

YKL40 in sporadic amyotrophic lateral sclerosis: cerebrospinal fluid levels as a prognosis marker of disease progression

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) has variable clinical course and fatal outcome. Since inflammation plays a role in the pathogenesis of ALS, chitinase-3-like protein 1 or YKL40 has been assessed as putative biomarker of disease progression. YKL40 mRNA levels are increased in anterior horn of the spinal cord ($P=0.004$) in sporadic ALS (sALS) cases when compared with age-matched controls. These correlate with increased mRNA expression of microglial markers *AIF1* and *CD68* in the spinal cord in sALS ($P=0.044$ and $P=0.000$, respectively). YKL40 mRNA and protein expression had a tendency to increase in post-mortem frontal cortex area 8 ($P=0.06$ and $P=0.08$, respectively). Yet YKL40 immunoreactivity is restricted to a subpopulation of astrocytes in these regions. YKL40 protein levels, as revealed by enzyme-linked immunosorbent assay (ELISA), are significantly increased in the CSF in sALS ($n=86$) compared with age-matched controls ($n=21$) ($P=0.045$). Higher levels are found in patients with fast progression when compared with patients with slow and normal progression ($P=0.008$ and $P=0.004$, respectively), and correlates with ALS-FRS-R slope ($P=0.000$). Additionally, increased protein levels of neurofilament light chain (NF-L) are also found in sALS ($P=0.000$); highest values are found in patients with fast progression when compared with cases with slow and normal progression ($P=0.005$ and $P=0.000$, respectively), and also correlate with ALS-FRS-R slope ($P=0.000$). Pearson's correlation test linked positively the increased levels of YKL40 with increased NF-L levels ($P=0.013$). These data point to YKL40 and NF-L protein levels in the CSF as a good biomarker combination of disease progression in sALS.

INTRODUCTION

Chitinase-3-like protein 1 (CHI3L1) or YKL40 is a glycoprotein with a molecular weight of about 40 kDa that belongs to the family of chitinase-like proteins. Chitinases break down glycosidic bonds in chitin, a component of the cell wall of fungi and the exoskeleton of arthropods [1-5]. YKL40 and other chitinases are also localized in various tissues in vertebrates but their function is not known; YKL40 shows no chitinase activity. Increased expression levels of certain chitinases, and particularly of YKL40, are linked to inflammation, injury, tissue remodelling and regeneration, angiogenesis, and abnormal cell proliferation in tumours [6, 7]. Focusing on neurologic diseases, increased YKL40 expression levels have been observed in encephalitis, stroke, traumatic brain injury, multiple sclerosis, and glioblastomas [8-17]. YKL40 expression is also increased in the cerebrospinal fluid (CSF) in neurodegenerative diseases such as Alzheimer's disease, frontotemporal dementia, and Creutzfeldt-Jakob disease, but not in Parkinson disease or dementia with Lewy bodies [18-36]. For this reason, determination of YKL40 in the CSF has been postulated as a new biomarker that may guide diagnosis in particular clinical settings. Since YKL40 is mainly expressed in astrocytes with only minor expression, if any, in microglia, increased YKL40 in CSF is interpreted as a reactive response of astrocytes linked to inflammation and regeneration [33,37-40].

Chitinases have also been assessed in the brain and biological fluids in amyotrophic lateral sclerosis (ALS). Chitotriosidase (CHIT1) activity is increased in blood in ALS cases when compared with controls, and CHIT1 levels are higher in patients with rapid progression [41]. Furthermore, CHIT1 is increased in microglia and macrophages in spinal cord in ALS, and CSF levels correlate with disease severity and progression [42]. YKL40 and chitinase-3-like protein 2 (CHI3L2) mRNA levels are increased in the motor cortex in ALS [43]. Finally, as determined with liquid chromatography/tandem mass spectrometry, elevated CHIT1, YKL40, and CHI3L2 levels in the CSF correlate with disease progression in ALS [44]. A parallel work presented by another group at the ALS Society Meeting (Amsterdam June 7-8, 2018) reported increased YKL40 in the CSF along the ALS-FTD spectrum [45].

The present study examines YKL40 mRNA and protein expression in brain, YKL40 mRNA levels in blood, and protein levels in the CSF in cases of sporadic ALS (sALS) to learn about the relation between mRNA and protein levels in the central nervous system, and those in CSF and peripheral blood. Based on the previous observations in several diseases, it is worth to have in

mind that that YKL40 is not looked as a putative specific biomarker of ALS but as a potential biomarker of prognosis. Therefore, the present study was geared to learn about the use of YKL40 as a possible biomarker of progression in this disease.

RESULTS

Increased *CHI3L1* mRNA expression levels in the anterior horn of the spinal cord and frontal cortex in sALS

Significantly increased expression of *CHI3L1* was found in the anterior horn of the spinal cord ($P=0.004$) in sALS (Figure 1A). Levels of transcripts coding for the main markers of astrocytes and microglia were also assessed in the anterior horn of the spinal cord. Significantly up-regulated levels of microglial markers *AIF1* and *CD68* were detected in the spinal cord in sALS ($P=0.044$ and $P=0.000$, respectively) (Figure 1B), which significantly correlated with *CHI3L1* mRNA expression ($P=0.043$ and $P=0.025$, respectively). However, *GFAP* and *ALDH1L1* mRNA levels did not show differences between sALS and control cases ($P=0.22$ and $P=0.77$, respectively) (Figure 1B). No correlations were detected with *CHI3L1* expression ($P=0.66$ and $P=0.88$, respectively) in the spinal cord region.

CHI3L1 mRNA expression had a tendency to increase in the frontal cortex area 8 in sALS ($P=0.06$) (Figure 1A). No changes were observed in the mRNA levels of *AIF1* ($P=0.32$), *CD68* ($P=0.89$), *GFAP* ($P=0.15$), and *ALDH1L1* ($P=0.15$) in sALS (Figure 1B). Finally, no correlations were found between *CHI3L1* mRNA levels, and astrocytic and microglial markers in frontal cortex area 8 of sALS cases.

Protein levels of YKL40 are increased in frontal cortex area 8 in sALS

Western blotting showed a tendency to increase YKL40 and IBA1 protein levels in the anterior horn of the spinal cord of sALS when compared with controls ($P=0.08$ and $P=0.07$, respectively). GFAP protein levels were significantly increased, particularly breakdown products (BDP) ($P=0.01$) in the spinal cord of sALS when compared with controls (Figure 1C). Increased GFAP low molecular weight bands (BDPs) have been previously reported in ALS [46]. In contrast, a significant increase in YKL40 ($P=0.03$) and GFAP ($P=0.02$) levels, but not in IBA1 ($P=0.62$), was found in frontal cortex area 8 in sALS when compared with controls (Figure 1C). YKL40 immunoreactivity was restricted to astrocytes in the frontal cortex and spinal cord in sALS and control cases (Figure 1D).

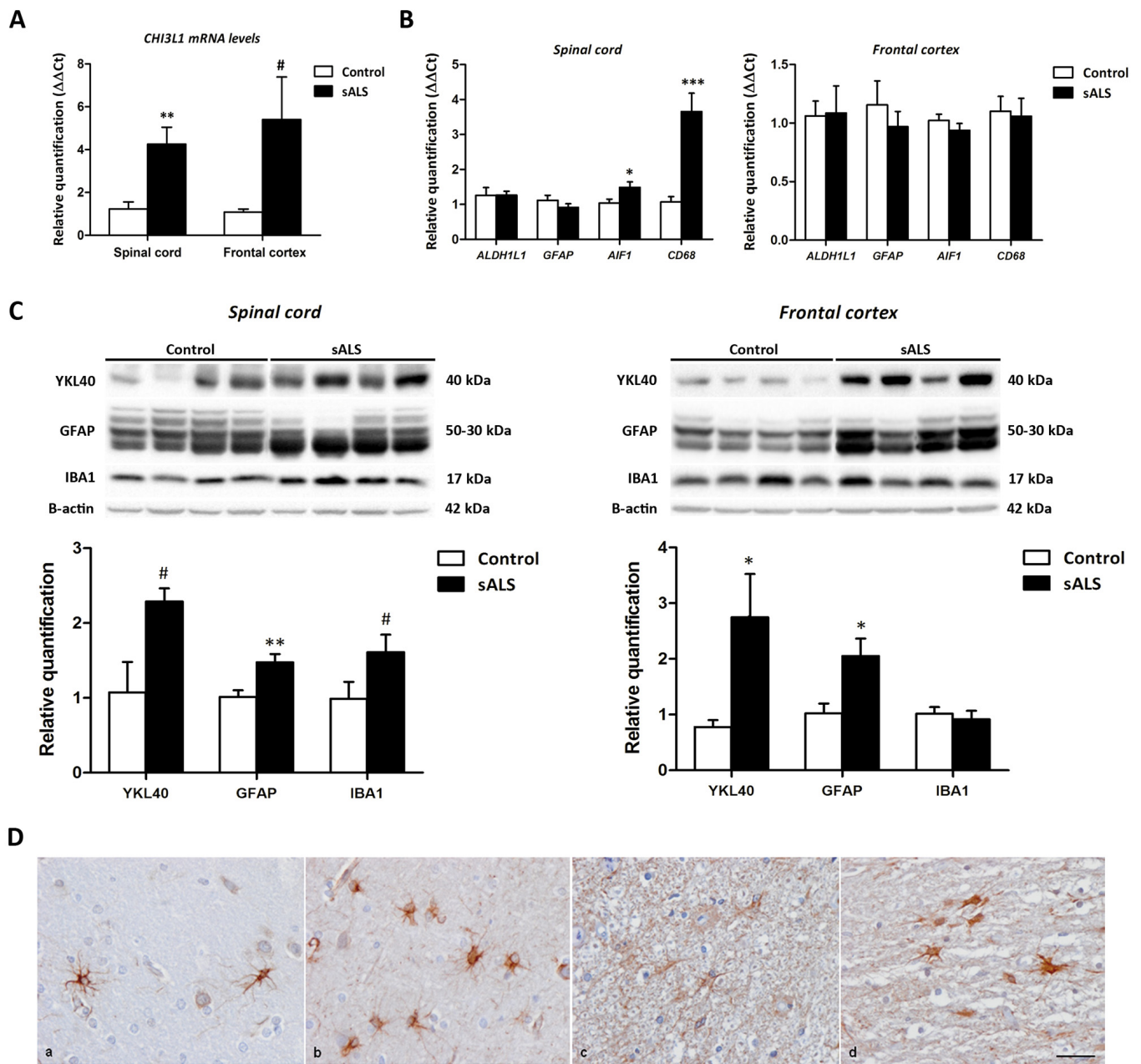


Figure 1. (A) *CHI3L1* mRNA expression levels in the anterior horn of the lumbar spinal cord and frontal cortex area 8 in sALS and control cases. *CHI3L1* is significantly up-regulated in the anterior spinal cord but has only a tendency to increase without significance in the frontal cortex in sALS compared with controls. (B) mRNA expression levels of microglial (*CD68* and *AIF1*) and astroglial (*GFAP* and *ALDH1L1*) markers in the anterior horn of lumbar spinal cord and frontal cortex area 8 in sALS and age-matched controls. Microglial markers *CD68* and *AIF1* are significantly up-regulated in the anterior horn of the spinal cord but not in the frontal cortex in sALS. The mRNA expression levels of astroglial markers in the spinal cord and frontal cortex are not modified in pathological cases when compared with controls. (C) Western blot analysis of YKL40 in the spinal cord (left panel) and frontal cortex area 8 (right panel) of control and sALS; β -actin was used for normalization. Graphical representation of western blot data; fold changes in the expression of protein are determined relative to the control cases. YKL40 and GFAP protein levels are increased in the spinal cord and frontal cortex in sALS when compared with controls. Due to individual variation, increased values in the anterior horn of the spinal cord showed only a tendency without statistical significance. In contrast, expression levels were not significantly modified in sALS. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, tendency # $P < 0.1$. (D) YKL40 expression in frontal cortex area 8 (a, b) and spinal cord (c, d) in control (a, c) and sALS (b, d) cases) is found in astrocytes; immunohistochemical sections lightly counterstained with haematoxylin, bar = 25 μ m.

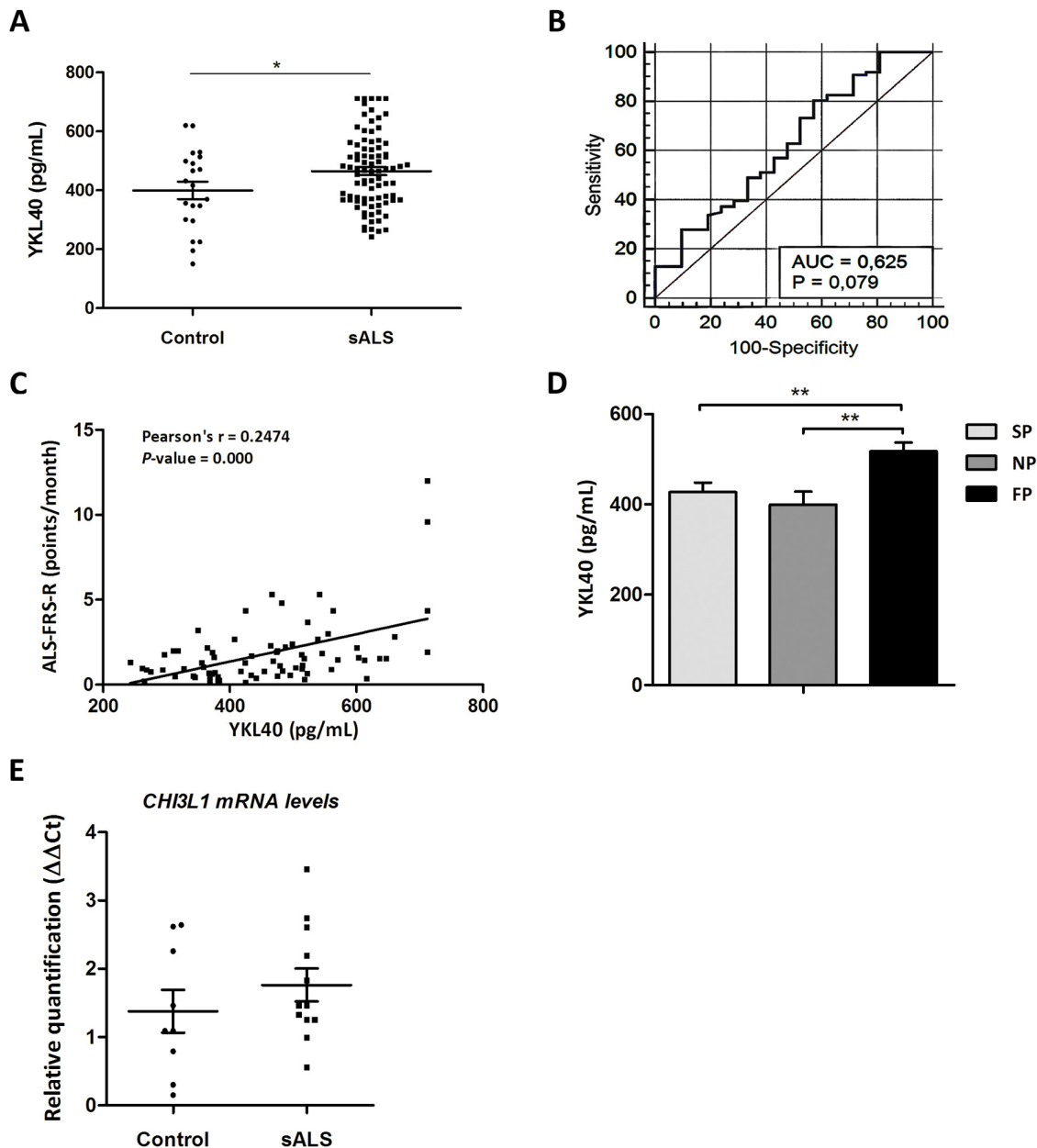


Figure 2. (A) Quantification of YKL40 protein levels in the CSF in sALS (n=85) and control (n=23) cases. (B) ROC curves for YKL-40 quantification in the differential diagnosis of sALS compared to control cases. In the legend, AUC values, corresponding to the area under ROC curves, and 95% confidence intervals are reported. (C) Positive correlation between ALS-FRS-R slope (point/month) and YKL40 levels (pg/mL) (Pearson's correlation, $P=0.000$). (D) Higher YKL40 protein levels in the CSF are found in cases with short survival (fast progression: FP) when compared with cases with slow and normal progression (SP and NP, respectively; $P = 0.008$, $P = 0.004$). (E) *CHI3L1* mRNA expression levels in whole-blood samples of sALS and control cases. *CHI3L1* is not deregulated in sALS.

Levels of YKL40 are increased in CSF of sALS patients and correlate with ALS-FRS evolution and fast disease progression

Significantly higher YKL40 levels were detected in sALS cases (465.41 ± 13.45 pg/mL) compared with controls (399 ± 29.52 pg/mL) ($P=0.045$) (Figure 2A).

To calculate the clinical accuracy of YKL-40 in discriminating between sALS and the control group, we estimated the AUC value (AUC: 0.6254, 95% CI: 0.52–0.72) (Figure 2B). Considering the optimal cut-off at 356.24pg/mL, defined by the Youden index, an overall sensitivity of 80% and specificity of 43% can be predicted. To demonstrate possible relations between

increased levels of YKL40 and the main clinical parameters, Pearson's correlation or parametric comparisons tests were applied. Clinical parameters such as age, gender, disease onset, disease progression, signs of frontotemporal lobar degeneration, and ALS-FRS-R score were examined. Pearson's correlation indicated a significant link between age and YKL40 levels, increasing with age in controls and sALS ($P=0.000$). Additionally, Pearson's correlation test demonstrated a positive correlation between ALS-FRS-R slope and YKL40 levels in CSF ($P=0.004$) (Figure 2C). Based on these observations, YKL40 levels were studied in function of the disease progression in every patient; a significant increase in YKL40 CSF levels was identified in those patients with fast progression when compared with patients with slow and normal progression ($P=0.008$ and $P=0.004$, respectively) (Figure 2D). Since at the end of the study only 28 of the 86 sALS cases assessed had died, no attempt was made to analyze the relationship between YKL40 levels in the CSF with survival.

***CHI3L1* mRNA levels in blood**

Additionally, *CHI3L1* mRNA levels were analyzed in whole peripheral blood samples of sALS at the time of diagnosis. Despite the relatively small number of control and disease cases, individual variations were frequent in the two groups and accounted for the lack of significant changes between control and sALS cases (Figure 2E).

Levels of NF-L are increased in CSF of sALS patients and correlate with ALS-FRS-R slope evolution, fast disease progression and YKL40 levels

Neurofilament light chain (NF-L) levels were quantified in CSF of the same cohort of control and sALS cases. Significant higher NF-L levels were detected in sALS cases (4637.55 ± 192.31 pg/mL) compared with controls (610.36 ± 81.11 pg/mL) ($P=0.000$) (Figure 3A). Additionally, NF-L levels were correlated with ALS-FRS-R slope using Pearson's test; positive correlation

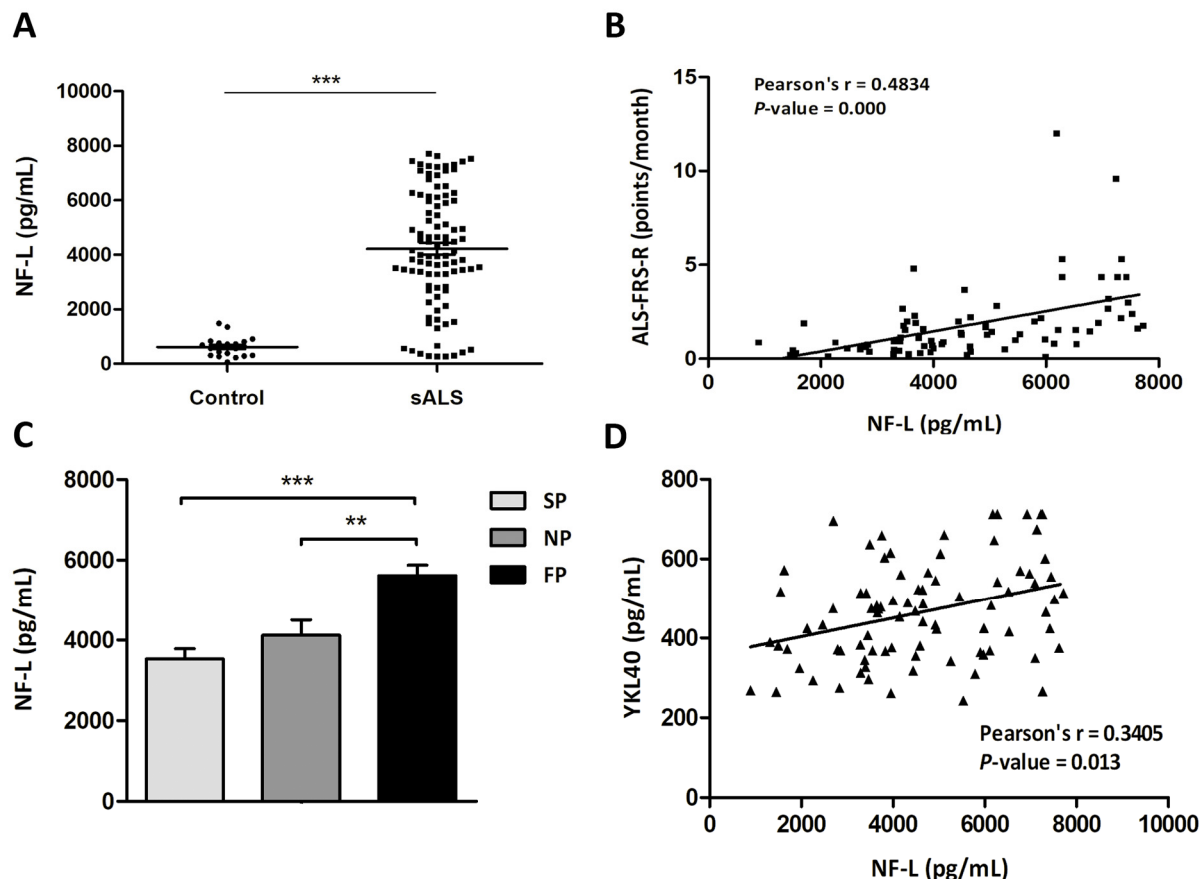


Figure 3. (A) Quantification of NF-L protein levels in the CSF in sALS (n=85) and control (n=23) cases. (B) Positive correlation between ALS-FRS-R slope (point/month) and NF-L levels (pg/mL) (Pearson's correlation, $P=0.000$). (C) Higher NF-L protein levels in the CSF are found in cases with fast progression (FP) when compared with cases with slow and normal progression (SP and NP, respectively; $P = 0.000$, $P = 0.005$). (D) Positive correlation between YKL40 levels (pg/mL) and NF-L levels (pg/mL) (Pearson's correlation, $P=0.013$).

was found between ALS-FRS-R and NF-L levels in CSF ($P=0.000$) (Figure 2B). NF-L levels were significantly increased in patients with fast progression when compared with patients with slow and normal progression ($P=0.000$ and $P=0.005$, respectively) (Figure 2C). Finally, positive significant correlation was observed between YKL40 and NF-L levels (Pearson's correlation test, $P=0.013$) (Figure 2D).

DISCUSSION

Inflammation involving microglial cells, macrophages, T cells, astrocytes, and neurons, and mediated by a plethora of mediators of the immune response including Toll-like receptors, members of the complement system, pro- and anti-inflammatory cytokines, chemoquines, and blood vessel factors, are activated in the anterior horn of the spinal cord and, to a lesser extent, in other brain regions in ALS [47-59].

Increased protein levels of several cytokines and mediators of the inflammatory response have also been reported in the CSF in ALS, including IL-1, IL-1 β , IL-6, IL-8, IL-12, IL-15, IL-17A, IL-18BP, IL-23, RANTES, chemokines, and MCP1 [60-69]. This heterogeneous representation indicates variations depending on the methods and products employed in the different laboratories. Moreover, CSF profiles of angiogenic and inflammatory factors are, at least in part, dependent on the respiratory status of ALS patients [70].

Increased levels of selected inflammatory markers are found in blood and serum in ALS, thus suggesting systemic inflammatory responses which roughly correlate with disease progression [71-80].

All these observations strongly support a role of inflammation in the pathogenesis of sALS. However, the identification of a biomarker of inflammation with practical prognosis value has been limited because of individual variation and variations between methods and laboratories.

Previous studies in ALS have shown YKL40 mRNA up-regulation in the motor cortex [43] and increased YKL40 protein levels in the CSF correlating with disease progression [44, 45]. Regarding brain tissue, the present observations show significant YKL40 mRNA up-regulation in the anterior horn of the spinal cord and frontal cortex area 8, accompanied by significantly increased YKL40 protein levels in the frontal cortex and a tendency to increased YKL40 in the spinal cord in sALS. Importantly, YKL40 is expressed in astrocytes, in agreement with other observations [33, 36-40], but in contrast to another description ascribing YKL40 expression to brain macrophages [44].

Up-regulation and increased YKL40 expression occurs in parallel with increased values of microglia markers in the spinal cord but not in the frontal cortex area in sALS, and with increased GFAP protein levels in the spinal cord and frontal cortex but not with GFAP mRNA up-regulation in these regions.

Together, these observations point to earlier responses in astrocytes when compared with microglial reactions in the frontal cortex in sALS, whereas microglial markers are strongly expressed in the spinal cord in the same group of patients.

Based on these findings, increased YKL40 protein levels in the CSF mirror YKL40 changes in the central nervous system, and they can be interpreted as the consequence of YKL40 delivery of astrocytes to the CSF. Unfortunately, no analysis of a possible correlation between YKL40 brain and spinal cord values, and disease progression/survival, was feasible in the present series because of the lack of sufficient clinical data. However, YKL40 CSF values negatively correlate with patient survival, thus indicating that higher YKL40 in the CSF likely occurs in patients with rapid disease progression.

We do not know at this time what the functional implications of elevated YKL40 expression in ALS and other neurological diseases are. Nor do we know whether YKL40, even considering this particular chitinase as a marker of astrocyte inflammation, has beneficial or deleterious effects. In this line, *chi3l1* KO mice have increased astrocytic responses (GFAP staining) and increased IBA1 microglial expression when compared with wild-type animals following traumatic brain injury, thus suggesting that YKL40 limits the extent of astroglial and microglial neuro-inflammation [38]. If this is the case then increased YKL40 expression *per se* would not be dangerous but rather a manifestation of increased beneficial response in the face of a more aggressive facet of ALS in a subgroup of patients.

The present findings point to the likelihood that increased YKL40 levels in the CSF are not disease specific but they are good biomarker of disease progression in sALS.

Previous studies have shown increased levels of neurofilaments in the CSF of ALS cases [81-84]. NF heavy chain levels in CSF were negatively correlated disease duration and ALS-FRS-R slope, and NF-L levels in CSF were negatively correlated with disease duration. Thus, NF heavy and light chain levels have potential use as markers of neural degeneration in ALS [85, 86]. Increased NF-L in the CSF are not either

specific for the disease, but they are more likely used as measures of disease progression [85, 86].

In the present work, YKL40 levels in the CSF were assessed in parallel with levels of NF light chain. As expected, our results are in line with previous observations by other authors; NF-light chain levels are significantly increased in ALS and levels negatively correlate with disease progression and ALS-FRS-R slope in our series.

In summary, the present findings point that YKL40 and NF-L levels in CSF constitute valuable combination of biomarkers for improving accuracy in the prognosis of patients with sALS.

MATERIALS AND METHODS

Tissue samples

Post-mortem fresh-frozen lumbar spinal cord (SC) and frontal cortex (FC) (Brodmann area 8) tissue samples were obtained from the Institute of Neuropathology HUB-ICO-IDIBELL Biobank following the guidelines of Spanish legislation on this matter and the approval of the local ethics committee. The post-mortem interval between death and tissue processing was between 2h and 17h. One hemisphere was immediately cut in coronal sections, 1-cm thick, and selected areas of the encephalon were rapidly dissected, frozen on metal plates over dry ice, placed in individual air-tight plastic bags, numbered with water-resistant ink, and stored at -80°C until use for biochemical studies. The other hemisphere was fixed by immersion in 4% buffered formalin for 3 weeks for morphologic studies. Transversal sections of the spinal cord were alternatively frozen at -80°C or fixed by immersion in 4% buffered formalin. The anterior horn of the lumbar spinal cord was dissected on a dry-ice frozen plate under a binocular microscope at a magnification x4.

The neuropathological study was carried out on paraffin sections of twenty-six selected regions of the cerebrum, cerebellum, brain stem, and spinal cord which were stained with haematoxylin and eosin, Klüver Barrera, periodic acid Schiff, and processed for immunohistochemistry with anti- β -amyloid, phospho-tau (clone AT8), α -synuclein, α B-crystallin, TDP-43, ubiquitin, p62, glial fibrillary acidic protein, CD68, and IBA1 [87]. All cases met the neuropathological criteria for classical ALS regarding involvement of motor cortex, pyramidal tracts, and selected motor nuclei of the cranial nerves and anterior horn of the spinal cord [88, 89]. In addition, TDP-43-immunoreactive small dys-trophic neurites and TDP-43-positive cytoplasmic neuronal inclusions in frontal cortex area 8 were observed in 11 of 18 cases, but

they were abundant only in three cases (cases 29, 30, and 31). Spongiosis in the upper cortical layers was found in only one case (case 28). Frontotemporal dementia was found in no cases of the present series.

Patients with associated pathology including Alzheimer disease (excepting neurofibrillary tangle, NFT, pathology stages I-II of Braak and Braak), Parkinson disease, tauopathies, vascular diseases, neoplastic diseases affecting the nervous system, metabolic syndrome, hypoxia, and prolonged axonal states such as those occurring in intensive care units were excluded. Cases with infectious, inflammatory, or autoimmune diseases, either systemic or limited to the nervous system, were not included. Age-matched control cases had not suffered from neurologic or psychiatric diseases, and did not have abnormalities in the neuropathological examination excepting NFT pathology stages I-II of Braak and Braak. A summary of sALS and control cases is shown in Table 1.

CSF collection

Cerebrospinal fluid (CSF) was collected prospectively from patients undergoing lumbar puncture due to clinical suspicion of motor neuron disease at the functional unit of amyotrophic lateral sclerosis (UFELA) of the Neurology Service of the Bellvitge University Hospital. Samples were obtained from 86 sALS patients (Table 2). In these patients, $1.5 \pm 0.5\text{mL}$ of CSF was collected in polypropylene tubes as part of the clinical routine investigation. CSF was centrifuged at 3,000 rpm for 15 min at room temperature. Supernatant was collected and aliquoted in volumes of 250 μL and stored at -80°C until use. All samples were analyzed after one freeze/thaw cycle.

Patients were evaluated clinically according to the main signs at onset (spinal, bulbar, and respiratory) and categorized according to disease progression as fast, expected, and slow progression depending on the survival or the clinical evolution in those still alive. Fast progression was considered in patients who survived less than 3 years; normal progression was considered between 3 and 5 years, and slow progression for those still alive after 5 years. The ALS Functional Rating Scale Revised (ALS-FRS-R, version May 2015) was used in every case. CSF from control cases was obtained from 21 healthy donors following the protocols for the use of biological samples for research (Table 2). No ALS cases or controls suffered from infection or inflammatory disorder at the time of sampling. CSF samples from sALS cases and age-matched controls were obtained after signed informed consent and approval by the Clinical Research Ethics Committee (CEIC) of the Bellvitge University Hospital.

Table 1. Summary of cases with tissue samples.

Case	Age	Gender	Diagnosis	PM delay	Initial symptoms	RIN value	
						SC	FC
1	49	F	Control	07 h 00 min	-	-	7.2
2	75	F	Control	03 h 00 min	-	-	7.2
3	55	M	Control	05 h 40 min	-	-	7.7
4	59	M	Control	12 h 05 min	-	6.4	-
5	59	M	Control	07 h 05 min	-	-	7.8
6	43	M	Control	05 h 55 min	-	6.6	7.7
7	53	M	Control	07 h 25 min	-	-	5.3
8	56	M	Control	03 h 50 min	-	-	7.6
9	47	M	Control	04 h 55 min	-	5.6	7.7
10	64	F	Control	11 h 20 min	-	6.2	-
11	46	M	Control	15 h 00 min	-	5.9	7.9
12	56	M	Control	07 h 10 min	-	6.1	-
13	71	F	Control	08 h 30 min	-	5.9	-
14	64	F	Control	05 h 00 min	-	7.0	-
15	79	F	Control	06 h 25 min	-	6.7	-
16	75	M	Control	07 h 30 min	-	5.0	-
17	55	M	Control	09 h 45 min	-	5.3	-
18	52	M	Control	03 h 00 min	-	-	8.3
19	52	M	Control	04 h 40 min	-	-	6.3
20	76	M	Control	06 h 30 min	-	6.6	-
21	60	F	Control	11 h 30 min	-	-	7.5
22	51	F	Control	04 h 00 min	-	6.3	7.9
23	54	M	Control	08 h 45 min	-	-	7.0
24	56	M	ALS	10 h 50 min	NA	7.1	-
25	70	M	ALS	03 h 00 min	Respiratory	7.3	7.0
26	77	M	ALS	04 h 30 min	NA	7.4	-
27	56	F	ALS	03 h 45 min	NA	8.2	7.7
28	59	M	ALS	03 h 15 min	NA	7.5	7.7
29	63	F	ALS	13 h 50 min	Bulbar	6.8	8.2
30	59	F	ALS	14 h 15 min	NA	6.4	6.7
31	54	M	ALS	04 h 50 min	Spinal	-	7.8
32	76	M	ALS	12 h 40 min	Spinal	-	7.4
33	64	M	ALS	16 h 30 min	NA	6.3	7.3
34	57	F	ALS	04 h 00 min	Bulbar	6.2	8.6
35	75	F	ALS	04 h 05 min	Bulbar	6.8	6.8
36	79	F	ALS	02 h 10 min	NA	7.0	-
37	57	F	ALS	10 h 00 min	Bulbar	6.5	7.1
38	50	M	ALS	10 h 10 min	Spinal	-	5.9
39	59	F	ALS	02 h 30 min	Spinal	-	7.5
40	46	M	ALS	07 h 00 min	Spinal	7.0	8.0
41	69	F	ALS	17 h 00 min	Spinal	6.4	6.3

Abbreviations: F: female; M: male; PM delay: post-mortem delay; SC: spinal cord; FC: frontal cortex; RIN: RNA integrity number.

Table 2. Summary of cases with CSF samples.

Group	n	Gender	Initial symptoms	
Control	21	9 (M) + 12 (F)	-	
sALS	86	47 (M) + 39 (F)	Spinal	55
			Bulbar	29
			Respiratory	2

Abbreviations: F: female; M: male; NA: not available.

Table 3. Summary of cases for whole peripheral blood mRNA studies.

Case	Age	Gender	Diagnosis	Initial symptoms	RIN value
1	60	M	Control	-	9.1
2	68	M	Control	-	9.2
3	66	F	Control	-	9.0
4	N/A	M	Control	-	8.9
5	74	M	Control	-	8.0
6	N/A	F	Control	-	8.3
7	67	M	Control	-	6.1
8	72	F	Control	-	6.0
9	44	F	Control	-	6.0
10	66	F	Control	-	6.1
11	60	M	ALS	Spinal	7.4
12	63	M	ALS	Spinal	8.7
13	66	F	ALS	Bulbar	8.9
14	53	F	ALS	Bulbar	7.3
15	73	M	ALS	Bulbar	8.6
16	65	M	ALS	Spinal	8.9
17	43	M	ALS	Bulbar	8.6
18	57	F	ALS	Bulbar	7.4
19	65	M	ALS	Bulbar	7.1
20	67	M	ALS	Bulbar	7.4
21	73	M	ALS	Spinal	6.1
22	73	F	ALS	Spinal	6.0

Abbreviations: F: female; M: male; NA: not available; RIN: RNA integrity value.

Whole blood samples were collected using PAXgene Blood RNA Tube (PAXgene Blood RNA Tube, PreAnalytiX, Qiagen® GmbH, Hilden, GE) collecting system. Two PAXgene Blood RNA tubes were obtained per case. Samples were collected at the first visit once the clinical diagnosis was established (n=12 sALS, n=10 controls). Tubes were kept for 2 h at room temperature to ensure lysis of blood cells, and then stored at -20°C for 24 h. Thereafter, tubes were stored at -80°C for at least 7 days prior to processing. A summary of sALS and control cases is shown in Table 3.

Genetic studies

Genetic testing was performed on genomic DNA isolated from blood or brain tissue. Informed consent for the chromosome9 open reading frame (*C9ORF72*), superoxide dismutase 1 (*SOD1*), TAR DNA binding protein (*TARDBP*), and FUS RNA binding protein (*FUS*) analysis was obtained from each patient or legal representative. Patients in this study did not show mutations in the assessed genes.

RNA extraction and RT-qPCR

RNA from dissected frozen anterior horn of the lumbar spinal cord (n=14 sALS, n=13 controls) and frontal cortex area 8 (n=15 sALS, n=14 controls) was extracted following the instructions of the supplier (RNeasy Mini Kit, Qiagen® GmbH, Hilden, Germany). PAXgene Blood RNA tubes were incubated overnight at 4°C in a shaker-plate to equilibrate the temperature and to increase yields, and then at room temperature for 2h before starting the procedure. RNA from frozen whole blood samples was extracted following the instructions of the supplier (PAXgene Blood RNA kit, PreAnalytiX, Qiagen® GmbH, Hilden, GE). RNA integrity and 28S/18S ratios were determined with the Agilent Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA, USA) to assess RNA quality, and the RNA concentration was evaluated using a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific). Complementary DNA (cDNA) preparation used the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) following the protocol provided by the supplier. Parallel reactions for each RNA sample were run in the absence of MultiScribe Reverse Transcriptase to assess the lack of contamination of genomic DNA. TaqMan RT-qPCR assays were performed in duplicate for each gene on cDNA samples in 384-well optical plates using an ABI Prism 7900 Sequence Detection system (Applied Biosystems, Life Technologies, Waltham, MA, USA). For each 10µL TaqMan reaction, 4.5µL cDNA was mixed with 0.5µL 20x TaqMan Gene Expression Assays and 5µL of 2x TaqMan Universal PCR Master Mix (Applied Biosystems). Taqman probes used in expression assays were: allograft inflammatory factor (*AIF1*) (Hs00741549_g1) coding for IBA1, aldehyde dehydrogenase 1 family member L1 (*ALDH1L1*) (Hs01003842_m1), glial fibrillary acidic protein (*GFAP*) (Hs00909233_m1), and *CH3L1* (Hs01072228_m1). Hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*) was used as internal control for normalization of spinal cord samples, whereas β -glucuronidase (*GUS- β*) was used as the internal control for normalization of frontal cortex samples [90, 91]. Mean values of two house-keeping genes, glucuronidase beta (*GUS- β*) [92] and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) [93], were used as internal controls for normalization of whole-blood mRNA expression studies.

The parameters of the reactions were 50°C for 2min, 95°C for 10min, and 40 cycles of 95°C for 15sec and 60°C for 1min. Finally, the capture of all TaqMan PCR data used the Sequence Detection Software (SDS version 2.2.2, Applied Biosystems). The double-delta cycle threshold ($\Delta\Delta CT$) method was utilized to analyze the data results with Student's *t* test.

Gel electrophoresis and immunoblotting

Frozen samples of frontal cortex area 8 (n=6 sALS, n=6 controls) and anterior horn of the spinal cord at the lumbar level (n=4 sALS, n=4 controls) were homogenized in RIPA lysis buffer composed of 50mM Tris/HCl buffer, pH 7.4 containing 2mM EDTA, 0.2% Nonidet P-40, 1mM PMSF, protease and phosphatase inhibitor cocktail (Roche Molecular Systems, USA). The homogenates were centrifuged for 20 min at 12,000 rpm. Protein concentration was determined with the BCA method (Thermo Scientific). Equal amounts of protein (12µg) for each sample were loaded and separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred onto nitrocellulose membranes (Amersham, Freiburg, GE). Non-specific bindings were blocked by incubation in 3% albumin in PBS containing 0.2% Tween for 1h at room temperature. After washing, the membranes were incubated overnight at 4°C with antibodies against glial fibrillary acidic protein (GFAP) (dilution of 1:500; rabbit polyclonal monoclonal, MO761, Dako, Agilent, Santa Clara, USA), ionized calcium binding adapter molecule 1 (IBA1) for microglia (diluted at 1:1,000; rabbit polyclonal, 019-19741, WAKO, Fujifilm, Tokyo, Japan), and YKL-40 (diluted 1:200; goat polyclonal, AF-2599, R&D Systems, Minneapolis, MN, USA). Protein loading was monitored using an antibody against β -actin (42 kDa, 1:30,000, Sigma). Membranes were incubated for 1h with appropriate HRP-conjugated secondary antibodies (1:2,000, Dako); the immunoreaction was revealed with a chemiluminescence reagent (ECL, Amersham). Densitometric quantification was carried out with the ImageLab v4.5.2 software (BioRad), using β -actin for normalization. Six samples per group were analyzed.

Immunohistochemistry

De-waxed sections, 4µm thick, of the lumbar spinal cord (n=6 sALS, n=6 controls) and frontal cortex area 8 (n=6 sALS, n=6 controls) were processed in parallel for immunohistochemistry. Endogenous peroxidases were blocked by incubation in 10% methanol-1% H₂O₂ for 15min followed by 3% normal horse serum. Then the sections were incubated at 4°C overnight with anti-YKL40 primary antibody (PA5-43746, ThermoFisher, Waltham, Massachusetts, USA) at a dilution of 1:200. Immediately afterwards, the sections were incubated with EnVision + system peroxidase (Dako, Agilent, Santa Clara, CA, USA) for 30min at room temperature. The peroxidase reaction was visualized with diaminobenzidine and H₂O₂. No signal was obtained following incubation with only the secondary antibody. Sections were slightly stained with haematoxylin.

Enzyme-linked immunosorbent assays (ELISA) in CSF

YKL40 protein levels were measured using the MicroVue YKL40 EIA ELISA kit (Quidel, San Diego, CA, USA) following the manufacturer's instructions. Receiver operating characteristic (ROC) curves and derived area under the curve (AUC) were calculated. The best cut-off value, sensitivity, and specificity were estimated based on the Youden index (point on a ROC curve providing the best balance of both sensitivity and specificity) [94]. NF-L levels were measured using the NF-light® (Neurofilament light) ELISA kit from UmanDiagnostics (Umea, Sweden) following the manufacturer's instructions.

Statistical analysis

The normality of distribution was analyzed with the Kolmogorov-Smirnov test. The unpaired Student's *t*-test was used to compare each group when values followed normal distribution, and statistical analysis of the CSF protein data between groups was carried out using one-way analysis of variance (ANOVA) followed by Tukey post-test, in both cases using the SPSS software (IBM Corp. Released 2013, IBM-SPSS Statistics for Windows, Version 21.0., Armonk, NY, USA). Graphic design was performed with GraphPad Prism version 5.01 (La Jolla, CA, USA). Outliers were detected using the GraphPad software QuickCalcs ($p < 0.05$). The data were expressed as mean \pm SEM, and significance levels were set at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, and tendencies at $\#P < 0.1$. Pearson's correlation coefficient was used to assess a possible linear association between two continuous quantitative variables.

AUTHOR CONTRIBUTIONS

PA-B carried out the analysis of YKL40 expression in brain tissue, CSF and peripheral blood; RD and MP examined the clinical characteristics and course of the patients, and obtained CSF and blood from sALS cases; MJC obtained the CSF from control individuals; FLI advised certain aspects of YKL40 in brain and CSF; IF directed and supervised the study, evaluated the results, and wrote the final version of the manuscript which was circulated for comments and suggestions, and approved by all the authors.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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