CSF Chitinase 3–Like 2 Is Associated With Long-term Disability Progression in Patients With Progressive Multiple Sclerosis

Manuel Comabella, MD, PhD, Jaume Sastre-Garriga, MD, PhD, Eva Borras, PhD, Luisa M. Villar, MD, PhD, Albert Saiz, MD, PhD, Sergio Martínez-Yélamos, MD, PhD, Juan Antonio García-Merino, MD, PhD, Rucsanda Pinteac, MSc, Nicolas Fissolo, PhD, Antonio J. Sánchez López, PhD, Lucienne Costa-Frossard, MD, Yolanda Blanco, MD, Sara Llufriu, MD, PhD, Angela Vidal-Jordana, MD, PhD, Eduard Sabidó, PhD, and Xavier Montalban, MD, PhD

Correspondence
Dr. Comabella
manuel.comabella@vhir.org

Abstract

Objective

This study aimed to identify long-term prognostic protein biomarkers associated with disease progression in patients with progressive multiple sclerosis (MS).

Methods

CSF samples were collected from a discovery cohort of 28 patients with progressive MS who participated in a clinical trial with interferon beta. Patients were classified into high and low disability progression phenotypes according to numeric progression rates (NPR) and step-based progression rates (SPR) after a mean follow-up time of 12 years. Protein abundance was measured by shotgun proteomics. Selected proteins from the discovery cohort were quantified by parallel reaction monitoring in CSF samples from an independent validation cohort of 41 patients with progressive MS classified also into high and low disability progression phenotypes after a mean follow-up time of 7 years.

Results

Of 2,548 CSF proteins identified in the discovery cohort, 10 were selected for validation based on their association with long-term disability progression: SPATS2-like protein, chitinase 3–like 2 (CHI3L2), plasma serine protease inhibitor, metallothionein-3, phospholipase D4, beta-hexosaminidase, neurexophilin-1, adipocyte enhancer-binding protein 1, cathepsin L1, and lipopolysaccharide-binding protein. Only CHI3L2 was validated, and patients with high disability progression exhibited significantly higher CSF protein levels compared with patients with low disability progression ($p = 0.03$ for NPR and $p = 0.02$ for SPR). CHI3L2 levels showed good performance to discriminate between high and low disability progression in patients with progressive MS (area under the curve 0.73; sensitivity 90% and specificity 63%).

Conclusions

Although further confirmatory studies are needed, we propose CSF CHI3L2 as a prognostic protein biomarker associated with long-term disability progression in patients with progressive MS.

From the Unitat de Neuroimmunologia Clínica (M.C., J.S.-G., R.P., N.F., A.V.-J., X.M.), Hospital Universitari Vall d´Hebron; Eva Borràs (E.B., E.S.), Proteomics Unit, Universitat Pompeu Fabra, Barcelona; Departments of Neurology and Immunology (L.M.V., L.C.-F.), Hospital Universitario Ramón y Cajal, Instituto Ramón y Cajal de Investigacion Sanitaria, Madrid; Service of Neurology (A.S., Y.B., S.L.), Hospital Clinic and Institut d'Investigacions Biomèdiques August Pi Sunyer, University of Barcelona; Department of Neurology (S.M.-Y.), Bellvitge University Hospital, Barcelona; and Neuroimmunology Unit (J.A.G.M., A.J.S.L.), Hospital Universitario Puerta de Hierro, Madrid, Spain.

Glossary

AEBP1 = adipocyte enhancer-binding protein 1; **AGC** = auto gain control; **CHI3L2** = chitinase 3–like 2; **CIS** = clinically isolated syndrome; **CTSL** = cathepsin L1; **EDSS** = Expanded Disability Status Scale; **HEXB** = hexosaminidase; **IFNβ** = interferon beta-1b; **MS** = multiple sclerosis; **MT3** = metallothionein-3; **NFL** = neurofilament light chain; **NPR** = numeric progression rates; **NXPH1** = neurexophilin-1; **PLD4** = phospholipase D4; **PPMS** = progressive MS; **PRM** = parallel reaction monitoring; **ROC** = receiver operating characteristic; **RRMS** = relapsing-remitting course; **SERPINA5** = plasma serine protease inhibitor; **SPATS2L** = SPATS2-like protein; **SPR** = step progression rates; **UPLC** = ultra-performance liquid chromatography.

Classification of Evidence

This study provides Class II evidence that high CSF CHI3L2 levels identified higher disability progression in patients with progressive MS.

Multiple sclerosis (MS) is a neurodegenerative immune-mediated disorder of the CNS and an important cause of disability among young adults.[1] Although the etiology of MS is unknown, it is assumed that both a complex genetic background and environmental triggers contribute to disease manifestation.[2-6] In approximately 80%–85% of patients, MS starts with acute episodes of neurologic dysfunction and follows a relapsing-remitting course (RRMS).[1] However, over time, a significant number of these patients will enter a secondary progressive phase of the disease which is characterized by maintained neurologic deterioration with or without further relapses.[7] The remaining 15%–20% of patients with MS follow a progressive course from disease onset, and they are referred to as primary progressive MS (PPMS).[8] Whereas currently available therapies in MS are highly effective to suppress the predominantly inflammatory component observed in patients with RRMS, these therapies have proven to be ineffective in patients with progressive forms of MS in whom a neurodegenerative component dominates.[9,10] In this setting, a more in-depth understanding of the prognostic factors associated with disability progression will be critical to stratify patients with progressive MS according to disease evolution and ultimately may set the rationale for the design of more specific therapeutic approaches to prevent disease progression. In this study, we aimed to identify long-term prognostic biomarkers predictive of heightened disability progression in patients with progressive MS.

Methods

Discovery Cohort

Twenty-eight patients with progressive MS (17 patients with primary progressive forms [PPMS][11] and 11 patients with transitional progressive forms[8]) who participated in a 2-year phase II randomized, double-blind, placebo-controlled trial of interferon beta (IFNβ)-1b[12] were included in this study. Selection of patients was performed based on the availability of CSF samples during the trial, and as shown in Table 1, this cohort was representative of the entire cohort that participated in the trial for demographics and clinical characteristics.

Thirteen patients (46.4%) received IFNβ during the 2-year trial. Except for 1 patient treated with IFNβ during the trial who received mitoxantrone for 1 year after trial completion and then was treated with IFNβ for 11 additional years, the remaining 27 patients included in this study were untreated during the whole follow-up period. Progression rate variables were created by dividing Expanded Disability Status Scale (EDSS) changes, confirmed after 6 months, by the time on follow-up. Because many patients were in the range of EDSS where a change in 0.5 points corresponds to 1 EDSS step increase, 2 different variables were computed using these 2 different approaches: numeric progression rates (NPR) and step-based progression rates (SPR). Progression rates were computed between the time of trial onset and the last visit, and between the time after trial completion and the last visit. High disability progression was defined by the presence of NPR or SPR above the 75th percentile of disability progression. Patients with progression rates below the 75th percentile were defined as low disability progression. Demographic and clinical characteristics of patients with high and low disability progression are summarized in eTable, links.lww.com/NXI/A581.

Chromatographic and Mass Spectrometric Analysis in the Discovery Cohort

CSF samples were collected by lumbar puncture during the trial both in placebo and IFNβ-treated patients (mean time [range] from trial onset: 13 months [8.8–17.9]). CSF samples were centrifuged to remove cells, and the remaining volume was aliquoted and conserved at −80°C until used. For each sample, 120 μL of CSF were used for protein precipitation in cold acetone overnight at 4°C. Pellets were then solubilized in 6 M urea in 200 mM ammonium bicarbonate, reduced with 100 mM dithiothreitol, and alkylated with 200 mM iodoacetamide. Proteins were then digested with LysC 1:10 ratio (w:w; enzyme:substrate) at 37°C overnight followed by trypsin 1:10 ratio (w:w; enzyme:substrate) at 37°C for 8 hours. Tryptic peptide mixtures were desalted using a C18 UltraMicroSpin column (The Nest Group).

Peptide mixtures were analyzed in an Orbitrap Fusion Lumos with an EASY-Spray source coupled to a nano-ultra-performance liquid chromatography (UPLC) system (EASY-nanoLC 1000 liquid chromatography) equipped with a reverse-phase chromatography 25-cm column with an inner diameter of 75 μm, packed with 1.9 μm C18 particles (Nikkyo Technos, Japan). Chromatographic gradients started at 7% buffer B with a flow rate of 250 nL/minute and gradually increased to 35% in 120 minutes. After each run, the column was washed for 15 minutes with 90% buffer B (buffer A: 0.1% formic acid in water and buffer B: 0.1% formic acid in acetonitrile). The mass spectrometer was operated in data-dependent acquisition mode, with full MS scans over a mass range of m/z 350–1,500 with detection in the Orbitrap (120K resolution) and with auto gain control (AGC) set to 100,000. In each cycle of data-dependent acquisition analysis, after each survey scan, the most intense ions above a threshold ion count of 10,000 were selected for fragmentation with HCD at normalized collision energy of 28%. The number of selected precursor ions for fragmentation was determined by the "top speed" acquisition algorithm (maximum cycle time of 3 seconds), and a dynamic exclusion of 60 seconds was set. Fragment ion spectra were acquired in the ion trap with an AGC of 4,000 and a maximum injection time of 300 ms.

Acquired data were analyzed using the Proteome Discoverer software suite (v2.0, Thermo Fisher Scientific), and peptides were identified using the Mascot search engine (v1.6.0.16, Matrix Science). Data were searched against a human protein database derived from the SwissProt database (as in April 2015) plus the most common contaminants. The precursor ion mass tolerance was 7 ppm at the MS1 level, and up to 3 missed cleavages for trypsin were allowed. The fragment ion mass tolerance was set to 0.5 Da, methionine oxidation was set as variable modification, and cysteine carbamidomethylation was set as fixed modification. The identified peptides were filtered by 5% false discovery rate. Peptide areas were obtained using the "precursor ions area detector" module in the Proteome Discoverer software suite (v2.0, Thermo Fisher Scientific). Protein abundance in each condition was estimated using the log-transformed average of the 3 most intense peptides per protein group.

Selection of Candidate Proteins Associated With Disability Progression for Validation

Selection of proteins for validation in an independent cohort of patients with progressive MS was performed based on the differences in CSF protein levels between patients with high and low disability progression according to NPR and SPR at trial onset and after trial completion, and correlations between CSF protein levels and numeric and step progression rates at trial onset and after trial completion. Those proteins exhibiting a statistically significant abundance change (p value <0.05) or significant correlation (p value <0.05) in most ($n \geq 5$) of the disability progression criteria reported in Table 3 were selected for further validation.

Validation Cohort

An independent cohort of 41 patients with PPMS was included in this study to validate selected proteins from the targeted quantitative proteomics approach. Patients were recruited from 4 MS centers (Barcelona—Hospital de Bellvitge [n = 7], Barcelona—Hospital Clinic [n = 11], Madrid—Puerta de Hierro [n = 6], and Madrid—Ramón y Cajal [n = 17]), and CSF sampling was performed as described above. NPR and SPR were calculated between the

Table 1 Demographic and Clinical Characteristics of Patients With Progressive MS Belonging to the Discovery Cohort

Characteristics	Discovery cohort	Whole cohort
N	28	72
Age (y)[a]	49.6 (9.4)	49.3 (8.2)
Female/male (% women)	12/16 (42.9)	37/35 (50.7)
PP/PT (% PP)	17/11 (61.0)	48/24 (66.0)
Follow-up time onset/off (y)[b]	12.5 (4.6)/10.9 (4.0)	12.1 (4.6)/10.3 (4.4)
EDSS onset/last[c]	6.0 (4.0–6.0)/8.0 (6.5–8.5)	6.0 (4.0–6.0)/8.0 (6.5–8.8)
Numeric progression rate onset/off[d]	0.3 (0.7)/0.2 (0.1)	0.3 (0.5)/0.2 (0.3)
Step progression rate onset/off[d]	0.6 (1.3)/0.3 (1.3)	0.5 (1.0)/0.4 (0.4)
Patients treated with IFNβ (%)[e]	13 (46.4)	35 (47.9)

Abbreviations: EDSS = Expanded Disability Status Scale, IFNβ = interferon beta-1b.
All data are expressed as mean SD unless otherwise stated. EDSS is expressed as median (interquartile range).
PP/PT refers to the number of patients with primary progressive and transitional progressive MS.
[a] Refers to age at the time of trial onset.
[b] Refers to time from trial onset to the last visit (onset) and from the time after trial completion to the last visit (off).
[c] Refers to EDSS at trial onset (onset) and at the time of last visit (last).
[d] Refers to numeric and step-based progression rates from trial onset to the last visit (onset) and from the time after trial completion to the last visit (off).
[e] Refers to patients under IFNβ treatment at the time of CSF collection. Whole cohort refers to all the patients who participated in the double-blind, placebo-controlled trial of interferon beta.

time of CSF collection performed at each recruiting center for diagnostic purposes and the time of last visit. As in the discovery cohort, patients in the validation cohort were classified according to the 75th percentiles of confirmed disability progression based on NPR and SPR. Patients were untreated for the whole follow-up period. A summary of demographics and clinical characteristics of the validation cohort is given in Table 2.

Standard Protocol Approvals, Registrations, and Patient Consents

Written informed consent was obtained from each participant. This study was approved by the corresponding local Ethics Committees, and written informed consent was obtained from each participant.

Quantification of CSF Levels of Selected Proteins in the Validation Cohort

Levels of selected proteins were measured after a targeted proteomics approach with parallel reaction monitoring (PRM). For each patient, 80 μL of CSF were used for protein precipitation in cold acetone overnight at 4°C. Pellets were then solubilized in 6 M urea in 200 mM ammonium bicarbonate, reduced with 100 mM dithiothreitol, alkylated with 200 mM iodoacetamide, and digested with LysC 1:10 ratio (w:w; enzyme:substrate) at 37°C overnight followed by trypsin 1:10 ratio (w:w; enzyme:substrate) at 37°C for 8 hours. Tryptic peptide mixtures were desalted using a C18 UltraMicroSpin column (The Nest Group, Inc). For each targeted peptide, an isotopically labeled counterpart ($^{13}C_6$, $^{15}N_2$-Lys and $^{13}C_6$, $^{15}N_4$-Arg, Pepotec Peptides; Thermo Fisher Scientific) was spiked in the peptide mixtures and used as internal standard for quantification purposes. Up to 2 unique peptides per protein were selected for targeted protein quantification. For each selected peptide, an isotopically labeled peptide ($^{13}C_6$, $^{15}N_4$-Arg and $^{13}C_6$, $^{15}N_2$-Lys) was spiked in the samples and used as internal standard for quantification by PRM. The amount of internal standard peptide to be spiked in each sample was evaluated using dilution curves, and the final concentration was chosen based on the following criteria: (1) to be within the concentration range in which a linear response of the peptide was observed and (2) to have an area as close to the endogenous peptide area as possible.

Digested samples were analyzed by PRM using an Orbitrap Eclipse coupled to an EASY-nanoLC 1200 UPLC system (Thermo Fisher Scientific) with a 50-cm C18 chromatographic column (Easy-Spray Column, PepMap RSLC C18). Peptide mixes were separated with a chromatographic gradient starting at 5% B with a flow rate of 300 nL/minute and going up to 22% B in 79 minutes and to 32% B in 11 minutes (buffer A: 0.1% formic acid in water and buffer B: 0.1% formic acid in acetonitrile). The Orbitrap Eclipse was operated in positive ionization mode with an EASY-Spray nanosource at 1.4 kV and at a source temperature of 275°C.

A scheduled PRM method was used for data acquisition with a quadrupole isolation window set to 1.4 m/z and MS2 scans over a mass range of m/z 300–2,000, with detection in the Orbitrap mass analyzer at a 30K resolution. MS2 fragmentation was performed using HCD fragmentation at 30 normalized collision energy; the AGC was set at 1E5 and the maximum injection time at 54 ms. The size of the scheduled window was 6 minutes, and the maximum cycle time was 1 seconds. All data were acquired using XCalibur software.

Product ion chromatographic traces corresponding to the targeted precursor peptides were evaluated using Skyline software v.20.1 based on (1) co-elution of endogenous and internal standard peptides, (2) the number of detected traces, (3) correlation of the trace relative intensities between endogenous and internal standard peptides, and (4) expected retention time. Normalization was performed based on the median abundance of the internal standard peptides across all runs. Protein abundance estimates were performed using the software package MSstats 3.8.2.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (proteomecentral.proteomexchange.org) through the Proteomics Identifications Database partner repository[13] with the data set identifier PXD022958.

Quantification of Neurofilament Light Chain Levels in CSF by Single-Molecule Array

Levels of neurofilament light chain (NFL) were measured in CSF samples from 36 patients with PPMS belonging to the validation cohort using commercially available NFL immunoassay kits (Quanterix, Billerica, MA, cat no. 103186) and run on a single-molecule array (Simoa) HD-1 Analyzer (Quanterix). Samples were run in duplicate in line with manufacturers' instructions using appropriate standards and

Table 2 Demographic and Clinical Characteristics of Patients With Progressive MS Belonging to the Validation Cohort

Characteristics	Validation cohort
N	41
Age (y)[a]	51.9 (9.1)
Female/male (% women)	28/13 (68.3)
Follow-up time (y)[b]	7.3 (5.1)
EDSS at CSF collection	3.0 (2.5–4.0)
EDSS at last visit	6.0 (3.5–6.8)
Numeric progression rate[c]	0.4 (0.5)
Step progression rate[c]	0.4 (0.6)

Abbreviation: EDSS = Expanded Disability Status Scale.
All data are expressed as mean SD unless otherwise stated. EDSS is expressed as median (interquartile range).
[a] Refers to age at the time of CSF collection.
[b] Refers to time from CSF collection to the last visit.
[c] Refers to numeric and step-based progression rates from the time of CSF collection to the last visit.

internal controls. The intra-assay coefficient of variation was 6%.

Classification of Evidence

Our primary research question was to identify CSF biomarkers associated with disability progression in patients with progressive MS. The classification of evidence assigned to this question is Class II.

Statistical Analysis

Differences in CSF protein abundances were assessed among patients with high and low disability progression according to NPR and SPR using an analysis of variance model. Linear association between CSF protein abundances and NPR and SPR during the follow-up was evaluated using the Spearman rank correlation coefficient. Considering that a proportion of patients were receiving IFNβ at the time of CSF collection, the potential effect of IFNβ in CSF protein levels was also evaluated including treatment as covariate in the analysis. Differences in progression rates between patients from the discovery and validation cohorts were assessed using a Mann-Whitney U test. Performance of CSF chitinase 3–like 2 (CHI3L2) levels to discriminate between patients with high and low disability progression was assessed using receiver operating characteristic (ROC) curve analysis.

Data Availability

All data analyzed during this study will be shared anonymized by request of a qualified investigator to the corresponding author.

Results

CSF Proteomic Analysis in the Discovery Cohort of Patients With Progressive MS Identifies Proteins Associated With Long-term Disability Progression

A schematic flowchart summarizing the main steps of the study design is represented in Figure 1. To identify proteins associated with disability progression, we first performed a quantitative proteomic analysis in CSF samples from a discovery cohort of 28 patients with progressive MS classified according to numeric and step progression rates. A total of 2,548 proteins were identified in CSF samples by mass spectrometric analysis (the full list of identified proteins is provided in eTable, links.lww.com/NXI/A582). Of these identified proteins, 10 proteins were prioritized for further validation because of their potential association with disability progression, as described in the Methods section: SPATS2-like protein (SPATS2L), CHI3L2, plasma serine protease inhibitor (SERPINA5), metallothionein-3 (MT3), phospholipase D4 (PLD4), and beta-hexosaminidase (HEXB), which were more abundant in the CSF of patients with high disability progression, and neurexophilin-1 (NXPH1), adipocyte enhancer-binding protein 1 (AEBP1), cathepsin L1 (CTSL), and lipopolysaccharide-binding protein (LBP), which were

more abundant in the CSF of patients with low disability progression (Table 3). Only NXPH1 showed significant changes in CSF protein levels in patients receiving IFNβ, although differences stayed significant after adjusting by treatment. The remaining detected proteins showed either a lack of statistically significant association with disability progression or were significantly associated with disability progression only in a minority (<5) of the classification criteria (eTable 3, links.lww.com/NXI/A583).

CSF CHI3L2 Is Validated as Long-term Prognostic Biomarker Associated With Disability Progression in Patients With PPMS

To validate the results obtained in the discovery cohort, levels of the 10 selected proteins were determined by targeted proteomics in CSF samples from an independent cohort of patients with progressive MS. Although the discovery cohort included patients with transitional progressive MS and PPMS, no statistically significant differences were observed in the CSF levels of the 10 selected proteins between both groups of patients (data not shown), results that supported the use of a validation cohort consisting exclusively of patients with PPMS. Follow-up time of patients with PPMS in the validation cohort was shorter and EDSS scores at onset lower compared with patients belonging to the discovery cohort (eTable 1, links.lww.com/NXI/A581 and eTable 2, links.lww.com/NXI/A582). However, there were no statistically significant differences in NPR and SPR between patients from the discovery and validation cohorts (0.3 [0.7] and 0.2 [0.1] for NPR at onset and off trial, respectively, in the discovery cohort vs 0.4 [0.5] in the validation cohort; 0.6 [1.3] and 0.3 [0.2] for SPR at onset and off trial, respectively, in the discovery cohort vs 0.4 [0.6] in the validation cohort; $p > 0.05$ for all comparisons).

Only CHI3L2 was validated, and similar to the findings in the discovery cohort (Table 3), CSF CHI3L2 levels were significantly higher in patients with PPMS who progressed faster during the follow-up based on both NPR and SPR compared with those patients having lower disability progression ($p = 0.03$ and $p = 0.02$, respectively) (Figure 2A). CSF CHI3L2 levels significantly correlated with NPR (r: 0.34, $p = 0.03$), whereas a trend for significant correlation was observed between CHI3L2 levels and SPR progression rates (r: 0.30, $p = 0.05$) (Figure 2a).

Figure 2B shows the potential for CSF CHI3L2 levels to discriminate between high and low disability progression in patients with PPMS. The area under the ROC curve (AUC) was 73% ($p = 0.03$) and a CSF CHI3L2 value of 17.1 (protein abundance estimate) resulted in the best cutoff to classify patients with PPMS with high and low disability progression according to both NPR and SPR, with a sensitivity of 90% and specificity of 63%.

Partial correlations adjusting for age did not reveal statistically significant associations between CSF CHI3L2 and NFL levels

($r = 0.02$, $p = 0.91$) or between CSF NFL levels and numeric progression rates ($r = 0.08$, $p = 0.62$) or SPR ($r = 0.09$, $p = 0.60$).

In the validation cohort, except for SPATS2L which could not be detected, the mean CSF levels of the remaining proteins did not significantly differ between high and low disability progression using NPR or SPR in patients with PPMS, and hence, they were not validated as biomarkers associated with disability progression in MS (Table 4; eFigure 1, links.lww.com/NXI/A579 and eFigure 2, links.lww.com/NXI/A580).

Discussion

Our understanding of disease progression is limited and still remains as one of the unmet needs in MS.[10] In this context, there is a lack of prognostic factors associated with disability progression in patients with progressive forms of MS. Bearing this in mind, we aimed to identify long-term prognostic biomarkers in the CSF of patients with progressive MS by conducting a 2-phase study. An initial or discovery cohort included patients with progressive MS with a follow-up longer than 10 years who participated in a single-center trial of IFNβ-1b.[12] By quantitative proteomics analysis of the CSF samples collected during the trial, we identified a number of candidate CSF proteins that were associated with long-term disability progression in patients classified according to 2 related progression rate indexes, which were applied at different time points during the trial. Although almost half of the patients were receiving IFNβ at the time of CSF collection, treatment did not seem to have a major impact on CSF protein levels.

A second or validation cohort included exclusively untreated patients with PPMS whose CSF samples were collected from different MS centers. Although in these patients follow-up time was shorter and EDSS scores were lower, the progression rates, which relate EDSS changes to time on follow-up, were

Figure 1 Flowchart Summarizing the Different Steps Undertaken in the Study
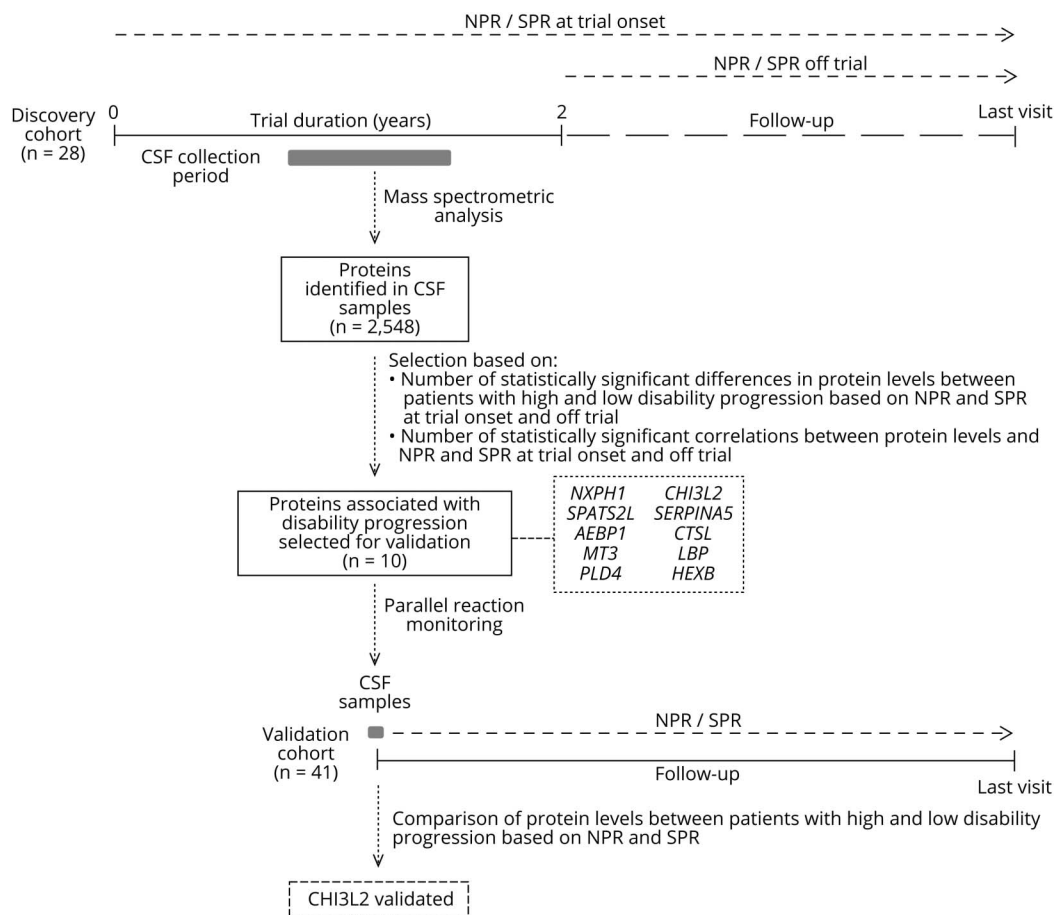
NPR and SPR were calculated in a discovery cohort of 28 patients with progressive MS who participated in a 2-year placebo-controlled trial of IFNβ-1b. Progression rates were computed between the time of trial onset and the last visit (NPR/SPR at trial onset), and between the time after trial completion and the last visit (NPR/SPR off trial). Based on these progression rates, patients with progressive MS were classified into high and low disability progression phenotypes. By means of a quantitative proteomic approach, a total of 2,548 proteins were identified in CSF samples from patients belonging to the discovery cohort, of which 10 proteins were found to be associated with disability progression in most of the selection criteria shown in the figure. Protein levels for these 10 proteins were measured by PRM in CSF samples from a validation cohort of 41 patients with progressive MS classified into high and low disability progression phenotypes according to NPR and SPR computed between the time of CSF collection and the time of the last visit.

Table 3 Selected CSF Proteins in the Discovery Cohort for Validation

Uniprot	Symbol	NPR "onset"[a] Levels H/L (*p*)	SPR "onset"[a] Levels H/L (*p*)	NPR "off"[b] Levels H/L (*p*)	SPR "off"[b] Levels H/L (*p*)	NPR "onset"[a] rho (*p*)	SPR "onset"[a] rho (*p*)	NPR "off"[b] rho (*p*)	SPR "off"[b] rho (*p*)	H/L[c]	N[d]
P58417	NXPH1[e]	25.3/26.8 (**0.007**)	25.4/26.8 (**0.018**)	25.7/26.9 (**0.037**)	25.3/26.8 (**0.012**)	−0.52 (**0.042**)	−0.62 (**0.023**)	−0.76 (**0.001**)	−0.79 (**0.008**)	Down	**8**
Q9NUQ6	SPATS2L	25.4/23.8 (**0.035**)	25.6/23.8 (**0.041**)	25.4/23.6 (**0.017**)	25.8/23.7 (**0.016**)	0.54 (**0.019**)	0.47 (**0.045**)	0.44 (0.066)	0.49 (**0.041**)	Up	**7**
Q8IUX7	AEBP1	24.2/25.0 (**0.003**)	24.2/25.1 (**0.002**)	24.5/25.0 (0.066)	24.5/25.0 (0.066)	−0.56 (**0.014**)	−0.62 (**0.005**)	−0.58 (**0.008**)	−0.61 (**0.006**)	Down	**6**
Q15782	CHI3L2	26.3/25.4 (**0.030**)	26.4/25.3 (**0.012**)	25.8/25.3 (0.202)	26.1/25.3 (**0.044**)	0.38 (0.111)	0.52 (**0.022**)	0.49 (**0.024**)	0.50 (**0.021**)	Up	**6**
P05154	SERPINA5	26.2/25.7 (0.073)	26.2/25.7 (0.051)	26.2/25.7 (**0.018**)	26.3/25.7 (**0.013**)	0.46 (**0.023**)	0.45 (**0.025**)	0.68 (**0.001**)	0.63 (**0.001**)	Up	**6**
P07711	CTSL	27.0/27.5 (0.090)	26.8/27.6 (**0.009**)	27.1/27.5 (0.206)	26.9/27.5 (**0.047**)	−0.37 (**0.033**)	−0.38 (**0.032**)	−0.32 (0.073)	−0.42 (**0.025**)	Down	**5**
P25713	MT3	21.5/20.9 (**0.010**)	21.5/20.9 (**0.010**)	21.2/20.8 (0.051)	21.2/20.9 (0.267)	0.69 (**0.007**)	0.79 (**0.001**)	0.49 (0.101)	0.60 (**0.047**)	Up	**5**
Q96BZ4	PLD4	25.9/24.2 (**0.002**)	25.7/24.3 (**0.038**)	25.7/24.1 (**0.017**)	25.6/24.3 (0.083)	0.66 (**0.028**)	0.59 (0.056)	0.75 (**0.016**)	0.61 (0.061)	Up	**5**
P18428	LBP	24.6/25.4 (**0.008**)	24.7/25.3 (0.070)	24.7/25.5 (**0.011**)	24.8/25.3 (0.192)	−0.62 (**0.012**)	−0.66 (**0.011**)	−0.59 (**0.043**)	−0.57 (0.064)	Down	**5**
P07686	HEXB	26.7/26.0 (0.078)	26.3/26.2 (0.796)	26.7/26.0 (**0.036**)	26.5/26.1 (0.302)	0.45 (**0.025**)	0.40 (**0.047**)	0.50 (**0.012**)	0.46 (**0.025**)	Up	**5**

Abbreviations: AEBP1 = adipocyte enhancer-binding protein 1; CHI3L2 = chitinase 3-like 2; CTSL = cathepsin L1; HEXB = hexosaminidase; IFNβ = interferon beta-1b; LBP = lipopolysaccharide-binding protein; MT3 = metallothionein-3; NPR = numeric progression rates; NXPH1 = neurexophilin-1; PLD4 = phospholipase D4; SERPINA5 = plasma serine protease inhibitor; SPATS2L = SPATS2-like protein; SPR = step-based progression rates. rho: Spearman rank correlation coefficient.
[a] Refers to NPR and SPR calculated from trial onset to the last visit.
[b] Refers to NPR and SPR calculated from the time after trial completion to the last visit.
[c] Refers to the direction in protein levels: up/down = increased/decreased levels in patients with high/low disability progression, respectively.
[d] Refers to the total number of statistically significant associations observed after applying the different classification methods. Levels H/L refers to mean protein levels in patients with high and low disability progression for each of the different indexes calculated from peptide intensities at log-scale (arbitrary units). Significant *p* values (p) are shown in bold.
[e] NXPH1 was the only selected protein showing significant changes in CSF abundance in patients treated with IFNβ, but differences remained significant after adjusting by this covariate.

Figure 2 Performance of CHI3L2 as a Biomarker of Disability Progression in Patients With Progressive MS
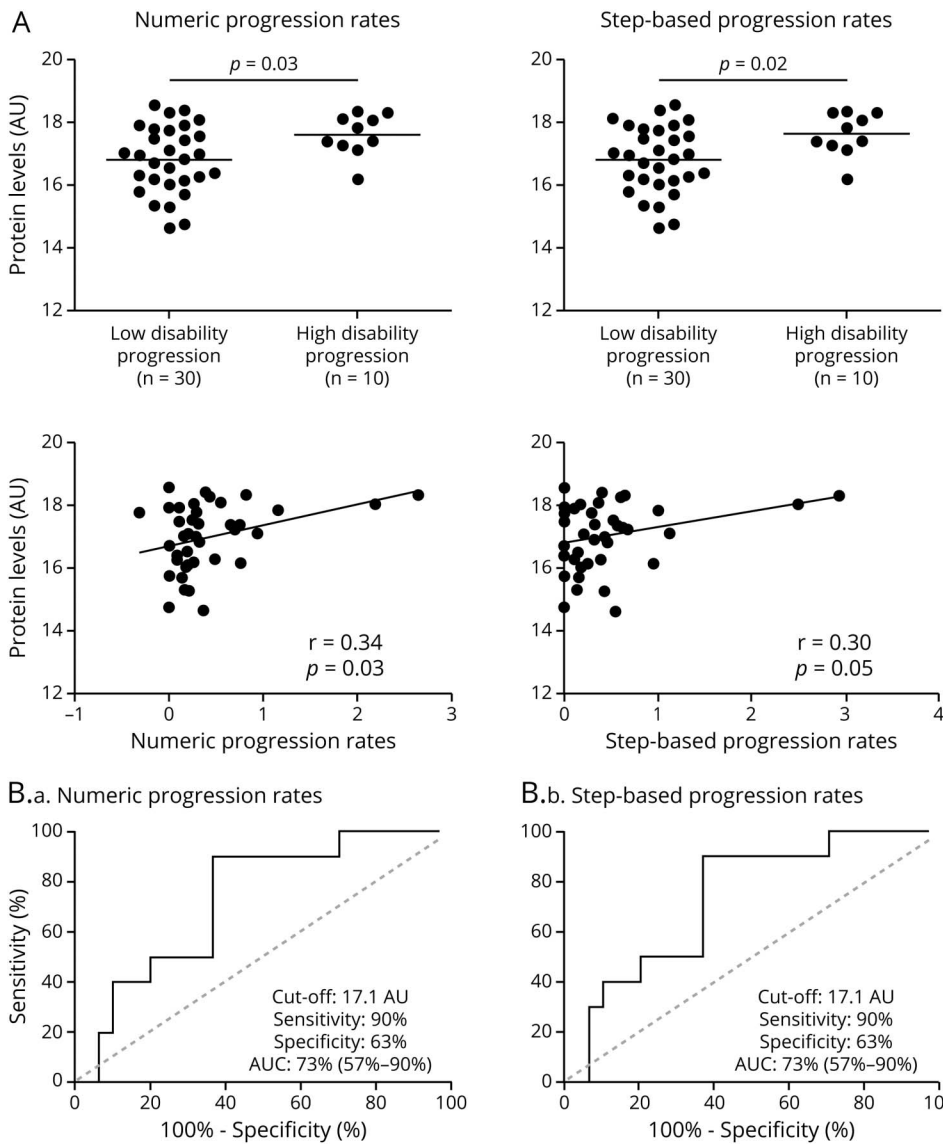
(A) Graphs comparing CSF CHI3L2 levels between high and low disability progression patients with progressive MS according to numeric and step-based progression rates, and correlation plots evaluating linear association between CSF CHI3L2 levels and progression rates, both in the validation cohort. (B) Performance of CSF CHI3L2 levels to discriminate between patients with high and low disability progression based on numeric (B.a) and step-based progression rates (B.b). AU = protein abundance estimate; AUC = area under the receiver operating characteristic curve. Numbers in parentheses represent 95% confidence intervals of the AUC; CHI3L2 = chitinase 3-like 2.

comparable among patients with progressive MS belonging to both study cohorts. Selected candidate CSF biomarkers identified in the initial cohort were measured in the validation cohort by means of PRM, a well-known specific and reproducible mass spectrometric technique for protein quantitation.[14] Only CHI3L2 was found to be significantly associated with disability progression in both cohorts of patients.

CHI3L2, also known as YKL39, is a member of the 18 glycosyl hydrolase family that can bind chitin with high affinity but lacks enzymatic activity.[15] CHI3L2 was first discovered in conditioned medium from primary cultures of human articular cartilage chondrocytes15, and its function has been related to the immune response and tissue remodeling. In this context, CHI3L2 was expressed by M2 polarized macrophages and was found to act as a pro-angiogenic and monocyte recruiting factor.[16] Little is known about the physiologic and pathologic roles

of CHI3L2 in the CNS. CSF CHI3L2 levels have been reported to be elevated in a number of neuroinflammatory conditions.[17-21] In patients with clinically isolated syndrome (CIS), high CSF CHI3L2 levels were associated with an increased risk for MS and predicted cognitive impairment evaluated by the paced auditory serial addition test.[18] In another study, CSF CHI3L2 levels were found to be significantly increased in patients with RRMS compared with patients with CIS, patients with progressive MS, and controls.[17]

In our study, CSF abundance for CHI3L2 was higher in patients with progressive MS who later progressed faster compared with those patients with lower progression rates. In addition, CSF CHI3L2 levels showed good potential to discriminate between high and low disability progression in patients with progressive MS after long-term follow-up, with excellent sensitivity and acceptable specificity. Although the

Table 4 CSF Proteins Levels of Selected Proteins in the Validation Cohort

Uniprot	Symbol	NPR		SPR	
		Levels H/L	*p* Value	Levels H/L	*p* Value
P58417	NXPH1	19.7 (19.2–20.1)/19.7 (19.2–20.2)	1.000	19.7 (19.2–20.1)/19.7 (19.2–20.2)	0.964
Q8IUX7	AEBP1	20.2 (19.5–20.9)/20.3 (19.7–20.6)	0.754	20.3 (20.0–20.9)/20.3 (19.6–20.6)	0.360
P05154	SERPINA5	20.1 (19.1–20.8)/19.7 (18.7–20.3)	0.376	20.2 (19.5–20.8)/19.7 (18.6–20.3)	0.112
P07711	CTSL	21.0 (20.8–21.2)/20.9 (20.6–21.3)	0.777	21.0 (20.8–21.2)/20.9 (20.6–21.3)	0.709
P25713	MT3	20.9 (20.5–21.3)/20.9 (20.1–21.4)	0.622	20.7 (20.4–21.1)/20.9 (20.1–21.4)	0.665
Q96BZ4	PLD4	20.4 (20.1–20.9)/20.4 (19.9–20.9)	0.777	20.5 (20.3–21.2)/20.3 (19.8–20.9)	0.235
P18428	LBP	17.9 (16.5–18.5)/17.8 (16.9–18.9)	0.893	18.1 (17.2.18.6)/17.8 (16.6–18.9)	0.622
P07686	HEXB	19.9 (19.7–20.2)/19.9 (19.5–20.3)	0.687	19.9 (19.7–20.2)/19.9 (19.5–20.3)	0.665

Abbreviations: AEBP1 = adipocyte enhancer-binding protein 1; CTSL = cathepsin L1; HEXB = hexosaminidase; LBP = lipopolysaccharide-binding protein; MT3 = metallothionein-3; NPR = numeric progression rates; SPR = step-based progression rates; NXPH1 = neurexophilin-1; PLD4 = phospholipase D4; SERPINA5 = plasma serine protease inhibitor.
Levels H/L refers to median protein levels (interquartile range) in patients with high and low disability progression for each of the different indexes.

underlying molecular mechanisms of the association observed between CSF CHI3L2 levels and disease progression are unknown, a previous study revealed CHI3L2 expression in astrocytes from white matter plaques and from normal appearing white matter.[17] In this context, several lines of evidence have pointed to astrocytes as important players in MS pathogenesis and suggested a contribution of these cells to the neurodegenerative component of the disease.[22-27] This astrocytic origin of CHI3L2 may explain the lack of correlation observed in our study between CSF CHI3L2 abundance and CSF NFL levels, as a representative biomarker of neuronal damage.[28]

CSF proteins that were significantly associated with long-term disability progression in a lower number of classification methods are also interesting candidate biomarkers that require further validation in independent cohorts of patients with progressive MS. Of note, one of these candidates is CHI3L1, a protein closely related to CHI3L2 in size and sequence, although with structural differences,[29] which has been extensively implicated in MS.[30] In contrast to CHI3L2, CHI3L1 was found significantly increased in the CSF of patients with progressive MS compared with patients with RRMS,[17] and high CSF CHI3L1 levels were identified as an independent predictor of disability worsening in patients with MS.[31]

The reasons for the lack of validation of the remaining proteins selected from the first phase of the study are unknown and probably manifold. They may be related to differences in the clinical characteristics of patients included in both cohorts such as the clinical form (patients with transitional progressive and PPMS in the discovery cohort vs patients with purely PPMS in the validation cohort), treatment with IFNβ in a subgroup of patients from the discovery cohort vs untreated patients in the validation cohort, and shorter follow-up and lower disability

scores in the validation cohort. However, as mentioned before, some of these differential factors were considered in the analysis, and also the progression rates that were used to classify patients into high and low disability progression phenotypes were comparable among patients from the discovery and validation cohorts. Other factors may relate to differences in the proteomic techniques used to determine protein abundance in the CSF of patients with MS. However, based on the results in the validation cohort, except for CHI3L2, the remaining candidate CSF proteins identified in the first phase of the study can probably be considered as false-positive results.

In conclusion, a proteomic approach in CSF samples from patients with progressive MS classified into high and low disability progression phenotypes has led to the identification and validation of CHI3L2 as a biomarker associated with long-term disability progression. Further mechanistic studies are needed to better understand the relationship between CSF CHI3L2 and disability progression in MS.

Disclosure

Publication History

Appendix Authors

Name	Location	Contribution
Manuel Comabella, MD, PhD	Servei de Neurologia-Neuroimmunologia. Centre d'Esclerosi Múltiple de Catalunya (Cemcat). Institut de Recerca Vall d'Hebron (VHIR). Hospital Universitari Vall d'Hebron. Universitat Autònoma de Barcelona, Spain	Design and conceptualized study, analyzed the data, and drafted the article for intellectual content
Jaume Sastre-Garriga, MD, PhD	Servei de Neurologia-Neuroimmunologia. Centre d'Esclerosi Múltiple de Catalunya (Cemcat). Hospital Universitari Vall d'Hebron. Universitat Autònoma de Barcelona, Spain	Study concept and design and revised the article for intellectual content
Eva Borras, PhD	Proteomics Unit, Universitat Pompeu Fabra (UPF), Barcelona, Spain	Study concept and design, analyzed the data, and revised the article for intellectual content
Luisa M. Villar, MD, PhD	Departments of Neurology and Immunology, Hospital Universitario Ramón y Cajal, Instituto Ramón y Cajal de Investigacion Sanitaria, Madrid, Spain	Data acquisition and revised the article for intellectual content
Albert Saiz, MD, PhD	Center of Neuroimmunology, Service of Neurology, Hospital Clinic and Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), University of Barcelona, Spain	Data acquisition and revised the article for intellectual content
Sergio Martínez-Yélamos, MD, PhD	Department of Neurology, Bellvitge University Hospital, Barcelona, Spain	Data acquisition and revised the article for intellectual content
Juan Antonio García-Merino, MD, PhD	Neuroimmunology Unit, Hospital Universitario Puerta de Hierro, Madrid, Spain	Data acquisition and revised the article for intellectual content
Rucsanda Pinteac, MSc	Servei de Neurologia-Neuroimmunologia. Centre d'Esclerosi Múltiple de Catalunya (Cemcat). Institut de Recerca Vall d'Hebron (VHIR). Hospital Universitari Vall d'Hebron. Universitat Autònoma de Barcelona, Spain	Data acquisition and revised the article for intellectual content

Appendix *(continued)*

Name	Location	Contribution
Nicolas Fissolo, PhD	Servei de Neurologia-Neuroimmunologia. Centre d'Esclerosi Múltiple de Catalunya (Cemcat). Institut de Recerca Vall d'Hebron (VHIR). Hospital Universitari Vall d'Hebron. Universitat Autònoma de Barcelona, Spain	Data acquisition and revised the article for intellectual content
Antonio J. Sánchez-López, PhD	Neuroimmunology Unit, Hospital Universitario Puerta de Hierro, Madrid, Spain	Data acquisition and revised the article for intellectual content
Lucienne Costa-Frossard, MD	Departments of Neurology and Immunology, Hospital Universitario Ramón y Cajal, Instituto Ramón y Cajal de Investigacion Sanitaria, Madrid, Spain	Data acquisition and revised the article for intellectual content
Yolanda Blanco, MD	Center of Neuroimmunology, Service of Neurology, Hospital Clinic and Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), University of Barcelona, Spain	Data acquisition and revised the article for intellectual content
Sara Llufriu, MD, PhD	Center of Neuroimmunology, Service of Neurology, Hospital Clinic and Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), University of Barcelona, Spain	Data acquisition and revised the article for intellectual content
Angela Vidal-Jordana, MD, PhD	Servei de Neurologia-Neuroimmunologia. Centre d'Esclerosi Múltiple de Catalunya (Cemcat). Hospital Universitari Vall d'Hebron. Universitat Autònoma de Barcelona, Spain	Data acquisition and revised the article for intellectual content
Eduard Sabidó, PhD	Proteomics Unit, Centre de Regulació Genòmica (CRG), Barcelona Institute of Science and Technology (BIST), Barcelona, Spain	Study concept and design, analyzed the data, and revised the article for intellectual content
Xavier Montalban, MD, PhD	Servei de Neurologia-Neuroimmunologia. Centre d'Esclerosi Múltiple de Catalunya (Cemcat). Hospital Universitari Vall d'Hebron. Universitat Autònoma de Barcelona, Spain	Study concept and design and revised the article for intellectual content

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