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ORIGINAL RESEARCH

Cross-sectional and longitudinal measures of chitinase proteins in amyotrophic lateral sclerosis and expression of CHI3L1 in activated astrocytes

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ABSTRACT

Objective Amyotrophic lateral sclerosis (ALS) is a complex disease with numerous pathological mechanisms resulting in a heterogeneous patient population. Using biomarkers for particular disease mechanisms may enrich a homogeneous subset of patients. In this study, we quantified chitotriosidase (Chit-1) and chitinase-3-like protein 1 (CHI3L1), markers of glial activation, in cerebrospinal fluid (CSF) and plasma and determined the cell types that express CHI3L1 in ALS.

Methods Immunoassays were used to quantify Chit-1, CHI3L1 and phosphorylated neurofilament heavy chain levels in longitudinal CSF and matching plasma samples from 118 patients with ALS, 17 disease controls (DCs), and 24 healthy controls (HCs). Immunostaining was performed to identify and quantify CHI3L1-positive cells in tissue sections from ALS, DCs and non-neurological DCs.

Results CSF Chit-1 exhibited increased levels in ALS as compared with DCs and HCs. CSF CHI3L1 levels were increased in ALS and DCs compared with HCs. No quantitative differences were noted in plasma for either chitinase. Patients with ALS with fast-progressing disease exhibited higher levels of CSF Chit-1 and CHI3L1 than patients with slow-progressing disease. Increased numbers of CHI3L1-positive cells were observed in postmortem ALS motor cortex as compared with controls, and these cells were identified as a subset of activated astrocytes located predominately in the white matter of the motor cortex and the spinal cord.

Conclusions CSF Chit-1 and CHI3L1 are significantly increased in ALS, and CSF Chit-1 and CHI3L1 levels correlate to the rate of disease progression. CHI3L1 is expressed by a subset of activated astrocytes predominately located in white matter.

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that is characterised by the degeneration of upper and lower motor neurons.[1] One of the challenges associated with ALS pathobiology stems from the heterogeneity of the disease arising from many genetic and pathological mechanisms. This inherently results in a heterogeneous patient population. Biomarkers that highlight specific pathogenic mechanisms would be valuable to stratify this heterogeneous patient population to enrich for a homogeneous subset of patients. Currently, the most promising protein biomarkers for ALS include phosphorylated neurofilament heavy chain (pNFH),[2–4] neurofilament light chain[4 5] and the extracellular domain of the neurotrophic receptor p75.[6 7] While these biomarkers have demonstrated potential diagnostic and prognostic utility, they reflect axonal injury and degeneration, which are not specific to a particular disease mechanism.

Biomarkers of neuroinflammation represent a promising avenue for continued development as there have been many recent and proposed ALS clinical trials using anti-inflammatory based therapeutics.[8–10] For example, recent early-phase clinical trials of NP001, an anti-inflammatory mediator that targets monocytes/macrophages, demonstrated modest but not statistically significant effects in patients with ALS.[9 10] However, a post hoc analysis identified the greatest benefit in patients with elevated systemic inflammation as determined by levels of C reactive protein (CRP). Interestingly, a follow-up phase IIB trial using patients with ALS with elevated CRP failed to demonstrate a reduction in disease progression rate. Though these trials ultimately failed, they demonstrated the use of an inflammatory biomarker to enrich for patients to treat with a specific therapy.

Recent studies explored chitinases, a class of secreted hydrolases that bind to and degrade chitin, as potential biomarkers for neurodegenerative diseases. Chitotriosidase (Chit-1) was the first identified mammalian chitinase that both binds and degrades chitin. Other members of the chitinase family, such as chitinase-3-like protein 1 (CHI3L1) or chitinase-3-like protein 2 (CHI3L2), bind chitin but do not exhibit enzymatic activity.[11] Recent evidence implicates chitinases in regulating the innate immune system[12] and modulating inflammation during the progression of many diseases.[13] Given that chitinases are expressed by activated microglia and/or astrocytes in the central nervous system (CNS), these biomarkers may be reflective of glial activation. Increased levels and activity of chitinases have been observed in biofluids from patients with multiple sclerosis (MS)[14 15] and Alzheimer's disease (AD).[15 16] Recently, Chit-1,[17–22] CHI3L1[18 20–23] and CHI3L2[18 20 21 23] were explored as potential ALS biomarkers in cerebrospinal fluid (CSF) and blood.

Table 1 Patient demographics

	ALS	DC	HC
Number of subjects with baseline visit	118	17	24
Number of subjects with two visits	80	2	10
Number of subjects with three visits	42	0	1
Number of subjects with four visits	17	0	0
Number of subjects with five visits	12	0	0
Gender (M/F)	70/49	11/6	11/13
Onset site (bulbar:limb)	29:88	N/A	N/A
Average age of onset (mean±SD)	54±10	N/A	N/A
Average age at baseline (mean±SD)	57±10	58±11	53±13
Number of *C9orf72* patients	14	N/A	N/A
Number of *SOD1* patients	1	N/A	N/A

ALS, amyotrophic lateral sclerosis; DC, disease control; F, female; HC, healthy control; M, male; N/A, not documented; *SOD1*, Superoxide dismutase 1.

Table 2 Demographics of the cases used for the immunohistochemical and immunofluorescent staining

Case	Sex	Age at death	Age at onset	Site of onset	Diagnosis	C9 (+ or –)
ALS 1	Female	61	58	Limb	ALS	–
ALS 2	Female	63	61	Limb	ALS	–
ALS 3	Male	74	70	N/A	ALS	–
ALS 4	Male	74	N/A	N/A	ALS	–
ALS 5	Male	83	81	N/A	ALS	–
ALS 6	Female	51	47	Bulbar	ALS	+
ALS 7	Male	44	40	Limb	ALS, CTE	Not tested
ALS 8	Female	68	60	Limb	ALS, scoliosis	–
ALS 9	Female	60	57	Limb	ALS	–
ALS 10	Male	39	35	N/A	ALS	–
ALS 11	Female	63	60	N/A	ALS	–
ALS 12	Male	72	N/A	N/A	ALS, FTLD	Not tested
DC 1	Male	81	N/A	N/A	AD	–
DC 2	Male	45	N/A	N/A	MSA	–
DC 3	Male	71	N/A	N/A	AD	–
NNDC 1	Female	74	N/A	N/A	NNDC	–
NNDC 2	Male	22	N/A	N/A	NNDC	–
NNDC 3	Female	70	N/A	N/A	NNDC	–
NNDC 4	Female	92	N/A	N/A	NNDC	–

N/A indicates that this information was not documented.
AD, Alzheimer's disease;ALS, amyotrophic lateral sclerosis; CTE, chronic traumatic encephalopathy;DC, disease control; FTLD, frontotemporal lobar degeneration; MSA, multiple system atrophy; NNDC, non-neurological disease control.

While these biomarkers were studied in various biofluids, few studies explored how levels of chitinases in biofluids change over time in the same individual. This information may provide insight into when and how these proteins modulate neuroinflammation in the periphery and CNS throughout the disease course. Furthermore, cell types expressing these proteins in ALS remain unclear. In this study, we performed both cross-sectional and longitudinal analysis of chitinases in CSF and matching plasma samples, and demonstrate that CSF chitinases can stratify fast-progressing and slow-progressing ALS. We also show that CHI3L1 is expressed by activated astrocytes in the white matter of the ALS motor cortex and spinal cord.

METHODS

Biofluid sample description

CSF and matching plasma from patients with ALS, neurological disease controls (DCs) and healthy controls (HCs) were obtained from the Northeast ALS Consortium (NEALS) Biofluid Repository and the Mayo Clinic Biorepository. All subjects provided institutional review board (IRB)-approved informed consent at either the Mayo Clinic or the site of participant enrolment for samples collected by the NEALS biorepository. All ALS subjects were defined by El Escorial criteria by experienced and licensed neurologists. CSF and plasma was collected from 118 patients with ALS and 41 controls (table 1).

No statistical differences were determined between age at baseline of ALS versus DCs (p=0.99), ALS versus HCs (p=0.74) or DCs versus HCs (p=0.84) as determined by the Kruskal-Wallis test with a Dunn correction applied. No sex differences were observed (p=0.41) as determined by χ^2 test.

The DC group (n=17) was composed of patients with a range of diseases including brain metastases, viral encephalitis, neuropathy, MS, upper motor neuron disease, primary lateral sclerosis, chronic inflammatory demyelinating polyneuropathy, idiopathic sensorimotor polyneuropathy, spinocerebellar ataxia, lymphoma and lower motor neuron disease. The 24 HCs lacked any identified neurological deficits. CSF and plasma samples were collected from these individuals using methods as previously described.[24 25] Longitudinal samples collected at clinic visits that were separated by at least 3 months were obtained, and the numbers of subjects providing longitudinal samples are shown in table 1. Tissue sections from 12 ALS, 3 DCs and 4 non-neurological disease controls (NNDCs) were obtained from the Barrow Neurological Institute and Target ALS postmortem tissue bank cores (subject demographics listed in table 2). Participants in the postmortem tissue bank cores provided IRB-approved informed consent for the collection of postmortem tissues.

Chitinase and pNFH measurements

A sandwich ELISA was used to quantify Chit-1 in CSF and matching plasma samples. Ninety-six well plates consisting of ImmunoClear standard module well strips for ELISAs (Thermo Fisher, Waltham, Massachusetts, USA) were coated with goat antihuman Chit-1 (R&D Systems, Minneapolis, Minnesota, USA) overnight at 4°C, washed and blocked. Purified recombinant Chit-1 protein (R&D Systems) was diluted in 1× Tris-buffered saline (TBS) (20 mM Tris, 150 mM sodium chloride, 0.05% ProClin 300, 1% bovine serum albumin, pH=7.6) to generate a standard curve from 0 to 10 ng/mL. CSF or plasma samples were also diluted in 1× TBS. For ALS samples, a dilution of 1:20 was used for CSF, and 1:100 was used for plasma. For control samples, a dilution of 1:20 was used for both CSF and plasma. Standards and samples were added to individual plate wells and were incubated at room temperature for 90 min. Mouse antihuman Chit-1 (R&D Systems) and goat antimouse IgG–horseradish peroxidase (Promega, Madison, Wisconsin, USA) were used for detection. The peroxidase reaction was developed using 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich, St. Louis, Missouri, USA) and stopped using 1 N hydrochloric acid (HCl). For the Chit-1 assay, average intra-assay and inter-assay coefficient of variations were below 15% for both CSF and plasma. Lower limit of quantification was 21.4 pg/mL for CSF and 20.3 pg/mL for plasma. Spike in recovery assays produced recoveries of 96% for CSF and 93% for plasma. Linearity test produced a regression coefficient (R^2) greater than 0.99 for both CSF and plasma. For CHI3L1, a human CHI3L1 DuoSet ELISA kit (R&D Systems) was used following manufacturer

instructions. For both ALS and control samples, a dilution of 1:400 was used for CSF and 1:100 was used for plasma. Assay performance characteristics for the commercial CHI3L1 immunoassay are available in the manufacturer's insert. All samples and standards were run in duplicate on each plate.

The Meso Scale Discovery immunoassay was employed to measure pNFH as previously described.[26] CSF samples were diluted 1:8 in 1× TBS, while plasma was diluted 1:4 in 1× TBS containing 30 mg/mL urea prior to measurement.

Immunostaining and analysis of human tissue

Immunohistochemistry (IHC) was performed on paraffin-embedded motor cortex tissue from ALS, DCs and NNDCs (see table 2 for demographics). Tissue sections were deparaffinised and rehydrated, and antigen retrieval was performed using a target retrieval solution, pH 9 (Dako, Santa Clara, California, USA). Tissues were blocked using Super Block (Scytek, Logan, Utah, USA) supplemented with Avidin (Vector Labs, Burlingame, California, USA). Tissue sections were incubated with rabbit anti-CHI3L1 antibody (Thermo Fisher) diluted in superblock supplemented with biotin (Vector Labs) overnight. An antirabbit biotinylated IgG secondary antibody (Vector Labs) was subsequently added and incubated for 1 hour at room temperature. Tissues were washed in phosphate-buffered saline (PBS) and immunostaining was visualised using the Vectastain Elite ABC reagent (Vector Labs) and Vector ImmPACT NovaRED peroxidase substrate kit (Vector Labs). Slides were counterstained with haematoxylin (Sigma-Aldrich). Images were acquired using an Olympus BX40 microscope. Images were deidentified and analysed in a blinded fashion using ImageJ to quantify the number of CHI3L1-positive cells with five images per condition and subject. Immunofluorescent (IF) staining was performed as previously described.[27] 4′,6-Diamidino-2-phenylindole was used to stain nuclei. Autofluorescence eliminator reagent (Sigma-Aldrich) was used to limit autofluorescence. IF images were acquired using a Zeiss LSM 710 confocal microscope (Zeiss, Thornwood, New York, USA). Details on antibodies used for IHC and IF are shown in online supplementary table S1.

Statistical analyses

Statistical analysis was performed using GraphPad Prism V.7.0. Cross-sectional analyses were performed using the baseline visit from both ALS and control groups. All CSF and plasma measurements were log transformed prior to analysis. Kruskal-Wallis tests were performed with a post hoc Dunn correction to assess statistical differences for each biomarker with $p<0.05$ considered significant unless specified otherwise. Correlations were analysed using the non-parametric two-tailed Pearson's correlation with a significance level set at 0.05. Receiver operator characteristic (ROC) curves were used to determine how Chit-1 and CHI3L1 were able to distinguish between ALS and control groups (both DCs and HCs) based on the area under the curve (AUC). Comparison of ROC curves was performed as previously described.[28] Optimal cut-off concentrations for each biomarker were selected based on the highest Youden index. A combination of biomarkers was calculated by the summation

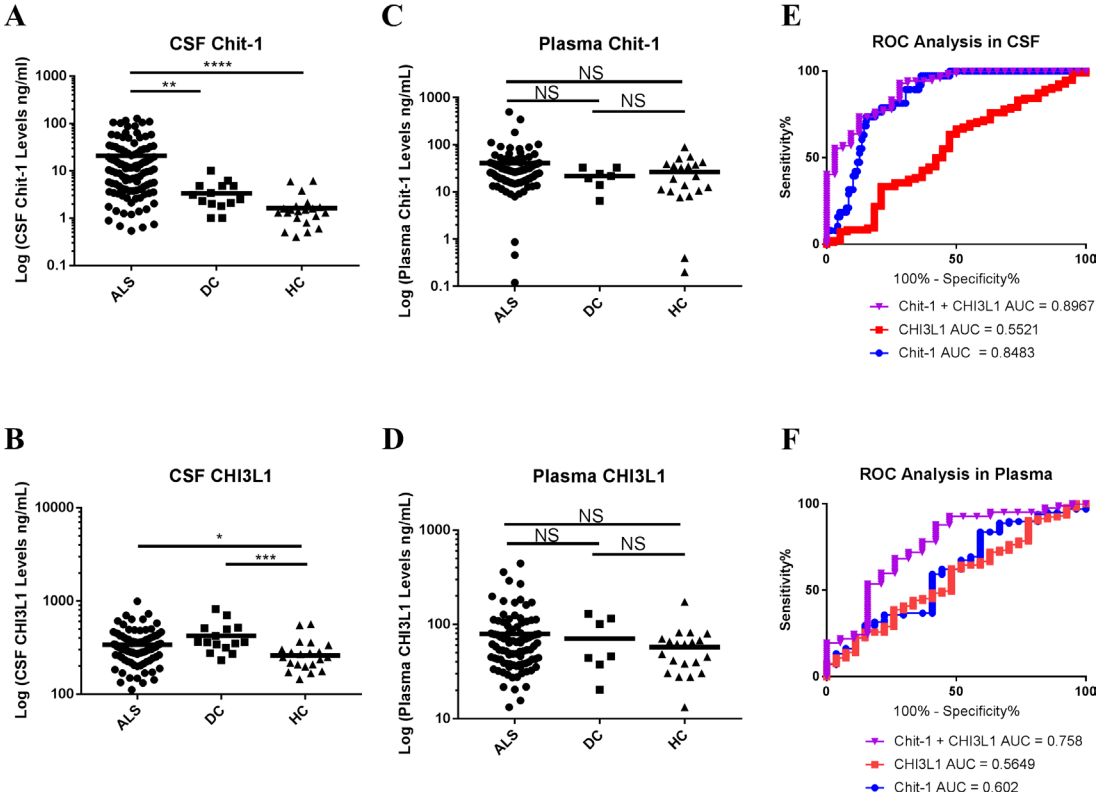

Figure 1 Concentration of (A) CSF Chit-1, (B) CSF CHI3L1, (C) plasma Chit-1 and (D) plasma CHI3L1 using data from baseline visits for patients with ALS, DCs and HCs. A Kruskal-Wallis test was used to assess differences between pairwise comparisons and p values were corrected using the Dunn correction. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ROC curves using baseline measures of CSF (E) and plasma (F) for Chit-1 (blue circles), CHI3L1 (red squares) and the combination of Chit-1 and CHI3L1 (purple triangles), comparing ALS with DC and HCs. ALS, amyotrophic lateral sclerosis; AUC, area under the curve; CHI3L1, chitinase-3-like protein 1; Chit-1, chitotriosidase; CSF, cerebrospinal fluid; DC, disease control; HC, healthy control; NS, not significant; ROC, receiver operator characteristic.

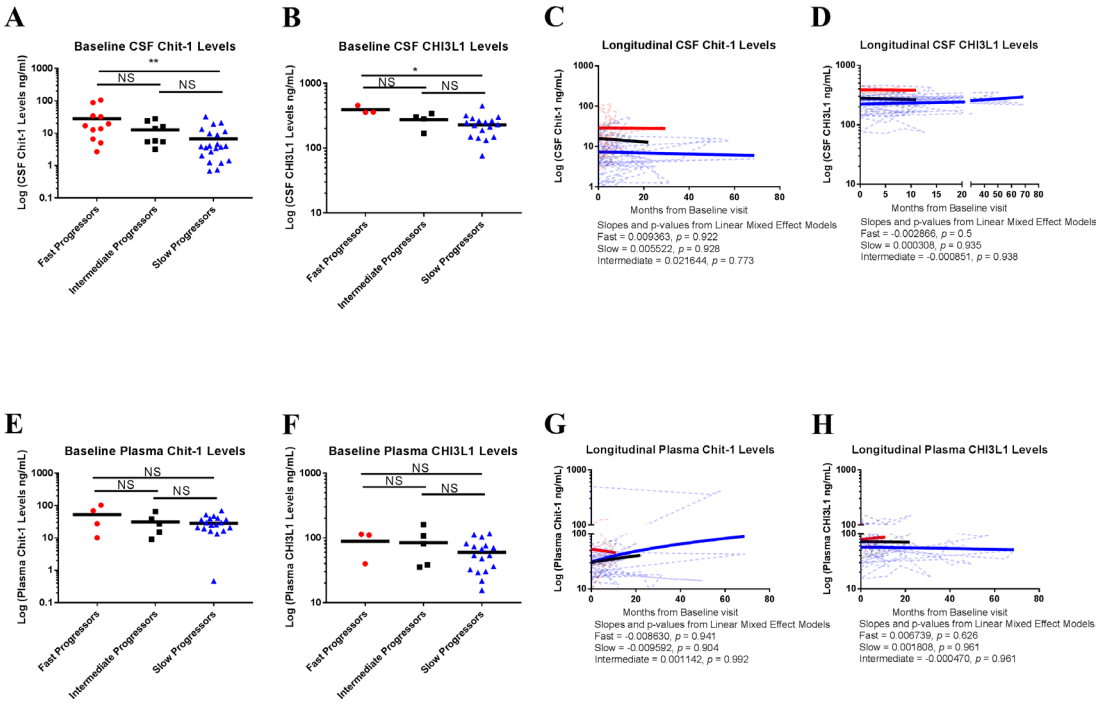

Figure 2 Longitudinal measures of chitinases from patients with amyotrophic lateral sclerosis with three or more clinic visits and biofluid collection. Patients were grouped based on disease progression rate, which was calculated by the change in the ALSFRS-r score/month between the baseline and last visit, where FPs exhibited a disease progression rate of ≥1 unit/month; SPs had a disease progression rate of <0.5 unit/month; and IPs had a disease progression rate of ≥0.5 unit/month but less than 1 unit/month. Baseline levels of (A) CSF Chit-1 (n=11 FPs, 8 IPs and 23 SPs) and (B) CSF CHI3L1 (n=3 FPs, 4 IPs and 17 SPs). Longitudinal levels of (C) CSF Chit-1 and (D) CSF CHI3L1, in which each dotted line represents an individual patient. The solid lines represent the overall linear fit of the longitudinal measurements of each chitinase in FPs (red), IPs (black) and SPs (blue). Baseline levels of (E) plasma Chit-1 (n=4 FPs, 5 IPs and 21 SPs) and (F) plasma CHI3L1 (n=3 FPs, 5 IPs and 17 SPs). Longitudinal levels of (G) plasma Chit-1 and (H) plasma CHI3L1, in which each dotted line represents an individual patient. The solid lines represent the overall linear fit of the longitudinal measurements of each chitinase in FPs (red), IPs (black) and SPs (blue). A Kruskal-Wallis test was used to assess differences in baseline measurements, and p values were corrected using the Dunn correction. **p<0.01, *p<0.05. P values from linear mixed effects modelling indicate the significance level in which the slopes differ from 0 as assessed by SPSS with p<0.05 being considered significant. CHI3L1, chitinase-3-like protein 1; Chit-1, chitotriosidase; CSF, cerebrospinal fluid; FP, fast progressor; IP, intermediate progressor; NS, not significant; SP, slow progressor.

of centre and scaled concentration values. For the longitudinal analyses, patients were segregated into fast progressors (FPs), slow progressors (SPs) and intermediate progressors (IPs) using the disease progression rate, which is defined by the change in ALS Functional Rating Scale Revised (ALSFRS-r) between the last and baseline visits/the amount of months between the visits. FPs were defined as those that had a disease progression rate of ≥1 unit/month; SPs had a disease progression rate of <0.5 unit/month; and IPs had a disease progression rate of ≥0.5 unit/month but less than 1 unit/month. Random slope and random intercept linear mixed effect modelling was performed using SPSS V.26 to evaluate the rate of change of each chitinase in FPs, SPs and IPs. To further assess differences between FPs, SPs and IPs, the baseline concentrations of Chit-1 and CHI3L1 were also compared using a Kruskal-Wallis test with p<0.05 considered as significant after Dunn correction.

RESULTS

Cross-sectional analysis of Chit-1 and CHI3L1 in ALS and controls

We previously identified increased abundance of Chit-1, CHI3L1 and CHI3L2 in ALS CSF as compared with HCs by mass spectrometry-based proteomics.[24] In our current study, we confirmed these results by measuring levels of Chit-1 and CHI3L1 in CSF and plasma by ELISA. CSF Chit-1 levels were significantly higher in patients with ALS compared with both DCs and HCs (figure 1A). Levels of CSF CHI3L1 were also higher in patients with ALS compared with HCs but not DCs (figure 1B). Increased CHI3L1 levels were also detected in DCs as compared with HCs. Levels of Chit-1 and CHI3L1 were assessed in matching plasma samples, but no significant differences were observed among groups for either chitinase (figure 1C,D). Additionally, neither Chit-1 nor CHI3L1 levels differed between sex or site of disease onset (data not shown). Interestingly, CHI3L1 but not Chit-1 levels were significantly higher in CSF from patients with the *C9orf72* repeat expansion (C9-ALS) as compared with non-C9-ALS cases (online supplementary figure S1B).

To assess if Chit-1 or CHI3L1 could distinguish between ALS and the combined control group, ROC curve analyses were employed. CSF Chit-1 levels discriminated patients with ALS and controls with an AUC of 0.8483, p<0.0001, while CSF CHI3L1 performed poorly with an AUC of 0.5521, p=0.34 (figure 1E). Based on the highest Youden index, a cut-off value of greater than 6.24 ng/mL of CSF Chit-1 yielded a sensitivity of 63.6% (95% CI 54.2% to 72.2%) and a specificity of 97.4% (95% CI 86.2% to 99.9%). At a cut-off value greater than 281.3 ng/mL of CSF CHI3L1, we observed a sensitivity of 66.3% (95% CI 55.9% to 75.7%) and a specificity of 50% (95% CI 33.4% to 66.3%). On combining the two biomarkers, no significant improvement in performance was achieved over Chit-1 alone (AUC Chit-1+CHI3L1=0.8967 vs AUC Chit-1=0.8483,

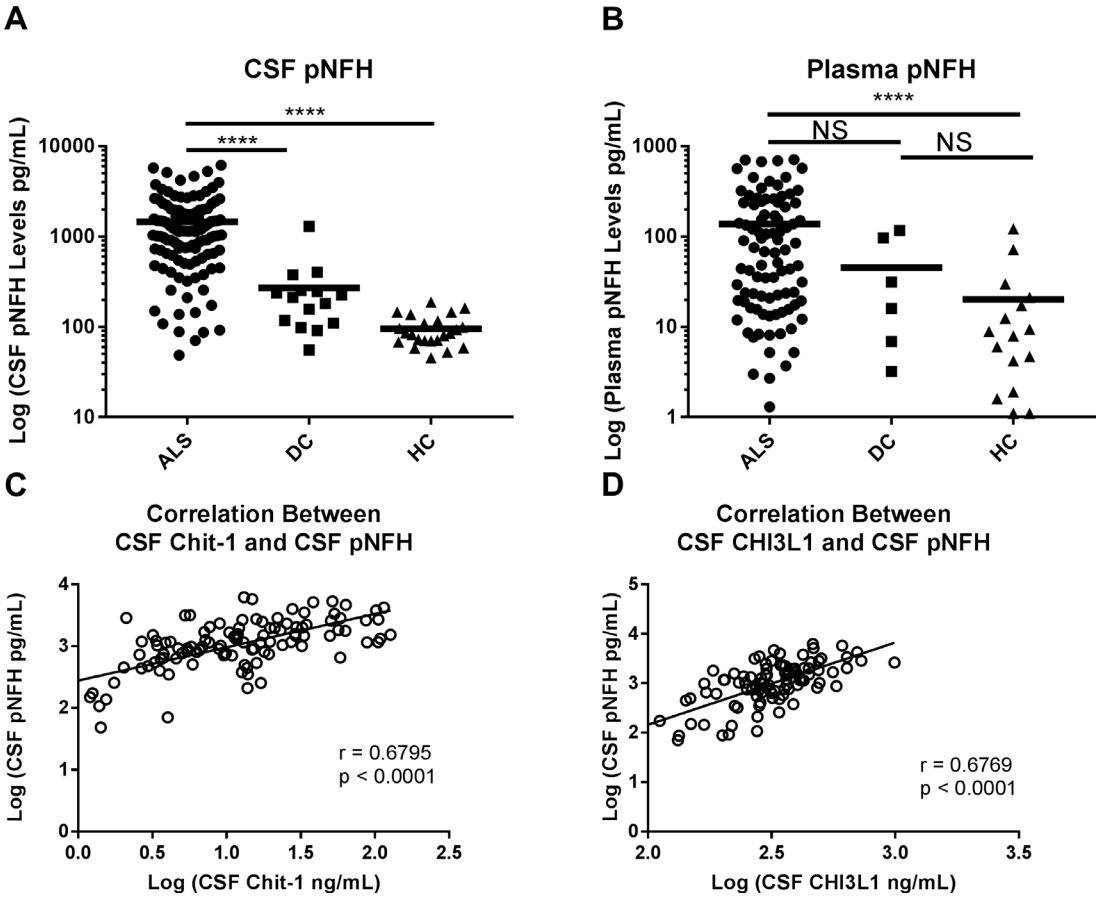

Figure 3 Levels of (A) CSF pNFH and (B) plasma pNFH at baseline visits for patients with ALS, DCs and HCs. A Kruskal-Wallis test was used to assess differences between pairwise comparisons, and p values were corrected using the Dunn correction. ****p<0.0001. Correlation analysis between (C) CSF Chit-1 and pNFH, or (D) CSF CHI3L1 and pNFH using data from the baseline visits in patients with ALS. Pearson's correlation coefficient (r) was used for all pair-wise analyses, with p<0.05 considered as significant. ALS, amyotrophic lateral sclerosis; CHI3L1, chitinase-3-like protein 1; Chit-1, chitotriosidase; CSF, cerebrospinal fluid; DC, disease control; HC, healthy control; NS, not significant; pNFH, phosphorylated neurofilament heavy chain.

p=0.05), but performance was improved over CHI3L1 alone (AUC Chit-1+CHI3L1=0.8967 vs AUC CHI3L1=0.5521, p<0.01) (figure 1E). A similar analysis was performed using plasma levels of Chit-1 and CHI3L1. The plasma AUC values were 0.602 (p=0.1053) for Chit-1, 0.5649 (p=0.3057) for CHI3L1 and 0.758 (p=0.0005) for the combination of Chit-1 and CHI3L1 (figure 1F). The combination of plasma Chit-1 and CHI3L1 significantly improved performance as compared with each biomarker alone (AUC Chit-1+CHI3L1 vs either AUC Chit-1 alone or AUC CHI3L1 alone, p<0.01).

Correlations between chitinases and clinical measures of ALS

CSF Chit-1 levels were negatively correlated with ALSFRS-r at the baseline visit (online supplementary figure S2A; r=−0.2402, p=0.02). There was no correlation between plasma Chit-1, CSF CHI3L1 or plasma CHI3L1 with ALSFRS-r (online supplementary figures S2B–D; plasma Chit-1; r=−0.2161, p=0.05; CSF CHI3L1 r=−0.1581, p=0.1561; and plasma CHI3L1 r=−0.06575, p=0.5598). There was no correlation between plasma Chit-1 and disease duration (online supplementary

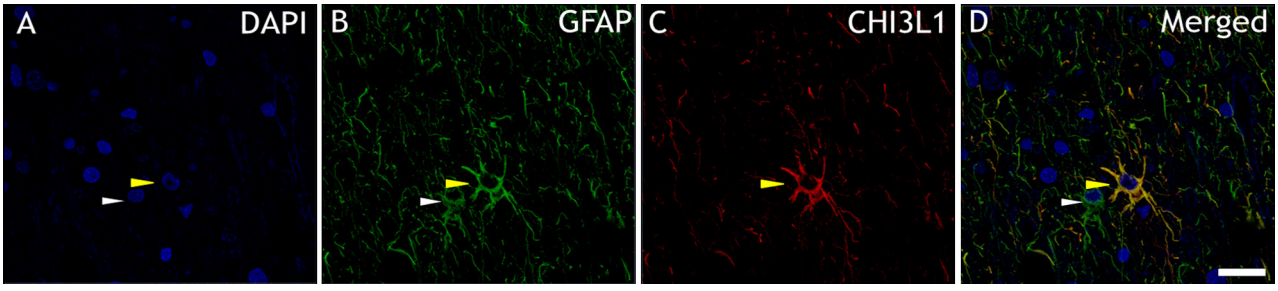

Figure 4 CHI3L1 expression in a subset of activated astrocytes. Double-label confocal microscopy for Glial fibrillary acidic protein (GFAP) (green) and CHI3L1 (red) in the motor cortex of a patient with amyotrophic lateral sclerosis, with nuclei counterstained using DAPI (blue). (A) DAPI, (B) GFAP, (C) CHI3L1 and (D) merged image. Images were acquired using 63× oil objective as described in the Methods section. Scale bar=20 µm for all panels. White arrowheads denote a GFAP-positive astrocyte and yellow arrowheads denote a GFAP-positive and CHI3L1-positive astrocyte. CHI3L1, chitinase-3-like protein 1; DAPI, 4′,6-diamidino-2-phenylindole.

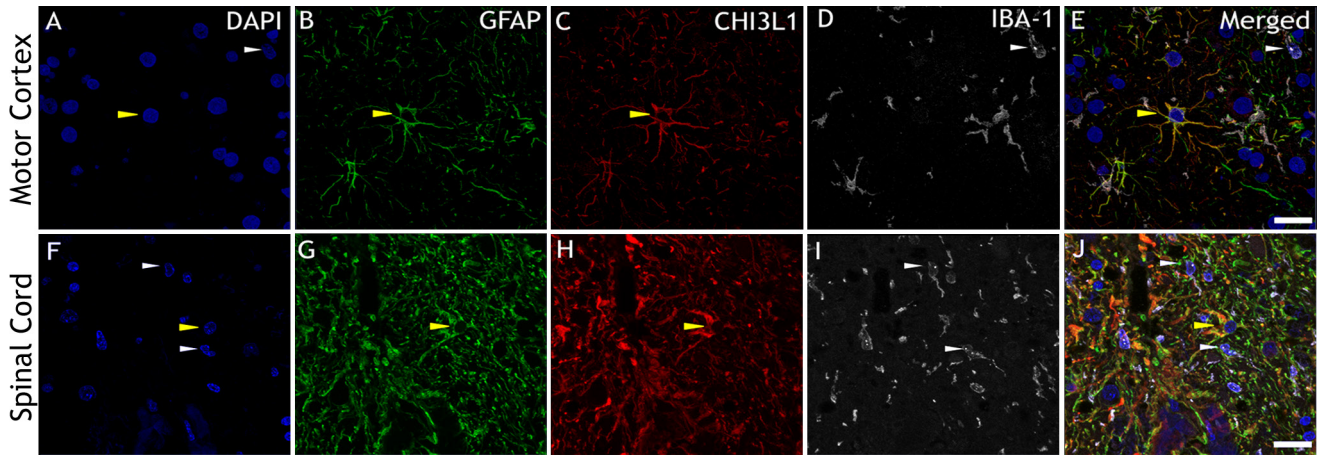

Figure 5 Triple-label immunofluorescence confocal microscopy of ALS motor cortex (A–E) and ALS lumbar spinal cord (F–J). (A,F) DAPI, (B,G) GFAP, (C,H) CHI3L1, (D,I) Iba-1 and (E,J) merged image demonstrate colocalisation of CHI3L1 in a subset of GFAP-positive astrocyte (yellow arrowheads) in both motor cortex and spinal cord, with no colocalisation of CHI3L1 and Iba-1 (white arrowheads) in either tissue. Images captured using 63× oil objective. Scale bar=20 µm for all panels. ALS, amyotrophic lateral sclerosis; CHI3L1, chitinase-3-like protein 1; DAPI, 4′,6-diamidino-2-phenylindole.

figures S2F; CSF r=−0.08614, p=0.4091). Similar results were also observed for plasma CHI3L1 (online supplementary figure S2H; r = 0.09351, p=0.3753); however, there were correlations between CSF Chit-1 and CHI3L1 with disease duration that reached statistical significance (online supplementary figure S2E, G; Chit-1 r=−0.257, p=0.0062, and CHI3L1 r=0.3083, p=0.0041).

Longitudinal analysis of Chit-1 and CHI3L1

Levels of Chit-1 and CHI3L1 were measured in matching CSF and plasma from 42 patients with ALS that had a minimum of 3 longitudinal time points (table 1). Baseline levels of both CSF Chit-1 and CSF CHI3L1 were significantly higher in FPs as compared with SPs (figure 2A,B). No significant difference was observed in SP versus IPs or between FPs versus IPs for either biomarker. To assess the rate of change of each chitinase over time, slopes from linear mixed effects modelling analyses were determined (figure 2C,D). No significant rise in the slopes of CSF Chit-1 or CSF CHI3L1 was observed. Collectively, these results, combined with the assessment of baseline levels, suggest that, over time, CSF Chit-1 and CSF CHI3L1 remain constant, but levels are significantly higher in fast FPs as compared with SPs, while no differences were observed in SPs versus IPs or FPs versus IPs.

These trends were not observed with plasma Chit-1 or plasma CHI3L1, where no differences in baseline levels and no changes in slopes were detected (figure 2E–H). These results indicate that no differences over time for either chitinase in plasma were observed. A similar analysis was performed using CSF and plasma pNFH levels, and no changes were observed over time in either biofluid (online supplementary figure S3), similar to previous observations in plasma.[29]

Chitinase levels correlate with pNFH in CSF

Similar to prior studies, levels of both CSF and plasma pNFH were significantly higher in patients with ALS as compared with control groups[2 3 29–31] (figure 3A,B). Interestingly, both CSF Chit-1 and CSF CHI3L1 correlated with CSF pNFH (figure 3C,D; Chit-1 vs pNFH, r=0.6795, p<0.0001; CHI3L1 vs pNFH, r=0.6769, p<0.0001). We detected no significant correlation between either chitinase to pNFH in plasma (data not shown). No significant correlation between Chit-1 and CHI3L1 was detected in either CSF or plasma (online supplementary figure S4).

CHI3L1 immunostaining in postmortem tissues

A prior study demonstrated Chit-1 colocalisation with Iba-1-expressing microglia in ALS spinal cord.[19] To determine cell type-specific expression of CHI3L1 in ALS, we performed double label immunofluorescence confocal microscopy in motor cortex tissue sections (figure 4A–D). Colocalisation of Glial fibrillary acidic protein (GFAP) and CHI3L1 confirmed that CHI3L1-positive cells are activated astrocytes (figure 4D, yellow arrowhead). Interestingly, not all GFAP-positive astrocytes exhibited CHI3L1 expression (figure 4D, white arrowhead). Triple-label confocal microscopy demonstrated that CHI3L1-positive cells were GFAP positive and Iba-1 negative in the white matter of both motor cortex (figure 5A–E) and lumbar spinal cord (figure 5F–J), indicating that CHI3L1 is a specific biomarker for a subset of activated astrocytes in the white matter of patients with ALS. To further assess differences in the amount and distribution of CHI3L1-positive cells in ALS and controls, we performed IHC for CHI3L1 in the motor cortex of ALS, DCs and NNDCs and observed CHI3L1-positive cells predominately in the subpial region and white matter (figure 6A). Interestingly, a similar staining pattern was observed in the frontal and occipital cortex of ALS cases (online supplementary figure S5). We next quantified CHI3L1-positive cells in both the grey and white matter of the motor cortex. In the grey matter, we did not observe any significant differences between ALS, DCs and NNDCs (data not shown). However, in the white matter, we observed approximately a 2.8-fold increase of CHI3L1-positive cells in ALS white matter as compared with NNDCs (figure 6B, C). No significant differences were observed in the white matter between ALS and DCs or between DCs and NNDCs.

DISCUSSION

We confirmed recent findings that CSF Chit-1 is significantly higher in ALS samples as compared with both DCs and HCs using unbiased proteomic[18 20 24] or targeted immunoassay methods.[18 19 21 22 32] CSF CHI3L1 was also higher in ALS as compared with HCs, confirming results from some studies[15 18] but contrasting with others.[20–22 33] We also determined that CSF CHI3L1 was higher in DCs as compared with HCs, supporting

a role for this protein as a candidate biomarker for multiple neurological diseases, including MS[14 15] and AD.[15 16] Plasma levels of both Chit-1 and CHI3L1 showed no significant difference between ALS and control groups. Similar observations were recently reported in the serum of patients with ALS.[21 22] Although several outliers with high levels of plasma Chit-1 or CHI3L1 were observed in the ALS group, patients with ALS with high-plasma Chit-1 did not exhibit high levels of CHI3L1. Based on ROC analysis, we demonstrate that a combination of Chit-1 plus CHI3L1 outperforms either chitinase alone in plasma but not CSF in classifying ALS versus controls. However, CSF Chit-1 alone can also classify ALS versus controls.

Elevated levels of Chit-1 and CHI3L1 in the CSF are indicative of glial activation that may potentiate neuroinflammation and motor neuron death in ALS. Prior studies demonstrated increased astrocyte infiltration in the spinal cord of Superoxide dismutase 1 (*SOD1*) transgenic mice,[34] and the presence of CHI3L1-positive cells in the lumbar spinal cord of patients with ALS.[35] Positron emission tomography demonstrated increased numbers of activated microglia in the motor cortex and corticospinal tract in patients with ALS.[36] A recent study identified increased Chit-1 immunoreactivity in activated microglia in the spinal cord white matter of patients with ALS.[19] Data are lacking on the cell-type expression of CHI3L1 in ALS. In our study, we identified a ~2.8-fold increase in CHI3L1-positive cells in the white matter of motor cortex of patients with ALS as compared with NNDCs. CHI3L1-positive cells were also evident in subpial layers, but only scattered positive cells were observed in the deeper layers of the grey matter. Similar results have also shown an increase in GFAP containing astrocytes in the white matter of ALS frontal cortex as compared with controls.[37] We demonstrate by confocal microscopy that CHI3L1 is expressed by a subset of activated astrocytes but not microglia located in the white matter of the motor cortex and spinal cord of patients with ALS (figures 4 and 5). While the consequence of increased CHI3L1 expressing astrocytes in the white matter of patients with ALS is unclear, we propose that increased CHI3L1 expressing astrocytes directly contribute to axonal degeneration in the white matter. This is supported by the correlation we observed between CSF CHI3L1 and CSF pNFH (a marker of axonal injury). Other studies have

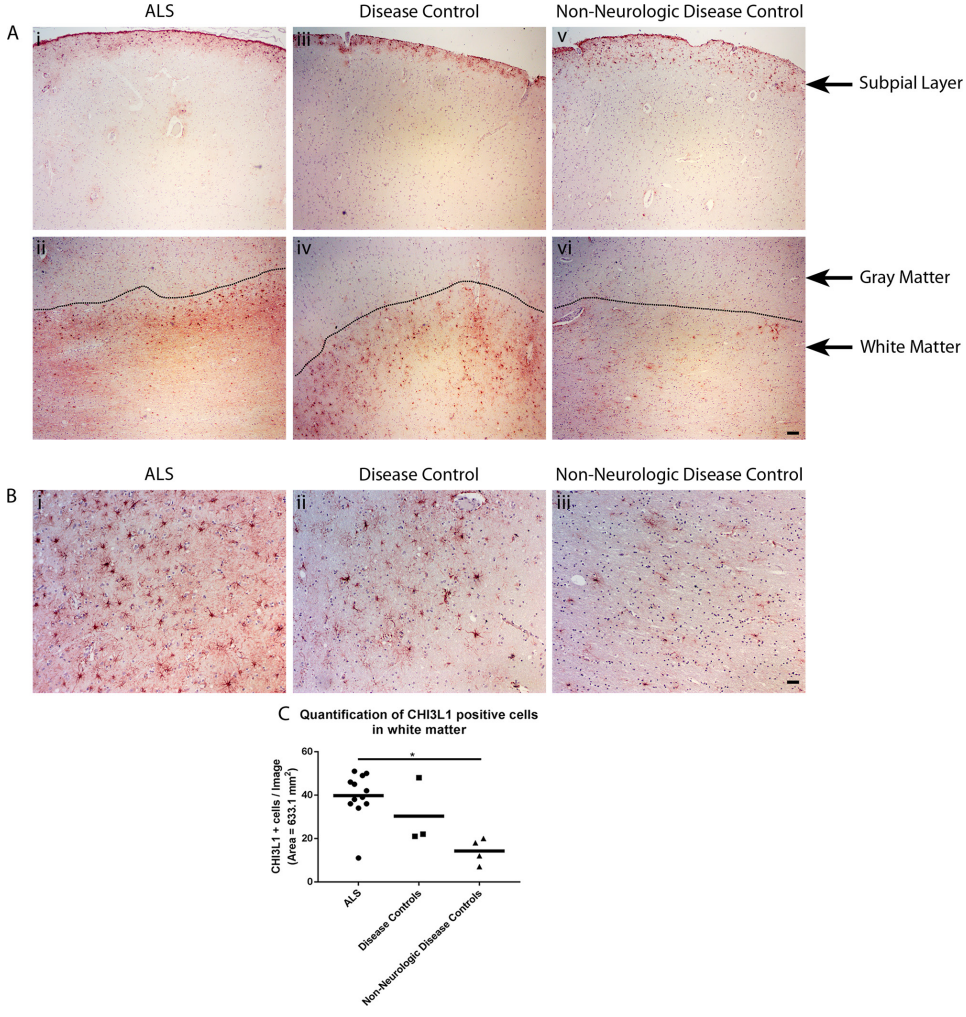

Figure 6 (A) Immunohistochemistry for CHI3L1 in motor cortex grey and white matter of (i and ii) patients with ALS, (III and iv) neurological DCs and (v and vi) NNDCs using a 4× objective. Dashed lines show the grey and white matter interface. (B) Representative images used for quantification of CHI3L1-positive cells in (i) ALS, (ii) neurological DCs and (iii) NNDCs using a 10× objective. (C) Quantification for the number of CHI3L1-positive cells in at least five fields of view of white matter (area=633.1 mm^2) for each case. Data analysis was performed in a blinded manner (n=12 ALS, 3 neurological DCs, 4 NNDCs). A Kruskal-Wallis test was used to assess differences between pairwise comparisons, and p values were corrected using the Dunn correction. *<0.05. scale bar=100 µm in (A) and 40 µm in (B). ALS, amyotrophic lateral sclerosis; CHI3L1, chitinase-3-like protein 1; DC, disease control; NNDC, non-neurological disease control.

proposed a link between activated white matter astrocytes and myelin degeneration[38] and a role for chitinases in modulating the extracellular matrix. Therefore, our results suggest that increased levels of CHI3L1 in the white matter may disrupt normal oligodendrocyte function or may act on extracellular matrix proteins to modulate axonal damage and further exacerbate neuroinflammation. Interestingly, only a subset of GFAP-positive astrocytes colocalised with CHI3L1, suggesting the presence of different astrocytic subtypes in the grey and white matter. Additionally, while CHI3L1 is expressed by activated astrocytes and not microglia in the frontal cortex of patients with AD,[39] CHI3L1 expression was detected in both astrocytes and microglia in MS.[14] Together, our results highlight a disease-specific expression pattern for chitinase proteins. Further studies are required to identify the pathological trigger inducing CHI3L1 expression and secretion from a subset of astrocytes. In addition, future studies will also evaluate cell type-specific expression of Chit-1 in cortical regions to complement prior observations in the spinal cord of patients with ALS.[19]

To assess longitudinal changes in protein levels, we determined the baseline levels and the rate of change of each chitinase over time. We demonstrated that, over time, both CSF Chit-1 and CSF CHI3L1 remain relatively constant, but segregate patients with ALS based on rate of disease progression. While CSF chitinase levels distinguish between FP and SP forms of ALS, plasma chitinase levels showed no significant differences over time and did not distinguish FP from SP patients with ALS. Similar trends were observed using serum from FP and SP patients with ALS.[19] Our data suggest the potential utility of CSF chitinases for segregation of patients with ALS based on disease progression rate, though these results must be validated in future studies.

A prior study demonstrated that patients with ALS carrying specific *CHIT1* polymorphisms exhibited lower CSF levels of Chit-1.[33] Since *CHIT1* genotype information was not available for our subject cohorts, we were not able to stratify our subject populations based on this polymorphism, representing a confounding factor in this study.

It remains unclear whether neuroinflammation is a cause or consequence of neuronal/axonal degeneration. We observed a strong correlation between CSF chitinase and CSF pNFH levels, suggesting concomitant neuroinflammation and axonal injury in the CNS. However, a recent study observed increased CSF levels of neurofilaments but no increases in Chit-1 or CHI3L1 levels in one asymptomatic mutation carrier that harboured a *SOD1* mutation and recently transitioned to the symptomatic phase of ALS, suggesting that axonal damage may precede inflammation.[33] To fully explore this important question, future studies must examine CSF levels of chitinases and neurofilaments in a large number of participants that harbour ALS disease-causing mutations at a presymptomatic stage and then follow these individuals in a longitudinal manner through their transition to the symptomatic phase. Additionally, transgenic mouse models of ALS could be used to assess cell type-specific expression of chitinases longitudinally both prior to symptom onset and throughout the disease course.

Overall, our study highlights that CSF rather than plasma chitinases are candidate ALS biomarkers, and levels correlate with the rate of disease progression as measured by the change in ALSFRS-r over time. Furthermore, CHI3L1 expression occurs in a subset of activated astrocytes predominately in the white matter of patients with ALS, warranting continued studies to better define the role of chitinases in the pathogenesis of ALS.

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Competing interests RB is founder of Iron Horse Diagnostics, Inc, a company commercialising assays for ALS and other neurological diseases.

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