.13 – 1.52], p<0.001), IL-17 (1.43 [1.07 – 1.91], p=0.015), LT-α (1.21 [1.00 – 1.47], p=0.046), TNF-α (2.01 [1.64 – 2.47], p<0.001), VEGF-A (1.30 [1.12 – 1.51], p<0.001).

The concentrations of these biomarkers of inflammation were also significantly higher in RRMS than in HCs, and for the following cytokines CSF concentrations were even higher patients with RRMS compared with patients with PPMS (mean fold increase in patients with RRMS vs patients with PPMS [95% CI]): IL-12p40 (2.52 [1.69 – 3.75], p<0.001), LT-α (1.24 [1.01 – 1.52], p=0.042) and TNF-α (1.27 [1.02 – 1.58], p=0.032). Furthermore, patients with RRMS had higher CSF concentrations of CCL-22 (1.60 [1.28 – 2.24], p<0.001), IFN-γ (1.81 [1.25 – 2.62], p=0.002) and IL-27 (1.35 [1.00 – 1.82], p=0.048) and lower concentrations of IL-7 (0.73 [0.57 – 0.95], p=0.017) than the HC group; there was no significant difference between patients with PPMS and HCs for these four cytokines.

Relationships between CSF biomarkers

We conducted further analyses of CSF biomarkers with increased concentrations in patients with PPMS compared with HCs (CCL-3, CXCL-10, CXCL-8, IL-10, IL12-p40, IL-15, IL-17, LT-α, TNF-α, and VEGF-A) to assess the associations with NFL, MBP, and the IgG-index (Figure 2). After correction for multiple comparisons, we found that IL-15 correlated significantly with NFL and MBP (both q<0.05), while IgG-index correlated significantly with IL-10, TNF-α, IL-12p40, and LT-α. We further analyzed correlations between CSF cytokines and found that several cytokines were closely and significantly correlated (Figure 3).
Relationships with MRI

We analyzed associations between CSF cytokines and MRI measures of 1-year disease activity (new and enlarging lesions) and cross-sectional MRI measures of disease severity (lesion volume, MTR in lesions, NAWM volume, fractional anisotropy and mean diffusivity in NAWM, CGM volume, and MTR in CGM). We found no significant associations between CSF cytokines and MRI-derived metrics of disease activity or severity after correction for multiple comparisons (q<0.05) (Supplementary Data, Figure S1).

Discussion

Our results show that intrathecal inflammation is present in patients with PPMS and, for many cytokines, the relative increase in CSF concentration was comparable to those observed in patients with newly diagnosed RRMS. These results expand our previously published analyses which revealed an increase in CSF concentrations of sCD27, sBCMA, CHI3L1, MBP, and NFL in the same PPMS cohort compared to a group of individuals who had neurologic symptoms but without any objective or paraclinical findings to define a specific neurologic disease.\(^{11}\) Notably, patients with SPMS with profound meningeal inflammation and gray matter demyelination had elevated mRNA expression and CSF concentrations of proinflammatory and B cell associated cytokines compared with SPMS patients with little meningeal inflammation and gray matter demyelination.\(^{18}\) Likewise, patients with RRMS with a high cortical lesion load at diagnosis had increased CSF concentrations of proinflammatory and B cell associated chemokines and cytokines (CXCL-8, CXCL-10, IL-10, LT-α, and TNF-α).\(^{18}\) We also show that proinflammatory and B cell-related biomarkers such as IgG-index, LT-α, and IL12-p40 are increased in patients with PPMS, indicating a pivotal role of B cell activity in the immune pathogenesis of both RRMS and progressive MS.\(^{19}\)

The concentrations of NFL and MBP in CSF have been shown to reflect neuroaxonal damage and demyelination, respectively.\(^{20,21}\) Surprisingly, patients with PPMS showed only weak and relatively few associations between NFL and MBP levels in CSF and CSF biomarkers of inflammation. Studies with mixed progressive MS patients have found contradictory results for NFL concentrations that were closely associated with several CSF biomarkers of inflammation.\(^{22,23}\) The patients in the present study were considerably older than the patients in the previous studies, and we hypothesize that age-dependent changes may contribute to the differences between the results of the present and previous studies.

Although we used an extensive and detailed MRI protocol to capture disease-related changes in brain structure, we found no associations between biomarkers previously related to disease activity and severity in patients with SPMS and MRI-metrics of disease activity and disease severity in this cohort of patients with PPMS.\(^{8}\) However, meningeal inflammation and cortical lesion load were not assessed in this study, and ultra-high field MRI as well as a prospective study of changes in MRI metrics over a longer period could have added valuable information to our findings.\(^{8,24}\)

We chose a conservative method of analysis regarding electrochemiluminescence values, but the low assay sensitivity may influence the results. However, we ensured an acceptable standard curve for all assays, and we excluded assays with a mean CV above 20% to account for high assay
variation. The exploratory nature of the study may also induce type I errors, but we chose a conservative definition of significant findings which, on the other hand, increases the risk of type II errors.

All PPMS data were collected from patients participating in a randomized, placebo-controlled trial that showed no difference between patients receiving DMF or placebo. The lack of an DMF treatment effect on CSF biomarkers of inflammation in PPMS does not seem to reflect lack of ongoing inflammation as concentrations were increased and comparable to levels in RRMS for many of the biomarkers studied. Histopathological and CSF studies have, indeed, provided strong evidence that there is widespread inflammation in the CNS in patients with PPMS involving both glial and innate immunity in patients with PPMS. The biomarkers of inflammation found to have increased concentrations in CSF in our patient cohort reflect both adaptive and innate immune cell activation.

In conclusion, we explored an extensive panel of biomarkers reflecting CSF inflammation in a cohort of patients with PPMS and found that although there was clear evidence of ongoing intrathecal inflammation, associations with biomarkers of demyelination and neuroaxonal damage were weak, and there were no associations with MRI measures of disease activity and severity. Although many of the analyzed biomarkers have previously been associated with disease severity and neuroaxonal damage in patients with progressive MS (primarily patients with SPMS), this could not be confirmed in the present study.

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Contributions

JT collected data, performed laboratory analysis and data analysis and composed the manuscript. HHC collected data and edited the manuscript. RHH collected data, performed laboratory analysis and edited the manuscript. MRM collected data, performed laboratory analysis and edited the manuscript. SB collected data and edited the manuscript. MNNH collected data and edited the manuscript. TV collected data and edited the manuscript. HL collected and analyzed MRI data and edited the manuscript. JEN collected data and edited the manuscript. HRS edited the manuscript. MRvE performed laboratory analyses and edited the manuscript. FS conceptualized the study, performed data analysis, and edited the manuscript.
Conflicts of interest

JT reports non-financial support from Biogen and Sanofi Genzyme outside the submitted work. HHC reports non-financial support from Merck, non-financial support from Teva, non-financial support from Biogen, non-financial support from Roche, outside the submitted work; RHH reports no conflicts of interest. MRvE reports no conflicts of interest. MRM reports non-financial support from Merck outside the submitted work. SB reports no conflicts of interest. MNNH reports no conflicts of interest. TV reports no conflicts of interest. HL reports no conflicts of interest. JEN reports no conflicts of interest. HRS has served on a scientific advisory board for Lundbeck A/S, Valby Denmark, and has received honoraria as speaker from Biogen Idec, Denmark A/S, Genzyme, Denmark and MerckSerono, Denmark, has received honoraria as editor from Elsevier Publishers, Amsterdam, The Netherlands and Springer Publishing, Stuttgart, Germany, has received travel support from MagVenture, Denmark, and has received a research fund from Biogen-idec. FS has served on scientific advisory boards for, served as consultant for, received support for congress participation or received speaker honoraria from Alexion, Biogen, Bristol Myers Squibb, H. Lundbeck A/S, Merck, Novartis, Roche and Sanofi Genzyme. His laboratory has received research support from Biogen, Merck, Novartis, Roche and Sanofi Genzyme.
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Figures

Figure 1. Concentrations of cytokines and chemokines in patients with PPMS, patients with RRMS, and healthy controls.
Legend (figure 1): Abbreviations: ng/L = nanograms/liter; IL = interleukin; LT = lymphotoxin; VEGF = vascular endothelial growth factor; CCL = chemokine ligand; CXCL = CXCL chemokine ligand; TNF = tumor necrosis factor; PPMS = primary progressive multiple sclerosis; HC = healthy controls; RRMS = relapsing-remitting multiple sclerosis. Boxplots show median and interquartile range, and whiskers show range excluding outliers. *p<0.05, **p<0.005, ***p<0.001, ns = not significant. P-values are derived from general linear model with log-transformed concentrations as dependent variable and group, age, and sex as independent variables (covariates). IL-5, IL-16 and IL-22 were not included in this analysis since no difference was found between groups in the pre-conducted ANOVA analysis (see methods).
Figure 2. Associations between CSF inflammation and MBP, NFL, and IgG-index

Legend (Figure 2): Abbreviations: CCL = chemokine ligand; CXCL = CXCL chemokine ligand; IL = interleukin; LT = lymphotoxin; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor; NFL = neurofilament light chain; MBP = myelin basic protein; IgG = immunoglobulin G. Heatmap based on correlation analyses of baseline concentrations of elevated CSF cytokines/biomarkers. *q<0.05, **q<0.005 (FDR corrected), spearman’s signed rank test.
Figure 3. Associations between CSF cytokines in patients with PPMS.

Legend (Figure 3): Abbreviations: CCL = chemokine ligand; CXCL = CXCL chemokine ligand; IL = interleukin; FA = fractional anisotropy; LT = lymphotoxin; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor. Heatmap based on correlation analyses of baseline concentrations of elevated CSF cytokines/biomarkers. *q<0.05, **q<0.01, ***q<0.001 (FDR corrected), spearman’s signed rank test.
### Tables

| Characteristic                      | PPMS, N = 59\(^1\) | HC, N = 21\(^1\) | RRMS, N = 40\(^1\) | p-value\(^2\) |
|------------------------------------|---------------------|------------------|---------------------|--------------|
| Sex, Female; No (%)                | 24 (41%)            | 11 (52%)         | 30 (75%)           | 0.003        |
| Age; mean (SD)                     | 54.5 (6.5)          | 48.7 (14.6)      | 36.4 (9.5)         | <0.001       |
| Disease duration, years; median [Q1 – Q3] | 11.1 [6.0 – 19.6] | NA               | 1.4 [0.7 – 3.8]    | <0.001       |
| Relapse within last year; n (%)    | 0 (0%)              | NA               | 26 (65%)           | >0.9         |
| EDSS; median [Q1, Q3]              | 4.00 [3.75, 6.00]   | NA               | 2.00 [1.00 – 2.75] | <0.001       |
| CSF cell count ≥5 cells/μL; n (%)  | 13 (22%)            | 0 (0%)           | 30 (75%)           | <0.001       |
| IgG index; mean (SD)               | 0.70 (0.30)         | 0.40 (0.07)      | 1.08 (0.59)        | <0.001       |
| missing                            | 1                   | 2                | 4                   |
| OCBs; n (%)                        | 52 (88%)            | 0 (0%)           | 39 (98%)           | <0.001       |

Table 1. Demographics and disease characteristics.

Legend (Table 1): Abbreviations: EDSS = expanded disability status scale; OCB = oligoclonal band. \(^1\)Statistics presented: n (%); Mean (SD); Median [Q1, Q3]; \(^2\)Statistical tests performed: chi-square test of independence; Kruskal-Wallis rank-sum test; Fisher's exact test.
Supplementary

Figure S1. MRI associations with CSF cytokines in patients with PPMS

Legend (Figure S1): Abbreviations: CCL = chemokine ligand; CGM = cortical gray matter; CXCL = CXCL chemokine ligand; IL = interleukin; FA = fractional anisotropy; LT = lymphotoxin; MTR = magnetization transfer ratio; NAWM = normal appearing white matter; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor. Heatmap based on correlation analyses of baseline concentrations of elevated CSF cytokines/biomarkers. *q<0.05, **q<0.005 (FDR corrected), spearman’s signed rank test.