RESEARCH PAPER

Chitotriosidase (CHIT1) is increased in microglia and macrophages in spinal cord of amyotrophic lateral sclerosis and cerebrospinal fluid levels correlate with disease severity and progression

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ABSTRACT

Objectives Neurochemical markers of amyotrophic lateral sclerosis (ALS) that reflect underlying disease mechanisms might help in diagnosis, staging and prediction of outcome. We aimed at determining the origin and differential diagnostic and prognostic potential of the putative marker of microglial activation chitotriosidase (CHIT1).

Methods Altogether 316 patients were included, comprising patients with sporadic ALS, ALS mimics (disease controls (DCo)), frontotemporal lobar degeneration (FTLD), Creutzfeldt-Jakob disease (CJD), Alzheimer's disease (AD), Parkinson's disease (PD) and healthy controls (Con). CHIT1 and neurofilament levels were determined in cerebrospinal fluid (CSF) and blood and analysed with regard to diagnostic sensitivity and specificity and prognostic performance. Additionally, postmortem tissue was analysed for CHIT1 expression.

Results In ALS, CHIT1 CSF levels were higher compared with Con ($p<0.0001$), DCo ($p<0.05$) and neurodegenerative diseases (AD $p<0.05$, PD $p<0.01$, FTLD $p<0.0001$) except CJD. CHIT1 concentrations were correlated with ALS disease progression and severity but not with the survival time, as did neurofilaments. Serum CHIT1 levels were not different in ALS compared with any other study group. In the spinal cord of patients with ALS, but not Con, AD or CJD cases, CHIT1 was expressed in the corticospinal tract and CHIT1 staining colocalised with markers of microglia (IBA1) and macrophages (CD68).

Conclusions CHIT1 concentrations in the CSF of patients with ALS may reflect the extent of microglia/macrophage activation in the white matter of the spinal cord. CHIT1 could be a potentially useful marker for differential diagnosis and prediction of disease progression in ALS and, therefore, seems suitable as a supplemental marker for patient stratification in therapeutic trials.

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease with a median survival time of 3 years.[1] It has been well recognised that in ALS non-cell autonomous mechanisms contribute to the pathogenesis and have a great impact on neurodegeneration (for review[2]) influencing onset and progression.[3] Immune modulatory therapies have been proposed to treat ALS; thus, tools are required to define the patients' inflammatory state.

When patients fulfil the clinical criteria of ALS, they are usually in an advanced disease stage.[4 5] Therefore, biomarkers are needed to aid early differential diagnosis, and as a tool to indicate the affected cell types during disease progression, outcome prediction and patient stratification for clinical trials.[6] At present, the most promising neurochemical biomarker for ALS is neurofilament, which reflects axonal dysfunction.[7–13]

In studies with small patient cohorts, it was recently reported that chitotriosidase (CHIT1) is increased in the cerebrospinal fluid (CSF) of patients with ALS compared with healthy and neurological controls[14 15] and that blood CHIT1 activity correlates with disease progression.[16] CHIT1 is expressed by cells of the monocyte/macrophage lineage and cleaves N-acetyl glucosamine polymers (mainly found in chitin). It is associated with innate immunity and conditions of acute or chronic inflammation.[17] Increased activity of plasma CHIT1 is used as a marker for lysosomal storage disorders.[18]

In this study, we analysed the potential of CHIT1 for its use as a differential diagnostic marker and the prognostic performance of CHIT1 in CSF and blood of patients with ALS in comparison to neurofilaments. In addition, we investigated spinal cord and brain tissue to examine the expression pattern and origin of CHIT1.

MATERIAL AND METHODS

Subjects and clinical characterisation

CSF and/or blood samples of 316 patients were investigated in this prospective study. Patients were

diagnosed at the Department of Neurology in Ulm from May 2010 to June 2015. Additional samples were obtained from the German frontotemporal lobar degeneration (FTLD) consortium, a quality controlled monitored multicentre initiative (www.ftld.de, December 2011 to September 2014) and from two centres for transmissible spongiform encephalopathies (Universities of Göttingen and Ulm 2002–2015). All studies with an overall aim to find novel biomarkers for neurological diseases were approved by the local ethics committees according to institutional guidelines, and patients provided written informed consent to participate in these studies (approval number 100305, 20/10, 39/11). Only patients not willing to participate were not included.

The CSF study cohort was composed of patients with ALS (n=60)[5] with slow and fast progression rates (PRs) (decrease of the revised ALS functional rating scale (ALSFRS-R)[19] score of <0.2 and >1.0 point/month, respectively), patients with initially suspected ALS but with other final diagnoses (disease controls (DCo), n=46), patients with other neurodegenerative diseases (n=135, spanning the spectrum of FTLD (patients with behavioural variant frontotemporal dementia, non-fluent variant of primary progressive aphasia (PPA), semantic variant of PPA, logopenic variant of PPA, progressive supranuclear palsy, corticobasal syndrome), patients with Alzheimer's disease (AD), sporadic Creutzfeldt-Jakob disease (CJD), and Parkinson's disease (PD)) and patients without signs of a neurodegenerative disease or an acute or chronic inflammatory process (controls (Con), n=25). A summary of patient group characteristics is given in table 1, detailed information about the DCo cohort is given in table 2.

Depending on the initial differential diagnosis, a standardised procedure of investigations and routine laboratory examinations were performed. For ALS, the physical function status was classified with the ALSFRS-R[19] with a maximum of 48 points, where lower values represented a more severe disease stage.

The serum study cohort included 40 patients with ALS, of whom 20 had a slow (PR<0.5) and 20 a fast (PR >1.0) progression of disease. Two serum samples were available for each patient, taken at an interval of 6 months. Patient characteristics are given in online supplementary table S1. For analysis of the differential diagnostic utility of serum CHIT1, protein concentrations were also measured in a single sample in 34 patients out of the DCo group and in 10 healthy volunteers.

Laboratory markers

CSF was obtained by lumbar puncture, centrifuged, aliquoted and stored within 2 hours at −80°C until analysis. Serum was extracted from blood (800 g, 5 min, 18°C), aliquoted and stored within 2 hours at −80°C until analysis. Analytes were handled by investigators blinded to patients' diagnoses.

ELISA kits were used for CHIT1 (MBL, Diegem, Belgium),[14] neurofilament light chain (NfL) (IBL, Hamburg, Germany) and phosphorylated neurofilament heavy chain (pNfH) (Biovendor, Heidelberg, Germany). For CHIT1, serum samples were added to the ELISA plate at 1:50 dilution, and CSF samples were diluted 1:5. Samples were incubated for 1 hour at room temperature. For NfL and pNfH, samples were diluted 1:1 and 1:3, respectively. For quality control, aliquots of the same six CSF samples were run on each plate. The mean interplate coefficient of variation was <20% for CHIT1, NfL and pNfH.

Immunohistochemistry and Immunofluorescence

Details on the antibodies used in this study are given in online supplementary table S2.

Table 1 Characteristics of the patient cohort

	Sex, f/m	Age (years)	CHIT1 (pg/mL)	NfL (pg/mL)	pNfH (pg/mL)	Disease duration (months)	ALSFRS-R	PR
ALS (n=60)	21/39	62.5 (52–70)	6978 (2806–18 829)	5417 (1571–10 302)	1958 (473–3854)	48 (29–134)	41.5 (28.5–46)	0.67 (0.04–1.74)
Slow progression (n=30)	8/22	55 (48–62)	3060 (2375–7705)	1818 (732–5527)	563 (232–2036)	133 (83–266)	46 (45–48)	0.04 (0–0.09)
Fast progression (n=30)	13/17	69 (63–73)	15 023 (5880–40 211)	8037 (4694–14 030)	3421 (1896–5425)	29 (21–42)	35 (27–39)	1.73 (1.6–3.0)
AD (n=25)	17/8	70 (63–74)	2470 (1523–4538)	1209 (1043–1640)	318 (234–421)			
PD (n=15)	10/5	69 (64–70)	1335 (605–6045)	1082 (727–1675)	512 (327–756)			
CJD (n=15)	9/6	62 (58–68)	2235 (1320–5640)	5450 (3610–12 370)	2670 (1353–5202)			
FTLD (n=80)	36/44	67 (58–73)	2470 (1289–5273)	2301 (1285–3640)	477 (284–669)			
bvFTD (n=31)	10/21	59 (54–72)	2050 (1130–6595)	1985 (1203–3000)	359 (256–656)			
PPA (n=23)	12/11	68 (61–75)	2190 (1380–3380)	3407 (2059–4250)	501 (324–648)			
nfvPPA (n=8)	3/5	76 (74–78)	2618 (1050–3575)	3466 (2583–4668)	5940 (330–1028)			
svPPA (n=10)	8/2	63 (56–69)	2065 (1211–3445)	4143 (3316–4371)	481 (236–671)			
lvPPA (n=5)	1/4	67(52–73)	2440 (2053–13 395)	1195 (1018–2197)	503 (294–537)			
CBS (n=7)	4/3	68 (57–70)	3460 (1610–8485)	1286 (1055–2326)	249 (243–519)			
PSP (n=19)	10/9	69 (64–74)	3195 (1460–6735)	2517 (1440–3232)	649 (468–965)			
Disease controls (DCo) (n=46)	11/35	58 (46–72)	2455 (1165–7850)	930 (658–1889)	361 (201–921)			
Controls (Con) (n=25)	15/10	68 (62–73)	1185 (730–1688)	742 (538–1083)	198 (157–198)			

Given are median values with IQR for age, disease characteristics, and measurements in cerebrospinal fluid.
AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS functional rating scale revised; bvFTD, behavioural variant frontotemporal dementia; CBS, corticobasal syndrome; CHIT1, chitotriosidase; CJD, Creutzfeldt-Jakob disease; FTLD, frontotemporal lobar degeneration; lvPPA, logopenic variant PPA; NfL, neurofilament light chain; nfvPPA, non-fluent variant PPA; PD, Parkinson's disease; pNfH, phosphorylated neurofilament heavy chain; PPA, primary progressive aphasia; PR, progression rate; PSP, progressive supranuclear palsy; svPPA, semantic variant PPA.

Table 2 Characteristics of initially suspected amyotrophic lateral sclerosis cases finally diagnosed with other diseases

Diagnosis	n	CSF CHIT1 (pg/mL)	Serum CHIT1 (pg/mL)	CSF NfL (pg/mL)	CSF pNfH (pg/mL)
Polyneuropathy	13	2440* (890–14 280)	32 900 (18 600–78 400)	922 (216–3535)	309† (63–1580)
Myopathy	7	2440* (795–58 260)	32 350* (25 350–54 950)	1202 (406–9165)	414† (62–2653)
Myositis	5	3165 (795–9960)	30 350 (13 850–123 400)	923 (405–3266)	374 (242–2096)
Hereditary spastic paraplegia‡	4	1725† (650–3990)	17 800† (17 450–73 650)	630 (168–937)	177† (100–244)
Cramp-fasciculation syndrome	4	1240 (281–14 130)	26 350† (2810–40 400)	595 (469–7622)	252 (90–4160)
Chronic inflammatory demyelinating polyneuropathy	3	8640 (1040–39 420)	n.a.	5104† (3548–6660)	1599† (1264–1934)
Somatization disorder	4	2495 (4205–6020)	34 125 (14 750–43 900)	921 (338–1502)	266 (93–225)
Anterior spinal artery syndrome	1	7820	27 300	891	274
Encephalitis	1	2970	n.a.	3498	1293
Myelitis	1	1000	32 100	921	252
Peroneal neuropathy	1	15 270	46 350	1534	384
Cervical myelopathy with radiculopathy	1	30 820	n.a.	4380	1923
Spinocerebellar ataxia	1	1165	60 300	1161	799

*Two values missing
†One value missing
Given are median values and ranges.
CHIT1, chitotriosidase; NfL, neurofilament light chain; pNfH, phosphorylated neurofilament heavy chain.

Cases with the following neuropathological diagnoses were stained by immunohistochemistry (IHC): n=3 ALS, n=2 CJD, n=2 AD, n=2 Con. Sections (4 µm) from different anatomical regions were automatically stained (Ventana Benchmark) applying IHC methodology. The following antibodies were used: CHIT1 (HPA010575 Sigma-Aldrich, St. Louis, Missouri, USA, 1:50), anti-pTDP43 (clone 1D3, 1:50), anti-PrP^{Sc} (clone L42, Insel Riems, 1:40), anti-HLA-DP/DQ/DR (clone CR3/43, DAKO, Hamburg, Germany, 1:100).

Paraffin-embedded spinal cord tissue from three patients with ALS and two Con was used for double immunofluorescence (IF) staining. Antigen retrieval was performed by heat mediation in a 10 mM citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked with 20 min incubation in 1% hydrogen peroxidase. Unspecific binding was blocked with 5% bovine serum albumin (BSA)+10% normal donkey serum for 1 hour at room temperature. Sections were incubated with rabbit anti-CHIT1 polyclonal antibody (HPA010575 Sigma-Aldrich, 1:50) and goat anti-IBA1 polyclonal antibody (Abcam, Cambridge, UK, 1:500) or monoclonal mouse anti-CD68 (DAKO, Hamburg, Germany, 1:200) overnight. The corresponding secondary antibodies (donkey anti-rabbit Alexa Fluor 594 (ab150064) and Alexa Fluor 488 (ab150129, Abcam, 1:200) or m-IgGk BP-CFL 488 (sc-516176, Santa Cruz, Heidelberg, Germany, 1:100) were incubated with the sections for 1 hour at room temperature. Cell nuclei were visualised with 4′, 6-diamidino-2-phenylindole, and Mowiol was used as mounting medium. Omitting the primary antibody resulted in lack of staining for all markers. In addition, the specificity of CHIT1 immunostaining was confirmed by incubating sections with primary antibody in the presence of excess CHIT1 immunogen (PrEST Antigen CHIT1 APrEST71944, Sigma-Aldrich) overnight at 4°C.

Calculations and statistical analyses

Statistical analysis was performed using GraphPad Prism 5.0. Assessment of normality of data with the D'Agostino-Pearson test revealed a non-Gaussian distribution. Therefore, the non-parametric two-tailed unpaired Mann-Whitney U test and Kruskal-Wallis test with Dunn's post hoc test were used to determine statistical differences. Correlations between parameters were calculated using the non-parametric two-tailed Spearman's rank correlation test at a 5% significance level. ALS disease duration was defined as time between self-reported date of first paresis to time point of sampling. Individual disease PRs were calculated by dividing the reduction in ALSFRS-R score (ALSFRS-R at onset, set to 48 minus present ALSFRS-R score) by the time in months between onset and sampling or by dividing the reduction in the ALSFRS-R between two examinations by the time of the interval in months according to established protocols.[11 20] The optimal cut-off level for dichotomising values was selected as the one resulting in the highest Youden index. The receiver operating characteristics curve was used for a graphical visualisation of the impact of the variation in the cut-off values.

RESULTS

Demographic and disease characteristics of the study groups

The age of patients in the diagnostic groups was different (p=0.0071), but post hoc testing only revealed significant differences between patients with AD and healthy controls. Patients with slow ALS progression were significantly younger, had a higher ALSFRS-R score at examination and longer disease durations than patients with fast disease progression (p<0.0001, respectively).

CHIT1 concentrations in the CSF of ALS compared with Con, DCo and other neurodegenerative diseases

A boxplot of CHIT1 concentrations measured in CSF of the diagnostic groups is given in figure 1A. In the group of patients with ALS, CHIT1 concentrations were higher than in other neurodegenerative diseases and Con (p<0.0001). Post hoc test revealed significant differences between ALS and FTLD, AD and PD, respectively, but not CJD due to exceptionally high CHIT1 concentrations in two samples. CSF-CHIT1 in DCo was significantly lower than in patients with ALS (p=0.0016), but higher than in Con (p=0.0034). Within the group of DCo, the highest CHIT1 levels were found in the CSF of a patient with limb-girdle muscular dystrophy (58 260 pg/mL), a patient with chronic inflammatory demyelinating polyneuropathy and monoclonal gammopathy (39 420 pg/mL), who also had extremely high levels of NfL and pNfH, and a patient with cervical myelopathy with radiculopathy (30 820 pg/mL). One

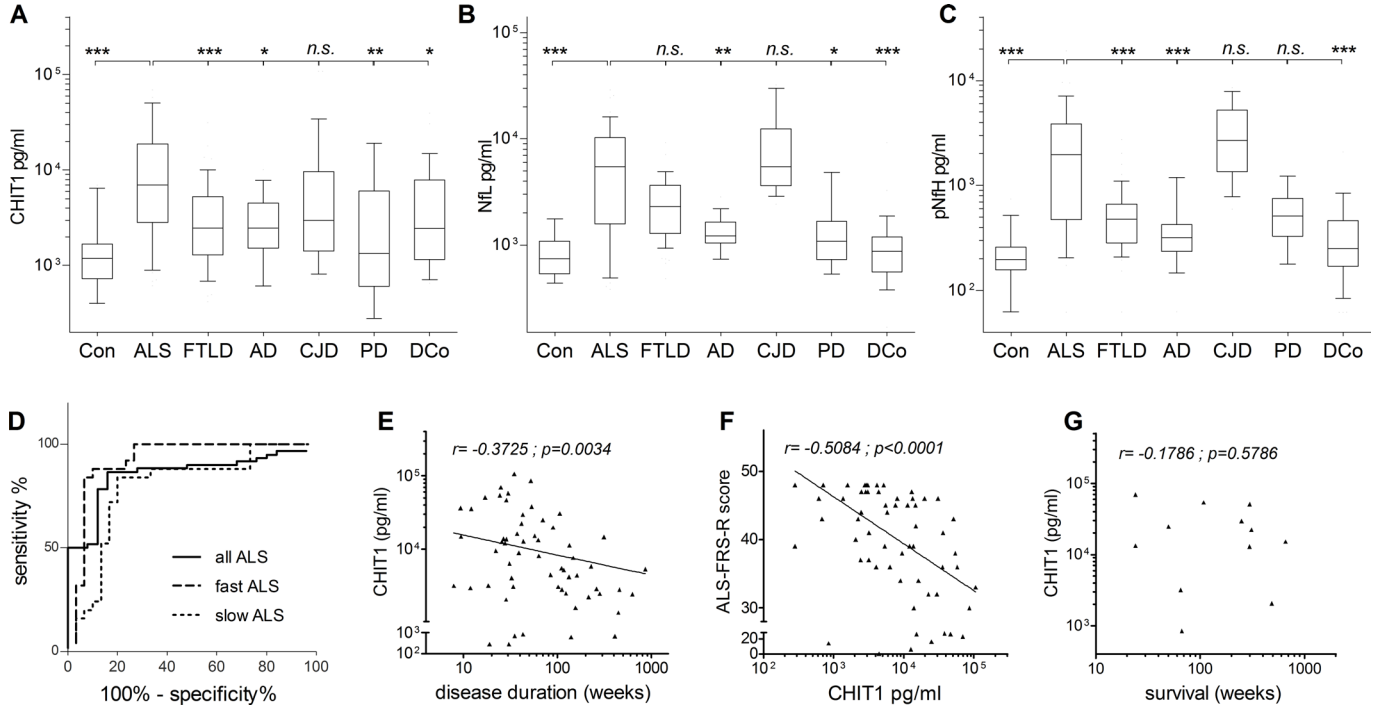

Figure 1 CHIT1 levels in the CSF for diagnosis and prognosis and comparison with neurofilament levels. (A) The concentration of CHIT1 is shown in the CSF of patients with ALS, healthy control volunteers (Con), a variety of neurodegenerative diseases and disease controls (DCo). In comparison, NfL and pNfH levels of the same cohort are given in panels (B) and (C). Significance values as indicated by stars (***p<0.0001, **p<0.01, *p<0.05) result from Dunn's post hoc test after the Kruskal-Wallis analysis (p<0.0001). (D) Receiver operating characteristic curves for discrimination of all patients with ALS (solid line), patients with ALS with slow progression (dotted line) and fast progression (dashed line) from control patients (Con) based on CSF CHIT1 levels. In panels E–G, CSF CHIT1 levels of patients with ALS are presented with regard to the disease duration from onset to sampling, disease severity as quantified by means of the ALSFRS-R and survival time after sampling, respectively. Triangles show individual measures; lines show linear/nonlinear regression fit. AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS functional rating scale revised; CHIT1, chitotriosidase; CJD, Creutzfeldt-Jakob disease; Con, non-neurodegenerative controls; CSF, cerebrospinal fluid; DCo, disease controls, patients under the direct differential diagnosis of ALS; FTLD, frontotemporal lobar degeneration; NfL, neurofilament light chain; PD, Parkinson's disease; pNfH, phosphorylated neurofilament heavy chain.

patient with glycogen storage disease type II had a CHIT1 CSF level of 2440 pg/mL and one patient with myopathy a level of 2385 pg/mL.

To see whether CHIT1 is increased early in the course of ALS, we analysed those patients separately who attended the clinic within 6 months from experiencing first symptoms (n=12) and still obtained significant results compared with Con (p=0.0002) and DCo (p=0.0059).

Compared with Con, CHIT1 concentrations were significantly higher in the CSF of FTLD (p=0.0017), AD (p=0.0055) and CJD (p=0.0387), but not of patients with PD (p=0.9889) in the Mann-Whitney U test. In the Kruskal-Wallis analysis, however, only the difference between Con and FTLD reached significance (p<0.05). This difference was not assigned to a particular FTLD subgroup (p=0.7674). Furthermore, there was no difference in the CHIT1 concentrations within PPA subgroups (p=0.6402).

CSF levels of NfL and pNfH in the diagnostic groups and comparison with CHIT1

Levels of NfL and pNfH in the CSF of the diagnostic groups are given in figure 1B and C. CSF NfL concentrations were significantly higher in patients with ALS compared with Con, AD, PD and DCo. There was also a tendency for higher NfL levels in ALS compared with FTLD. No differences were seen between the NfL levels measured in ALS and CJD. Concentrations of pNfH were significantly higher in ALS compared with Con, FTLD, AD and DCo, whereas no statistically significant differences were detected when comparing ALS with PD or CJD.

In comparison, all three markers performed similar in the differentiation of ALS and Con, but CHIT1 and pNfH discriminated better between ALS and FTLD than NfL, and NfL and pNfH performed better than CHIT1 in the differentiation of ALS and DCo.

Diagnostic performance of CSF CHIT1 for the diagnosis of ALS

At a cut-off concentration of 2003 pg/mL CHIT1, a sensitivity of 87% (95% CI 75% to 94%) and a specificity of 84% (95% CI 64% to 96%) were reached for the discrimination of ALS from Con with an area under the curve (AUC) of 0.8567 (figure 1D, solid line). The sensitivity and specificity for discrimination of ALS cases with fast PRs were 88% and 90% (AUC 0.7927) at a cut-off of 2848 pg/mL, respectively (figure 1D, dashed line), and for discrimination of slowly progressing cases at a cut-off of 2088 pg/mL 84% and 80%, respectively, with an AUC of 0.9207 (figure 1D, dotted line). For the discrimination between ALS and DCo, a sensitivity of 82% and a specificity of 51% at a cut-off of 2463 pg/mL CHIT1 were calculated.

The positive and negative likelihood ratios were 13 (95% CI 5.0 to 33.5) and 0.38 (95% CI 0.21 to 0.71) for the discrimination between ALS and Con. The likelihood ratios for discrimination of ALS and DCo were 1.89 (95% CI 1.33 to 2.68) and 0.58 (95% CI 0.35 to 0.96).

Correlation of CHIT1 CSF levels with ALS disease parameters

CHIT1 CSF levels of patients with ALS weakly correlated with age (r_S=0.4, p=0.0015) but not the blood–CSF barrier function as assessed by the albumin ratio. High CHIT1 concentrations were associated with a short disease duration (r_S=−0.3725, p=0.0034) (figure 1E) resulting in a positive correlation with the PR (r_S=0.5516, p=4.9×10^{-6}). Additionally, high CHIT1 levels correlated with low ALSFRS-R scores (r_S=−0.5084, p=3.4×10^{-5}) (figure 1F). The survival time from the date of sampling did not correlate with CSF CHIT1 concentrations (r_S=−0.1786, p=0.5786) (figure 1G).

Neurofilaments in the prognosis of ALS compared with CHIT1

Similar to CHIT1, neurofilament levels in CSF correlated with the age of patients with ALS (NfL: r_S=0.41, p=0.0012; pNfH: r_S=0.453, p=0.0003) and not with the albumin ratio. There was a correlation of CHIT1 with NfL levels (r_S=0.61, p<0.0001) and with pNfH levels (r_S=0.60, p<0.0001) in the ALS group.

As calculated for CSF CHIT1, NfL and pNfH also correlated negatively with the ALSFRS-R score (NfL: r_S=−0.536, p<0.0001; pNfH: r_S=−0.55, p<0.0001) and with disease duration (NfL: r_S=−0.507, p<0.0001; pNfH: r_S=−0.591, p<0.0001), and positively with clinical disease PR (NfL: r_S=0.638, p<0.0001; pNfH: r_S=0.667, p<0.0001). Like CHIT1, neither NfL nor pNfH correlated with survival (n=12, NfL: r_S=−0.116, p=0.721; pNfH: r_S=−0.158, p=0.625).

We found no correlation between levels of any of the markers with disease duration, ALSFRS-R score or PR in the group of fast progressors. Disease PRs in the group of slow progressors correlated with CHIT1 levels (r_S=0.442, p=0.045), NfL levels (r_S=0.618, p=0.003) and pNfH levels (r_S=0.466, p=0.033), while disease duration only correlated with neurofilament levels (NfL: r_S=−0.451, p=0.012; pNfH: r_S=−0.508, p=0.004), but not with CHIT1 levels (r_S=−0.1987, p=0.293). Furthermore, none of the markers correlated with the ALSFRS-R scores in the slow progression group.

For all markers, higher levels were determined for fast progressors than for slow progressors (figure 2A–C).

CHIT1 concentrations in the blood of patients with ALS at baseline examination and follow-up

The characteristics of patients with ALS in the serum follow-up study are summarised in supplementary table S1.

We determined CHIT1 concentrations in serum samples of 40 patients with ALS at baseline examination and compared them with the levels measured in disease controls and healthy volunteers. No differences in concentrations could be found (figure 2D).

In follow-up samples taken 6 months after the baseline examination, we could not see a consistent change of CHIT1 levels, neither in the patient subgroup with slow progression nor in that with fast progression (figure 2E and F). Furthermore, there was no correlation of CHIT1 serum levels with ALS PR (all: r_S=−0.126, p=0.4386; slow progressors r_S=−0.2709, p=0.2349; fast progressors: r_S=−2667, p=0.2697) (figure 2G).

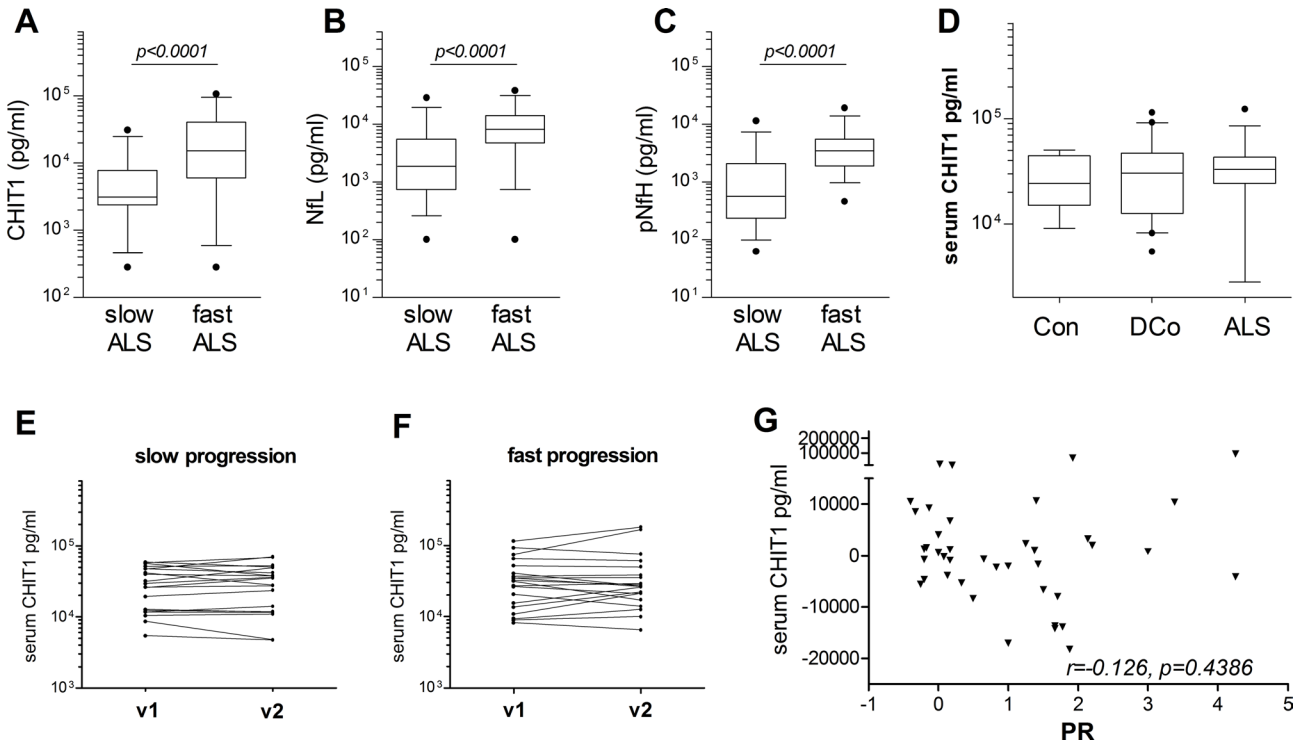

Figure 2 CSF/serum CHIT1 and CSF neurofilaments dependent on ALS progression. Graphs (A–C) show the levels of CHIT1, NfL and pNfH, respectively, in the CSF of patients with ALS with slow and fast disease progression. Mann-Whitney p values are given. (D) CHIT1 in the serum of patients with ALS (n=40; for details, see online supplementary table S1) compared with Con (five females and five males, median age 68 years, IQR 62–72 years) and DCo (10 females and 24 males, median age 55 years, IQR 46–69 years). In panels (E) and (F), the change of serum CHIT1 concentration from visit 1 (v1) to visit 2 (v2) of individual patients with ALS with slow and fast disease progression is given, respectively. (G) CHIT1 serum level in relation to disease progression rate (PR). Boxplots show median values and IQR, whiskers indicate 5%–95% percentile and symbols show outliers. ALS, amyotrophic lateral sclerosis; CHIT1, chitotriosidase; Con, non-neurodegenerative controls; CSF, cerebrospinal fluid; DCo, disease controls; NfL, neurofilament light chain; pNfH, phosphorylated neurofilament heavy chain.

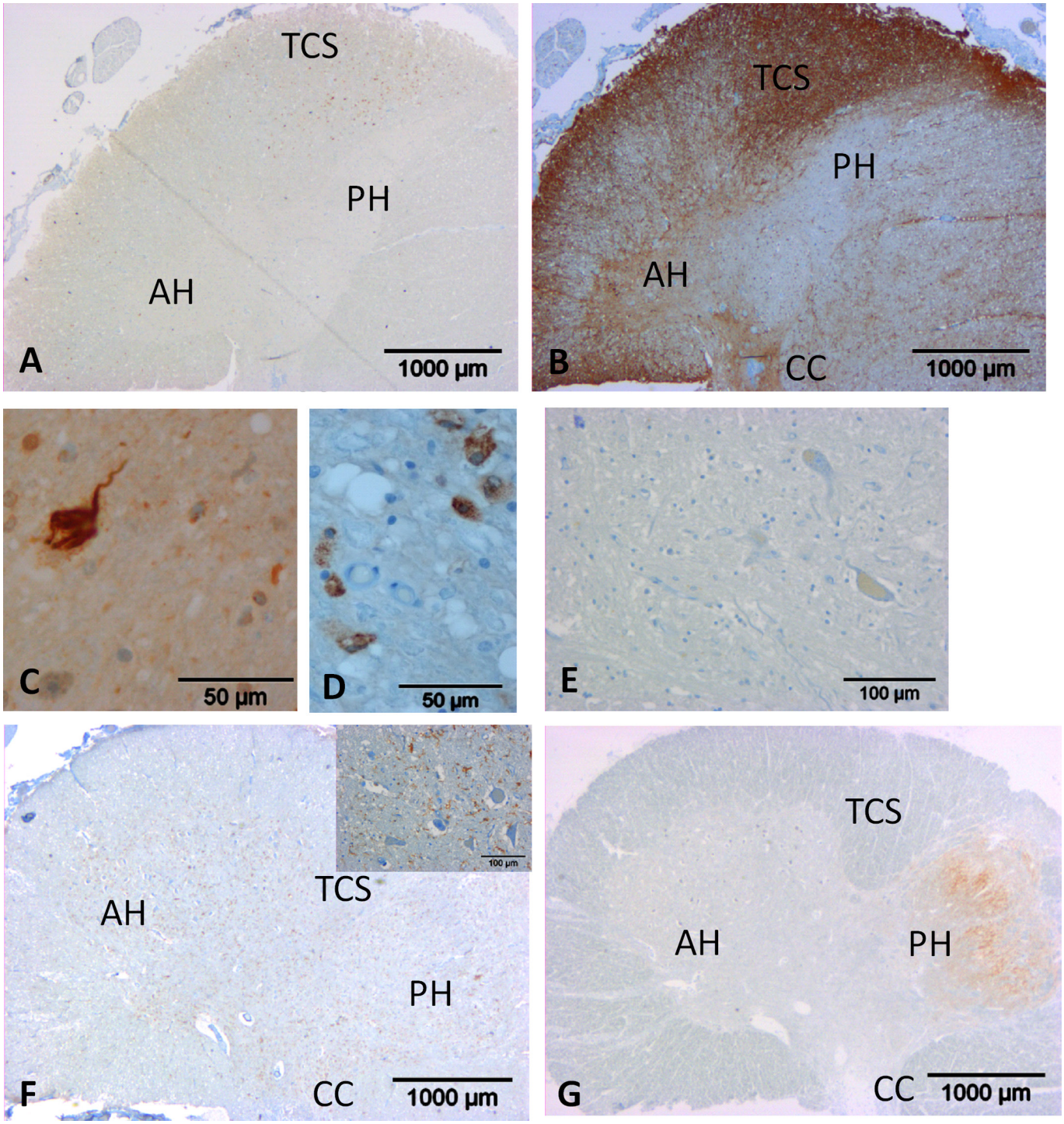

Figure 3 CHIT1 staining by IHC in human *postmortem* spinal cord. (A–D) Patient with ALS. In (A) one-half spinal cord, prominent CHIT1 immunostaining is found in the lateral and anterior corticospinal tract (TCS). Panel B shows activated microglia stained by CR3/43 mainly in the lateral and anterior TCS, less in the anterior horn (AH). In panel C, a pTDP43 (1D3)-positive cytoplasmatic inclusion is seen in a spinal cord motoneuron. Panel D shows CHIT1 appearing as fine granular cytoplasmic staining in numerous cells with a morphology resembling macrophages. (E–G) Patient with CJD. The spinal cord section shown in panel E is CHIT1 negative, whereas microglial activation is mainly seen in the grey matter as indicated by CR3/43 immunoreactivity (F and insert). (G) Prion immunohistochemistry (L42) shows a synaptic staining pattern in the posterior horn (PH) of the spinal cord. ALS, amyotrophic lateral sclerosis; CC, central canal; CHIT1, chitotriosidase; CJD, Creutzfeldt-Jakob disease.

CHIT1 staining in human postmortem central nervous system tissue

In IHC of ALS spinal cord, CHIT1 immunoreactivity was observed in the lateral corticospinal tract (TCS) and to a lesser extent also in the anterior TCS (figure 3A), only partially corresponding to the staining pattern of activated microglia, which is more widespread including both the white and grey matter (figure 3B). In our pTDP-43-positive ALS samples (figure 3C), CHIT1 staining was mainly seen in putative macrophages with a fine granular cytoplasmic staining (figure 3D). In

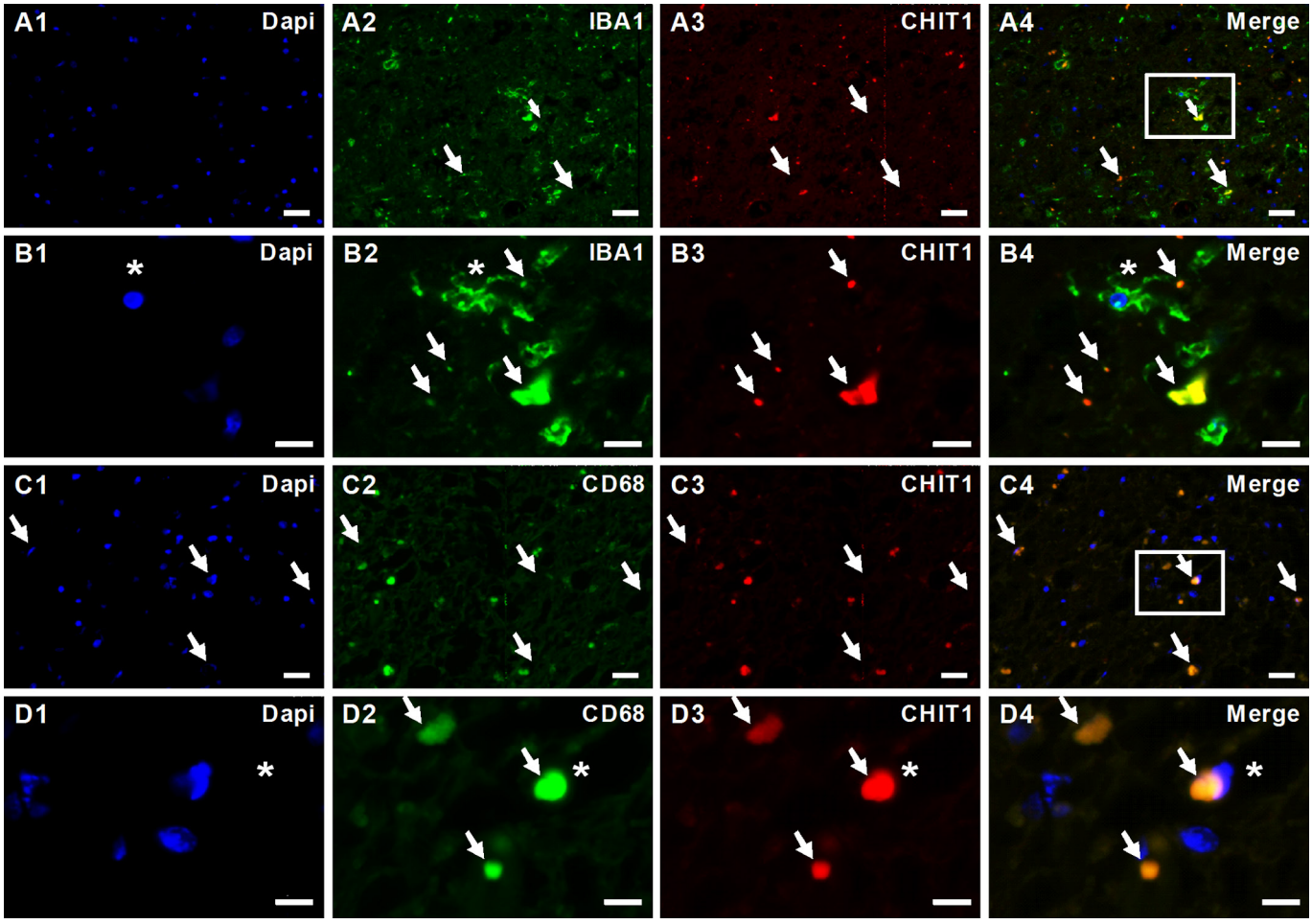

Figure 4 Colocalisation of CHIT1 with markers of microglia and macrophages in the spinal cord of ALS cases. (A, B) Double-labelling immunofluorescence for the microglial marker IBA1 (green) and CHIT1 (red) shows colocalisation of the two proteins (yellow to orange) as indicated by arrows. Unlabelled cells with clearly visible nuclei and cellular processes indicative of classical microglial morphology are also evident (star). (C, D) Double labelling for CD68 (green) and CHIT1 (red) indicates an activated state of CHIT1-expressing cells compatible with macrophage origin (arrows). Images in B1–B4 and D1–D4 show higher magnification of insets in A4 and C4, respectively, for each channel (blue: 4′, 6-diamidino-2-phenylindole, green: microglial/macrophage marker, red: CHIT1, combined: merged image). Bars in A and C 25 µm, bars in B and D 10 µm. ALS, amyotrophic lateral sclerosis; CHIT1, chitotriosidase.

CJD, no CHIT1 staining was found in spinal cord areas with microglial activation or pathological prion protein expression (figure 3E–G). Examination of brain sections revealed occasional and sparse CHIT1 staining in all diseases tested, again mainly in blood vessel-associated macrophages, but much weaker than in ALS spinal cord tissue (data not shown).

To characterise CHIT1-expressing cell type(s), we carried out double IF staining for CHIT1 in combination with IBA1 and CD68 antibodies. Thereby, it could be demonstrated that in the TCS of ALS spinal cord tissue CHIT1 colocalised with subpopulations of microglia, where it was expressed in processes and somata of IBA1-positive cells (figure 4A and B), and in CD68-positive macrophages (Figure 4C and D). Specificity of CHIT1 labelling in the TCS was confirmed with a competitive immunoreaction using excess of recombinant CHIT1 peptide in the primary antibody solution, which resulted in diminished staining in these areas.

DISCUSSION

In our study, we could confirm higher CHIT1 concentrations in the CSF of patients with ALS compared with non-neurodegenerative controls as well as to differential diagnostically relevant diseases.[14 15] In addition, analysing CSF samples of patients with a variety of neurodegenerative diseases, we found the highest CHIT1 concentrations in patients with ALS, whereas CHIT1 in AD, FTLD, PD and most CJD cases was only moderately increased.

A slight increase in CHIT1 was reported before in multiple sclerosis and AD, where it was regarded as an indicator of microglial activation and/or accumulation of alternative substrates of CHIT1 in amyloid beta plaques.[21–26] As microglial activation is a key hallmark common to the majority of neurodegenerative diseases,[27–30] we wondered about the source of differences in CHIT1 levels between different disease entities and performed IHC on human postmortem tissue. Despite strong microglial activation in both the grey and white matter of ALS spinal cord, CHIT1-positive subpopulations of microglia and macrophages were present mainly in the lateral and anterior TCS. In contrast, CHIT1 staining was not observed in the spinal cords of healthy controls, and CJD or AD cases. Therefore, we hypothesise that in ALS the increase of CHIT1 in CSF reflects a disease-specific activation of microglia/macrophages.

What is the significance of increased CHIT1 expression in ALS? Here, we can only speculate. Investigations in AD revealed chitin-like glucosamine polymers in amyloid plaques, which have been proposed to account for increased CHIT1 mRNA levels

in macrophages and protein levels in postmortem brains.[21 23 31] It is not known whether the aggregates characteristic for ALS also contain chitin-like polysaccharides, which could explain the pronounced upregulation of the processing enzyme CHIT1. In AD, levels of glucosamines may be increased due to impaired glucose utilisation in AD leading to the mentioned chitin-like polymers.[21] Likewise, in ALS levels of sialylated N-glycans were found to be increased,[32] supporting the notion that similar mechanisms may underlie the increased expression of CHIT1 in ALS. The predominant expression of CHIT1 in spinal cord white matter points to a reaction towards axonal constituents or clearance of damaged axons of affected TCS neurons. However, our results reflect only the situation at terminal disease stage. Here, analysis of tissue from ALS mouse models at different disease stages might help to clarify the pathophysiological mechanisms underlying CHIT1 upregulation.

Notably, as the CHIT1 concentration correlated inversely with the disease duration, we provide preliminary evidence that CHIT1 could qualify as an early ALS biomarker, in accordance with an early activation of the immune system in ALS observed both in animal models[33 34] and humans,[35 36] which could help to reduce diagnostic delay. Because CHIT1 concentrations also correlated with the ALSFRS-R score, its measurement could aid in early diagnosis of patients with rapid deterioration. Further investigations using alternative clinical criteria[37 38] or comparing symptomatic and asymptomatic gene carriers may help to optimise the timing for the detection of an early CHIT1 elevation in ALS.

Furthermore, CHIT1 correlated with the PR of the disease in our cohort of patients with ALS and therefore could aid in prognosis, which is required for a suitable ALS biomarker.[6 39] Compared with neurofilaments, we observed only minor differences regarding diagnostic and prognostic performance and a combined analysis might not be necessary. However, as there are marked differences in CHIT1 levels in the neurodegenerative diseases investigated, CHIT1 might be a more specific biomarker for a reliable detection of immune activation, and therefore might be used in upcoming therapeutic trials addressing this mechanism. Despite the previously reported marked increase of CHIT1 activity in the blood of patients with ALS,[16] we could not detect differences in concentrations of this enzyme in the serum of ALS, DCo and Con cases. Although data in paired CSF-serum samples are currently unavailable, different and potentially independent mechanisms may drive changes in CHIT1 protein levels and activity in CSF and blood. The upregulation of CHIT1 expression in ALS seems to be specifically triggered in neuronal tissue rather than blood, leading to the observed increase in CSF CHIT1 concentration.

Our pilot study has also limitations. The diagnostic groups were not age -matched. Therefore, the significant differences in CHIT1 concentrations found between patients with AD and healthy controls might have been influenced by age, which also affect CHIT1 activity in healthy elderly individuals.[40] A difference in the patients age was also present in the slow and fast progressor subgroups of patients with ALS. Here, a bias cannot be excluded especially because a correlation of CHIT1 levels with age, although weak, was found in our entire ALS group. Additional studies in a large cohort of age-matched patients with ALS will be necessary to clarify the impact of age on CHIT1 expression. Another limitation of our study was the low number of patients within some subgroups with neurodegenerative diseases. Furthermore, the molecular pathology for the patients was unknown and postmortem analyses were carried out only on a sample basis. Here, extended investigations of molecularly characterised patients are necessary to gain insight into the relationship between CHIT1 expression and inflammatory processes or presence of glucosamine substrates.

Taken together, we provide evidence that CSF CHIT1 could qualify as a diagnostic and prognostic marker for ALS. CHIT1 also distinguishes between ALS and other neurodegenerative diseases, presumably due to a specific activation of macrophages along the TCS.

Further studies are needed to elucidate exactly the timing and origin of CHIT1 in the CSF and to clarify the potential for patient stratification for upcoming therapeutic trials addressing immune and inflammatory processes in ALS.

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