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ORIGINAL COMMUNICATION

Exploiting the role of CSF NfL, CHIT1, and miR-181b as potential diagnostic and prognostic biomarkers for ALS

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Abstract

Amyotrophic lateral sclerosis (ALS) is a rare neurodegenerative disorder characterized by relentless and progressive loss of motor neurons. A molecular diagnosis, supported by the identification of specific biomarkers, might promote the definition of multiple biological subtypes of ALS, improving patient stratification and providing prognostic information. Here, we investigated the levels of neurofilament light chain (NfL), chitotriosidase (CHIT1) and microRNA-181b (miR-181b) in the cerebrospinal fluid (CSF) of ALS subjects ($N=210$) as well as neurologically healthy and neurological disease controls ($N=218$, including $N=74$ with other neurodegenerative diseases) from a large European multicentric cohort, evaluating their specific or combined utility as diagnostic and prognostic biomarkers. NfL, CHIT1 and miR-181b all showed significantly higher levels in ALS subjects compared to controls, with NfL showing the most effective diagnostic performance. Importantly, all three biomarkers were increased compared to neurodegenerative disease controls and, specifically, to patients with Alzheimer's disease (AD; $N=44$), with NfL and CHIT1 being also higher in ALS than in alpha-synucleinopathies ($N=22$). Notably, ALS patients displayed increased CHIT1 levels despite having, compared to controls, a higher prevalence of a polymorphism lowering CHIT1 expression. While no relationship was found between CSF miR-181b and clinical measures in ALS (disease duration, functional disability, and disease progression rate), CSF NfL was the best independent predictor of disease progression and survival. This study deepens our knowledge of ALS biomarkers, highlighting the relative specificity of CHIT1 for ALS among neurodegenerative diseases and appraising the potential diagnostic utility of CSF miR-181b.

Keywords ALS · CSF · Biomarker · NfL · CHIT1 · MiR-181b

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Introduction

Amyotrophic lateral sclerosis (ALS) is a complex neurological disorder characterized by the gradual and selective loss of both upper and lower motor neurons (MNs), leading to death from respiratory failure within 3–5 years after the onset of symptoms[1]. The disease shows a variety of clinical presentations and a highly heterogeneous molecular and pathological landscape, resulting from the interplay between a susceptible genetic background—with over 40 genes linked to both sporadic and familial forms [2]—and environmental risk factors in a time-locked exposure [1].

So far, ALS treatment mainly relies on symptom management and supportive care [3]. Indeed, the approved drugs riluzole and edaravone exert a modest effect on disease progression [3], while the combination of sodium phenylbutyrate and taurursodiol (AMX0035) has given disappointing results in the recent PHOENIX trial [4]. Although effective therapy is still lacking, several molecules acting on different pathological mechanisms are under clinical investigation [3]. Recently, the American Food and Drug Administration (FDA) granted accelerated approval to tofersen, a novel antisense oligonucleotide (ASO) targeting the *SOD1* gene, paving the way for future genetic therapies [5]. The European Medicines Agency (EMA) issued its marketing authorization for the drug as well.

A significant challenge in diagnosing ALS is that a large proportion of affected subjects either do not meet the necessary criteria for a definitive diagnosis during their lifetime or only meet them in the advanced phases of the disease [6–8]. ALS often presents initially with signs and symptoms that necessitate continuous observation over time to track clinical progression. This results in a substantial delay in diagnosis, hindering patient enrolment in clinical trials. In addition, a more precise stratification of disease subtypes would help not only patient recruitment to forthcoming clinical trials, but also prediction of disease outcome and evaluation of treatment efficacy. This segregation cannot merely rely on clinical features, but rather it must be supported at least by reliable disease biomarkers, able to provide possible pharmacodynamic measures for response to future proposed therapies. Beyond neurophysiological examination and neuroimaging, circulating biomarkers are among the most promising tools for diagnosis, prognosis and monitoring of treatment efficacy [9].

To date, neurofilaments (Nfs) are the molecules on which most of the efforts of the scientific community have been focused and, among circulating biomarkers, are the ones holding the highest potential of translation to clinical practice [10]. They represent neuron-specific cytoskeletal components, and their levels in biological fluids increase proportionally to the extent of axonal damage [11]. Their levels have been shown to be increased in ALS to a greater extent compared to most other neurological disorders, which makes them useful for the differential diagnosis with mimic disorders [12, 13]. A huge body of evidence has shown that increased expression of Nf light chain (NfL) and phosphorylated Nf heavy chain (pNfH) in cerebrospinal fluid (CSF) and blood of ALS subjects correlates with shorter life expectancy and more rapid disease progression [14–22]. Beyond its prognostic value, NfL seems to exhibit stable levels over time, with practical advantages in pharmacodynamic monitoring [16].

Nevertheless, Nfs are not able to recapitulate the whole spectrum of ALS pathology. Since inflammation is a significant pathological hallmark of the disease, the neuroinflammatory response was investigated by assessing chitotriosidase or chitinase 1 (CHIT1) as a marker associated with microglia activation and neuroinflammation in ALS [23, 24]. Indeed, CHIT1 is the main human chitinase protein, and catalyses the degradation of pathogenic chitin-like substances, exerting a neuroprotective role [25]. Although CSF levels of CHIT1 have been demonstrated to correlate with ALS progression [26–28] and independently predict survival in late symptomatic patients [29], a few studies suggest that NfL may outperform inflammatory markers in terms of both diagnostic and prognostic performance [15, 30]. The lower accuracy of CHIT1 seems to be ascribable to a lack in specificity, representing a common neuroinflammatory response to protein misfolding and aggregation [31]. Despite this, CHIT1 correlation with NfL levels may still yield diagnostic and prognostic utility, but this requires further investigations [28].

It is well recognized that multiple alterations of microRNA (miRNA) expression occur in neurodegenerative disorders, including ALS [2]. Beyond playing a crucial role in the post-transcriptional regulation of gene expression, miRNAs may have a great potential as disease biomarkers and promising tools for molecular intervention [32]. Among a plethora of dysregulated miRNAs, miR-181b may be particularly relevant as a CSF biomarker. Indeed, miR-181b belongs to the miR-181 family, which is particularly expressed in the central nervous system (CNS). Alterations of miR-181 family members have already been reported to occur in neurodegeneration, yielding new potential therapeutic targets [33]. Notably, circulating plasma levels of miR-181b were able to predict disease progression in a large cohort of ALS patients, with similar performance to NfL when taken alone, and a superior prognostic capacity when combined with NfL [34].

The aim of our work was to assess the expression levels of NfL, CHIT1, and miR-181b in the CSF from a large multicentric cohort of ALS patients and controls in order to

evaluate their single or combined utility as diagnostic and prognostic biomarkers for ALS.

Materials and methods

Ethical statement

This study was conducted in agreement with the ethical standards of the Declaration of Helsinki and with national legislation and institutional guidelines. All subjects enrolled in this study provided written informed consent approved by the local ethical committees (S51125, S58248, S60768, CE REBISLA 238_2023 19/04/2023 Policlinico EC, ALS-PHENO 2023_03_21_18) for the collection, storage and analysis of biological samples as well as clinical data. This experimental study was conducted in agreement with the international GLP and GCP guidelines.

Cohort definition

Patients were recruited in the Neurology Units of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, of IRCCS Istituto Auxologico Italiano, Milan, Italy, and of University Hospitals Leuven, Leuven, Belgium. The diagnosis of ALS was made according to current diagnostic criteria [35, 36] and lumbar puncture (LP) for CSF collection was performed as part of the diagnostic assessment. The onset of symptoms was defined as the initial complaint of weakness by the patient, and disease duration was estimated at the time of presentation to medical consultation. Disease progression rate (DPR) was calculated as 48 minus the ALS Functional Rating Scale-Revised (ALSFRS-R) score at the time of presentation, divided by disease duration in months. Patients with DPR under and above the median value were defined as slow or fast progressors, respectively. Clinical phenotypes [37], neuropsychological assessment, body mass index (BMI), and forced vital capacity (FVC, expressed as the percentage of the predicted value) were collected, when available.

A group of controls who underwent a LP as part of a normal diagnostic workup was included in this study. Control individuals were grouped into three categories similarly to a previous investigation [19]: (i) non-inflammatory controls (CTRL-1), including individuals without evidence of a neurological disease, patients with chronic non-inflammatory neuropathies, chronic vascular encephalopathy, normal pressure hydrocephalus (NPH), headache or other craniofacial pain, mild cognitive impairment (MCI), and epilepsy; (ii) inflammatory controls (CTRL-2), including patients with acute inflammatory diseases of the CNS and peripheral nervous system (PNS), and individuals with CNS tumours or metastases; and (iii) neurodegenerative controls (CTRL-3), including patients with neurodegenerative disorders other than ALS (Alzheimer's disease (AD), synucleinopathies, others).

Measurement of CSF NfL and CHIT1

CSF samples obtained by LP were collected and stored at − 80 °C until analysis. CSF NfL and CHIT1 levels were measured using commercially available ELISA kits according to the manufacturers' instructions (NfL: UmanDiagnostics AB, Umeå, Sweden; CHIT1: Cloud-Clone Corp., Houston, TX, USA). For NfL and CHIT1, specimens were diluted at a ratio of 1:1 and left undiluted, respectively, with measurements conducted in duplicate. Plates were read using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific) and standard curves were fitted with four-parameter logistic regression using SkanIt data analysis software (Thermo Fisher Scientific). For patients in whom CHIT1 levels were under the limit of detection, the lower limit of quantification (390 pg/mL) was considered as concentration for further analyses.

Measurement of CSF miR-181b

Circulating miRNAs were isolated from 300 µL of CSF using NucleoSpin® miRNA plasma kit (Macherey–Nagel). Reverse transcription was performed through the TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific), using 10 ng of RNA as a template. Reverse transcription products were pre-amplified by using the TaqMan® PreAmp Master Mix (Thermo Fisher Scientific). Real-time PCR experiments were set up using the TaqMan® Universal Master Mix II, no AmpErase® UNG (Thermo Fisher Scientific) and the specific TaqMan® assays for miR-181b and miR-125b (Thermo Fisher Scientific). The expression levels of hsa-miR-181b were normalized to the average levels of hsa-miR-125b using the ΔCt method, as previously shown [38]. Only Ct values < 35 were considered in the analysis. All data are mean of triplicates.

Genetic analysis

Genomic DNA was extracted from 3 mL of peripheral blood of patients and controls using standard procedures. The majority of patients were screened for mutations in the main four ALS-related genes (*C9ORF72* hexanucleotide repeat expansion, *SOD1*, mutational hotspots in *TARDBP* and *FUS*). To detect the 24-bp duplication polymorphism within exon 10 of *CHIT1*, the following primers were used for PCR amplification: CHIT_ex10for 5'-AGCTATCTG AAGCAGAAG and CHIT_ex10rev 5'-GGAGAAGCCGGC AAAGTC [39]. Electrophoresis on a 4% agarose gel allowed for the detection of 75 and/or 99 bp fragments.

Statistical analysis

Baseline demographic and clinical features of ALS and control subjects were analyzed through descriptive statistical methods. Categorical variables were compared using the chi-square test. After assessing for normality, continuous variables were reported as mean ± standard deviation (SD) or median and interquartile range [IQR]. Mann–Whitney and Kruskal–Wallis tests were employed to perform comparisons between two and more groups, respectively. Receiver Operating Characteristic (ROC) curves were generated and the areas under the curves (AUCs) were calculated to assess the accuracy of CSF biomarkers in discriminating between patients with ALS and controls. Best cut-off values were selected as those with the highest Youden's index (calculated as sensitivity + specificity − 1). In order to assess the diagnostic performance of the combination of CSF NfL, CHIT1 and miR-181b, we considered the subcohort in which all three biomarkers had been quantified and transformed the values of each biomarker into z-scores. For every subject, composite biomarker levels were computed as the sums of the z-scores of the three single biomarkers as well as of two biomarkers at a time. The levels of these virtual composite biomarkers enabled us to produce ROC curves for the discrimination between ALS and control groups [40].

Correlation analyses were performed using Spearman's test. Multiple linear regression models were used to evaluate the potential utility of biomarkers and clinical variables as predictors of DPR in ALS.

In order to compare survival from disease onset between groups defined by biomarker levels, Kaplan–Meier curves were plotted and compared by the log-rank test. A Cox proportional hazards model was used to assess the association of multiple covariates with survival.

Statistical analyses were performed with Prism 10.2 (GraphPad Software, Boston, MA, USA). The level of statistical significance for all tests was set at $p < 0.05$.

Results

Demographic and clinical characteristics of ALS patients and controls

A cohort of 210 ALS patients (86 females and 124 males) with median age at onset of 61 [54–68] years and 218 controls (112 females and 106 males) were included in this study. Three groups of controls were set up: CTRL-1 included 106 individuals, with a median age of 58 [45–71] years, CTRL-2 was made up of 38 patients, with a median age of 58 [47.5–72] years, while CTRL-3 was formed by 74 subjects, with a median age of 76 [71–79] years. Table 1 shows demographic features and biomarker values of all subjects involved in this study. The numerical breakdown

Table 1 Demographic and biochemical features of ALS patients and controls

	ALS (*N* = 210)	CTRL-1 (*N* = 106)	p-value ALS vs. CTRL-1	CTRL-2 (*N* = 38)	*p*-value ALS vs. CTRL-2	CTRL-3 (*N* = 74)	p-value ALS vs. CTRL-3	Controls (*N* = 218)	*p*-value ALS vs. Controls
Gender, *N* (%)									
Female	86 (41)	57 (53.8)	**0.030**	18 (47.4)	0.737	37 (50)	0.176	112 (51.4)	**0.030**
Male	124 (59)	49 (46.2)		20 (52.6)		37 (50)		106 (48.6)	
Age at evaluation (ys)	62 [55–69]	58 [45–71]	**0.022**	58 [47.5–72.2]	0.262	76[71–79]	**< 0.0001**	69 [52–76]	**0.013**
CSF NfL (pg/mL)	5620 [2564–10711]	650 [372–1018]	**< 0.0001**	1005 [403.3–2632]	**< 0.0001**	974 [753–1693]	**< 0.0001**	833.7 [480–1399]	**< 0.0001**
CSF CHIT1 (pg/mL)	1040 [390–3930]	390 [390–390]	**< 0.0001**	390 [390–1564]	0.440	390 [390–390]	**< 0.0001**	390 [390–390]	**< 0.0001**
CSF miR-181b (fold change)	0.13 [0.02–1.19]	0.046 [0.005–0.842]	0.399	0.03 [0.005–0.613]	> 0.999	0.054 [0.008–0.147]	**0.016**	0.046 [0.007–0.183]	**0.012**

Median [IQR] and number (%), as appropriate. *P*-values refer to Mann–Whitney test for continuous variables and χ^2 test for categorical variables

ALS amyotrophic lateral sclerosis; *ALSFRS-R* amyotrophic lateral sclerosis functional rating scale revised; *CHIT1* chitinase 1; *CSF* cerebrospinal fluid; *CTRL-1* control group 1; *CTRL-2* control group 2; *CTRL-3* control group 3; *miR-181b* microRNA 181b; *NfL* neurofilament light chain

*Spinal onset included also one patient with respiratory onset

P-values under the threshold for significance (<0.05) are marked in bold

of individuals comprising groups CTRL-1, CTRL-2 and CTRL-3 is shown in Supplementary Table 1.

Sex distribution was significantly different between ALS patients and controls ($p=0.030$), due to the preponderance of males in the ALS cohort, while controls had an older age at evaluation compared to ALS counterparts ($p=0.013$) (Table 1).

Demographic, clinical and biochemical features of patients with ALS are reported in Table 2. When the DPR was available, ALS subjects were grouped into slow ($N=102$) and fast progressors ($N=102$), depending on

Table 2 Demographic, clinical and biochemical features of ALS patients

	Slow progressing ALS ($N=102$)	Fast progressing ALS ($N=102$)	*p*-value Slow vs. Fast progressing ALS	ALS ($N=210$)
Gender, *N* (%)				
Female	32 (31.4)	52 (51)	**0.007**	86 (41)
Male	70 (68.6)	50 (49)		124 (59)
Age at onset (ys)	59.5 [52–67]	63 [55–69]	**0.021**	61 [54–68]
Age at evaluation (ys)	60.5 [54–68.25]	64 [56–69.25]	0.091	62 [55–69]
Site of onset, *N* (%)				
Spinal*	75 (73.5)	68 (66.7)	0.284	147 (70)
Bulbar	27 (26.5)	34 (33.3)		63 (30)
Disease duration at baseline (months)	16 [10–28.25]	9 [6–12]	**< 0.0001**	12 [7–18]
ALSFRS-R	44 [41–45]	37 [32–40.25]	**< 0.0001**	41 [36–44]
Progression rate	0.275 [0.17–0.42]	1.135 [0.87–1.78]	**< 0.0001**	0.616 [0.27–1.14]
BMI	24.2 [21.2–27.4]	24 [21–26.2]	0.564	24.1[21.2–26.4]
FVC	102 [83–115]	81 [64–103]	**0.0007**	91 [71–108]
Neuropsychological assessment, *N* (%)				
Normal	69 (71.9)	53 (56.3)	**0.026**	124 (63.9)
Cognitive impairment	12 (12.5)	17 (18.1)	0.285	29 (14.9)
Behavioral impairment	8 (8.3)	9 (9.6)	0.764	17 (8.8)
Cognitive + behavioral impairment	0	3 (3.2)	0.078	5 (2.6)
Dementia	7 (7.3)	12 (12.8)	0.209	19 (9.8)
Clinical phenotypes, *N* (%)				
Classic	43 (42.2)	49 (48)	0.399	94 (44.8)
Bulbar	24 (23.6)	28 (27.5)	0.521	53 (25.2)
Flail arm	8 (7.8)	3 (2.9)	0.121	11 (5.2)
Flail leg	8 (7.8)	5 (4.9)	0.390	14 (6.7)
Pyramidal	5 (4.9)	5 (4.9)	> 0.999	10 (4.8)
Respiratory	2 (2)	2 (2)	> 0.999	4 (1.9)
PLMN	9 (8.8)	10 (9.8)	0.810	19 (9)
PUMN	3 (2.9)	0	0.081	5 (2.4)
Genetic				
C9ORF72	9/100	8/102	0.735	17/208
SOD1	8/92	4/93	0.240	12/187
TARDBP	4/92	1/93	0.170	5/187
FUS	0/91	0/92	–	0/184
CSF NfL (pg/mL)	3856 [1527–7777]	8508 [4163–14640]	**< 0.0001**	5620 [2564–10711]
CSF CHIT1 (pg/mL)	390 [390–2810]	2150 [390–4950]	**0.029**	1040 [390–3930]
CSF miR-181b (fold change)	0.12 [0.01–2.07]	0.24 [0.025–1.06]	0.814	0.13 [0.02–1.19]

Median [IQR] and number (%), as appropriate. *P*-values refer to Mann–Whitney test for continuous variables and χ^2 test for categorical variables

ALS amyotrophic lateral sclerosis; *ALSFRS-R* Amyotrophic Lateral Sclerosis Functional Rating Scale—Revised; *CHIT1* chitinase 1; *CSF* cerebrospinal fluid; *CTRL-1* control group 1; *CTRL-2* control group 2; *FVC* forced vital capacity; *miR-181b* microRNA 181b; *NfL* neurofilament light chain

*Spinal onset included also one patient with respiratory onset

P-values under the threshold for significance (<0.05) are marked in bold

whether DPR was below or above the median value in this sub-cohort. While sex was homogeneously distributed in fast progressors, male patients were overrepresented in slow progressors ($p = 0.007$). ALS patients with a higher DPR had a significantly older age at onset ($p = 0.021$), a shorter disease duration ($p < 0.0001$), a lower ALSFRS-R score ($p < 0.0001$) and a lower FVC ($p = 0.0007$) compared to slow progressors, while age at evaluation and site of onset did not significantly differ (Table 2). FVC was available for 135 patients. Fast progressors were more significantly impaired at neuropsychological assessment ($p = 0.026$; $N = 194$). No significant differences were found between slow and fast progressing patients in terms of clinical phenotypes, BMI ($N = 148$), or presence of a causative genetic mutation in one of the four main ALS genes.

CSF biomarkers in ALS patients and controls

CSF levels of NfL, CHIT1 and miR-181b were measured for $N = 390$, $N = 226$, and $N = 176$ patients, respectively.

CSF levels of NfL, CHIT1 and miR-181b were significantly higher in ALS compared to all 218 control individuals ($p < 0.0001$, $p < 0.0001$, and $p = 0.001$, respectively). The difference was still significant when comparing the CSF levels of the three biomarkers between the ALS and CTRL-3 group (NfL, $p < 0.0001$; CHIT1, $p < 0.0001$; miR-181b, $p = 0.016$) (Fig. 1 A–C), while only CSF NfL and

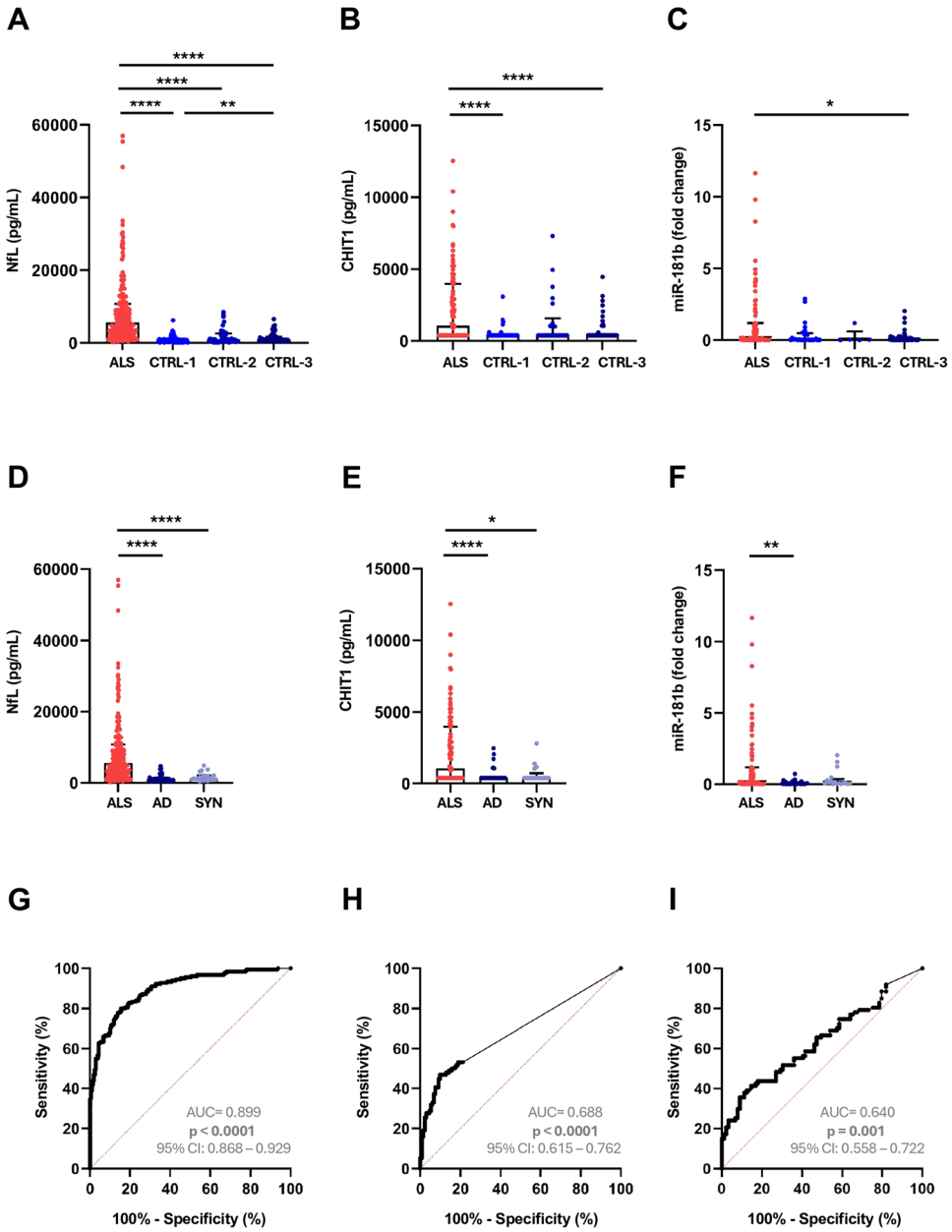

Fig. 1 CSF NfL, CHIT1 and miR-181b distribution in ALS and controls. **A**–**C** CSF NfL, CHIT1 and miR-181b levels in ALS compared to all control individuals. **D**–**F** CSF NfL, CHIT1 and miR-181b levels in ALS compared to patients with AD and synucleinopathies (CTRL-3 group). Scatter dot plot values represent median and interquartile range. Symbols of statistically significant differences: *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ (Kruskal–Wallis test). **G**–**I** ROC curves of CSF NfL, CHIT1, miR-181b in ALS patients vs. all control individuals

CHIT1 concentrations, and not miR-181b, were significantly increased in ALS patients compared to CTRL-1 group (NfL, $p < 0.0001$; CHIT1, $p < 0.0001$). Conversely, only CSF NfL levels were significantly higher in ALS compared to the CTRL-2 group ($p < 0.0001$), while CSF CHIT1 and miR-181b levels showed no significant difference between the two cohorts. This finding may be at least in part due to the considerable number of patients with an inflammatory disease included in the CTRL-2 group and the relatively small number of individuals with measurements of CSF miR-181b levels (Supplementary Table 1).

In order to assess whether the biomarker profile differed among different neurodegenerative disorders, we directly compared ALS with patients with AD and with patients affected by synucleinopathies (Parkinson's disease, multiple system atrophy, dementia with Lewy bodies). Strikingly, we found that ALS patients had significantly elevated concentrations of CSF NfL, CHIT1 and miR-181b compared to AD ($p < 0.0001$, $p < 0.0001$, and $p = 0.002$, respectively) and increased CSF levels of NfL and CHIT1 compared to synucleinopathies ($p < 0.0001$ and $p = 0.017$, respectively; Fig. 1 D–F).

The diagnostic performance of these biomarkers was assessed using ROC curves. CSF NfL levels displayed a high accuracy in discriminating ALS from controls with an AUC of 0.899 (95% confidence interval (CI): 0.868 to 0.929, $p < 0.0001$), corresponding to a sensitivity of 80% (95% CI: 73.6–85.2%) and a specificity of 84.3% (95% CI: 78.6–88.6%) at a cut-off of 2079 pg/mL (Fig. 1G). Conversely, CSF CHIT1 had a lower ability to predict the diagnosis of ALS vs. controls with an AUC of 0.688 (95% CI: 0.615–0.762, $p < 0.0001$), showing poor sensitivity (46.8%; 95% CI: 37.1–56.8%) and high specificity (90.2%; 95% CI: 83.9–94.2%) at a cut-off of 1564 pg/mL (Fig. 1H). CSF miR-181b had low sensitivity (41.4%; 95% CI: 31.6–51.9%) and high specificity (85.4%; 95% CI: 76.6–91.3%) at a cut-off of 0.424. The AUC for CSF miR-181b was 0.640 (95% CI: 0.558 to 0.722, $p = 0.001$) (Fig. 1 I).

In order to assess whether combining the three biomarkers improved the diagnostic performance, we computed values of a z-score-based composite biomarker for the sub-cohort for which levels of all three biomarkers were available ($N = 58$ ALS patients and $N = 76$ controls). We compared ALS patients to the whole control group ($N = 76$) and to the neurodegenerative group (CTRL-3, $N = 56$), assessing combinations of two biomarkers at a time. For both comparisons, the best diagnostic performance was obtained when combining NfL and CHIT1 levels (ALS vs. all controls: AUC = 0.848; 95% CI: 0.785–0.911; $p < 0.0001$; ALS vs. CTRL-3: AUC = 0.826; 95% CI: 0.753–0.899; $p < 0.0001$). ROC analyses are shown in Supplementary Fig. 1 (Figure S1A–F). However, the combination of the three biomarkers does not seem to significantly improve the discrimination between ALS and controls (AUC = 0.829; 95% CI: 0.761–0.898; $p < 0.0001$) or neurodegenerative disorders (AUC = 0.809; 95% CI: 0.731–0.886; $p < 0.0001$) (Figure S1 G, H).

Indeed, NfL alone has the best diagnostic performance in differentiating ALS from controls (AUC = 0.889; 95% CI: 0.836–0.942; $p < 0.0001$) and neurodegenerative disorders (AUC = 0.871; 95% CI: 0.808–0.933; $p < 0.0001$).

ALS patients show elevated CSF CHIT1 levels despite higher frequency of CHIT1 polymorphism

In a substantial subset of our cohort ($N = 163$ subjects, including $N = 86$ ALS patients and $N = 77$ controls), we assessed the presence of the 24-bp duplication polymorphism in *CHIT1*, which was reported to lower the levels of CHIT1 protein in the biofluids [41]. In this subgroup, CHIT1 measurement in the CSF was available for 153 individuals.

We split our cohort according to the median CHIT1 value (390 pg/mL) and we demonstrated that heterozygous and homozygous carriers of the mutated allele were significantly more represented among patients with CHIT1 levels lower than or equal to the median value ($p = 0.004$), confirming literature data [41]. In terms of allelic frequency, 52 mutated alleles were present in patients with lower CHIT1 levels, compared to only 14 mutated alleles in those with CHIT1 concentrations above the median value ($p < 0.0001$; Fig. 2A).

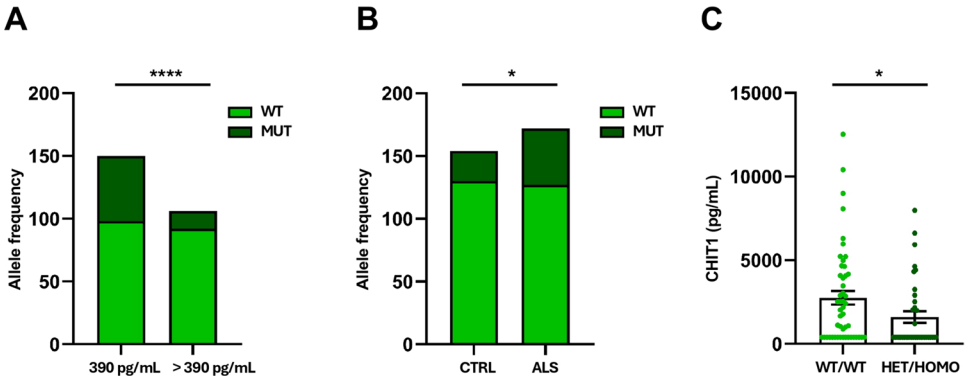

Fig. 2 Analysis of *CHIT1* polymorphism. **A** distribution of wild-type and mutated alleles in individuals with CHIT1 levels equal to 390 pg/mL vs. above 390 pg/mL (****$p < 0.0001$). **B** distribution of wild-type and mutated alleles in ALS patients and controls (*$p = 0.021$). **C** comparison of CSF CHIT1 levels in wild-type homozygotes vs. heterozygous/homozygous polymorphism carriers in ALS cohort (*$p = 0.014$)

Thus, we investigated whether *CHIT1* polymorphism was responsible for the decreased CHIT1 levels in the control group. Interestingly, we found that ALS patients had an over-representation of the mutated allele (ALS: 127 wild-type alleles and 45 mutated alleles; controls: 130 wild-type alleles and 24 mutated alleles; $p = 0.021$; Fig. 2B). Analysis of the distribution of CSF CHIT1 concentrations across ALS patients with different genetic backgrounds demonstrated that patients with wild-type homozygosity had significantly higher CHIT1 levels compared to heterozygous and homozygous polymorphism carriers (2037 vs. 390 pg/mL; $p = 0.014$; Fig. 2C). These findings suggest that ALS patients showed significantly increased levels of CHIT1 in the CSF despite a higher prevalence of *CHIT1* polymorphism.

CSF NfL, CHIT1 and miR-181b levels correlate with clinical variables in ALS patients

No significant difference was found in CSF NfL, CHIT1 and miR-181b with respect to site of onset. CSF CHIT1 levels were significantly increased in male compared to female ALS patients (median values, 1783 pg/mL vs. 390 pg/mL; $p = 0.038$), while CSF NfL and miR-181b levels were equally represented in both sexes. However, when comparing male patients to male controls and female patients to female controls, CSF CHIT1 levels were higher in the ALS groups, irrespective of sex (male ALS patients vs. male controls, median values: 1783 pg/mL vs. 390 pg/mL; $p < 0.0001$; female ALS patients vs. female controls, median values: 390 pg/mL vs. 390 pg/mL; $p = 0.005$). No differences were identified between males and females in the control category. Altogether, the above findings suggest that the presence of higher CHIT1 concentrations in ALS patients is not simply ascribable to the preponderance of men in the ALS group.

When we analyzed the distribution of the three biomarkers across different clinical phenotypes (classic, bulbar, flail arm, flail leg, respiratory, pyramidal, pure lower motor neuron [PLMN], pure upper motor neuron [PUMN]), we found a trend towards higher NfL in classic and bulbar forms compared to others ($p < 0.011$), but no statistically significant differences were evident after multiple comparisons. No difference in the levels of the three biomarkers was identified across different neuropsychological phenotypes (purely motor ALS, ALS with cognitive impairment (ALSci), ALS with behavioural impairment (ALSbi), ALS with cognitive and behavioural impairment (ALScbi), and ALS-frontotemporal dementia (ALS-FTD)). However, when comparing the concentrations of the three biomarkers among patients with different genetic backgrounds (*C9ORF72* repeat expansion carriers, *SOD1* mutation carriers, *TARDBP* mutation carriers, patients tested but not carrying any causative genetic mutations in these genes), CSF miR-181b levels were significantly higher in *SOD1* carriers vs. non-mutated patients ($p = 0.004$). However, patients with *SOD1* mutations had a younger median age at evaluation (51.5 vs. 62 years, $p = 0.0007$), therefore, the higher miR-181b levels found in these patients could be at least partly explained by this age difference (see below).

In order to investigate the relationships between biomarkers and clinical variables within the ALS cohort, we performed a correlation analysis using a Spearman non-parametric test. A positive correlation was observed for CSF NfL levels with both CSF CHIT1 ($r = 0.568$, $p < 0.0001$) and CSF miR-181b levels ($r = 0.272$, $p = 0.029$); on the contrary, no significant correlation between CSF CHIT1 and miR-181b was found. In the ALS group, there was a significant inverse correlation of age at evaluation with both CSF NFL ($r = -0.178$, $p = 0.017$) and CSF miR-181b levels ($r = -0.233$, $p = 0.030$); a negative trend was also observed for CSF CHIT1, albeit without statistical significance (Fig. 3 **A–C**). Conversely, age at evaluation positively correlated with CSF NfL levels in the control group ($r = 0.525$, $p < 0.0001$), while no correlation was found with CSF CHIT1 and miR-181b levels. Moreover, CSF NfL levels inversely correlated with disease duration ($r = -0.331$, $p < 0.0001$) (Fig. 3D), while CSF CHIT1 and miR-181b concentrations did not (Fig. 3E–F). An inverse correlation existed between ALSFRS-R scores and both CSF NfL levels ($r = -0.219$, $p = 0.004$) (Fig. 3G) and CSF CHIT1 levels ($r = -0.265$, $p = 0.012$) (Fig. 3H), while there was no significant correlation with CSF miR-181b levels (Fig. 3 I). FVC showed a significant inverse correlation only with CSF NfL ($r = -0.218$, $p = 0.019$) (Fig. 3L–N). No correlation was identified between BMI and any of the three biomarkers.

DPR significantly correlated with both CSF NfL ($r = 0.393$, $p < 0.0001$) and CSF CHIT1 concentrations ($r = 0.274$, $p = 0.009$), but not with CSF miR-181b levels (Fig. 4A–C). Accordingly, CSF NfL levels were significantly higher in fast vs. slow progressors (median values: 8508 pg/mL vs. 3856 pg/mL; $p < 0.0001$) (Fig. 4D). The same was observed for CSF CHIT1 levels (median values: 2153 pg/mL vs. 390 pg/mL; $p = 0.029$), while no significant difference was found between fast and slow progressors in terms of CSF miR-181b levels (Fig. 4E, F). Using the three biomarkers as variables in a multiple linear regression model, CSF NfL was the only independent predictor of DPR (OR: $9.70*10^{-5}$; 95% CI, $7.13*10^{-5}$–$1.23*10^{-4}$; $p < 0.0001$). However, when adding age at onset, site of onset, FVC and BMI as covariates to the model, no variable showed significant association with DPR. In linear regression models assessing each individual biomarker together with the previously mentioned other covariates, CSF NfL and FVC were able to independently predict DPR (NfL: OR = $2.90*10^{-5}$; 95% CI: $9.74*10^{-6}$ to $4.84*10^{-5}$; $p = 0.004$; FVC: OR = −0.009; 95% CI: −0.02 to $2.10*10^{-3}$; $p = 0.013$) (Supplementary Table 2).

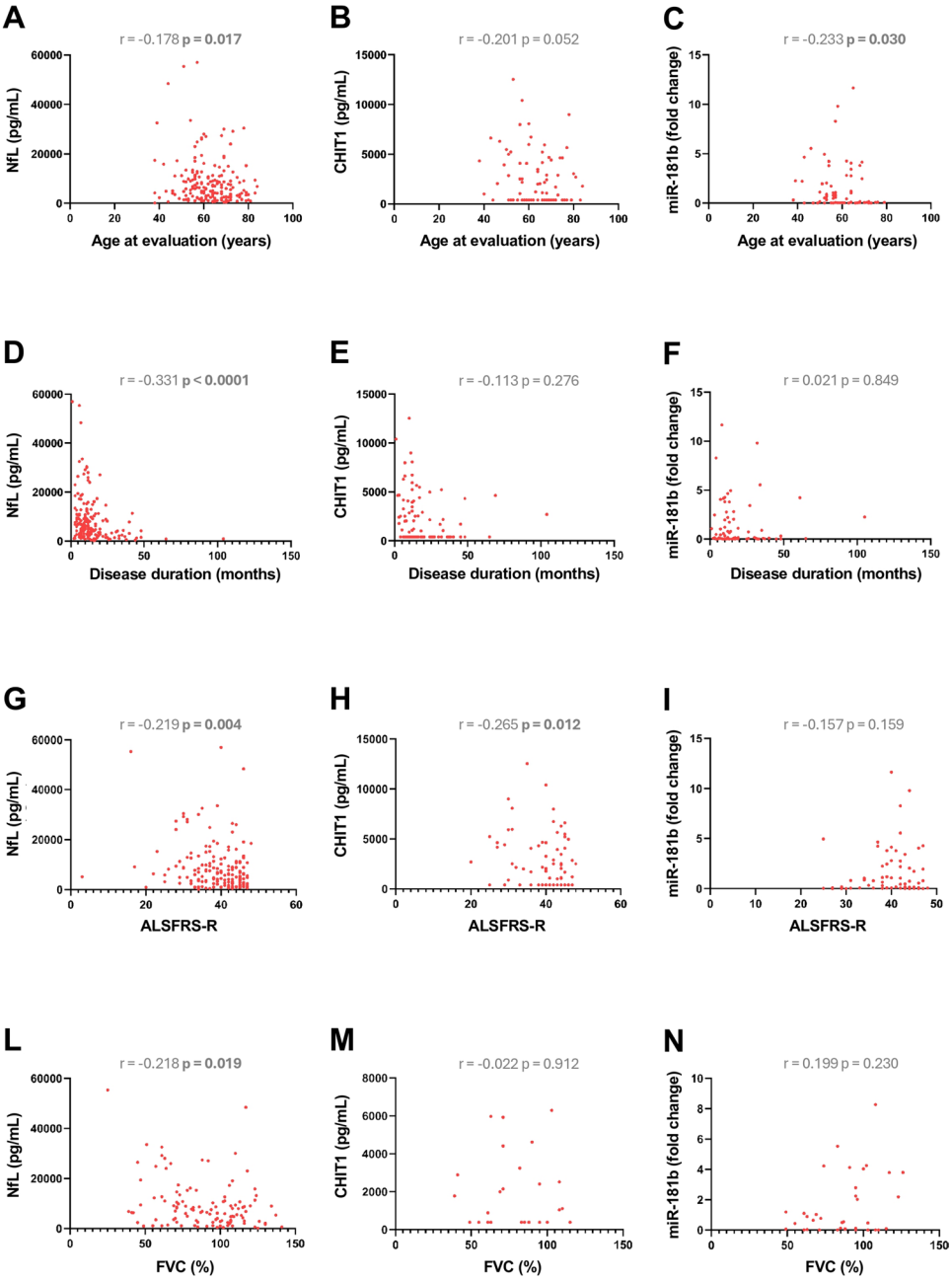

Fig. 3 Correlation between biomarkers and clinical variables. Correlation analyses of CSF NfL, CHIT1 and miR-181b with age at evaluation (**A**-**C**), disease duration (**D**-**F**), ALSFRS-R (**G**-**I**) and FVC (**L**-**N**) (r = Spearman's coefficient). *ALSFRS-R* Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised. *FVC* forced vital capacity (expressed as % of predicted value)

CSF NfL levels, but not CHIT1 and miR-181b levels, are associated with survival in ALS patients

Median survival time was 33 months [21.0–60.5, $N = 144$]. We investigated a possible association between CSF biomarkers and survival time using Kaplan–Meier analysis. CSF NfL levels higher than or equal to 5620 pg/mL (median value in ALS patients) were associated with a significantly shorter survival time (chi-square 39.79, $p < 0.0001$) (Fig. 5 A); conversely, neither CSF CHIT1 levels above or equal to/below 1042.3 pg/mL (median value in ALS patients) nor CSF miR-181b levels above or equal to/below 0.133 (median value in ALS patients) were associated with survival ($p = 0.155$ and $p = 0.951$, respectively) (Fig. 5B, C). In Cox proportional hazards models considering age at onset, site of onset (bulbar vs. spinal), ALSFRS-R, BMI and each biomarker separately (NfL, CHIT1 and miR-181b) as covariates, CSF NfL levels and ALSFRS-R independently predicted survival in the first model (i.e. that including NfL as biomarker) and all variables, including CHIT1, were significantly associated with survival in the second model (i.e. that including CHIT1) (Table 3).

To further explore the prognostic role of CSF CHIT1 in ALS patients not captured by an increased value of CSF

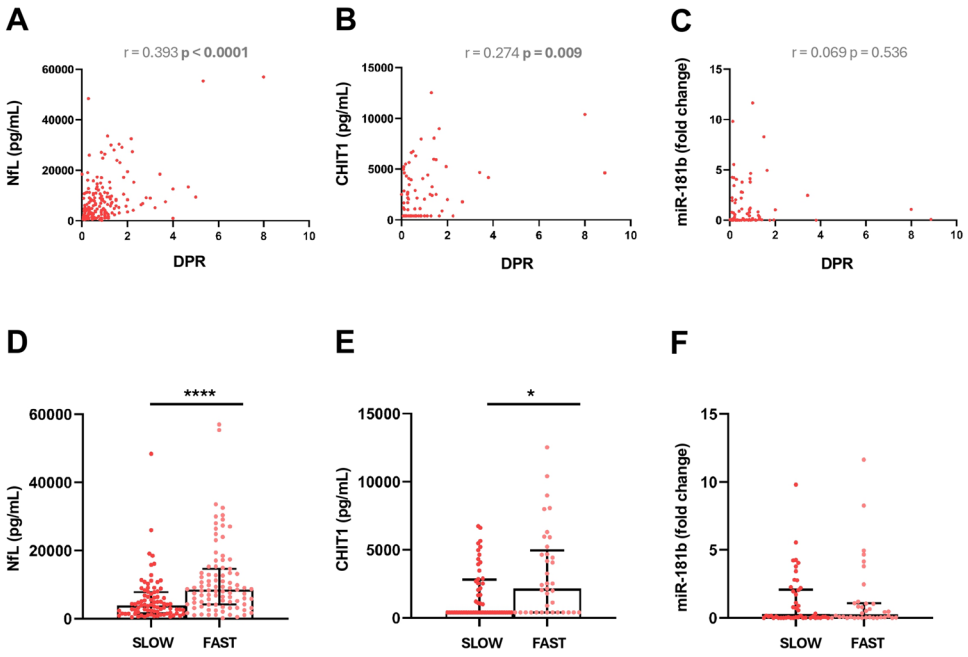

Fig. 4 Correlation between biomarkers and disease progression rate. A-**C** correlation of CSF NfL, CHIT1 and miR-181b with disease progression rate (DPR) (r = Spearman's coefficient). **D-F** CSF NfL, CHIT1 and miR-181b levels in fast progressing ALS cases compared to slow progressing patients (*$p < 0.05$; ****$p < 0.0001$; Mann–Whitney test). Scatter dot plot values represent median and interquartile range

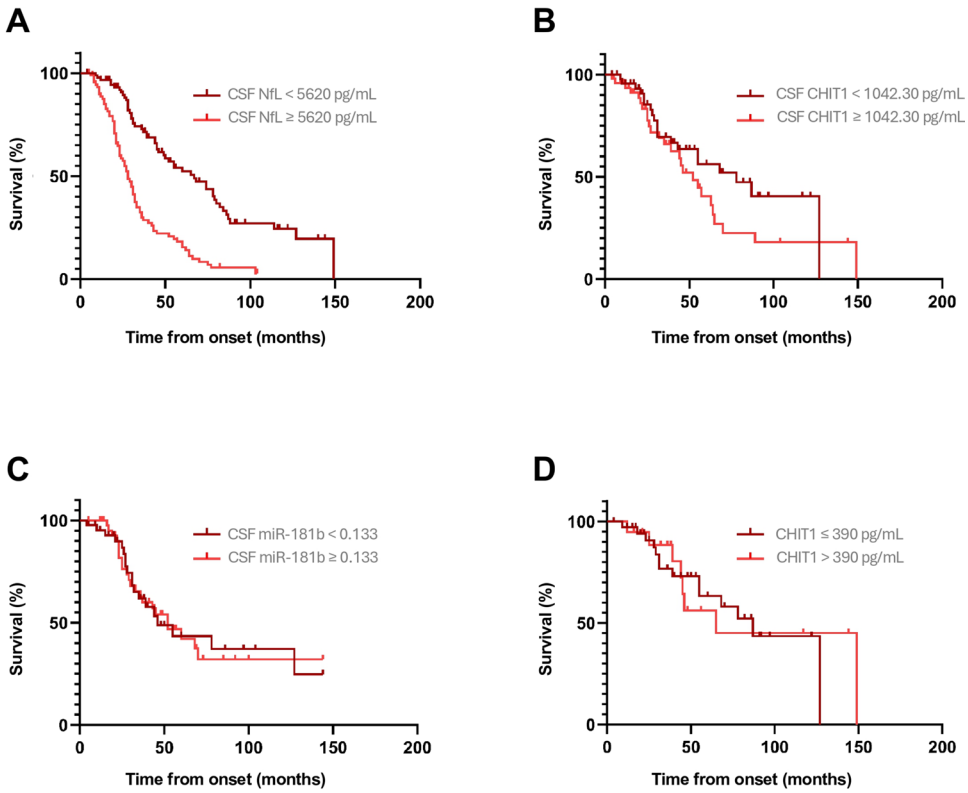

Fig. 5 Survival analysis. **A-C** Kaplan–Meier curves according to CSF NfL, CHIT1, and miR-181b levels. **D** survival estimates according to CHIT1 levels in patients with CSF NfL levels below the median. Survival time was calculated from disease onset and median values of these biomarkers were used as cut-offs

NfL, we analyzed CSF CHIT1 measurements in patients with CSF NfL values below the median. Within this cohort ($N = 55$), no correlation was found between CSF CHIT1 measurements and DPR ($p = 0.965$). Accordingly, no difference in CSF CHIT1 values between slow and fast progressors was observed ($p > 0.999$). Finally, we considered patients with low CSF NfL (i.e. CSF NfL below the median) and subdivided them according to the median CHIT1 level recalculated in this group (390 pg/mL). However, among these patients with low NfL, the presence of high vs. low levels of CHIT1 was not significantly associated with survival ($p = 0.841$) (Fig. 5D).

Table 3 Cox proportional hazards models with biomarkers and clinical variables

Variable	HR [95%CI]	*p*-value
CSF NfL (pg/mL)	1.000 [1.000–1.000]	**< 0.0001**
Age at onset	1.010 [0.989–1.032]	0.365
Site of onset	1.175 [0.780–1.693]	0.414
ALSFRS-R	0.941 [0.910–0.973]	**0.0003**
BMI	0.995 [0.981–1.005]	0.404
CSF CHIT1 (pg/mL)	2.334 [1.256–5.141]	**0.016**
Age at onset	1.60 [1.027–1.347]	**0.027**
Site of onset	25.99 [3.757–266.6]	**0.002**
ALSFRS-R	0.867 [0.767–0.967]	**0.014**
BMI	0.681 [0.493–0.878]	**0.008**
CSF miR-181b	0.958 [0.657–1.292]	0.799
Age at onset	1.027 [0.981–1.077]	0.267
Site of onset	1.775 [0.724–4.266]	0.199
ALSFRS-R	0.921 [0.856–0.991]	**0.023**
BMI	0.936 [0.830–1.043]	0.250

ALSFRS-R ALS functional rating scale revised; *BMI* body mass index; *CSF* Cerebrospinal fluid; *CHIT1* chitinase 1; *HR* Hazard Ratio; *miR-181b* microRNA181-b; *NfL* neurofilament light chain; Site of onset refers to bulbar

P-values under the threshold for significance (<0.05) are marked in bold

Discussion

In this study, we assessed the relationship between CSF levels of three different molecules, namely NfL, a marker of axonal degeneration, CHIT1, associated with microglia activation and neuroinflammation, and miR-181b, a neuron-specific miRNA, and demographic and clinical variables in a large multicenter cohort of ALS patients and controls (including patients with other neurodegenerative diseases), and investigated their potential role as diagnostic and prognostic biomarkers. Indeed, while a huge body of evidence supports the relevance of CSF and blood NfL both in aiding diagnosis and in predicting disease progression and survival in ALS [16–19, 21, 22, 42, 43], few studies have explored the association between CSF CHIT1 and the disease so far [28–30, 44]. To date, although multiple studies on the role of miRNAs in ALS have been published, the prognostic power of circulating plasma miR-181b in ALS has been investigated to a lesser extent [34]. To our knowledge, our study is the first to demonstrate a significant difference in CSF miR-181b levels between ALS and controls. Indeed, differently from miR-181a [45], the diagnostic performance of CSF miR-181b has never been previously investigated in ALS.

Although our results were partially undermined by the presence of several values under the limit of detection for both CHIT1 and miR-181b, the three biomarkers displayed significantly increased levels in the CSF of ALS patients and were able to significantly distinguish them from controls, including patients with other neurodegenerative disorders. Indeed, while significantly higher CSF NfL and CHIT1 concentrations have been observed in ALS patients compared to those with AD and PD [42, 44], we reported for the first time a significant difference in CSF miR-181b levels compared to patients with AD as well as elevated CHIT1 concentrations in ALS compared to synucleinopathies. The latter finding suggests that CHIT1, and possibly other microglial neuroinflammatory markers, might be relatively specific to ALS or TDP-43 proteinopathies, successfully discriminating between them and other neurodegenerative diseases/proteinopathies (AD and synucleinopathies). However, NfL has the highest diagnostic performance, showing superiority compared to the combination of the three biomarkers.

As expected, CHIT1 levels did not significantly differ between ALS and CTRL-2, likely because inflammation is a fundamental pathogenic process of most of the disorders included in this control group. In addition, inflammation is not necessarily an invariable element in the pathogenesis of every form of ALS [46]. Therefore, given that CHIT1 measured in the CSF is produced to a large extent by microglia [44, 47], one can expect that ALS cases with less prominent microglial activation are not well captured by this biomarker.

It is known that a common 24-bp duplication in *CHIT1* gene lowers CHIT1 concentrations in biological fluids [41]. In addition to confirming this data in our cohort, we demonstrated that ALS patients displayed significantly increased CHIT1 concentrations in the CSF compared to controls despite having a higher frequency of the polymorphism. The latter finding has not been reported previously and is in apparent contradiction with that of increased CSF CHIT1 levels in ALS. On one hand, the combination of these seemingly discordant results further supports the pathophysiological relevance of microglial activation in ALS, as this acquired process seems to prevail over a genetically determined tendency towards a lower production of the molecule. On the other hand, one could speculate that the genetically determined tendency towards lower CHIT1 production could play a role in the early, preclinical phases of ALS pathogenesis, for example hindering an initial beneficial intervention of microglia, whereas in later, symptomatic phases (those captured by our investigation), acquired mechanisms promoting an increased CHIT1 production (e.g., microglial activation as a reaction to motor neuron degeneration) prevail.

When investigating the relationships between CSF biomarkers and clinical variables, we surprisingly found a weak inverse correlation between CSF NfL and age at evaluation. Similar results were obtained for CSF miR-181b. This is in apparent contradiction with evidence that NfL levels increase with age in neurologically healthy controls, probably reflecting a progressive, despite modest, burden of

subclinical neurodegeneration over time [48]. Conversely, a positive correlation was observed, as expected, between CSF NfL levels and age at evaluation within the control group. However, the lack of a positive correlation between age and CSF NfL levels in ALS is likely due to the fact that the huge amount and speed of motor neuron loss mask the subclinical age-related neurodegeneration. To the best of our knowledge, a correlation between miR-181b levels in the CSF and age at evaluation has never been reported before, while other authors found no correlation between circulating levels of this biomarker and age at onset [34]. Furthermore, we identified a previously unreported difference in the sex distribution of CSF CHIT1, which was more represented in male patients compared to females. This result may suggest that male ALS patients have a greater component of microglial inflammation compared to females.

In line with previous findings [42, 44], CSF NfL concentrations inversely correlated with disease duration and FVC, while CSF NfL and CHIT1, but not CSF miR-181b, were negatively associated with ALSFRS-R. Consistently, the former two biomarkers showed a strong direct correlation with disease progression rate, thus distinguishing fast progressors from slow progressors. However, when considering each biomarker as well as site of onset, age at onset, FVC and BMI as covariates in a multivariate regression, CSF NfL and FVC remained the only independent predictors of disease progression. Notably, ALS patients with rapid disease progression were more likely to have cognitive or behavioural impairment at neuropsychological assessment, compared to slow progressors. Similarly, CSF NfL concentrations, together with ALSFRS-R, predicted survival in a Cox regression model. Furthermore, CSF CHIT1, age and site of onset, as well as BMI and ALSFRS-R, all exhibited an association with survival, as previously shown [23].

It is worth noting that several ALS cases, including some with rapid progression, did not show markedly increased CSF NfL levels. This suggests that, despite being one of the most promising prognostic indicators among neurochemical biomarkers, NfL is not always effective in capturing ALS patients with poor prognosis and short survival. Indeed, since multiple pathophysiological mechanisms are involved in ALS, a further stratification using a combination of biomarkers might more accurately reflect disease activity. For instance, it has been elegantly demonstrated that serum UCHL1 serves as an additional tool to stratify and predict prognosis in ALS patients with low serum NfL levels [20]. However, in our cohort, among patients with CSF NfL concentrations under the median value, CHIT1 was not able to accurately identify patients with higher DPR and shorter survival. Thus, we believe that measurement of CSF CHIT1 in ALS patients with low NfL levels does not significantly improve prediction of disease progression and survival. Altogether, our study, in line with others investigating the prognostic performance of NfL and other measures [29, 30], suggests that NfL outperforms CHIT1 as a biomarker of disease progression and survival.

Using next-generation sequencing, miR-181 has been recently identified among miRNAs with stable levels over time in plasma samples of ALS patients [34]. In the same study, miR-181a was detected in somata and neurites of neurons of the motor cortex and in spinal cord ventral horns in mice [34], suggesting a potential role as a marker of axonal damage. Elevated plasma miR-181 levels were predictive of an increased risk of mortality in two distinct patient cohorts, displaying similar capacity to NfL; in addition, when combined, the two molecules served as more powerful predictors of survival [34]. In our cohort, CSF miR-181b levels did not show a significant correlation with disease duration and DPR, nor did they predict survival. These findings may be, at least in part, due to the limited number of patients with a detectable level and the even smaller number of patients for whom both CSF NfL and miR-181b measurements were available.

Our work is not devoid of limitations. The most important are the following: 1. We did not include a group of ALS-mimic conditions, therefore, we were not able to evaluate the discriminative performance of NfL, CHIT1 and miR-181b in the context of the proper differential diagnosis of ALS, which is ideally one of the most important applications of neurochemical biomarkers; 2. As the included ALS patients had by definition a diagnosis which was mainly based on neurological examination and electromyographic findings as per current diagnostic criteria [35, 36], our study did not enable us to compare the diagnostic accuracy of biomarkers with that of more traditional resources (namely, clinical examination and electromyography themselves); on the other hand, most of our patients had not undergone ^{18}F-fluorodeoxyglucose positron emission tomography (PET) imaging, therefore, a direct comparison between the diagnostic accuracy of this technique and that of biomarkers could not be conducted; 3. Despite the large sample size, measurements of all three biomarkers were available for only a subset of patients, which prevented us from fully exploiting the additive value of combining the three biomarkers; 4. Since many patients had CSF CHIT1 values under the threshold of detectability, possible small differences among patients with low CHIT1 levels could not be identified; 5. As the study was retrospective and cross-sectional, we were not able to perform longitudinal neurochemical assessments.

In conclusion, we appraised the CSF levels of three different molecules, NfL, CHIT1 and miR-181b, in a large multicentric European ALS cohort, confirming previous literature data and reporting new findings, including a potential role for miR-181b in discriminating ALS patients from neurodegenerative controls—although the prognostic value of

this miRNA needs to be further evaluated—as well as the observation that CSF levels of CHIT1 are increased in ALS in spite of a higher prevalence of the genetic polymorphism associated with reduced levels of the molecule. Overall, this investigation contributes to deepening our knowledge in the field of neurochemical biomarkers of ALS.

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Author contributions D.G. conceived and designed the study. D.G., P.M., M.M., S.S., N.T., V.S., P.V.D., G.P.C., S.C., and F.V. collected the CSF samples. M.R., P.M., D.S., R.D.B., L.S., I.M., and N.H. performed the experiments. D.G. and F.V. carried out all the statistical analysis. D.G., M.R., and F.V. drafted the manuscript with input from all authors. A.R., N.T., V.S., K.P., P.V.D., G.P.C., and S.C. critically revised the manuscript for intellectual content. All the authors read and approved the submitted version.

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Data availability Data are available upon reasonable request.

Declarations

Conflicts of interest N.T. received compensation for consulting services and/or speaking fees from Amylyx Pharmaceuticals, Biogen, Italfarmaco, and Zambon Biotech SA. He is Associate Editor for Frontiers in Aging Neuroscience. V.S. received compensation for consulting services and/or speaking activities from AveXis, Cytokinetics, Italfarmaco, Liquidweb S.r.l., and Novartis Pharma AG; he receives or has received research supports from the Italian Ministry of Health, AriSLA, and E-Rare Joint Transnational Call; he is in the Editorial Board of Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration, European Neurology, American Journal of Neurodegenerative Disease, and Frontiers in Neurology. F.V. is Associate Editor of Journal of Alzheimer's Disease. The other authors report no relevant competing interests.

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