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Cerebrospinal fluid GAP-43 in early multiple sclerosis

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Abstract

Background/Objective: Novel biomarkers identifying and predicting disease activity in multiple sclerosis (MS) would be valuable for primary diagnosis and as outcome measures for monitoring therapeutic effects in clinical trials. Axonal loss is present from the earliest stages of MS and correlates with disability measures. Growth-associated protein 43 (GAP-43) is a presynaptic protein with induced expression during axonal growth. We hypothesized this protein could serve as a biomarker of axonal regeneration capacity in MS.

Methods: We developed a novel GAP-43 enzyme-linked immunosorbent assay for quantification in cerebrospinal fluid (CSF) and measured GAP-43 levels in 71 patients with clinically isolated syndrome, 139 MS patients and 51 controls.

Results: GAP-43 concentrations were similar in patients and controls. Nevertheless, GAP-43 levels were higher in patients with >10 T2-magnetic resonance imaging (MRI) lesions ($p = 0.005$). CSF GAP-43 concentrations correlated with CSF mononuclear cell counts ($p = 0.031$) and were inversely correlated with patient age ($p = 0.038$) with a trend for higher CSF GAP-43 concentrations in patients with gadolinium-enhancing MRI lesions and positive CSF oligoclonal immunoglobulin G status.

Conclusion: Our results suggest that axonal regeneration capacity is relatively preserved in early MS. CSF GAP-43 concentration is positively associated with markers of inflammation, suggesting possible inflammatory-driven expression of this growth-associated protein in early MS.

Keywords: Multiple sclerosis, cerebrospinal fluid, biomarkers, axonal loss, axonal regeneration, growth-associated protein 43

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Introduction

Multiple sclerosis (MS) is a chronic, inflammatory and degenerative disease of the central nervous system (CNS) that affects young people and is a major cause of disability in this age group. MS usually starts with relapses and remissions and progression occurs later, while clinically isolated syndrome (CIS) refers to the first attack of the disease.[1] Many immunomodulatory agents that reduce the relapse rate and disability progression are available for MS and often are also prescribed to patients with CIS.[1]

Because most disease-modifying drugs for MS have safety and tolerability issues (for example, increased risk for progressive multifocal leukoencephalopathy in patients treated with natalizumab), treatment decisions are sometimes difficult. Biomarkers that could predict disease course and response to treatment are needed and would help in the clinical management of MS patients.

Different neuronal and glial proteins in cerebrospinal fluid (CSF) have been investigated for their diagnostic and prognostic potential in MS.[2] Axonal loss, which is present from the earliest stages of the disease, correlates importantly with clinical disability and is crucial for disease progression.[3,4] Correspondingly, increased concentration of neurofilament light chains (NFLs) released into the CSF following axonal damage was shown to predict relapses, disability progression and development of gadolinium (Gd)-enhancing lesions. [2,4,5]

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Complete recovery observed during early remissions is mainly due to remyelination and resolution of inflammation.[1] However, some studies indicate that compensation of axonal damage may also occur in MS.[6,7] Axonal remodeling and concurrent expression of growth-associated protein 43 (GAP-43) were demonstrated in an animal model of MS.[7] GAP-43 is a known marker of growth cones, synaptic plasticity and synaptic and axonal regeneration.[8] It was shown to drive spontaneous sprouting of axons in transgenic mouse lines overexpressing GAP-43.[9] GAP-43 synthesis was demonstrated in developing neurons and was upregulated after axotomy simultaneously with or prior to initiation of axonal outgrowth.[8,10] Alongside traumatic injury inflammatory lesions may as well induce changes in axonal connectivity with parallel expression of GAP-43.[7] In the post mortem MS brain increased levels of GAP-43 were observed in remyelinated white matter, which possibly reflects regeneration attempts of the damaged axons.[6] In the same study CSF GAP-43 concentrations were negatively correlated with magnetic resonance imaging (MRI) measures of atrophy, indicating lower numbers of axons in the brain.[6] Furthermore, lower CSF GAP-43 levels were found in patients with secondary progressive MS compared with relapsing–remitting MS (RRMS), implying lost or reduced regenerating capacity of axons in late MS.[11]

We measured CSF GAP-43 concentration in a large number of CIS and early RRMS patients as well as in control individuals to test whether CSF GAP-43 concentration could serve as an axonal regeneration marker in MS. Most CIS and MS patients were clinically assessed by Expanded Disability Status Scale (EDSS) score, CSF and MRI analysis, enabling us to study possible associations between GAP-43 concentration and disease activity and severity. If axonal regeneration capacity, as evidenced by CSF GAP-43, is preserved in early MS, similar concentrations in patients and control individuals (or even higher in patients) would be observed. Thus, measurement of CSF GAP-43 could possibly be used as a biomarker for axonal regeneration potential in an individual with MS.

Materials and methods

Patient selection

A total of 210 patients, 71 with CIS (49 (69%) women), and 139 with MS (92 (66%) women) and 51 control individuals (30 (59%) women) were included in the study. Among MS patients 118 had RRMS, 19 primary progressive MS and two secondary progressive MS. The diagnosis of MS was established according to the McDonald criteria, and hospital charts of CIS and MS patients from the Department of Neurology, University Medical Centre, Ljubljana, were reviewed for clinical and paraclinical data at the time of lumbar puncture. Outpatient charts were also reviewed for clinical data at one-year follow-up (EDSS increase of $\geq$0.5 points, relapses, disease-modifying treatments). The control group included patients with degenerative spine diseases, primary headaches and psychiatric symptoms. The study was approved by the Republic of Slovenia National Medical Ethics Committee (number 0120-150/2017-3). All participants provided written informed consent for lumbar puncture and permitted future research with the remaining samples.

Routine CSF analysis

CSF samples were also collected at the Department of Neurology, University Medical Centre, Ljubljana, from 2013 to 2016 for routine diagnostic procedures in all patients and controls. The remaining CSF was centrifuged and afterward stored at −80°C. Total CSF protein, albumin, and immunoglobulin (Ig)G and IgM levels were determined with nephelometry (Siemens BN ProSpec). Oligoclonal IgG bands (OBs) were detected by isoelectric focusing and immunoblot with alkaline phosphatase-labeled anti-human IgG.

GAP-43 determination

Nunc-Immuno Polysorp microwell modules (Thermo Fisher Scientific, MA, USA) were coated with mouse anti-GAP-43 antibody (0.77 µg/ml NM4, Fujirebio, Tokyo, Japan) in carbonate buffer pH 9.6, overnight at 4°C. After washing, wells were blocked with phosphate-buffered saline (PBS)/0.05%Tween/1x Casein (10x Casein blocking buffer, B6429, Sigma-Aldrich, MO, USA) for one hour at room temperature. Thereafter, full-length, in-house recombinant GAP-43 calibrators (78 pg/ml–5000 pg/ml), blanks, prediluted control samples and CSF samples in assay diluent (1%BSA/PBS/0.05%Tween) were coincubated with a rabbit anti GAP-43 detector antibody (0.14 µg/ml Cat. no: ABB-135, produced through immunization with aa216-238 of GAP-43, Nordic Biosite, Täby, Sweden) overnight at 4°C. After additional washes, plates were incubated with anti-rabbit horseradish peroxidase (1:30,000, Promega, WI, USA) for 1.5 hours. After subsequent washes, wells were incubated for 20 minutes with 3,3′,5,5′-tetramethylbenzidine (TMB, KemEnTech Diagnostics, Taastrup, Denmark) in the dark.

The color reaction was stopped by addition of 0.2 M H_2SO_4 and the absorbance was read in a Sunrise™ microplate absorbance reader (Tecan group, Männedorf, Switzerland) at 450 nm (650 nm as reference value). CSF sample concentration was calculated via interpolation from the calibrator curve (four-parameter logistic fit weighted $1/Y^2$).

In-house GAP-43 enzyme-linked immunosorbent assay (ELISA) characterization

The performance of the novel ELISA was evaluated in leftover CSF samples from clinical routine. The assay precision was determined by measuring the GAP-43 concentration in two CSF pools, aliquoted and stored at −80°C, in five to seven duplicates on three different occasions. The lower limit of quantification was calculated as the mean of 16 blank duplicates plus 10 times its standard deviation. Sample dilution linearity was measured by dilution of three CSF samples with endogenously high GAP-43 levels and diluting 1:1–1:4. Recovery was evaluated by dividing two CSF samples in four aliquots and spiking with 0, 500, 750 or 1000 pg/ml calibrator before analysis. As GAP-43 shares a protein domain with neurogranin,[12] specificity of the assay was assessed by spiking full-length, in-house recombinant neurogranin at 1000–50,000 pg/ml into aliquots of two CSF samples that were analyzed simultaneously with a neat aliquot. Sample stability was evaluated by dividing six samples into nine aliquots, where one was directly placed at −80°C, and the others stored at –20°C or at 4°C overnight or for one week, at room temperature for 24 hours, or freeze-thawed one to four times.

NFL immunoassay

CSF NFL quantification was performed in a subgroup of CIS/MS patients using Uman Diagnostics NF-light® ELISA according to the manufacturer's instructions. The assay is based on anti-NFL monoclonal antibodies and has a detection limit of 32 pg/ml. Intraassay coefficient of variation (CV) was <5% and an internal control sample was used to check the validity of results.

Statistics

Statistical analysis was performed with GraphPad Prism, version 6 (GraphPad Software, San Diego, CA, USA). Fisher, Mann–Whitney *U* and Kruskal–Wallis tests were used as appropriate for comparison of the data, and Spearman coefficient was calculated for correlation analyses.

Results

Demographic and clinical characteristics

Patients with CIS and MS were slightly younger than controls ($p = 0.030$) with a median age of 36 years (range, 21–55), 38 years (range, 15–70) and 46 years (range, 15–72) in the CIS, MS and control groups, respectively. Median duration of the disease was 0 years (range, 0–3) in CIS and one year (range, 0.5–25) in MS patients. Median EDSS score in patients was 2 (range, 0–7), in patients with CIS 1.25 (range, 0–3.5) and in patients with MS 2 (range, 0–7).

Out of 210 patients, 33 were lost to follow-up and 88 patients (as expected in a real-life setting) started with disease-modifying treatment. From 89 untreated patients (47 MS and 42 CIS patients), 13 (15%, 11 with MS and two with CIS) developed disability progression (>0.5 points on EDSS score) and 16 (18%, seven with MS and nine with CIS) had one or more relapses. None of the patients had both relapses and disability progression.

In-house GAP-43 ELISA characterization

The repeatability and interassay precision of a CSF pool with a concentration of 2992 pg/ml was 7.3% CV and 7.8% CV respectively, and for a CSF pool of 719 pg/ml, the repeatability was 9.8% CV and the interassay precision was 13.7% CV. The lower limit of quantification was 156 pg/ml and sample dilution linearity was 95% on average. Recovery of calibrator standard was 106% on average. Both dilution linearity and recovery were within recommended levels according to Andreasson et al.[13] Spiking in recombinant neurogranin (1–50 ng/ml) did not affect the GAP-43 concentration (11% CV, recovery of neurogranin –3% to 0%). CSF GAP-43 measurements were stable after one to four freeze-thaw cycles ($n = 9$; 100.9%–107.9%) and various storage temperatures ($n = 7$; 94.8%–103.5%, percentage of −80°C stored aliquot concentration).

GAP-43 and main CSF characteristics

Similar CSF GAP-43 levels were observed in controls and patients ($p = 0.516$). Median concentrations were 1701 pg/ml (range, 318–2987), 1497 pg/ml (range, 183–4573) and 1622 pg/ml (range, 426–7108) in the control, CIS and MS groups, respectively (Figure 1). There was no correlation between GAP-43 and CSF/serum albumin ratio ($p = 0.3551$).

CSF mononuclear white cell counts were elevated in 34 out of 70 (49%) CIS patients and in 58 out of 135 (43%) MS patients. Median GAP-43 concentrations

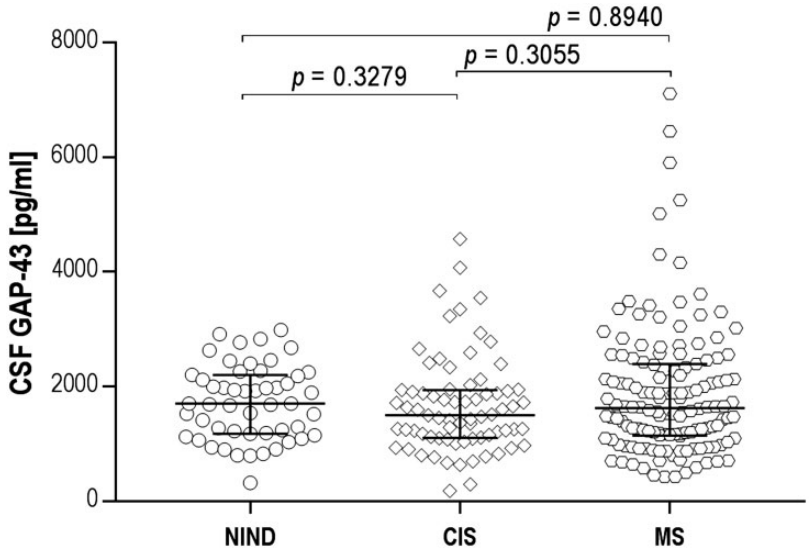

Figure 1. CSF GAP-43 in patients and control individuals.
CIS: clinically isolated syndrome; CSF: cerebrospinal fluid; GAP-43: growth-associated protein 43; MS: multiple sclerosis; NIND: noninflammatory neurological disease.

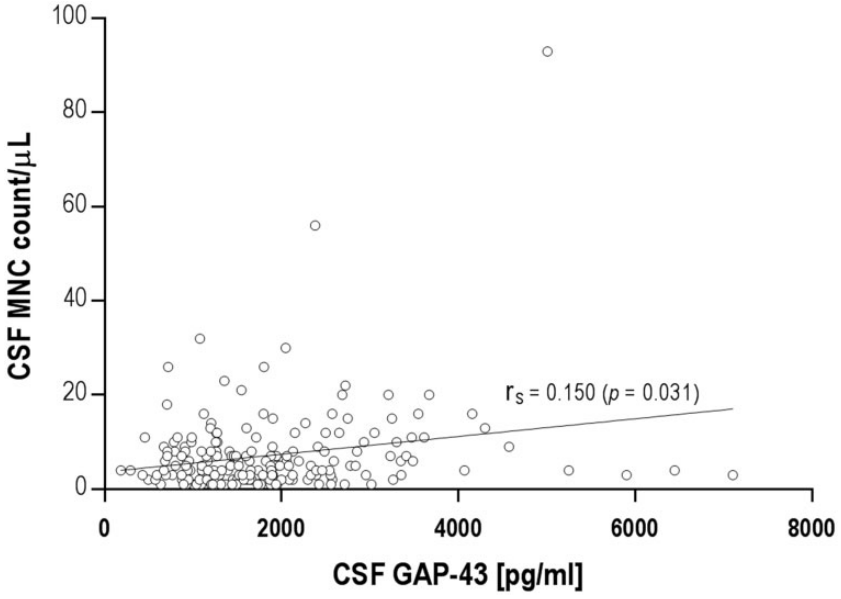

Figure 2. GAP-43 concentrations are in correlation with CSF MNC.
CSF: cerebrospinal fluid; GAP-43: growth-associated protein 43; MNC: mononuclear cell counts.

were 1649 pg/ml (range, 453–5011) in patients with elevated CSF cell counts compared with 1516 pg/ml (range, 182–7108) in patients with normal CSF cell counts; $p = 0.1310$. A weak but statistically significant correlation of CSF GAP-43 concentration with CSF mononuclear cell count was found ($r = -0.150$, $p = 0.031$; Figure 2). OBs were detected in 77% (55/71) of CIS patients and 94% (128/136) of MS patients. Median GAP-43 levels trended higher in OB-positive than in OB-negative patients; 1598 pg/ml (range, 293–7108) compared with 1380 pg/ml (range, 1832–3262); $p = 0.080$. Intrathecal IgM synthesis was present in 12/67 (18%) CIS and 32/131 (24%) MS patients. Median GAP-43 concentration did not differ between IgM-negative (1638 pg/ml; range, 182–7108) and IgM-positive patients (1504 pg/ml; range, 453–4573, $p = 0.7918$).

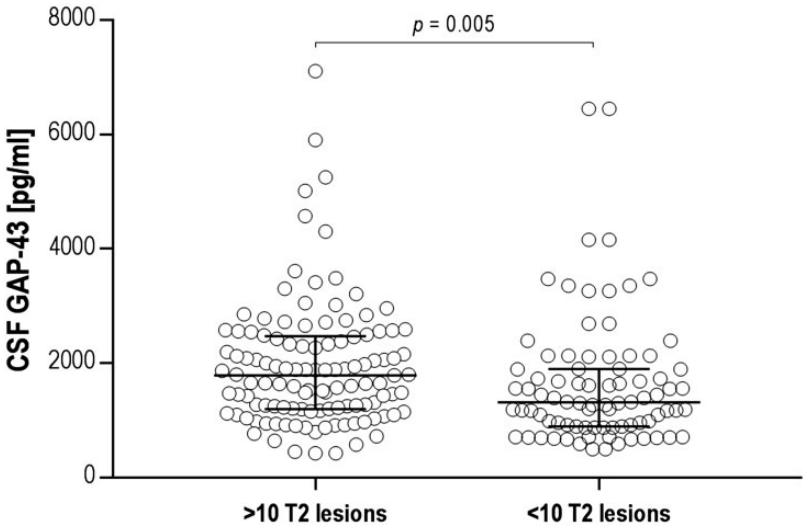

Figure 3. GAP-43 and T2-brain MRI lesions.
CSF: cerebrospinal fluid; GAP-43: growth-associated protein 43; MRI: magnetic resonance imaging.

GAP-43 and MRI lesions

A high number of T2 lesions (>10) were present in 24/66 (36%) CIS patients and in 94/136 (70%) MS patients. Significantly higher CSF GAP-43 concentration was observed in patients with >10 T2 lesions (median 1788 pg/ml, range, 426–7108) compared with patients with lower numbers of T2 lesions (median 1310 pg/ml, range, 495–6447; $p = 0.005$, Figure 3). Nevertheless, when patients with high numbers of T2 lesions were further divided into an MS and CIS group, the difference was significant only for MS patients ($p = 0.026$). Forty-three percent of all CIS and MS patients (77/181) had Gd-enhancing lesions, while spinal cord lesions were present in 20/24 (83%) CIS patients and 58/65 (89%) MS patients. There was a trend for higher GAP-43 concentrations in patients with Gd-enhancing lesions (median 1727 pg/ml, range, 426–5906) compared with patients without Gd-enhancing lesions (1504 pg/ml, range, 293–7108; $p = 0.2002$). Median CSF GAP-43 levels did not differ significantly between patients with or without spinal cord lesions ($p = 0.588$).

GAP-43 and clinical features

Overall there was no correlation between GAP-43 concentrations and age ($r = -0.078$; $p = 0.2106$), but a weak negative correlation between GAP-43 levels and age was found in patients ($r_s = -0{,}145$; $p = 0.038$), which was not seen in controls ($r_s = 0.145$; $p = 0.312$). GAP-43 concentrations were not associated with gender ($p = 0.745$). There was no correlation between GAP-43 and disability according to EDSS score ($r_s = -0.101$; $p = 0.150$) and no association with disease duration ($p = 0.909$). Prolonged latencies of visual evoked potentials (VEP) were found in 4/13 (31%) CIS and 27/40 (67%) MS patients, but median GAP-43 concentrations were not significantly different among patients with prolonged and normal VEP ($p = 0.499$). Patients who progressed had lower median GAP-43 concentrations, 1033 pg/ml (range, 495–2715) than patients without progression, 1555 pg/ml (range, 293–6447); $p = 0.0269$. Median GAP-43 concentrations trended higher in patients with relapses, 1897 pg/ml (range, 691.5–4573) compared with patients without relapses at one year, 1446 pg/ml (range, 293–6447); $p = 0.1059$.

GAP-43 and NFL. In addition to GAP-43, CSF NFL were measured in 85 MS and CIS patients with one-year follow-up data. Median NFL concentrations were 552 pg/ml (range, 46–3200). There was a trend for patients with CIS to have lower concentrations of NFL, 494.7 pg/ml (range, 46.3–3200) than patients with MS, 582.9 pg/ml (range, 154.2–2060); $p = 0.1405$. Median CSF NFL concentrations were similar in patients with relapses, 468 pg/ml (range, 46–1784) and in patients without relapses, 566 pg/ml (range, 76–3200); $p = 0.5112$ in the first year. There was also no difference between patients with progression in the first year, whose median NFL were 557 pg/ml (range, 360–2360), and patients without progression, whose median NFL were 548 pg/ml (range, 46–3200); $p = 0.1916$. Moreover, no correlation between GAP-43 and NFL concentrations was observed ($r = 0.090$; $p = 0.4050$) in this subgroup of patients.

Discussion

CSF GAP-43 concentrations in our CIS and RRMS patients were not different compared with control individuals without neurodegeneration and CNS inflammation. This suggests that axonal regeneration capacity is relatively preserved in early MS.

Our findings are in contrast with the results of Gnanapavan et al., who found low GAP-43 concentrations in different stages of MS in comparison with controls.[14] Gnanapavan et al. used NM2 as capture antibody, and another commercial C-terminal GAP-43 antibody as detector in their in-house ELISA. NM2 is known to have an epitope spanning an IQ domain and thereby binding both GAP-43 and neurogranin,[12] which is not the case for NM4. In addition, Gnanapavan and coauthors used a modified recombinant protein as calibrator. These differences may explain why our results are not directly comparable. On the other hand, in a study by Häggmark et al. in which antibody-based CSF profiling was used there was a difference between CSF GAP-43 concentrations between secondary progressive and RRMS patients. Patients with progressive MS had lower CSF GAP-43 concentrations, indicating lost or reduced regenerative potential in late MS.[11] In a neuropathological study by Teunissen et al., however, increased GAP-43 expression in remyelinated or nondemyelinated white matter in the vicinity of MS plaques was found and may confirm regenerative attempts by the damaged axons. The authors also report a negative correlation between CSF GAP-43 concentrations and brain atrophy measures.[6] Another pathological study showed that neuroaxonal regeneration was more pronounced in early MS compared with traumatic brain injury.[15] CSF GAP-43 increases that may reflect inherent attempts to limit axonal damage were also observed in primarily neurodegenerative diseases.[16,17] For example, tissue-specific changes in GAP-43 expression and higher CSF GAP-43 concentrations were found in patients with Alzheimer disease compared with controls.[16,17] This in contrast was not seen in patients with frontotemporal dementia.[18]

In our study, CSF GAP-43 concentrations were significantly higher in patients with more than 10 T2-MRI lesions compared with those with a lower number of T2 lesions. They correlated weakly with CSF mononuclear cell counts and were inversely correlated with patient age. There was also a trend for higher GAP-43 levels in patients with Gd-enhancing lesions and positive OBs, which could suggest that more pronounced inflammation in MS induces more axonal regeneration. Though this hypothesis might appear speculative, beneficial effects of inflammation on endogenous repair mechanisms in MS and experimental demyelination have already been reported. For example, it was shown that inflammation associated with CNS demyelination provided a stimulus for the activation of oligodendrocyte precursor cells with subsequent remyelination.[19] One could argue that higher levels of CSF GAP-43 were found in patients with high numbers of T2 lesions in whom plaques could consist mainly of demyelination, neurodegeneration or gliosis. But it is also known that in early plaques (our CIS and MS patients had median disease duration of 0 or 1.25 years, respectively), inflammation and demyelination processes are predominant.[20] Unfortunately MRI data on brain atrophy were not available for our patients, thus we were not able to examine the association between CSF GAP-43 and brain atrophy.

Our follow-up data are short (one year) and incomplete as is expected in a real-life setting. Fifteen percent of patients were lost to follow-up and more than 40% of the included patients immediately started with disease-modifying agents. The number of patients with relapses or disability progression in our untreated patients was low. This is not surprising because patients with higher risk of clinical activity started disease-modifying treatment at the diagnosis. The untreated patients who progressed in the first year had lower GAP-43 levels, which confirms the role of GAP-43 as an axonal regeneration marker. On the other hand, GAP-43 concentrations trended higher in patients with relapses in the first year, which is in agreement with our cross-sectional findings showing that the expression of GAP-43 could be inflammatory driven. It would be very interesting to see whether the effect seen on relapses was also present on MRI, but unfortunately our control MRI data were not good enough for inclusion in the study.

CSF NFLs were also measured in a selected group of patients with follow-up information and trended lower in CIS compared with MS patients, which is expected—less axonal loss is present in patients with the earliest MS. Absence of correlation between GAP-43 and NFLs does not come as a surprise. Namely, GAP-43 levels were shown to be at the same time positively associated with inflammatory activity of early MS (high brain MRI lesions at the diagnosis and relapses in first year) but on the other hand negatively associated with disability progression. We found no correlation between NFL and

clinical activity in MS, which was disappointing. Absence of correlation could be due to biased selection of patients—CSF NFLs were determined only in untreated MS (even more often CIS) patients with follow-up data who had milder clinical course and fewer MRI lesions. Furthermore, the duration of follow-up was too short.

Lumbar puncture with CSF examination is an important part of diagnostic workup in patients with suspected MS and was again included in the latest revisions to the McDonald criteria.[21] Besides biomarkers that support the diagnosis of MS (e.g. OBs), there is room for biomarkers that could predict the clinical course in an individual. This is extremely important because more than 15 drugs with different efficacy, safety and tolerability profiles are now available for MS. With the CSF markers of axonal loss (NFLs) or microglial activation (YKL-40, TREM-2), one can predict early unstable disease and late disability in a patient with MS.[2,4,22] Perhaps biomarkers for axonal regeneration capacity, GAP-43 or others, could in the future provide additional useful information in treatment decision making.

Some of our MS patients, as seen in Figure 1, had very high concentrations of GAP-43. Longitudinal studies should be undertaken to reveal whether these individuals are better off over time and more responsive to immunomodulatory treatment.

If remodeled axonal connections are proven to be sufficient for functional recovery, further research on GAP-43 and axonal regeneration potential in MS will also be justified in view of novel therapeutic strategies.

To conclude, CSF levels of GAP-43 were similar in patients with CIS, RRMS and controls, indicating that axonal regeneration potential is preserved in early MS. We also found that indicators of CNS inflammation are associated with CSF GAP-43, suggesting that expression of this growth-associated protein may be inflammatory driven in early MS.

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Conflict of Interests

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Supplemental material

Supplementary material is available for this article online.

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