



# OPEN Inflammatory signature in amyotrophic lateral sclerosis predicting disease progression

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Experimental studies identified a role of neuroinflammation in the pathogenesis of neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS). However, the role of inflammatory molecules as diagnostic and prognostic biomarkers in patients with ALS is unclear. In this cross-sectional study, the cerebrospinal fluid (CSF) levels of a set of inflammatory cytokines and chemokines were analyzed in 56 newly diagnosed ALS patients and in 47 age- and sex-matched control patients without inflammatory or degenerative neurological disorders. The molecules analyzed included: interleukin (IL)-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-17, granulocyte colony stimulating factor (GCSF), macrophage inflammatory protein (MIP)-1a, MIP-1b, tumor necrosis factors (TNF), eotaxin. Principal component analysis (PCA) was used to explore possible associations between CSF molecules and ALS diagnosis. In addition, we analyzed the association between CSF cytokine profiles and clinical characteristics, including the disease progression rate score, and peripheral inflammation assessed using the Neutrophil-to-lymphocyte ratio (NLR). PCA identified six principal components (PCs) explaining 70.67% of the total variance in the CSF cytokine set. The principal component (PC1) explained 26.8% of variance and showed a positive load with CSF levels of IL-9, IL-4, GCSF, IL-7, IL-17, IL-13, IL-6, IL-1 $\beta$ , TNF, and IL-2. Logistic regression showed a significant association between PC1 and ALS diagnosis. In addition, in ALS patients, the same component was significantly associated with higher disease progression rate score and positively correlated with NLR. CSF inflammatory activation in present in ALS at the time of diagnosis and may characterize patients at higher risk for disease progression.

**Keywords** Amyotrophic lateral sclerosis (ALS), Neuroinflammation, Disease progression, Cerebrospinal fluid (CSF), Cytokines, Neutrophil-to-lymphocytes ratio (NLR)

## Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ALSFRS-R	ALS functional rating scale-revised
B-H	Benjamini-Hockberg
CI	Confidence interval
CSF	Cerebrospinal fluid
GCSF	Granulocyte colony-stimulating factor
IFN	Interferon
IL	Interleukin
LP	Lumbar puncture
MIP	Macrophage inflammatory protein

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NLR	Neutrophil-to-lymphocyte ratio
OR	Odds ratio
PC	Principal component
PCA	Principal component analysis
PD	Parkinson's disease
TNF	Tumor necrosis factor

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting upper and lower motoneurons, characterized by progressive course and unfavorable prognosis<sup>1</sup>. The pathogenesis of ALS is not fully understood, although several mechanisms potentially involved in motor neuron degeneration have been identified, including neuronal hyperexcitability, mitochondrial dysfunction, oxidative stress, dysregulated vesicular transport, impaired DNA repair, and altered protein homeostasis<sup>2</sup>.

A role of neuroinflammation has been proposed in different neurodegenerative conditions, including ALS. Clinical and neuropathological data<sup>3</sup> proposed the activation of both the innate and the adaptive immune response in animal models and in patients with ALS<sup>4,5</sup>. Notably, mutations typically associated with ALS, including SOD1 and c9orf72, have been also associated with enhanced immune activation and cytokine expression<sup>6</sup>.

Previous studies pointed to increased expression of inflammatory mediators in patients with ALS<sup>7</sup>. High blood and CSF levels of several inflammatory cytokines and chemokines, including interleukin (IL)-1 $\beta$ , IL-4, IL-6, IL-8, IL-17, tumor necrosis factor (TNF), and granulocyte colony stimulating factor (GCSF) have been reported in ALS patients compared with controls<sup>8–15</sup>. In addition, some associations have been reported between the CSF concentrations of specific proinflammatory molecules, such as IL-2, IL-6, and interferon (IFN) $\gamma$ , and parameters of disease progression<sup>12,13,16</sup>.

While recent studies suggest that indexes of systemic inflammation could be useful biomarkers related to disease progression in ALS<sup>17</sup>, the role of CSF inflammatory cytokines as diagnostic and prognostic biomarkers in ALS is still poorly defined<sup>7,18</sup>. Significant variability exists among studies in the cytokines analyzed, and it is unclear whether a specific cytokine profile could characterize ALS patients and help to predict the disease course<sup>7</sup>.

In this study, we analyzed a large set of inflammatory CSF mediators in a group of newly diagnosed ALS patients and in a group of control patients without inflammatory or degenerative neurological disorders. Principal component analysis (PCA) was used to explore with an unbiased approach the possible synergistic effects of different molecules and identify specific CSF cytokine profiles associated with ALS. We identified a main component (PC1), reflecting the combined effect of different inflammatory cytokines, particularly IL-9, IL-4, GCSF, IL-7, IL-17, IL-13, IL-6, IL-1 $\beta$ , TNF, IL-2, which is associated with ALS diagnosis. This component was also significantly associated with higher disease progression rate score calculated at the time of ALS diagnosis and positively correlated with markers of peripheral inflammation.

## Methods

### ALS and control patients

In this cross-sectional studies, 56 patients with definite, clinical or laboratory supported probable ALS according to El-Escorial criteria<sup>19</sup>, were consecutively recruited from April 2016 to September 2020 at the Neurology Unit of IRCCS Neuromed hospital in Pozzilli (IS) Italy. We excluded patients with other neurological diseases, relevant medical conditions or inflammatory diseases. A control group of 47 patients without degenerative/inflammatory diseases, including vascular leukoencephalopathy (N = 19 patients), metabolic and hereditary polyneuropathies (N = 14), normal pressure hydrocephalus (N = 3), functional neurological disorder (N = 6), migraine (N = 1), spondylotic myelopathy (N = 2) and spastic paraparesis (N = 2) was also enrolled. The research was conducted according to the principles expressed in the Declaration of Helsinki. Written consent was obtained from each participant. The study was approved by the Ethics Committee of IRCCS Neuromed Research Institute (cod. 10–17).

### Clinical assessment

Clinical disability status was evaluated in all ALS patients at the time of diagnosis using the ALS Functional Rating Scale-Revised (ALSFRS-R) total score and subscores<sup>20</sup>. As a reliable prognostic biomarker, we used the disease progression rate (DPR) that expresses the ALSFRS-R as a function of the disease duration<sup>21</sup>. We calculated the DPR at the time of diagnosis, defined as (ALSFRS-R total score – ALSFRS-R patient's total score at time of diagnosis)/patient's disease duration from onset to diagnosis in months. ALS patients were also divided into different disease progression groups according to disease progression rate scores (low < 0.47, medium 0.47–1.11, high > 1.11) as in Labra et al.<sup>21</sup>. Patients were not treated with riluzole, edavarone or anti-inflammatory drug therapy.

### Blood and CSF collection and analysis

Blood and CSF samples were collected at the time of diagnosis during hospitalization at the Neurology Unit of IRCCS Neuromed hospital in Pozzilli (IS) Italy. Blood samples were collected from all subjects by venipuncture performed in the morning following overnight fasting. The Neutrophil-to-lymphocytes ratio (NLR) was calculated as absolute peripheral neutrophil count divided by absolute periphery lymphocyte count. CSF was collected by Lumbar puncture (LP), centrifuged (1300 rpm, 10 min) to remove cellular elements, and stored at – 80 °C until being analyzed using a Bio-Plex multiplex cytokine assay (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. The CSF molecules examined included: interleukin (IL)-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-17, granulocyte colony stimulating factor (GCSF), Macrophage inflammatory protein (MIP)-1a, MIP-1b, Tumor necrosis factors (TNF), Eotaxin. Concentrations

were calculated according to a standard curve generated for the specific target and expressed as picograms/ml. All samples were analyzed in triplicate.

### Statistical analysis

Kolmogorov–Smirnov test was applied to verify normality of data distribution. Data were expressed as mean (standard deviation, SD) or as median (25–75th percentiles) if not normally distributed.

We applied the Principal Component Analysis (PCA) to the sample of the 18 CSF cytokines to reduce the dimensionality of the cytokine data set and explore possible synergic effects of CSF cytokines. Logistic regressions were used to test the association between PCA components and group (ALS patients vs control patients) and disease progression rate. Non-parametric Spearman's correlation was used to evaluate the correlation between CSF cytokines levels and demographic/clinical variables. Non-parametric Mann–Whitney test was applied to evaluate differences in CSF cytokines levels between groups. A  $p$  value  $< 0.05$  was considered significant. The Benjamini–Hochberg (B–H) correction was applied when analyzing individual CSF cytokines to control the false discovery rate and the Type I errors (false positives). Box plots were used to depict statistically significant differences in cytokine levels between groups.

All analyses were performed using IBM SPSS Statistics for Windows (IBM Corp., Armonk, NY, USA). Missing data: NLR in 4 control patients (8.5%).

## Results

### CSF inflammatory molecules in ALS and controls

The clinical characteristics of ALS and control individuals are shown in Table 1. No significant differences were found in age and sex distribution between the two groups ( $p = 0.109$ , and  $p = 0.496$ , respectively). In addition, peripheral white blood cells and NLR did not differ between ALS and control patients (see Table 1).

PCA was performed on a set of 18 CSF cytokines from 56 ALS patients and 47 control patients. The first 6 principal components (PCs) explained 70.67% of the variance in the whole cytokine set, suggesting a synergistic effect of the different cytokines. The association of individual CSF cytokines with the first 6 PCs are shown in Fig. 1A and Supplementary Table 1.

We used logistic regression to test the association between group (ALS patients vs controls) and the first 6 PCs (Fig. 1B). A negative association was found between PC1 and group (ALS vs controls) (OR 0.429, 95% CI 0.232–0.792,  $p = 0.007$ ), indicating that this component is associated with ALS diagnosis.

As shown in Fig. 1A, PC1 was the main component explaining the 26.8% of variance in the cytokine set. The inflammatory cytokines IL-9, IL-4, GCSF, IL-7, IL-17, IL-13, IL-6, IL-1 $\beta$ , TNF IL-2, and IL-10 showed a significant positive load with this component.

The association between PC1 and group (ALS patients vs controls) was significant also considering possible effects of sex and age (OR 0.342, 95% CI 0.168–0.696,  $p = 0.003$ ).

When comparing the CSF levels of single inflammatory molecules in the ALS and control groups, we found higher CSF concentrations of several cytokines associated with PC1, including IL-1 $\beta$  ( $p = 0.031$ ), IL-2 ( $p = 0.007$ ), IL-4 ( $p = 0.037$ ), IL-6 ( $p = 0.01$ ), IL-9 ( $p = 0.008$ ), IL-13 ( $p = 0.01$ ), IL-17 ( $p = 0.006$ ), GCSF ( $p < 0.001$ ) in ALS patients compared with control patients (Fig. 2, and Supplementary Table 2). After controlling for multiple comparisons, differences in CSF levels of IL-2, IL-6, IL-9, IL-13, IL-17, and GCSF were statistically significant (all B–H  $p < 0.05$ ).

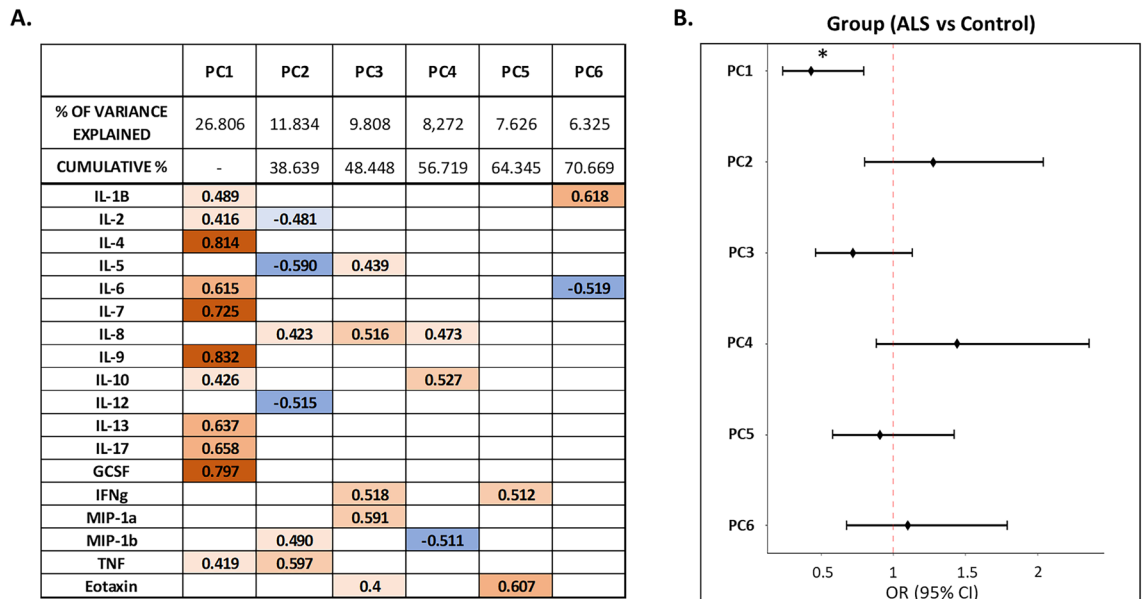
Considering that IL-7 levels were particularly low in both patients and controls (Supplementary Table 2), we evaluated whether this finding might have influenced the PCA analysis. The PCA analysis was performed excluding IL-7. The analysis confirmed 6 PCs explaining 71.1% of the variability, showing an association with individual cytokines comparable to the previous analysis. The significant association between PC1 and group (ALS vs controls) was confirmed (OR 0.399, 95% CI 0.213–0.749,  $p = 0.004$ ).

### CSF inflammatory molecules and clinical characteristics

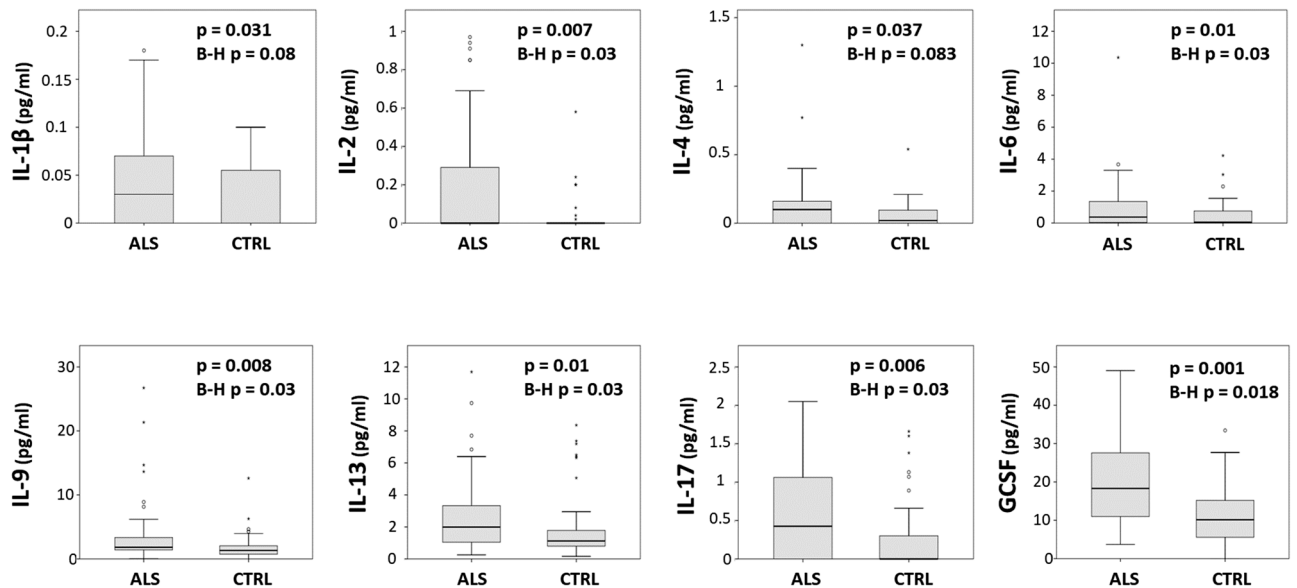
In ALS patients and controls, we analyzed possible associations between CSF cytokines and demographic characteristics at diagnosis, including peripheral inflammatory markers.

		ALS patients (N = 56)	Control patients (N = 47)	
Sex, F	N (%)	19/56 (33.9)	19/47 (40.4)	0.496*
Age at LP	Median (IQR)	62.7 (55.6–72.1)	59.1 (51.9 – 65.5)	0.109 <sup>§</sup>
Disease duration, months	Median (IQR)	8.5 (6–12)	–	–
ALSFRS-R total score	Median (IQR)	44 (42–45)	–	–
ALSFRS-R bulbar subscore	Median (IQR)	12 (11–12)	–	–
White blood cells total count	Median (IQR)	6.7 (5.7–8)	7.34 (5.6–8.42)	0.353 <sup>§</sup>
Neutrophils	Median (IQR)	4.15 (3.4–5)	4.5 (3.3–5.6)	0.507 <sup>§</sup>
Lymphocytes	Median (IQR)	1.79 (1.47–2.2)	1.9 (1.6–2.3)	0.333 <sup>§</sup>
NLR	Median (IQR)	2.4 (1.76–3.07)	2.29 (1.82–2.9)	0.808 <sup>§</sup>

**Table 1.** Clinical characteristics of ALS and control patients. \*Pearson's Chi-square  $p$ ; <sup>§</sup>Mann–Whitney  $p$ . ALSFRS-R ALS Functional Rating Scale-Revised, LP Lumbar puncture, NLR neutrophil-to-lymphocyte ratio.



**Figure 1.** Association of individual CSF cytokines with the first 6 PCs. **(A)** Principal component analysis (PCA) results: load of individual cytokines with the first 6 PCs, significant associations (cut-off=0.4) are shown (red = positive; blue = negative). **(B)** Logistic regression: associations between diagnosis group (ALS vs controls) and the first 6 PCs, OR and 95% CI are shown, \* $p < 0.05$ . ALS amyotrophic lateral sclerosis, CI confidence interval, CSF cerebrospinal fluid, GCSF granulocyte colony-stimulating factor, IFN $\gamma$  interferon, IL interleukin, MIP macrophage inflammatory protein, OR odds ratio, PC principal component, TNF tumor necrosis factor.



**Figure 2.** CSF cytokines in ALS and control patients. Boxplot showing the CSF levels of inflammatory cytokines in ALS and controls. The circles represent outlier patients. The star marks the extreme values. Mann-Whitney  $p$  and B-H corrected  $p$  are shown. ALS amyotrophic lateral sclerosis, B-H Benjamini-Hockberg, CSF cerebrospinal fluid, GCSF granulocyte colony-stimulating factor, IL interleukin.

In the control group, no significant associations were found between PCA components and either demographic characteristics (sex and age at LP) or peripheral inflammatory indexes (NLR) (all  $p > 0.05$ ).

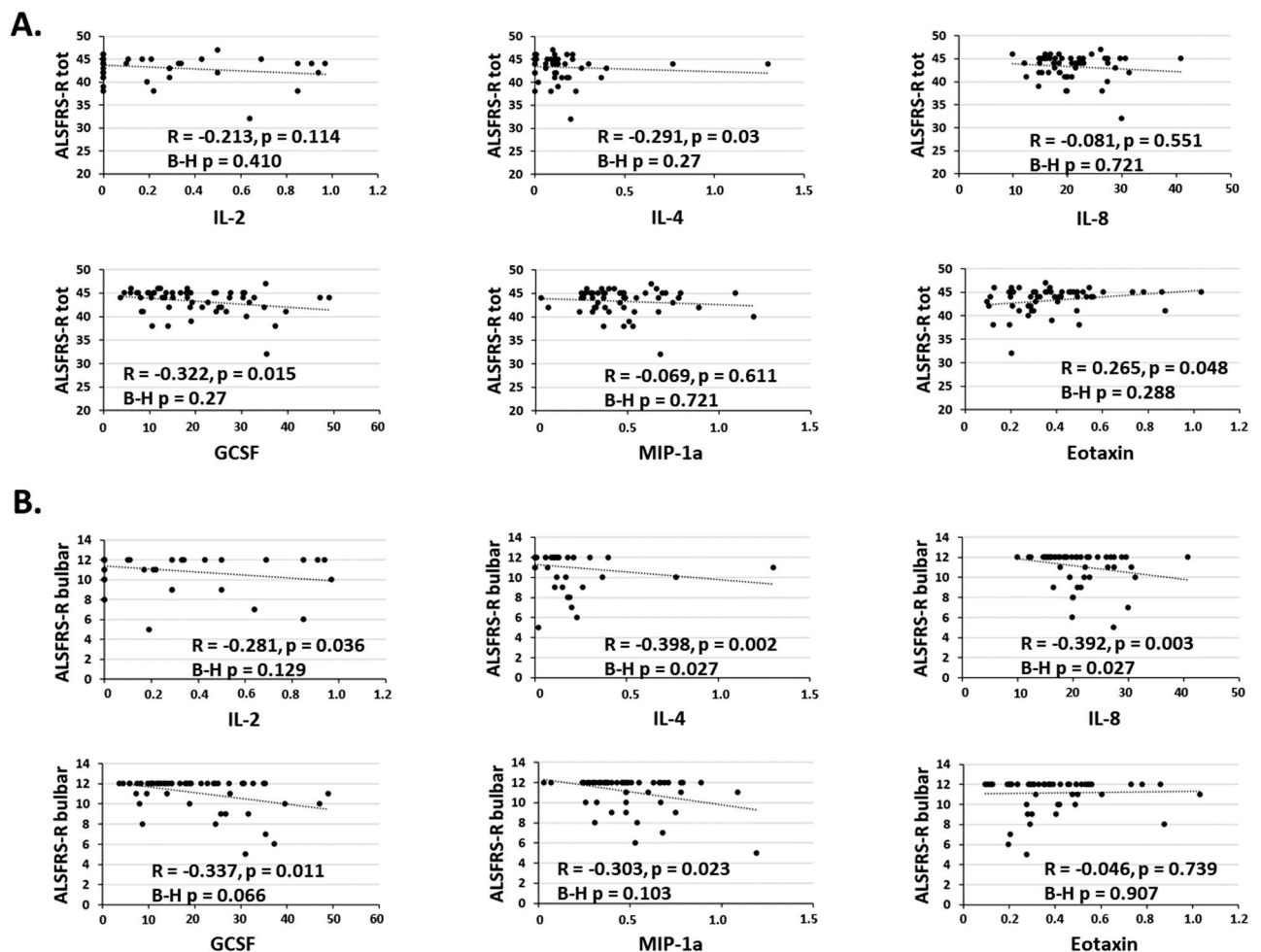
No significant associations were found between PCA components and demographic parameters (sex and age at LP) in ALS patients (all  $p > 0.05$ ), except a positive correlation between age at LP and PC3 (Spearman's  $Rho = 0.291$ ,  $p = 0.03$ ,  $N = 56$ ). This PC, explaining the 9.8% of the variability in the cytokine set, was positively associated with IL-5, IL-8, IFN $\gamma$  and MIP-1a. A negative correlation was found between PC1 and disease duration (Spearman's  $Rho = -0.279$ ,  $p = 0.037$ ,  $n = 56$ ). In addition, a positive correlation was observed between disease duration and PC5 (Spearman's  $Rho = 0.426$ ,  $p = 0.001$ ,  $n = 56$ ). PC5 was a minor component of the PCA, explaining only the 7.6% of variance. The CSF levels of Eotaxin and IFN $\gamma$  showed a significant positive load with this

component (see Fig. 1). Conversely, no significant correlations were found between PCs and disease severity evaluated using the ALSFRS total score and bulbar subscale (all  $p > 0.05$ ). Finally, in the ALS group, a significant positive correlation was found between PC1 and NLR (Spearman's  $Rho = 0.309$ ,  $p = 0.021$ ). No significant correlations were found between NLR and other PCs.

When analyzing individual CSF cytokines, negative correlations were found between disease duration and IL-8 (Spearman's  $Rho = -0.288$ ,  $p = 0.031$ ,  $n = 56$ ), MIP-1b (Spearman's  $Rho = -0.477$ ,  $p < 0.001$ ,  $n = 56$ ), TNF (Spearman's  $Rho = -0.350$ ,  $p = 0.008$ ,  $n = 56$ ), and MIP-1a (Spearman's  $Rho = -0.267$ ,  $p = 0.047$ ,  $n = 56$ ) (Supplementary Table 3). However, after controlling for multiple comparisons, only the negative correlation between disease duration and MIP-1b was statistically significant ( $B-H p = 0.004$ ). In addition, some correlations were also observed between specific cytokines and ALSFRS-R total and bulbar scores (Fig. 3 and Supplementary Table 3). In particular, negative correlations were found between ALSFRS-R total score and IL-4 (Spearman's  $Rho = -0.291$ ,  $p = 0.030$ ), GCSF (Spearman's  $Rho = -0.322$ ,  $p = 0.015$ ), and between ALSFRS-R bulbar score and IL-2 (Spearman's  $Rho = -0.281$ ,  $p = 0.036$ ), IL-4 (Spearman's  $Rho = -0.398$ ,  $p = 0.002$ ), IL-8 (Spearman's  $Rho = -0.392$ ,  $p = 0.003$ ), GCSF (Spearman's  $Rho = 0.337$ ,  $p = 0.011$ ), and MIP-1a (Spearman's  $Rho = -0.303$ ,  $p = 0.023$ ). However, after controlling for multiple comparisons, only the negative correlations between ALSFRS-R bulbar subscale and both IL-4 ( $B-H p = 0.027$ ), and IL-8 ( $B-H p = 0.027$ ) were statistically significant. Positive correlations were observed also between NLR and CSF cytokines including IL-6 (Spearman's  $Rho = 0.283$ ,  $p = 0.034$ ), G-CSF (Spearman's  $Rho = 0.291$ ,  $p = 0.029$ ), MIP-1a (Spearman's  $Rho = 0.268$ ,  $p = 0.046$ ), and MIP-1b (Spearman's  $Rho = 0.311$ ,  $p = 0.020$ ), although not significant after controlling for multiple comparisons (all  $B-H p > 0.05$ ).

### CSF inflammatory molecules and disease progression rate

We explored in ALS patients the association between CSF inflammatory molecules and disease progression rate. A significant positive correlation was found between disease progression rate at diagnosis and PC1



**Figure 3.** Correlations between CSF cytokines and ALSFRS-R total and bulbar scores. Cytokine concentrations are expressed in pg/ml. Spearman's  $Rho$ ,  $p$ , and  $B-H$  corrected  $p$  are shown. *ALSFRS-R* ALS Functional Rating Scale-Revised, *CSF* cerebrospinal fluid, *B-H* Benjamini–Hockberg, *CSF* cerebrospinal fluid, *GCSF* granulocyte colony-stimulating factor, *IFN* interferon, *IL* interleukin, *MIP* macrophage inflammatory protein, *TNF* tumor necrosis factor.

and (Spearman's  $Rho = 0.332$ ,  $p = 0.012$ ,  $n = 56$ ), and a negative correlation was found with PC5 (Spearman's  $Rho = -0.425$ ,  $p = 0.002$ ,  $n = 56$ ). No significant correlations were found with other PCs (all  $p > 0.1$ ).

To better explore the association between CSF inflammation and disease progression rate, patients were divided into three groups according to disease progression rate score at diagnosis (see methods). 29 ALS patients showed low disease progression rate ( $< 0.47$ ), 19 patients medium progression rate ( $0.47-1.11$ ), and 8 patients high progression rate ( $> 1.11$ ).

Multiple logistic regression evidenced a positive association between disease progression group (low vs medium) and PC1 (OR 3.226, 95% CI 1.412–7.374,  $p = 0.005$ ) and a negative association with PC5 (OR 0.211, 95% CI 0.072–0.614,  $p = 0.004$ ). A significant negative association was also found between disease progression group (low vs high) and PC5 (OR 0.159, 95% CI 0.044–0.581,  $p = 0.005$ ), conversely the association with PC1 was not significant (OR 2.796, 95% CI 0.998–7.834,  $p = 0.051$ ).

Considering the low number of patients in the high disease progression rate group ( $n = 8$ ), we merged the medium and high groups for further analyses. The clinical characteristic of ALS patients in the two disease progression groups (low and medium/high) are shown in Table 2. Significant differences were found between the two groups in age at LP ( $p = 0.008$ ), and as expected in both disease duration and clinical severity (see Table 2). In addition, the NLR was significantly different in the two groups, being higher in patients with medium/high disease progression rate scores.

Multiple logistic regression confirmed a positive association between disease progression group (low vs medium/high) and PC1 (OR 3.126, 95% CI 1.406–6.950,  $p = 0.005$ ), and a negative association with PC5 (OR 0.195, 95% CI 0.070–0.541,  $p = 0.002$ ) (Fig. 4). These associations were significant also considering the effect of sex and age at LP (PC1: OR 3.725, 95% CI 1.426–9.726,  $p = 0.007$ ; PC5: OR 0.086, 95% CI 0.018–0.403,  $p = 0.002$ ). No significant associations were found with other PCs.

Finally, comparing single CSF cytokines in the two disease progression groups (Fig. 5 and Supplementary Table 4), higher levels of IL-2 ( $p = 0.037$ ), IL-5 ( $p = 0.031$ ), IL-6 ( $p = 0.016$ ), IL-8 ( $p = 0.045$ ), GCSF ( $p = 0.001$ ), MIP-1a ( $p = 0.025$ ), and lower levels of Eotaxin ( $p = 0.011$ ) emerged in ALS patients with medium/high progression rate. After controlling for multiple comparisons, the association with GCSF was statistically significant (B–H  $p = 0.002$ ).

## Discussion

In recent years, experimental and clinical studies suggested that neuroinflammation may play an important role in the pathogenesis of ALS<sup>3</sup>. Although increased expression of various CSF inflammatory mediators was previously reported in patients with ALS<sup>7</sup>, it is unclear whether a specific cytokine profile could characterize ALS patients at diagnosis and help to predict the disease course.

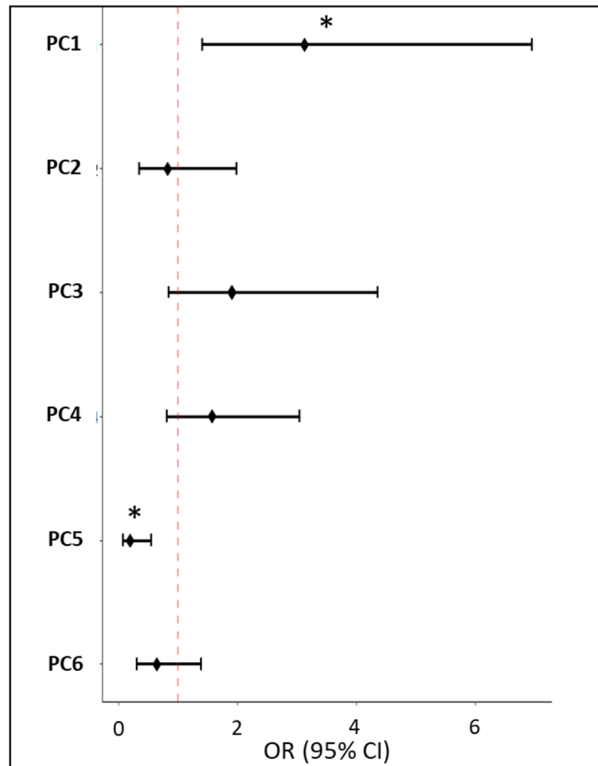
To compare the profile of CSF molecules between ALS patients and controls, and to explore possible associations with clinical characteristics, PCA was applied to identify specific components that reflect the synergistic effect of different molecules. A significant association was found between ALS diagnosis and the first PC (PC1), which is the largest source of variability in our cytokine set, representing the combined effect of multiple inflammatory molecules, specifically IL-9, IL-4, GCSF, IL-7, IL-17, IL-13, IL-6, IL-1 $\beta$ , TNF, and IL-2. These findings suggest that a specific group of CSF inflammatory cytokines could be differently expressed in newly diagnosed ALS patients.

Previous studies explored CSF levels of inflammatory cytokines in ALS patients and controls<sup>8,10,13,15,22</sup>. Despite considerable variability between studies, some inflammatory cytokines, such as IL-4, IL-7, IL-17, and GCSF, were more consistently elevated in ALS patients<sup>8,13,22</sup>, while other molecules, including IFN $\gamma$  and TNF, yielded more inconsistent results<sup>7</sup>. Interestingly, a meta-analysis of CSF cytokine data in patients with different neurodegenerative conditions, such as Parkinson's disease (PD), Alzheimer's disease (AD) and ALS, evidenced that some cytokines, including GCSF, IL-2, IL-15, IL-17, MCP-1, MIP-1a, TNF and VEGF, may be specifically associated with ALS<sup>15</sup>. Our results could therefore provide further evidence in favor of the existence of a specific CSF cytokine profile associated with ALS. In fact, the set of CSF inflammatory cytokines identified in our study by

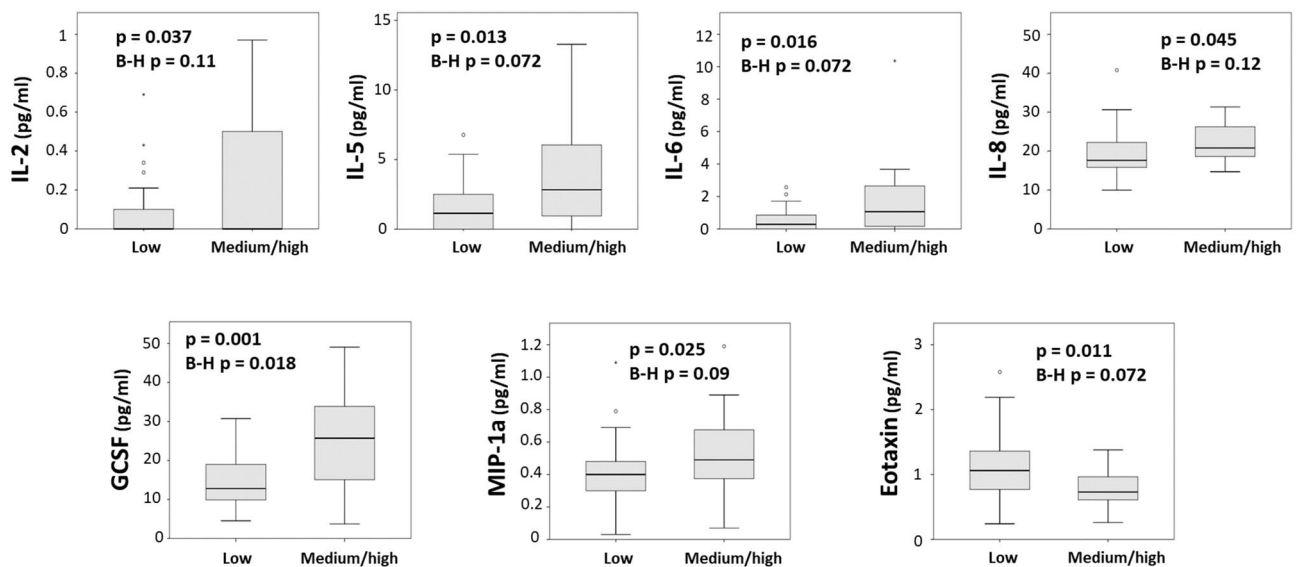
		Low disease progression rate	Medium/high disease progression rate	
ALS patients	N	29	27	
Sex, F	N (%)	8/29 (27.6)	11/27 (40.7)	0.299*
Age at LP	Median (IQR)	58.6 (50.8–68.8)	65.9 (60.03–75.22)	<b>0.008<sup>§</sup></b>
Disease duration, months	Median (IQR)	12 (11–16)	6 (5–7)	<b>&lt; 0.001<sup>§</sup></b>
ALSFRS-R total score	Median (IQR)	45 (44–45)	42 (41–44)	<b>&lt; 0.001<sup>§</sup></b>
ALSFRS-R bulbar subscore	Median (IQR)	12 (12–12)	12 (9–12)	<b>0.014<sup>§</sup></b>
White blood cells total count	Median (IQR)	6.86 (5.8–7.68)	6.57 (5.53–8.78)	0.928 <sup>§</sup>
Neutrophils	Median (IQR)	4.1 (3.35–4.9)	4.3 (3.4–5.6)	0.384 <sup>§</sup>
Lymphocytes	Median (IQR)	2.06 (1.64–2.45)	1.6 (1.3–1.9)	<b>0.007<sup>§</sup></b>
NLR	Median (IQR)	1.92 (1.63–2.51)	2.81 (1.06–3.45)	<b>0.003<sup>§</sup></b>

**Table 2.** Clinical characteristics of ALS patients at diagnosis according to disease progression rate group. Significant values are in bold. \*Pearson's Chi-square p; <sup>§</sup>Mann–Whitney p. *ALSFRS-R* ALS Functional Rating Scale-Revised, *LP* Lumbar puncture, *NLR* neutrophil-to-lymphocyte ratio.

**Disease progression rate score  
(low vs medium/high)**



**Figure 4.** PCA components and Disease progression rate. Logistic regression: associations between ALS disease progression rate group (low vs medium/high) and the first 6 PCs, OR and 95%CI are shown, \*\* $p < 0.05$ . ALS amyotrophic lateral sclerosis, CI confidence interval, GCSF granulocyte colony-stimulating factor, OR Odds ratio, PC principal component.



**Figure 5.** CSF cytokines and disease progression rate. Boxplot showing the CSF levels of inflammatory cytokines in ALS and controls. The circles represent outlier patients. The star marks the extreme values. Mann-Whitney p and B-H corrected p are shown. ALS amyotrophic lateral sclerosis, B-H Benjamini-Hockberg, CSF cerebrospinal fluid, GCSF granulocyte colony-stimulating factor, IL interleukin, MIP macrophage inflammatory protein.

an unsupervised method (PCA) showed high concordance with previous studies, suggesting that this approach may be useful to reduce the variability of results.

Experimental studies in different neurodegenerative disorders have demonstrated that neuroinflammation may be directly involved in neuronal damage and disease progression<sup>23</sup>. In animal models of ALS, microglial activation and increased expression of proinflammatory mediators and have been associated with neuronal damage<sup>24</sup>. Inflammation may critically interact with different pathogenetic mechanisms involved in ALS progression, exacerbating oxidative damage and promoting protein misfolding<sup>23,25</sup>. In addition, neurophysiological studies have demonstrated that inflammatory molecules, may directly exacerbate excitotoxic damage altering the activity of glutamatergic and GABAergic synapses<sup>26,27</sup>.

An association between CSF inflammatory biomarkers at diagnosis and parameters of clinical progression has been reported in other neurodegenerative disorders, such as multiple sclerosis and AD<sup>28,29</sup>. Previous studies explored in ALS patients the association between CSF inflammatory molecules and clinical parameters<sup>7</sup>, reporting correlations between specific CSF cytokines, such as IL-8, MCP-1, MIP-1a, IFN $\gamma$  and disease severity<sup>8–10,12,13,16,30</sup>.

When evaluating possible associations between CSF molecules and clinical characteristics of ALS patients, a significant positive association was found between PC1 and disease progression rate. In addition, a negative association was observed between disease progression rate and PC5. This component showed positive associations only with Eotaxin and IFN $\gamma$ .

The analysis of individual cytokines yielded more variable results. Although several CSF cytokines associated with PC1, including IL-2, IL-6, and GCSF, were higher in ALS patients with medium/high progression rate, after controlling for multiple comparisons only GCSF remained significant. In addition, some of these molecules, particularly IL-2, IL-4, IL-8, GCSF, MIP1a showed also negative correlations with ALSFRS-R total and bulbar subscales, although only IL-4 and IL-8 CSF levels resulted significantly associated after controlling for multiple comparisons.

Cytokines related with PC1 include a heterogeneous group of pro- and anti-inflammatory molecules. IL-1 $\beta$ , IL-2, IL-6, TNF, and IL-17 have been implicated in the pathogenesis of various neurological disorders and represent important pro-inflammatory cytokines involved in the chemotaxis and activation of immune cells<sup>31</sup>. CSF levels of these molecules were previously associated with a more severe course in patients with neurodegenerative and neuroinflammatory diseases<sup>32–35</sup>. Conversely, IL-4, IL-5 and IL-13 have been associated with T helper type 2 responses and with the activation of anti-inflammatory processes and the resolution of inflammation<sup>36</sup>. Also IL-9 has been associated with anti-inflammatory activities modulating the expression of regulatory T cells<sup>37</sup>. Finally, GCSF is a growth factor released by various immune cells, which stimulates the production of granulocytes and monocytes<sup>38</sup>. GCSF receptors are expressed by peripheral immune cells, microglial cells and neurons<sup>39</sup>. Protective effects of this molecule have been reported in animal models of stroke and neurodegenerative disorders<sup>39,40</sup>, and GCSF has thus been proposed as a therapeutic intervention in patients with ALS<sup>41–45</sup>.

Taken together, these results suggest a heterogeneous activation of the immune response in newly diagnosed ALS patients, with concurrent elevation of both pro- and anti-inflammatory cytokines. Although our results, together with previous data, support the existence of a CSF inflammatory activation in ALS, it is unclear whether this inflammatory process represents an unspecific response to brain damage or is directly involved in neurodegeneration. While CSF correlation studies are inconclusive in this regard, several lines of evidence from genetic studies and animal models suggest a direct role for inflammation in the pathogenesis and progression of ALS. Indeed, while genes classically associated with ALS (e.g., SOD1, C9orf72) have been shown to influence inflammatory responses<sup>6</sup>, it has been also demonstrated that polymorphisms in cytokine genes can influence the course of ALS<sup>46–48</sup>.

An important aspect limiting the clinical utility of CSF markers is the invasive collection procedure. For prognostic purposes, indices of peripheral inflammation appear more suitable and have been evaluated in several diseases in recent years. NLR is a recognized marker of systemic inflammation and correlates with worse prognosis in different clinical conditions, including neurodegenerative disorders as PD and AD<sup>49,50</sup>. Studies in ALS evidenced that increased NLR was associated with fast progression and shorter survival<sup>17</sup>. In our study, NLR was significantly higher in ALS patients with medium/high disease progression rate scores and was also positively associated with PC1, suggesting a link between peripheral and central inflammatory markers in ALS.

Limitations of the present study include the lack of follow up clinical evaluations and the enrollment of a control group composed of patients with neurological non-inflammatory/non-degenerative disorders. We used disease progression rate which is a reliable measure of progression risk in ALS patients at diagnosis, however, further studies with prospective data are needed to confirm our findings. Indeed, variability and inconsistency in previous findings could be due to different reasons, including difference in the cytokines analyzed, in the demographic and clinical characteristics of the ALS and control patients. In our study we included newly diagnosed and untreated ALS patients with early stage of disease and relatively short disease duration.

Overall, our findings are in line with a role of neuroinflammation in ALS pathogenesis and progression. The unsupervised approach employed in our study suggests possible synergistic effects of different inflammatory CSF cytokines particularly IL-9, IL-4, GCSF, IL-7, IL-17, IL-13, IL-6, IL-1 $\beta$ , TNF, IL-2, Eotaxin, and IFN $\gamma$ . However further studies are needed to clarify whether this CSF inflammatory profile is specifically associated with ALS, and to define the specific contribution of different molecules.

### Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.



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### Author contributions

Conceptualization: CF, DC, MSB; writing—original draft preparation: CF, MSB; writing- review and editing: ABr, LG, DC; data collection/curation: CF, LG, FB, ED, GG, FA, ABo; Analysis or interpretation of data: RF, AF, AM, GM, MS; funding acquisition: FB, AM, GM, DC; statistical analysis: MSB. All authors have read and agreed to the published version of the manuscript.

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### Competing interests

FB acted as advisory board members for Teva and Roche and received honoraria for speaking or consultation fees from Merck Serono, Teva, Biogen Idec, Sanofi, and Novartis, and non-financial support from Merck Serono, Teva, Biogen Idec, and Sanofi. DC is an Advisory Board member of Almirall, Bayer Schering, Biogen, GW Pharmaceuticals, Merck Serono, Novartis, Roche, Sanofi-Genzyme, and Teva and received honoraria for speaking or consultation fees from Almirall, Bayer Schering, Biogen, GW Pharmaceuticals, Merck Serono, Novartis, Roche, Sanofi-Genzyme, and Teva. He is also the principal investigator in clinical trials for Bayer Schering, Biogen, Merck Serono, Mitsubishi, Novartis, Roche, Sanofi-Genzyme, and Teva. His preclinical and clinical research was supported by grants from Bayer Schering, Biogen Idec, Celgene, Merck Serono, Novartis, Roche, Sanofi-Genzyme and Teva. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-67165-9>.

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