Potential Role of CHI3L1+ Astrocytes in Progression in MS

Laura Cubas-Núñez, PhD,* Sara Gil-Perotín, MD, PhD,* Jéssica Castillo-Villalba, BSc, Verónica López, BSc, Luis Solís Tarazona, MD, Raquel Gasqué-Rubio, BSc, Sara Carratalá-Boscá, MA, Carmen Alcalá-Vicente, MD, PhD, Francisco Pérez-Miralles, MD, PhD, Hans Lassmann, MD, PhD, and Bonaventura Casanova, MD, PhD

Correspondence
Dr. Gil-Perotín
sara.garcia@uv.es

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Abstract

Objective

Neurofilament light protein (NfL) and chitinase 3–like 1 (CHI3L1) are biomarkers for acute neuroaxonal damage and local inflammation, respectively. Thus, we set out to evaluate how these biomarkers were associated with clinical features of demyelinating diseases in parallel with the expression in brain autopsies from patients with similar disease stages, assuming their comparability.

Methods

NfL and CHI3L1 in CSF and serum CHI3L1 were assessed retrospectively in a cross-sectional cohort of controls (n = 17) and patients diagnosed with MS (n = 224), relapsing (n = 163) or progressive (n = 61); neuromyelitis optica (NMO, n = 7); and acute disseminated encephalomyelitis (ADEM, n = 15). Inflammatory activity was evaluated at the time of sampling, and CSF biomarker levels were related to the degree of inflammation in 22 brain autopsy tissues.

Results

During a clinical attack, the CSF NfL increased in MS, NMO, and ADEM, whereas CHI3L1 was only elevated in patients with NMO and ADEM and in outlier MS patients with extensive radiologic activity. Outside relapses, CHI3L1 levels only remained elevated in patients with progressive MS. CHI3L1 was detected in macrophages and astrocytes, predominantly in areas of active demyelination, and its expression by astrocytes in chronic lesions was independent of lymphocyte infiltrates and associated with active neurodegeneration.

Conclusions

Both CSF NfL and CHI3L1 augment during acute inflammation in demyelinating diseases. In MS, CHI3L1 may be associated with low-grade nonlymphocytic inflammation and active neurodegeneration and therefore linked to progressive disease.

Classification of Evidence

This study provides Class III evidence that CSF NfL and CHI3L1 levels increase in inflammatory brain diseases during acute inflammation.

*These authors contributed equally to this work.

From the Neuroimmunology Unit (L.C.-N., S.G.-P., J.C.-V., V.L., R.G., S.C., C.A., F.P.-M., B.C.), Polytechnic and University Hospital La Fe; Neurology Department (L.S.T.), University Hospital Dr Peset, Valencia, Spain; and Department of Neuroimmunology (H.L.), Center for Brain Research, Vienna, Austria.

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Glossary

ADEM = acute disseminated encephalomyelitis; **AMS** = acute MS; **APP** = amyloid precursor protein; **BBB** = blood-brain barrier; **DMT** = disease-modifying therapy; **EDSS** = Expanded Disability Status Scale; **GFAP** = glial fibrillary acidic protein; **IQR** = interquartile range; **LP** = lumbar puncture; **NAGM** = normal-appearing gray matter; **NAWM** = normal-appearing white matter; **NfL** = neurofilament light protein; **NMO** = neuromyelitis optica; **PMS** = progressive MS; **PPMS** = primary progressive MS; **SEL** = slowly expanding lesion; **SPMS** = secondary progressive MS; **RRMS** = relapsing-remitting MS.

MS is a pathology that can be considered a continuum, in which neuronal damage occurs in parallel to inflammation from the onset of the disease.¹ This neuronal damage might be not perceived clinically due to compensation by functional neuronal reserves of the relatively young individuals affected.² Thus, there is currently much interest in identifying biomarkers that will enable disease progression to be evaluated, even before a clinical diagnosis, and personalized treatment strategies to be adopted. We recently proposed that the combined assessment of 2 biomarkers in the CSF might identify patients with subclinical disease progression: neurofilament light protein (NfL) and chitinase 3-like 1 (CHI3L1).³ CHI3L1 is a member of the chitin family of proteins that are related to chronic inflammation.⁴ Within the brain, CHI3L1 is expressed by astrocytes^{5–7} and microglia/macrophages,^{6–9} and its levels in the CSF reflect its endogenous synthesis.⁶ Indeed, elevations in CHI3L1 have prognostic implications in MS.^{3,10,11} NfL is released into the CSF and serum on axonal damage, and in serum, it is considered a surrogate marker of disease activity in MS with potential prognostic value.^{12,13} NfL and CHI3L1 levels also appear to be elevated during clinical attacks in other demyelinating diseases, such as neuromyelitis optica (NMO)¹⁴ and acute disseminated encephalomyelitis (ADEM).¹⁵ However, unlike MS, the accrual of neurologic disability independent of relapses is rare or absent in these entities.^{16,17}

In the present study, we analyzed CHI3L1 and NfL levels in the CSF of patients with MS, ADEM, and NMO, and we compared these data with the expression of CHI3L1 and the degree of inflammation and neurodegeneration in autopsy tissue obtained from patients at similar disease stages. In terms of the clinical data, our primary end point was to assess the differences in the levels of these biomarkers between patients with distinct demyelinating diseases and in relation to their disease activity. From a pathologic viewpoint, the aim was to study the differential expression of CHI3L1 by distinct cell types and its relationship to the surrounding inflammation.

Methods

Participants and Brain Samples

Brain autopsy tissue was analyzed from 22 patients with MS and 6 age-matched controls ($n = 6$): acute MS (AMS)/relapsing-remitting (RRMS: $n = 6$); primary progressive (PPMS: $n = 5$); secondary progressive (SPMS: $n = 11$), and 1

patient with NMO (AQP4+) and another 1 with ADEM (see patient characteristic and lesions analyzed in table 1). The clinical part of the study was performed on a cross-sectional cohort of 224 patients with MS (163 RRMS; 37 SPMS; 24 PPMS), 7 NMO (AQP4+), and 15 ADEM. Patients were attended at the Hospital Universitari i Politècnic La Fe between 2008 and 2017, and they were included in the study when CSF and serum samples, MRI, and longitudinal disability data were available. Demographic and clinical data were collected retrospectively and prospectively, with the last update in December 2019 (table 2). Non-MS control CSF samples ($n = 17$) were selected from patients with no evidence of infection, inflammation, autoimmunity, or known neurodegenerative disease.

Standard Protocol Approvals, Registrations, and Patient Consents

This clinical study was approved by the Institutional Ethics Committee in Hospital La Fe (reference number PI17/01544). The material for pathologic analysis was collected from the archive of the Center for Brain Research of the Medical University of Vienna (ethics committee number: 535/2004/2019).

Data Availability

The data sets analyzed in this current study are available from the corresponding author on reasonable request.

Definitions

Diagnosis of clinically definite MS was made prospectively according to the 2017 McDonald criteria.¹⁸ An active disease was considered when a clinical attack occurred and/or at least 1 gadolinium-enhanced (Gd+) lesion was present in T1-weighted MRI. An increase in T2 lesions was not considered as activity because the temporal relationship with acute inflammation was not always well defined. A clinical attack or relapse was defined as acute worsening of neurologic activity lasting more than 24 hours, not explained by fever or physical stress, and followed by a varying degree of recovery. Urinary symptoms alone were not considered for a diagnosis of relapse. CSF samples were considered contemporary to active disease when a lumbar puncture (LP) was performed within 90 days of the assessment of clinical attack. Clinical phenotypes were classified according to the modified Lublin criteria.¹⁹ Patients were considered as SPMS when they had an Expanded Disability Status Scale (EDSS) score ≥ 3.0 with a 6-month confirmed increase to an EDSS score of ≥ 4.0 , and in whom the pyramidal functional system was ≥ 2.0 and there

Table 1 Brain Specimen Characteristics

Case	Type	Sex	Age	Lesion	Region of interest	Disease duration (mo)
C1	Control	F	30		NWM	
C2	Control	F	36		NWM	
C3	Control	F	39		NWM	
C4	Control	M	46		NWM	
C5	Control	M	65		NWM	
C6	Control	M	70		NWM	
MS1	AMS	M	35	Act. Lesion III	NAWM, initial, EA, and active center	1.5
MS2	AMS	F	45	Act. Lesion III, SEL	NAWM, initial, EA, LA, SEL: edge, and SEL: core	0.2
MS3	AMS	M	45	2× Act. Lesion III	NAWM, initial, 2× EA, LA, and active center	0.6
MS4	AMS	M	59	Act. Lesion II	NAWM, EA, and active center	5
MS5	AMS	F	69	Act. Lesion III	NAWM, initial, EA, and LA	2
MS6	RRMS	F	40	Act. Lesion III	NAWM, initial, EA, and active center	120
MS7	PPMS	F	34	2× inactive lesion	NAWM, 2× inactive core	204
MS8	PPMS	M	53	SEL	NAWM, SEL: edge, and SEL: core	168
MS9	PPMS	F	55	Active lesion I	NAWM, EA, and active center	168
MS10	PPMS	M	67	SEL	NAWM, SEL: edge, and SEL: core	87
MS11	PPMS	F	77	SEL	NAWM, SEL: edge, and SEL: core	168
MS12	SPMS	F	52	SEL and inactive	SEL and inactive	30
MS13	SPMS	M	34	SEL	NAWM, SEL: edge, and SEL: core	120
MS14	SPMS	M	41	SEL	NAWM, SEL: edge, and SEL: core	137
MS15	SPMS	F	46	SEL	NAWM, SEL: edge, and SEL: core	444
MS16	SPMS	F	48	Active lesion I	NAWM, EA, SEL, and inactive	410
MS17	SPMS	F	53	SEL	NAWM, SEL: edge, and SEL: core	241
MS18	SPMS	F	53	Inactive lesion	NAWM and inactive core	360
MS19	SPMS	F	61	Inactive lesion	NAWM and inactive core	288
MS20	SPMS	F	62	Inactive lesion	NAWM and inactive core	144
MS21	SPMS	F	81	Inactive lesion	NAWM and inactive core	432
MS22	SPMS	F	45	Active lesion	NAWM, EA, and SEL	240
NMO	—	F	20	Active/inactive lesion	Active and inactive	48
ADEM	—	M	13	Inf; act PV DM	NAWM; active lesions	0.06

Abbreviations: Act. = classical active lesions; ADEM = acute disseminated encephalomyelitis; AMS = acute MS; EA = early active lesion; LA = late active lesion; NAWM = normal-appearing white matter; NMO = neuromyelitis optica; NWM = normal white matter of controls; PPMS = primary progressive MS; RRMS = relapsing-remitting MS; SEL = slowly expanding lesion; SPMS = secondary progressive MS.

was no evidence of relapse. The PPMS phenotype was assigned to those patients who fulfilled the 2017 McDonald criteria for PPMS. Patients with MS were treated with a first-line disease-modifying therapies (DMTs) at the physician's discretion, unless one of the following circumstances occurred: (1) 2 clinical attacks in 1 year; (2) a clinical attack and/or a new Gd+ lesion within 3 months after a bout; and (3) a disabling clinical attack with residual EDSS of at least 2

points. In these cases and in those with treatment failure, a second-line DMT was administered. Nonresponders to first- and second-line DMT proceeded to autologous stem cell transplantation.

NMO was diagnosed according to the 2015 Wingerchuk criteria,²⁰ and only seropositive patients for NMO-IgG were included in the study. ADEM was diagnosed according to the

Table 2 Clinical and Demographic Characteristics of Patients

Variable	Controls (n = 17)	RRMS (n = 163)	PMS (n = 61)	NMO (n = 7)	ADEM (n = 11)	P Value
Age at disease onset (y)	—	30 (24–37)	35 (25–43)	42 (31–48)	38 (20–52)	0.405
Age at the time of LP (y)	35 (31–36)	34 (28–41)	46 (40–53)	42 (35–50)	43.50 (22–54)	0.273
Females	9 (53%)	132 (81%)	28 (47%)	7 (100%)	3 (23%)	0.002
Time from diagnosis to LP (y)	—	0.5 (0.1–5)	10.5 (5.9–16.7)	1 (0–10)	0 (0–1)	<0.001
Follow-up from LP (y)	—	3.7 (1.8–5.4)	3.5 (2.1–5.8)	6.6 (4–9.9)	2.2 (0.7–4)	<0.001
Clinical relapse at the time of LP	—	93 (57%)	3 (5%)	5 (72%)	7 (62%)	0.007
Gd+ in MRI at the time of LP	—	77 (48%)	15 (25%)	2 (29%)	7 (58%)	0.524
OCB-IgG	0 (0%)	142 (88%)	55 (91.6%)	2 (29%)	2 (18%)	<0.001
OCB-IgM	0 (0%)	81 (50%)	28 (46.7%)	2 (29%)	1 (9%)	0.007
Baseline EDSS score	0	2 (1–3)	5 (4–6)	7 (3–8)	5 (3–6)	<0.001
EDSS score ≥3.5	0	32 (19.6%)	57 (93.4%)	5 (71.4%)	4 (36.3%)	—
Treatment at LP (1st-line DMT, 2nd-line DMT, and ASCT)	—	30 (20, 9, 1)	25 (8, 17, 0)	4 (3, 1, 0)	0	—
NfL in CSF (pg/mL)	148.3 (119.8–185.5)	561.9 (269–1,480)	479.1 (286–704.5)	534 (233–9,150)	1,111 (201–6865)	0.6
CHI3L1 in CSF (ng/mL)	64.8 (47.4–78.3)	115.6 (80.5–186.1)	159.93 (104–229.5)	208.2 (111–448)	374.2 (109–634)	0.01

Abbreviations: ADEM = acute disseminated encephalomyelitis; ASCT = autologous stem cell transplantation; CHI3L1 = chitinase 3-like 1; DMT = disease-modifying therapy; EDSS = Expanded Disability Status Scale; Gd+ = gadolinium-enhancing lesion; LP = lumbar puncture; NfL = neurofilament light chain; NMO = neuromyelitis optica; OCB = oligoclonal band; PMS = progressive MS; RRMS = relapsing-remitting MS.

Categorical variables are described as counts (n) and percentages (%) and continuous and ordinal variables by median (first and third quartiles).

2012 IPMSSG criteria.²¹ In all cases, disability was estimated according to the EDSS²² at the time of LP and every 6 months until the last visit. AMS for pathologic studies was diagnosed in the case of rapidly progressive and malignant forms of MS that led to death within weeks to months from onset, without any signs of remission.²³ Most patients died due to cardiac or respiratory complications.

Biomarker Measurement

CSF and serum samples were stored at –80°C in the Biobank at the La Fe hospital and with the approval of the Ethics and Scientific Committees (PT17/0015/0043). CHI3L1 and NfL CSF levels were assessed as reported previously following consensus guidelines for CSF collection and biobanking.²⁴ The mean intraassay coefficients of variation for NfL and CHI3L1 were 4.5% and 6.5%, respectively, and the interassay coefficients were 3.3% and 5.2%, respectively. We considered patients with biomarker levels more than 1.5 times the interquartile range (IQR) above the Q3 (CHI3L1 >367 ng/mL; NfL >2027 pg/mL) calculated in the global MS cohort as outliers. Serum CHI3L1 levels (sCHI3L1) were measured at a 1:50 dilution in 88 CSF samples using the same kit (DC3L10, R&D systems, Minneapolis, MN). To assess blood-brain barrier (BBB) permeability, the albumin index (Q-alb) was calculated for all the samples as the ratio of CSF (mg/L) to the serum albumin concentration (g/L).

MRI Acquisition and Analysis

Brain, cervical, and dorsal spine MRI after a first clinical attack were analyzed. As the initial studies were from different centers and over a long time period (2006–2019), the acquisition protocol and the sequences used were variable. Nevertheless, the FLAIR sequence (transverse or sagittal planes with a thickness between 1 and 3 mm), turbo spin echo T2 sequence (3–5 mm thick), proton density sequence (3–5 mm thick), and T1 sequence after administration of gadolinium (transverse plane and 3–5 mm thick) were available in all cases for analysis. Lesions were classified according to their location, number, and postgadolinium enhancement.

Immunohistochemistry

Histochemistry and immunohistochemistry were performed on sections to distinguish: normal-appearing white matter (NAWM) at least 1 cm from any lesion; classical active lesions following the type I, II, or III pattern of demyelination²⁵; slowly expanding lesions (SELs); and inactive lesions. All these lesions were defined according to previously published criteria.^{26–28} Immunohistochemistry was performed using the biotin-streptavidin method, the details of which were provided previously.²⁹ Sections were incubated with a CHI3L1 antibody (AF2599, R & D Systems) diluted in Dako buffer (S3006, Dako, Agilent, Santa Clara, CA), with 10% horse serum to block nonspecific protein binding, whereas the rest of the antibodies were diluted in Dako buffer

containing 10% fetal calf serum. For triple immunofluorescence, Dako diluent (Dako S3022) was used as the blocking buffer and to dilute the primary antibodies. Secondary antibodies were added sequentially. Images were acquired with an optical microscope Nikon Eclipse Ci (Nikon, Tokyo, Japan) and for immunofluorescence with a confocal microscope Olympus FV1000 (Olympus, Tokyo, Japan). The features of the antibodies used and the staining conditions (e.g., concentration, antigen retrieval) are summarized in tables e-1, links.lww.com/NXI/A427 and e-2, links.lww.com/NXI/A428, and for further details about CHI3L1 assessment, see Appendix e-1, links.lww.com/NXI/A422. As controls, immunohistochemistry was performed in the absence of the primary antibody. Images were obtained with a confocal (Olympus FV1000) and optical microscope (Nikon Eclipse Ci).

Statistical Analysis

Statistical analysis was performed with IBM SPSS v 26.0 software. Regarding clinical data, our primary end point was to assess differences in the levels of biomarkers between patients with distinct demyelinating diseases to relate those to disease activity. From a pathologic viewpoint, the aim was to study the differential expression of CHI3L1 by distinct cell types and its relationship to the inflammatory milieu. The descriptive analysis included the median value and IQR. Differences between the groups in the histologic studies were assessed with a Mann-Whitney *U* test due to the non-normal distribution of the data. For clinical studies, the logarithmic NfL and CHI3L1 values were used and parametric tests were run using an analysis of covariance, adjusting for baseline covariates. In the case of multiple testing (comparisons between more than 2 groups), significant values were corrected with the Bonferroni-Holm procedure. Reported *p* values were the result of 2-sided tests, and a *p* value <0.05 was considered statistically significant. There were no missing values among the variables analyzed because the inclusion criteria required all values to be collected for a patient to be selected.

Results

NfL and CHI3L1 Are Elevated in the CSF of Patients With MS, With Distinct Concentrations Associated With Different Lesion Stages

When measured in the CSF of patients with MS, the median values of CHI3L1 (CHI3L1^{CSF}) and NfL (NfL^{CSF}) were higher than in the controls (*p* < 0.001: table 2), although there were differences among the MS patients in terms of the expression and release of these markers (figure 1). An elevation in NfL^{CSF} levels was mainly evident in patients with MS with disease activity (*p* < 0.001: figure 1A), and it was higher in the RRMS subgroup compared with patients with progressive MS (PMS) (*p* = 0.003). These differences were irrespective of sex, disease duration, or age at the time of LP. Elevated NfL^{CSF} levels were also associated with the incidence (figure 1E, *p* < 0.001) and number of Gd+ lesions in MRI (*p* < 0.001; *r* = 0.495: supplementary figure 1A, links.lww.com/NXI/A423).

In contrast to NfL, CHI3L1^{CSF} levels were higher in patients with PMS than in patients with RRMS (*p* = 0.009) after

adjusting for sex, disease duration, or age (table 1). The EDSS at the time of LP was mildly correlated with the NfL^{CSF} and CHI3L1^{CSF} values (*r* = 0.190; *p* = 0.03; *r* = 0.308; *p* < 0.001, respectively). However, no correlations were evident between the NfL^{CSF} or CHI3L1^{CSF} values and overall lesion load (*p* = 0.349 and *p* = 0.331, respectively) or the location of inactive T2 lesions, and these values only increased moderately with radiologic activity (figure 1E and supplementary figure 1B, links.lww.com/NXI/A423). There was no difference in the CHI3L1^{CSF} levels between patients with or without disease activity (*p* = 0.969; figure 1, B and C), although CHI3L1 outliers (more than 1.5 times the IQR above the Q3) corresponded to patients with a confirmed clinical attack, elevated NfL^{CSF}, and widespread Gd-enhanced lesions concentrated in periventricular and juxtacortical locations (figure 1F).

Pathologic Correlations in Part Explain the Differences Between NfL^{CSF} and CHI3L1^{CSF} in MS Clinical Phenotypes

Global expression of CHI3L1 in MS lesions was determined through the optical density that measured antibody binding in sections, which followed the patterns of CHI3L1 release into the CSF described above. In MS brains, there was stronger CHI3L1 expression than in the control's white matter (figure 2, A–C) and cortex (figure 2, D–F), and although CHI3L1 expression was most pronounced in early active lesions, it was also very prominent in PMS (figure 3; table e-3, links.lww.com/NXI/A429). Indeed, the strongest expression was seen at active lesions and in the NAWM of patients with AMS (figures 2, B, G–I and 3A). Strong expression was also evident in the chronic active lesions and SEL of PMS tissue (figures 3A and 4J–L). A more detailed analysis showed that CHI3L1 was mainly expressed by 2 cell types, microglia/macrophages and astrocytes (supplementary figure 2A–C, links.lww.com/NXI/A424). The expression of CHI3L1 in CD68⁺ macrophages/microglia was mainly seen at early stages of disease activity (figure 2H–I). The CHI3L1+/Iba1+ cells were round, whereas only rarely was CHI3L1 detected in Iba1+ cells with a ramified morphology. At later stages of disease activity (chronic active lesions), CHI3L1 expression by astrocytes in SEL and in the NAWM or normal-appearing gray matter (NAGM) predominated (figures 2B–C, E–F and 4J–L). Although its expression was diffuse in the NAWM or NAGM, in late active and chronic lesions, including SEL, CHI3L1+ astrocytes were found at the periphery of the lesions forming an active border (see arrows in figures 2I and 4J–L). Only very weak expression was seen in inactive lesions (supplementary figure 3A–F, links.lww.com/NXI/A425).

Regarding the association of CHI3L1-expressing cells (figure 3B–E) with inflammatory infiltrates in late active lesions, the number of these cells correlated with the density of CD68⁺ macrophages/microglia (*r* = 0.626; *p* < 0.001) (figure 3D). Although there was some correlation

Figure 1 NfL and CHI3L1 Levels in the CSF of Patients With MS, NMO, and ADEM Relative to Disease Activity

(A) NfL levels in the CSF increased significantly in patients with MS in the context of disease activity alone. In remission, the levels of NfL in the patients with ADEM differed significantly to those in the patients with MS and NMO. (B) Increased CHI3L1 levels in the CSF of patients with MS, NMO, and ADEM during disease activity, and they were higher in the NMO and ADEM samples than in those from patients with MS. The levels of CSF CHI3L1 in ADEM were significantly higher than in the remission phase. (C) There was significantly more CHI3L1 in progressive MS (PMS) irrespective of disease activity. (D) There was a trend toward more CHI3L1 in patients with PMS than in patients with NMO and ADEM in remission. (E) NfL levels in the CSF were associated with radiologic activity, with a trend toward an association with CHI3L1. (F) The average number of Gd+ lesions in MS and the distribution of these lesions in patients with high levels of NfL and CHI3L1 (oNfL/oCHI3L1) or high NfL and low CHI3L1 (oNfL). Median levels of NfL and CHI3L1 in RMS outside relapse are represented as a red line for reference. A p value of <0.05 was considered statistically significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). ADEM = acute disseminated encephalomyelitis; CHI3L1 = chitinase 3-like 1; Gd+ = gadolinium-enhanced lesion; NfL = neurofilament light protein; NMO = neuromyelitis optica; oCHI3L1 = outlier CHI3L1; oNfL = outlier NfL; RMS = relapsing MS.

between the density of CD68⁺ cells and lymphocyte infiltration into the tissue (figure 3E), no association was found between CD68⁺ cells and meningeal follicles ($p =$

0.621) or between the CHI3L1⁺ cells and lymphocytic aggregates in the NAWM ($p = 0.116$) or meninges ($p = 0.627$).

Figure 2 Distribution of CHI3L1 Expression in the Brain of Controls, Patients With AMS, and Patients With RRMS

Light microscope micrographs of AMS and RRMS specimens (A–I). NAWM (A–C). CHI3L1 expression was not found in the NAWM of controls (A), whereas diffuse expression was detected in patients with AMS (B) and RRMS (C). Cortex (D–F). Similarly, no CHI3L1 expression was detected in control specimens (D), whereas it was weakly expressed in the cortex of patients with AMS (E) but not patients with RRMS (F). Active lesion (G–I). A typical early active white matter lesion in a patient with AMS with profuse perivenular infiltrates of CD68⁺ cells (H) and demyelination, as shown by Klüver-Barrera staining (G). CHI3L1 expression (I) was intense within the lesion, expressed in the center by round cells that correspond to microglia, and at the periphery or active border by larger ramified cells corresponding to astrocytes (arrows in I). CHI3L1, chitinase 3-like 1; AMS = acute MS; NAWM = normal-appearing white matter; RRMS, relapsing-remitting MS.

The pathologic correlates of NfL in the CSF were acute neuronal and axonal damage. The best pathologic indicator was axonal dystrophy, reflected by the accumulation of amyloid precursor protein (APP) in dystrophic axons and neurons, due to a disturbance of acute axonal transport. A considerable number of APP⁺ spheroids were found in active lesions (figure 2I left detail), and they were spatially related to the CHI3L1 expressed by astrocytes, yet not by microglia, at the edge of the active and chronic active lesions or SELs (figure 4G–I; detail in fig 4H; supplementary figure 4, links.lww.com/NXI/A426).

CHI3L1 Is Not Expressed Outside of Relapses in Monophasic or Relapsing Inflammatory Demyelinating Diseases That Lack a Progressive Stage

In addition to the very high NfL levels (figure 1A), the most characteristic change in the CSF of AQP4⁺-NMO patients during a clinical attack was the extreme increase in CHI3L1, which was two-fold higher than in MS and that remained significantly different after adjusting for sex, age, and disease duration ($p < 0.001$; figure 1B). CHI3L1^{CSF} levels did not correlate with the number of contrast-enhancing lesions ($r = 0.632$; $p =$

0.253), and these values normalized in remission when they were lower than in PMS. However, the small sample size precluded assessing the statistical significance of these changes (figure 1D).

In the brain specimen of NMO analyzed, 2 active lesions and an inactive lesion were identified, with AQP4 staining showing the lesion center to be devoid of astrocytes (figure 5A–B). The active center was infiltrated by CHI3L1⁺/CD68⁺ cells and characterized by a ribbon of hypertrophic astrocytes at the border of the lesion, cells that coexpressed CHI3L1 and glial fibrillary acidic protein (GFAP) strongly (figure 5C–E). CHI3L1⁺ astrocytes were enlarged and had lost their expansions, a process known as clasmotodendrosis (figure 5E, inset), normally followed by necrosis. CHI3L1⁺ astrocytes were absent from the inactive center, NAWM, and NAGM. However, CHI3L1 immunoreactivity persisted in the extracellular space of some inactive lesions (figure 5H), which could be explained by massive release from necrotic astrocytes into the extracellular space.

ADEM is a short-lasting inflammatory disease in which a monophasic attack is followed by the clearance of inflammation,

Figure 3 CHI3L1-Expressing Cells in MS and Their Relationship to the Immune Response in MS Lesions

(A) Quantification of CHI3L1 expression, measured by the OD of staining in different areas of all MS and control specimens. (B) Density of CHI3L1+ cells in each area and type of lesion. (C) Density of CD68+ cells in the distinct types of lesions. (D) There was a positive correlation between CHI3L1 and CD68 expressing cells in all the regions analyzed. (E) There was a positive correlation between perivascular infiltrates and CD68+ cells but not with CHI3L1-expressing cells (not shown). CHI3L1 density: AL cortex vs IL cortex ($p = 0.053$), AL WM vs IL WM ($p = 0.083$), and SEL edge vs SEL core ($p = 0.053$). *CD68 density: AL cortex vs IL cortex ($p = 0.000$), AL WM vs IL WM ($p = 0.083$), and SEL edge vs SEL core ($p = 0.053$). AL = active lesion; AMS = acute MS; CHI3L1 = chitinase 3-like 1; IL = inactive lesion; OD = optical density; PMS = progressive MS. SEL = slow-expanding lesion; WM = white matter.

some perivascular axonal loss, astrocyte gliosis, and remyelination of the remaining demyelinated axons. Extremely high NfL values were registered in the CSF (figure 1A), reflecting the extensive and acute axonal damage that was associated with a worse EDSS ($r = 0.622$; $p = 0.031$). Likewise, CSF CHI3L1 reflected the intense inflammatory activity in the early stages of disease (figure 1B) and its later normalization compared with PMS (figure 1D), which was correlated with the time from the clinical attack ($r = -0.649$; $p = 0.012$). The early liberation of

CHI3L1 into the CSF was apparently due to perivascular activated microglial infiltration into the active lesions, NAWM, or NAGM (figure 5I–J), as no CHI3L1-expressing astrocytes were found in the ADEM case analyzed.

Serum CHI3L1 Levels Are High in PPMS but They Do Not Reliably Reflect CHI3L1^{CSF} Levels

As CHI3L1^{CSF} levels might be informative of innate inflammatory disease activity in patients with RRMS or PMS, we

Figure 4 Distribution of CHI3L1 Expression in the Brain of PMS Cases

Light microscopy micrographs of active and inactive lesions in patients with PMS. Active lesions in the WM (left column), cortex (medium column), and SEL (right column) (A–L). All active lesions displayed extensive demyelination, evident as a loss of reactivity to PLP (A–C), intense inflammatory infiltrates of CD68⁺ cells (D–F), and an accumulation of APP in transected axons and spheroids as a sign of neurodegeneration (G–I). A detail of the spatial relationship between CHI3L1⁺ astrocytes and APP⁺ spheroids is shown as a subset in H; APP: red, GFAP: white, CHI3L1: green, DAPI: blue. CHI3L1 expression was less diffuse and more restricted to the expanding border of the lesions (arrows), and most cells were morphologically identified as astrocytes (see details in J–L). Scale bar: 100 μ m (A–L), 20 μ m (details J–L); 10 μ m (detail in H). APP = amyloid precursor protein; CHI3L1 = chitinase 3-like 1; GFAP = glial fibrillary acidic protein; PLP = myelin proteolipid protein; PMS = progressive MS; SEL = slow-expanding lesion; WM = white matter.

studied its correlation with sCHI3L1 levels and its value as a potential surrogate marker for low-grade ongoing inflammation. Serum CHI3L1 levels tended to be higher in patients with PMS than in patients with RRMS (31.9 ng/mL [25–40.1] and 28.63 ng/mL [22.5–37.6], respectively; $p = 0.062$, and they were significantly higher in patients with PPMS [38.2 ng/mL, 26.7–49.8]; $p = 0.014$). However, the sCHI3L1 values were not correlated with the CHI3L1^{CSF} values ($r = 0.150$; $p = 0.162$), and they were not associated with disease activity ($p = 0.1$), Gd⁺ lesions ($p = 0.397$), or EDSS at the time of LP ($p = 0.188$).

Discussion

In this study, we show that CHI3L1 and NfL are released to the CSF in the context of acute inflammation in all the demyelinating diseases analyzed: MS, NMO, and ADEM. CHI3L1^{CSF} values only remained elevated outside the

periods of relapse in patients with MS and, in particular, in those with a progressive disease. From the pathologic studies, it appears that elevated NfL values are correlated with neuroaxonal damage, evident through the presence of APP⁺ axons, and CHI3L1 in MS might be a marker for more global tissue injury, induced not only in macrophages/microglia as a probable reaction to adaptive inflammation but also, in astrocytes, possibly as a stress response to low-grade chronic inflammation and ongoing neurodegeneration.

Although in all inflammatory demyelinating diseases CHI3L1 was expressed strongly in active lesions, only NfL CSF levels corresponded to disease activity. Thus, we infer from our data that the CSF concentration of a biomarker may not only be proportional to the extent of its local production in the focal lesion but also it may depend on other factors. An important feature that may influence the concentration of a CNS protein in the CSF is its ability to diffuse through the narrow

Figure 5 CHI3L1 Expression in NMO and ADEM

Light microscopy micrographs of a case of NMO and of ADEM. NMO (A–H). Active lesions in NMO were devoid of GFAP (A) and AQP4 (B), reflecting astrocyte damage. Loss of PLP reactivity at the active border of the lesion is related to active demyelination (C). The active edge showed CD68⁺ (D) and CHI3L1 (E) infiltrates. All CHI3L1⁺ astrocytes had a disrupted morphology as a result of a process referred to as clasmotodendrosis (detail in E). At the inactive center of the lesion, residual demyelination could be observed in the absence of inflammatory cells (F–G), and CHI3L1⁺ extracellular debris was seen (asterisk in H). Scale bar: 250 μ m (A, B); 100 μ m (C–J); 10 μ m (detail in E and K). ADEM = acute disseminated encephalomyelitis; AQP4 = aquaporin type 4; CHI3L1 = chitinase 3–like 1; GFAP = glial fibrillary acid protein; NMO = neuromyelitis optica; PLP = myelin proteolipid protein.

extracellular space of the brain and spinal cord. We only saw elevated CHI3L1^{CSF} in patients with intense radiologic activity and predominant periventricular or cortical/juxtacortical lesions, i.e., with active inflammation. The compartmentalization of the brain and crosstalk between the CSF brain and CSF pial barriers could explain this phenomenon, as suggested previously.³⁰ The diffusion rate of a protein in the brain extracellular space is determined by its molecular size and molecular charge. Although the molecular weights of NfL and CHI3L1 are similar, there is an important difference in their molecular charge. CHI3L1 is a strongly cationic protein³¹ that may bind to the anionic surface of astrocytes within the brain extracellular space, restricting its diffusion in the CNS like other cationic proteins.³² Likewise, and unlike NfL, the sCHI3L1¹² levels are

not correlated with those in the CSF, which may again be due to more restricted diffusion through the brain extracellular space and across the BBB.

In NMO and ADEM, CHI3L1 is expressed and released into the CSF in association with clinical bouts.^{14,15} Although in these diseases CHI3L1 was present in activated macrophages and microglia at active lesions, the extremely high levels of CHI3L1^{CSF} detected in the case of NMO may be related to the release of CHI3L1 from damaged astrocytes. Indeed, CHI3L1 was expressed in degenerating astrocytes at the active lesion edge, and it also formed aggregates in the extracellular space around the inactive lesion center. A similar pattern has been described for other astrocyte proteins, such

as GFAP and S-100b, which are also released into the extracellular space by degenerating astrocytes³³ and that accumulate to very high levels in the CSF during the acute phase of the disease.³⁴ Clinical ADEM is characterized by diffuse brain inflammation at several locations and numerous Gd+ lesions. This widespread inflammation might explain the increase in CHI3L1^{CSF} during a clinical attack. In active ADEM lesions, CHI3L1 appears to be expressed by macrophages/microglia but not by astrocytes. The absence of CHI3L1-expressing astrocytes in the patient with ADEM could, at least in part, be due to a later induction of CHI3L1 expression by astrocytes as the patient died 2 days after onset. Alternatively, it could have been due to a distinct pathogenic mechanism of lesion production mediated by other immune cell types.

Outside of relapse, median CHI3L1^{CSF} levels were higher in patients with PMS than in patients with RRMS, and sCHI3L1 levels were also higher in patients with PMS, as seen previously.³⁵ These discrepancies between RRMS and PMS may be due to differences in BBB permeability and/or the location of lesions, suggesting a different pattern of release that may be related to the lesion location or to the diffuse changes representative of the progressive disease.

The relationship between CHI3L1^{CSF} and active neuroinflammation, and the elevation of CHI3L1^{CSF} in PMS, implies a possible relationship between this biomarker and the progression of disability. The pathologic changes in patients with PMS indicate that CHI3L1 is expressed by astrocytes at the edge of chronic active lesions and that it is not correlated with lymphocytic infiltrates in the NAWM or in meningeal follicles in the vicinity of lesions. Hence, CHI3L1 in PMS might be linked to the innate immunity associated with low-grade inflammation. In fact, intrathecal B-cell accumulation, a reflection of adaptive immunity, was not correlated with CHI3L1^{CSF} levels.³⁶ Whether CHI3L1 expression fulfills a protective or pathogenic role in disease is still unclear. Although CHI3L1 expression implies a worse prognosis in several cancers and chronic inflammatory processes outside the nervous system, while there is also evidence of an anti-inflammatory role for CHI3L1.³⁷ In support of the benefits that CHI3L1 may provide, demyelination and neuronal injury were exacerbated in a mouse experimental autoimmune encephalomyelitis model of MS lacking the *CHI3L1* gene.³⁸ In fact, the *CHI3L1* knockout mice displayed extensive astrogliosis and were in a continuous proinflammatory state, suggesting that they underwent immune deregulation in the absence of the protein.³⁹ In contrast, within the CNS, a pathogenic role for CHI3L1 in perpetuating inflammation or inflicting neuronal damage has been proposed. The CHI3L1 protein induced cytotoxicity in cultured neurons in vitro through a mechanism that is as yet unknown.⁴⁰ Moreover, CHI3L1^{CSF} levels increase in neurodegenerative diseases like Alzheimer disease as patients deteriorate,^{41–43} and high levels of this protein in amyotrophic lateral sclerosis imply a more accelerated disease course.⁴⁴ We showed previously that CHI3L1 was the only independent factor associated with a 1-point EDSS progression and the possibility of receiving a diagnosis of progressive disease

in patients with RRMS.³ It was also recently reported that astrocytes might have a neurotoxic and gliotoxic effect in acute and chronic MS lesions.⁴⁵ Indeed, APP staining was spatially and temporally related to astrocytic CHI3L1 in active MS lesions, including chronic cortical lesions. Thus, the hypothesis that CHI3L1+ astrocytes might play a direct role in MS neurodegeneration is feasible. In fact, another astrocytic protein, the GFAP, has been proposed as a reliable biomarker of progressive damage in MS both in serum and CSF.⁴⁶ The difference between these biomarkers lies in the fact that GFAP is a structural pan-astrocytic marker, whereas CHI3L1 expression is restricted to reactive astrocytes and microglia/macrophages, such as those seen in active inflammatory lesions, and that it might therefore provide additional pathogenic information.

Ongoing neurodegeneration does not occur in demyelinating diseases like NMO and ADEM, and indeed, we found that both entities had low CHI3L1^{CSF} levels during remission. Axonal damage related to astrocytes expressing CHI3L1 could explain the absence of progressive injury when these astrocytes suffer necrosis. Inactive lesions in NMO lack CHI3L1+ astrocytes because these cells had died due to disease-specific mechanisms. In the case of ADEM, CHI3L1 astrocytes might not be induced or present in the acute phase, although we lack an autopsic case in remission to confirm its absence.

Our study has some limitations. The assessment of CHI3L1 in CSF was cross-sectional, and the RRMS population with severe clinical conditions in the clinical study might be underrepresented as there were only 6 patients with EDSS score >5 (3.7%). Nevertheless, all cases were accompanied by complete clinical, radiologic, and biochemical data. The pathologic samples of patients with RRMS were cases of aggressive disease or AMS, and thus, these are cases of extreme inflammation, however, an extensive clinical sample with well-characterized histologic MS lesions was analyzed. Only single cases of NMO and ADEM were available for the pathologic study.

In summary, both NfL and CHI3L1 are released into the CSF during acute inflammation in the demyelinating diseases studied, yet in MS, the elevation of CHI3L1 might also be linked to the progressive form of the disease. This is paralleled by pathologic evidence of low-grade non-lymphocytic inflammation by CHI3L1+ astrocytes in chronic lesions that are associated with active neurodegeneration. It would be interesting to perform a spatial-temporal assessment of CHI3L1 expression to elucidate whether it is an early or late event in the inflammatory process that triggers or maintains disease. Indeed, further prospective studies might help to strengthen the link between CHI3L1, disease progression, and neurodegeneration.

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Disclosure

L. Cubas-Núñez reports no disclosures. S. Gil-Perotín has received compensation for serving on scientific advisory boards or speaking honoraria from Biogen Idec and Sanofi-Aventis, outside the submitted work. J. Castillo-Villalba, V. López, L. Solís-Tarazona, R. Gasqué-Rubio, S. Carratalá-Boscá, and C. Alcalá-Vicente report no disclosures. F. Pérez-Miralles was part of the steering committee of the UPPMS study and has received compensation for serving on scientific advisory boards or speaking honoraria from Almirall, Biogen Idec, Genzyme, Merk-Serono, Mylan, Novartis, Roche, Sanofi-Aventis, and Teva, outside the submitted work. H. Lassmann reports no disclosures. B. Casanova has received compensation for serving on scientific advisory boards or speaking honoraria from Biogen Idec, Merk-Serono, Mylan, Novartis, Roche, and Sanofi-Aventis, outside the submitted work. Go to Neurology.org/NN for full disclosures.

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Appendix Authors

Name	Location	Contribution
Laura Cubas Núñez, PhD	Neuroimmunology Unit, Polytechnic and University Hospital La Fe, Valencia, Spain	Performed the immunohistochemistry and obtained the optical densitometry data and ran the statistical analyses on the data; revised the manuscript for intellectual content
Sara Gil-Perotín, MD, PhD	Neuroimmunology Unit, Polytechnic and University Hospital La Fe, Valencia, Spain	Designed the study and analyzed the data, collected and analyzed the clinical data, ran the statistical analyses on these data, supervised the results, wrote the manuscript, and revised the final version
Jéssica Castillo-Villalba, BSc	Neuroimmunology Unit, Polytechnic and University Hospital La Fe, Valencia, Spain	Helped in performing biomarker measurements and revised the manuscript
Verónica López, BSc	Neuroimmunology Unit, Polytechnic and University Hospital La Fe, Valencia, Spain	Performed the cell counting, helped design the figures, and revised the manuscript
Luis Solís-Tarazona, MD	Neurology Department, University Hospital Dr Peset, Valencia, Spain	Helped to collect radiologic and clinical data and revised the manuscript

Appendix (continued)

Name	Location	Contribution
Raquel Gasqué-Rubio, BSc	Neuroimmunology Unit, Polytechnic and University Hospital La Fe, Valencia, Spain	Helped in performing biomarker measurements and revised the manuscript
Sara Carratalá-Boscá, MA	Neuroimmunology Unit, Polytechnic and University Hospital La Fe, Valencia, Spain	Helped to collect radiologic clinical data and revised the manuscript
Carmen Alcalá-Vicente, MD, PhD	Neuroimmunology Unit, Polytechnic and University Hospital La Fe, Valencia, Spain	Attending physician of patients included in the study, helped to register the clinical data, and revised the manuscript
Francisco Pérez-Miralles, MD, PhD	Neuroimmunology Unit, Polytechnic and University Hospital La Fe, Valencia, Spain	Attending physician of patients included in the study, helped to register the clinical data, and revised the manuscript
Hans Lassmann, MD, PhD	Department of Neuroimmunology, Center for Brain Research, Vienna, Austria	Helped to design the pathologic data collection, ceded brain samples and the optical densitometry plug-in to analyze the images, and assisted with the interpretation of histologic data and writing of the manuscript
Bonaventura Casanova, MD, PhD	Neuroimmunology Unit, Polytechnic and University Hospital La Fe, Valencia, Spain	Head of the Neuroimmunology laboratory and attending physician of the patients included in the study, helped to register the clinical data, study design, and revised the manuscript

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