Glial and neuronal markers in cerebrospinal fluid in different multiple sclerosis types.

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Objective: To investigate glial, neuronal and amyloidal biomarkers in CSF samples from patients with different multiple sclerosis (MS) types.

Methods: CSF levels of neurofilament light protein (NFL), total-tau (t-tau), tau phosphorylated at threonine 181 (p-tau), glial fibrillary acidic protein (GFAP), S-100B protein (S-100B), chitinase 3-like 1 protein (YKL-40), monocyte chemoattractant protein (MCP-1), α-cleaved soluble amyloid-precursor protein (α-sAPP), β-cleaved soluble amyloid-precursor protein (β-sAPP), and 38, 40 and 42 amino acid long fragments of amyloid β (Aβ38, Aβ40, Aβ42) were analysed in 109 clinically isolated syndrome (CIS) patients, 192 relapsing-remitting MS (RRMS), 6 secondary progressive MS (SPMS) and 17 primary progressive MS (PPMS) patients recruited between 20XX and 20YY at the XXX clinic.

Results: The CSF levels of NFL were significantly higher in relapsing-remitting forms of MS, while GFAP levels were higher in progressive forms of MS. No significant differences were found for any of the biomarkers between the RRMS and CIS groups. YKL-40, MCP-1, S-100B, t-tau, p-tau and amyloidal cleavage proteins Aβ38, Aβ40, Aβ42 showed no significant differences among the different MS types. CSF levels of NFL and YKL-40 correlated in CIS patients while GFAP levels and YKL-40 levels correlated in RRMS patients. In relation to relapses, NFL levels were higher when the relapse period was extended to 3 month prior to lumbar puncture. By contrast, GFAP, MCP-1, tau proteins and amyloid-related markers showed increased levels during stable stages; while YKL-40 and S-100B were the less influenced by relapses.

Conclusion: The CSF biomarker profile was similar among different types of MS, but NFL levels were higher in relapsing remitting forms of MS and CSF GFAP was higher in progressive MS (?).

BRIEF ABSTRACT FOR JOURNAL OF NEUROIMMUNOLOGY (100 words)

In the present study, CSF concentrations of NFL, t-tau, p-tau, GFAP, S-100B, YKL-40, MCP-1, α-sAPP, β-sAPP, and Aβ38, Aβ40, Aβ42 were measured in 109 clinically isolated syndrome (CIS) patients, 192 relapsing-remitting MS (RRMS), 6 secondary progressive MS (SPMS) and 17 primary progressive MS (PPMS) patients. The CSF levels of NFL were significantly higher in RRMS while CSF levels of GFAP were higher in PPMS. CSF levels of NFL and YKL-40 correlated in CIS patients while GFAP levels and YKL-40 levels correlated in RRMS patients.

INTRODUCTION

Multiple sclerosis (MS) is classically described as a demyelinating disease of the central nervous system (CNS) due to the histological findings of white matter lesions in MS brains. However, there is evidence that not only myelin and oligodendrocytes are implied in MS pathogenesis, but also neuronal damage and astroglial activation which are important features in the pathogenesis of MS [1-4]. Clinically, MS is considered a chronic autoimmune condition. Even though, the involvement of immune system has been thoroughly described in MS, there is also evidence that diffuse neurodegenerative processes are implied in the MS pathogenesis from the early stages of the disease [5]. Probably, the predominance of autoimmune activation at the onset of the disease explains the inflammatory course of the relapsing-remitting forms of MS. While the

predominance of neuronal and glial degeneration would be associated with progressive forms of MS. Ideally, knowing the underlying histopathological process in each MS patient could lead to an individual treatment for a specific target. Notwithstanding, it is not feasible to obtain brain biopsies. Thus, MS patients are classified by their clinical course as clinically isolated syndrome (CIS), relapsing-remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS), and they are treated according to the international standards.

Cerebrospinal fluid (CSF) is the closest body fluid to brain tissue (ref: Blennow K et al., Nat Rev Neurol 2010). Therefore, CSF from a diagnostic lumbar puncture could reflect the features of brain damage at MS onset and could differentiate the different patterns of MS.

The purpose of the present study was to investigate glial and neuronal biomarkers in CSF samples from patients with different MS types and to test whether a correlation among the biomarkers exists and whether the profile of CSF biomarkers varies among the different MS types. Hence, we analysed biomarkers related to axonal damage (neurofilament light protein: NFL) [3], neuronal injury (total-tau: t-tau and tau phosphorylated at threonine 181: p-tau) [6], glial activation (human chitinase 3-like 1 protein: YKL-40 and monocyte chemoattractant protein: MCP-1) [2,7], astrocytic damage (glial fibrillary acidic protein: GFAP and S-100B protein: S-100B) [1,8], and amyloid metabolism (α-cleaved soluble amyloid-precursor protein: α-sAPP; β-cleaved soluble amyloid-precursor protein: β-sAPP; 38, 40 and 42 amino acid long fragments of amyloid β: Aβ38, Aβ40, Aβ42) [9,10].

MATERIALS AND METHODS

Patients and clinical assessments

The present observational study was approved by the ethics committee of Bellvitge University Hospital, L'Hospitalet de Llobregat, Spain and informed consent was

obtained from all patients. Samples were obtained from the Bellvitge Biomedical Research Institute (IDIBELL), the CSF biobank MS Unit collection. Samples were matched with clinical data from patients recruited and prospectively followed at the MS Unit, Bellvitge University Hospital. All clinical data were entered into the European Database for Multiple Sclerosis (EDMUS) [11]. Patients were diagnosed according to the Poser and McDonald criteria as appropriate and were classified as having CIS, RRMS, SPMS or PPMS according to the disease course when the lumbar puncture was performed [12-14]. For the inclusion into the relapsing group, the first signs of relapse had to start within one month of sampling. The neurological deficits were scored with the Expanded Disability Status Scale (EDSS) [15].

CSF sampling and biochemical analyses

CSF samples were collected in 109 CIS patients, 192 RRMS patients, 6 SPMS patients and 17 PPMS patients by lumbar puncture into polypropylene tubes, centrifuged at 2200 x g for 10 min, aliquoted into 1 mL cryo tubes that were stored at -80^{o}C pending analyses. Concentrations of CSF biomarkers were analysed in blind fashion at the Clinical Neurochemistry Laboratory, Institute of Neuroscience and Physiology, University of Gothenburg, using enzyme-linked immunosorbent assays as described [16].

Statistics

Continuous variables were described by their mean and standard deviation or median and interquartile range, depending on their distribution, and categorical variables by numbers and percentages. Differences between groups were analysed with Kruskal-Wallis test followed by pairwise post hoc comparisons using the Mann-Whitney *U* test, p values ≤ 0.05 were considered significant. Spearman's test was used to analyse

correlations between demographics and CSF biomarker levels, p values ≤ 0.05 were considered significant. Correlations among different biomarkers were analysed using Spearman's test and p values were adjusted using Bonferroni (Holms) correction (p*). Univariate statistical analyses were prepared using Statistical Package for the Social Sciences 20.0 (SPSS Inc, Chicago, IL). Multivariate analysis was performed using orthogonal projection to latent structures discriminant analysis (OPLS-DA) implemented in the software SIMCA-P+ v. 12 (Umetrics, Umea, Sweden) [17].

RESULTS

Demographics and clinical characteristics of MS patients are shown in Table 1.

CSF biomarker levels and demographics

The correlation between age at lumbar puncture and CSF biomarker levels was evaluated in a total of 324 patients. Spearman's test correlation index: NFL: - 0.21, p < 0.0001; GFAP: 0.24, p < 0.0001; YKL-40: 0.16, p = 0.004; S-100B: 0.14, p = 0.01; p-tau: 0.15, p = 0.007; α-sAPP: 0.16, p = 0.004, β-sAPP: 0.15, p = 0.005; no significant correlation was found for any other biomarker. Similar correlations were found in the subgroup of CIS and RRMS patients (n = 301) (Spearman's test correlation index: NFL: - 0.16, p = 0.003; GFAP: 0.21, p < 0.0001; YKL-40: 0.14, p = 0.02; S-100B: 0.15, p = 0.01; p-tau: 0.14, p = 0.02; α-sAPP: 0.12, p = 0.04, β-sAPP: 0.12, p = 0.03). In relation to gender, significantly higher CSF levels of MCP-1, YKL-40, GFAP, p-tau, α-sAPP, β-sAPP, Aβ38, Aβ40, Aβ42 were found in males vs females (Table 2). The subgroup of relapsing-remitting patients (n = 301) showed similar data except for YKL-40 (males: 105 ng/mL (80-165); females 94 ng/mL (67-146), p = 0.04) and GFAP (no significant differences in CSF levels between males and females). There were no significant correlations between the EDSS at lumbar puncture time and CSF biomarker levels except for YKL (CI: 0.17, p = 0.002); α-sAPP (CI: -0.15, p = 0.006) and β-sAPP

(CI: -0.14, p = 0.01). Similar correlations for EDSS were found in the subgroup of relapsing-remitting patients.

CSF biomarker levels in different MS patients

Table 2 shows the CSF biomarker levels for each group of MS patients. NFL levels were significantly higher in relapsing-remitting forms, while GFAP levels were significantly higher in progressive forms (Table 2). YKL-40 levels were higher in the PPMS group compared to the CIS group (Table 2).

CSF biomarker levels and relapses.

Patients from CIS and RRMS groups were evaluated together (n = 301) and were classified to be in the relapsing or remitting phase. CSF levels of GFAP, MCP-1, t-tau, p-tau, α-sAPP, β-sAPP, Aβ38, Aβ40, Aβ42 were significantly lower during relapse vs remission (Table 2). There were no differences in YKL-40 and S-100B levels between the relapsing and remitting stages (Table 2). CSF NFL levels were significantly increased only when the relapse period was extended to three months prior to sampling (relapse: 1455 ng/L, 677 – 2670; remission: 830 ng/L, 520 – 1835; p = 0.009). Hence, the difference in CSF biomarker levels between the relapsing and remitting phase was larger for GFAP, MCP-1 and NFL, while smaller for YKL-40 (Figure 1). Patients of the CIS group were classified depending on their first neurological relapse: long tracts, brainstem, spinal cord and optic neuritis. No association was found between biomarker levels and the type of clinical presentation at the time of first relapse (data not shown).

Correlations among glial markers

In RRMS and CIS patients, significant correlations were observed between GFAP and YKL-40 (CI = 0.52, p = 0.006*), between GFAP and MCP-1 (CI = 0.46, p = 0.006*) and GFAP and S-100B (CI = 0.31, p = 0.006*). In progressive forms of MS no correlation among glial biomarkers was found.

Correlations among neuronal markers

There was a weak but significant correlation between NFL ant t-tau (CI = 0.26, p = 0.006*), while no correlation was found between NFL and p-tau (CI = 0.03, p = 0.57 before Bonferroni correction). P-tau and t-tau had a strong correlation (CI = 0.77, p = 0.004*).

Correlations between glial and neuronal markers

Significant correlations were observed between YKL-40 and NFL, the strongest correlation in the CIS group (CI = 0.59, p = 0.006*). GFAP correlated weakly but significantly with NFL in RRMS (CI 0.25, p = 0.006*) while no other correlation between GFAP and NFL was found after Bonferroni correction. No correlations were found between MCP-1 and NFL. In relapsing-remitting forms of MS, t-tau and p-tau significantly correlated with GFAP (t-tau: CI = 0.49, p = 0.005*; p-tau: CI = 0.42, p = 0.005*) and MCP-1 (t-tau: CI = 0.31, p = 0.005*; p-tau: CI = 0.28, p = 0.005*), while no significant correlations were found after Bonferroni correction between t-tau or p-tau and YKL-40. In PPMS patients, no correlations were found between GFAP, YKL-40 or MCP-1 and NFL, while t-tau and p-tau showed a strong correlation with GFAP and MCP-1 (CI>0.58, $p < 0.02$ for all comparisons, but they were not significant after Bonferroni correction).

Correlations among neuronal, glial and amyloidal biomarkers.

No correlations were found between NFL and amyloidal metabolism biomarkers. While t-tau and p-tau showed strong and significant correlations with α-sAPP, β-sAPP, Aβ38, Aβ40 and Aβ42 (t-tau: CI > 0.45, p-tau: CI > 0.62, p = 0.004* for all correlations). No correlations were found between YKL-40 and amyloidal biomarkers. Furthermore, correlations between GFAP or MCP-1 and amyloidal markers were weak and only significant in the RRMS group (data not shown). Levels of α-sAPP and β-sAPP correlate strongly (CI = 0.93, p = 0.002*). Aβ38, Aβ40 and Aβ42 correlate strongly

amongst themselves (CI > 0.92, p = 0.002* for all correlations) and with α-sAPP (CI > 0.54, p = 0.002* for all correlations) and β-sAPP (CI > 0.58, p = 0.002* for all correlations).

DISCUSSION

In the present study we analysed 12 different biomarkers related to neurons, glia and amyloid metabolism in 324 CSF diagnostic samples of different MS types. The CSF biomarker profile was similar among different types of MS except for NFL, which showed higher levels in relapsing-remitting forms of MS, and GFAP and YKL-40 which showed higher levels in progressive forms of MS. These results are in agreement with previously published data [18,19]. The CIS and RRMS groups showed the same profile of biomarkers. Our cohort of CIS patients included not only patients that presented a first neurological episode suggestive of MS but also fulfilled MR Barkhof criteria (ref). We also evaluated the influence of the relapses in the CSF biomarker levels. NFL showed the highest levels in the relapsing group when the relapse period was extended to 3 months prior to lumbar puncture. This finding is in agreement with previous reports and could be explained because the inflammatory process, first provokes demyelination and after, axonal degeneration that ends with the release of NFL to the environment. Then, the increasing of NFL levels in the CSF happens with a brief delay in time from the onset of the relapse [20]. In contrast, MCP-1 showed increased levels in the stable stages. There is evidence that in MS lesions, MCP-1 is mainly expressed by reactive astrocytes and its overexpression mediates the recruitment of brain resident immune cell and the recruitment of infiltrating monocytes from the systemic bloodstream to the lesion sites as an initial mechanism of inflammatory response to a brain injury [21]. However, the sustained release of MCP-1 prolongs the inflammation and provokes cytotoxicity [21]. Therefore, we could hypothesize that CSF levels of MCP-1 increase during the relapse period as a response to tissue damage and they continue increasing beyond the period of time

considered as clinical relapse. GFAP, tau proteins and amyloidal markers also showed increased levels during the stable stages, probably as a product of cell death after severe inflammatory processes or continuous neurodegeneration as explained above. YKL-40 and S-100B were the biomarkers that were the least influenced by relapses as we previously reported [16]. Some studies have proposed the regulation of inflammation and the inhibition of apoptosis as the main roles of YKL-40 in inflammatory diseases [2, 22]. Moreover, YKL-40 seems to be involved in tissue remodelling [22]. Thus, the biological functions of YKL-40 could explain its continuous release by activated astrocytes in MS, independently of the MS stage. The influence of relapses on CSF biomarker levels should be confirmed in future studies, since it could be useful to know whether to consider the time of the lumbar puncture is important or not for sampling in clinical trials or MS studies. The influence of age in CSF biomarker levels in healthy controls and MS patients has been described with controversial results, probably because the influence of the disease on biomarker levels is stronger than the influence of the age [2, 16, 20, 23]. In the age range of our study, we observed weak correlations between age and the different biomarkers, being still less significant in relapsing-remitting forms of MS. However, neither older nor younger ages have been explored. Therefore, we observed a negative correlation between NFL levels and age, while GFAP showed a positive correlation with age. This could be explained because younger patients were those in the early stages of the relapsing-remitting forms of MS and they probably were in a more inflammatory phase of the disease, while older patients were those in the progressive forms of MS. In relation to gender, glial and amyloidal markers showed higher levels in males, however these differences were less significant in the relapsing-remitting group, except for MCP-1 which showed significantly higher levels in males. We could hypothesize that these differences could be related to the higher prevalence of more severe MS forms in males associated with a prolonged inflammation and neurodegeneration [24]. Contrary to what we expected, the influence of the disability rate on the CSF biomarker levels was spurious. This

could be explained because CSF biomarker levels could reflect more accumulative underlying pathological processes than disability scores at a specific point in time, especially if the scores are focused on motor tasks as EDSS [15]. CSF samples were obtained close to the time of MS diagnosis. Only 11 patients (7 RRMS and 4 SPMS) were under treatment at the time of lumbar puncture. Although we can not exclude that the treatment influenced biomarker levels in SPMS group, we consider that MS treatment did not influence the results of the whole study. Looking at the correlations among different biomarkers, we have to consider that some biomarkers are products of axonal degeneration (NFL), astrogliosis (GFAP) or glial activation (YKL-40 and MCP-1), products that are released into the environment and the CSF; while other markers like tau proteins and amyloid metabolism products aggregate and their influx to CSF is reduced. This could explain stronger correlations among glial markers than those between axonal and neuronal markers. Another factor to take into account is the size of the cohorts, which could explain, for instance, that no correlations were found among glial markers in progressive forms of MS. Nevertheless, in spite of these limitations, interesting correlations were found. Thus, levels of YKL-40 correlated significantly with NFL levels, especially in the CIS group. This finding could be explained because both markers are associated with early immune processes since YKL-40 is secreted by activated astrocytes to regulate the inflammation and NFL are a product of axonal degradation after demyelination. This finding is in agreement with previous studies, which showed that CIS patients with high levels of both NFL and YKL-40 were associated with a shorter time before a second relapse [16, 25]. Moreover, significant correlations between YKL-40 and GFAP were observed in CIS and RRMS patients, which were expected since YKL-40 is released by activated astrocytes and GFAP is a product of mature astrocyte degradation after immune responses. Furthermore, this finding is in agreement with our previous work which showed that high levels of both YKL-40 and GFAP were independent risk factors for disability progression in RRMS patients [16].

In conclusion, the present study is the first that evaluates a variety of biomarkers from the same CSF sample from patients with different MS types and analyses demographic, clinical and biological data. We observed interesting results in the group of relapsing-remitting forms of MS, which showed higher CSF levels of NFL and a strong correlation between YKL-40 and NFL and between YKL-40 and GFAP.

Figure1

Title: Importance of CSF biomarkers to discriminate active relapse from stable phase in relapsing-remitting MS.

Footnote: Variable importance on the projection plots. The graph shows the relative contribution of the biomarkers to discriminate between patients in active relapse vs. stable phases in the relapsing-remitting forms of MS group. The error bars represent 95% confidence intervals.#