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**Clinical and biological predictors for
Amyotrophic Lateral Sclerosis:
a monocentric experience**

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ABSTRACT

Background: While the defining clinical characteristics of amyotrophic lateral sclerosis (ALS) primarily revolve around the degeneration of motor neurons, the increasingly recognized role of neuroinflammatory players is currently consolidated. Moreover, significant variability exists in disease expression, limiting accurate prediction of life expectancy, confounding design and evaluation of ALS treatments. Although our understanding of contributions from non-neuronal cells and mechanisms involved in ALS pathogenesis is improving, a representative and reliable panel of prognostic biomarkers is still lacking. In this context, we aim to describe in our population the behavior of certain clinical and biological ALS predictors, starting from the targetable players in ALS pathophysiology.

Methods: This retrospective study investigates clinical data and biomarkers of 182 ALS patients recruited from the ALS Center of the Modena University Neurological Department between January 1st, 2007 and December 1st, 2023. Biomarkers of axonal injury (NfL and pNfH) and microglial and astrocytic activation (Serpina1, TREM2, CHI3L1, OPN and S100B, respectively) were measured both in CSF and in serum. In a subpopulation of patients, we examined complete blood count (CBC)- derived parameters, including the neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), systemic-immune-inflammation index (SII), systemic inflammation response index (SIRI) and Aggregate Systemic Inflammation Index (AISII). All variables were analyzed for association with clinical features in the entire cohort and then in sex- and age-based subgroups.

Results: Serpina1_{CSF} was significantly lower in females [4.43 ug/ml (IQR 2.76-6.33 ng/ml) vs 5.61 ug/ml (IQR 3.39-9.16 ng/ml), $p=0.032$], while SII indexes was oppositely distributed [higher in females, 540.67 (IQR 363.05-807.24) vs 384.89 (307.5-578.52), $p=0.015$]. Neither cognitive involvement or functional scores (King's staging system and MiToS) were significantly correlated with biomarkers' panel. Although we observed scattered correlations between each biomarker and specific ALS surrogates of disease progression, neurofilaments more persistently demonstrate their impact on these variables, as well as they retain high discriminative power relative to different classes of progression (for NfL both on serum and CSF $p<0.001$, pNfH_{CSF} $p<0.001$). Serum Serpina1 levels act differently across the three classes, being more elevated in patients with very slow progression, and decreasing with faster progression in a significant manner ($p=0.043$). In univariate survival analysis, without

overcoming neurofilaments, several indicators of inflammation emerged as impacting negatively on survival, namely SIRI (HR 1.43, 95% CI 1.03-1.966, $p=0.03$), AISI (HR 1.002, 95%CI 1.001-1.003, $p=0.002$) and SII (HR 1.001, 95% CI 1.0003-1.001, $p=0.003$). Interestingly, among them AISI retained its power also at multivariate analysis (HR 1.002, 95% CI 1.0004-1.001, $p=0.012$). When considering sex, SerpinA1_{CSF} levels influenced survival only for females (HR 1.042, 95%CI 1.0065-1.078, $p=0.02$), in a similar manner of other inflammatory biomarkers, namely NLR (1.154, 95% 0.099-1.333, $p=0.05$), MLR (HR 8.572, 95%CI 1.046-70.23, $p=0.045$), SIRI (HR 1.483, 95% 1.082-2.0334, $p=0.014$) and SII (HR 1.00099, 95% 1.0008-1.003, $p=0.001$). Finally, after stratification of ALS patients by age, SerpinA1_{CSF} acted as a negative prognostic factor in patients older than 65 years old (HR for age class 65-75 years old: 1.030, 95% CI 1.0005-1.059, $p=0.046$; HR for patients older than 75 years old: 1.087, 95% CI 1.016-1.16, $p=0.015$) while AISI and SII influenced more patients younger than 75 years old [for patients < 65 years HR 1.002 (95% CI 1.0005-1.003, $p=0.009$) for AISI and HR 1.0008 (95%CI 1.0001-1.0015, $p=0.02$) for SII; for patients 65-75 years, HR 1.002 (95%CI 1.0006-1.003, $p=0.005$) for AISI and HR 1.001 (95%CI 1.0003-1.002, $p=0.005$) for SII].

Conclusions: One of the novel findings in our research is the influence of sex on several biomarkers explored, since for females we observed a more impactful action by CSF SerpinA1, CSF CHI3L1 and systemic biomarkers on survival. Moreover, SerpinA1 impact negatively on survival in older patients, while AISI and SII acted mainly in case of younger patients. However, both neurofilament or CHI3L1 CSF levels outperform the other neuroinflammatory biomarkers at predicting rate of disease progression in our entire cohort of patients, so the prognostic meaning of these measure alone remains inconclusive. Larger collaborative longitudinal studies will be necessary to explore the origins of these markers and the activation patterns of various glial and systemic cells over the course of the disease, shedding light on the role of inflammation in ALS and clarify their role in patients' survival.

SOMMARIO

Background: Sebbene le caratteristiche cliniche distintive della sclerosi laterale amiotrofica (SLA) riguardino principalmente la degenerazione dei motoneuroni, il ruolo della neuroinfiammazione è attualmente consolidato. Inoltre, esiste una significativa variabilità nell'espressione della malattia, che limita la previsione accurata dell'aspettativa di vita e complica la prognosi e la valutazione dei trattamenti per la SLA. Sebbene la nostra comprensione del contributo delle cellule non neuronali e dei meccanismi coinvolti nella patogenesi della SLA stia via via crescendo, manca ad oggi un pannello rappresentativo e affidabile di biomarcatori prognostici. In questo contesto, ci siamo posti l'obiettivo di descrivere nella nostra popolazione monocentrica il comportamento di alcuni predittori clinici e biologici della SLA, partendo da alcuni dei noti bersagli fisiopatologici.

Metodi: Questo studio retrospettivo analizza i dati clinici e biologici di 182 pazienti affetti da SLA arruolati dal Centro SLA del Dipartimento di Neurologia dell'Università di Modena tra il 1° gennaio 2007 e il 1° dicembre 2023. I biomarcatori di danno assonale (NfL e pNfH) e di attivazione microgliale e astrocitaria (rispettivamente SerpinA1, TREM2, CHI3L1, OPN e S100B) sono stati misurati sia nel liquido cerebrospinale (CSF) che nel siero. In una sottopopolazione di pazienti, abbiamo studiato il ruolo del sistema immunitario sistemico analizzando i parametri derivati dall'emocromo completo, inclusi il rapporto neutrofili/linfociti (NLR), il rapporto monociti/linfociti (MLR), l'indice di infiammazione immunitaria sistemica (SII), l'indice di risposta all'infiammazione sistemica (SIRI) e l'indice aggregato di infiammazione sistemica (AIS). Tutte le variabili sono state analizzate per l'associazione con le caratteristiche cliniche nell'intero gruppo di studio e successivamente nei sottogruppi divisi in base al sesso ed età.

Risultati: I livelli di SerpinA1 nel CSF sono risultati significativamente inferiori fra le pazienti di sesso femminile [4.43 ug/ml (IQR 2.76-6.33 ng/ml) vs 5.61 ug/ml (IQR 3.39-9.16 ng/ml), $p=0.032$], mentre l'indice SII era distribuito in modo opposto [più alto nelle donne, 540.67 (IQR 363.05-807.24) vs 384.89 (307.5-578.52), $p=0.015$]. Né il coinvolgimento cognitivo né i punteggi funzionali (King's staging system e MiToS) erano significativamente correlati con il nostro pannello di biomarcatori. Sebbene abbiamo osservato correlazioni sporadiche tra ciascun biomarcatore e specifici surrogati di progressione della malattia SLA, i neurofilamenti dimostrano più costantemente il loro impatto su queste variabili e mantengono un elevato potere discriminante rispetto a diverse

classi di progressione (per NfL sia nel siero che nel CSF $p < 0.001$, pNFH nel CSF $p < 0.001$). I livelli sierici di SerpinA1 agiscono in modo diverso nelle tre classi, essendo più elevati nei pazienti con progressione molto lenta e diminuendo con una progressione più rapida in modo significativo ($p = 0.043$). Nell'analisi univariata, senza superare i neurofilamenti, diversi indicatori sono emersi come negativi per la sopravvivenza, in particolare SIRI (HR 1.43, 95% CI 1.03-1.966, $p = 0.03$), AISI (HR 1.002, 95%CI 1.001-1.003, $p = 0.002$) e SII (HR 1.001, 95% CI 1.0003-1.001, $p = 0.003$). Interessante è che tra questi, l'AISI ha mantenuto il suo potere anche nell'analisi multivariata (HR 1.002, 95% CI 1.0004-1.001, $p = 0.012$). Considerando il sesso, solo per le donne i livelli di SerpinA1CSF hanno influenzato la sopravvivenza (HR 1.042, 95%CI 1.0065-1.078, $p = 0.02$), in modo simile a NLR (1.154, 95% 0.099-1.333, $p = 0.05$), MLR (HR 8.572, 95%CI 1.046-70.23, $p = 0.045$), SIRI (HR 1.483, 95% 1.082-2.0334, $p = 0.014$) e SII (HR 1.00099, 95% 1.0008-1.003, $p = 0.001$). Infine, dopo aver stratificato i pazienti SLA per età, SerpinA1CSF ha agito come fattore prognostico negativo nei pazienti di età superiore ai 65 anni (HR per la fascia di età 65-75 anni: 1.030, 95% CI 1.0005-1.059, $p = 0.046$; HR per i pazienti di età superiore ai 75 anni: 1.087, 95% CI 1.016-1.16, $p = 0.015$) mentre AISI e SII hanno influenzato maggiormente i pazienti più giovani di 75 anni [per i pazienti < 65 anni HR 1.002 (95% CI 1.0005-1.003, $p = 0.009$) per AISI e HR 1.0008 (95% CI 1.0001-1.0015, $p = 0.02$) per SII; per i pazienti 65-75 anni, HR 1.002 (95%CI 1.0006-1.003, $p = 0.005$) per AISI e HR 1.001 (95% CI 1.0003-1.002, $p = 0.005$) per SII].

Conclusioni: Uno dei nuovi risultati della nostra ricerca è l'influenza del sesso verso i diversi biomarcatori esplorati, poiché per le donne abbiamo osservato un'azione più incisiva sulla sopravvivenza da parte dei biomarcatori sistemici, di SerpinA1 e CHI3L1 su liquor. Inoltre, SerpinA1 ha un impatto negativo sulla sopravvivenza nei pazienti più anziani, mentre AISI e SII agiscono principalmente nei pazienti più giovani. Tuttavia, i livelli di neurofilamenti o CHI3L1 nel CSF superano gli altri biomarcatori neuroinfiammatori nel predire il tasso di progressione della malattia nella nostra intera coorte di pazienti, quindi il valore prognostico di queste misure da sole rimane inconclusivo. Saranno necessari studi longitudinali collaborativi più ampi per esplorare le origini di questi marcatori e i modelli di attivazione di varie cellule gliali e sistemiche durante il corso della malattia, facendo luce sul ruolo dell'infiammazione nella SLA e chiarendo il loro ruolo nella sopravvivenza dei pazienti.

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LIST OF ABBREVIATIONS

AAT = alpha-1 antitrypsin

AD = Alzheimer's Disease

ASI= Aggregate Systemic Inflammation Index

ALS = Amyotrophic Lateral Sclerosis

ALS-MiToS = ALS Milano-Torino Staging

ALSbi = ALS with behavioural impairment

ALScbi = ALS with combined cognitive and behavioural impairment

ALSci = ALS with cognitive impairment

ALSFRS-R = ALS Functional Rating Scale Revised

ALS-FTD = ALS with frontotemporal dementia

BBB = blood-brain barrier

BMI = Body Mass Index

C9orf72 = Chromosome 9 open reading frame 72

CBC = Cell Blood Count

CHI3L1 = Chitinase-3-like protein 1

CJD = Creutzfeldt–Jakob disease

CNS = Central Nervous System

COPD = chronic obstructive pulmonary disease

CSF = Cerebrospinal Fluid

CS =corticospinal

DPR = disease progression rate

ELISA = Enzyme-Linked Immunosorbent Assay

fALS = familial Amyotrophic Lateral Sclerosis

FLAIR = Fluid Attenuated Inversion Recovery

FTD = Frontotemporal dementia

FTLD = frontotemporal lobar degeneration

FUS = FUS RNA Binding Protein

FVC = Forced Vital Capacity

HR = Hazard Ratio

IV = invasive ventilation

LMN = Lower Motor Neuron

LO = Last Observation

LP = lumbar puncture

MEPs = Motor-Evoked Potentials

MLR =monocyte-to-lymphocyte ratio

MNs = Motorneurons

MND = Motor Neuron Disease

MRI = Magnetic Resonance Imaging

MS = Multiple Sclerosis

NfL = Neurofilament Light Chain

NFs = Neurofilaments

NIV = Non-Invasive Ventilation

NGS = next generation sequencing

NLR =neutrophil-to-lymphocyte ratio

OPN = Osteopontin

PCR = Polymerase Chain Reaction

PEG = percutaneous endoscopic gastrostomy

PLS = Primary Lateral Sclerosis

PMA = Progressive Muscle Atrophy

pNfH = Phosphorylated Neurofilament Heavy Chain

rEEC = El Escorial criteria revised

RBC = red blood cells

ROADS = Rasch-Built Overall ALS Disability Scale

sALS = sporadic Amyotrophic Lateral Sclerosis

SII = systemic-immune-inflammation index

SIRI= systemic inflammation response index

SOD1 = Superoxide Dismutase 1

S100B = S100 calcium-binding protein B

TARDBP = TAR DNA binding protein

TNF α = Tumor necrosis factor α

TREM2 = triggering receptor expressed on myeloid cells 2

UMN = Upper Motor Neuron

WBC = white blood cells

1. INTRODUCTION

1.1. Amyotrophic Lateral Sclerosis: clinical and biological heterogeneity

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative motor neuron disease (MND) that irreversibly causes death of the lower motor neurons in the brainstem and spinal cord and of the upper motor neurons in the motor cortex. ALS was historically considered a relatively uniform disease of progressive voluntary muscle weakness, spreading to adjacent body segments, typically leading to death from respiratory failure within two to four years of diagnosis (Ahmed et al., 2020). However, the disease may also selectively or differentially affect the upper or lower motor neurons (respectively UMN and LMN), and may even include an extra-motor involvement (Beeldman et al., 2016; Hardiman et al., 2017). Expanded knowledge of the phenotypic variability of the disease suggests the possibility of different pathogenic trajectories, ranging from extremes within the MND spectrum with selective UMN or LMN involvement, such as primary lateral sclerosis (PLS) or progressive muscular atrophy (PMA). Consistently, the rate of disease progression from symptom onset to death varies greatly, with survival anywhere between a few months and more than 10 years (Knibb et al., 2016). Therefore, studies over the past decades have redefined ALS as a complex disorder with significant heterogeneity in clinical presentation in the site of disease onset, distribution of upper and lower neuron motor signs and survival (Goutman et al., 2022).

Overall, ALS is considered a systemic disease and exhibits significant variations in clinical presentations, including non-motor symptoms, cognitive and behavioral changes in up to 60% of cases, and frontotemporal dementia (FTD) in 15% of cases (Phukan et al., 2012; Montuschi et al., 2015). Consequently, diagnosing ALS can be challenging, and the process has remained largely unchanged in clinical practice over the last decade, relying on clinical history and examination. The typical median time between initial symptoms and a definitive diagnosis is between 10 and 16 months (Richards et al., 2020), due to the rarity and limited awareness of the disease, incomplete recognition of symptoms, and a lack of early and appropriate specialist involvement (Galvin et al., 2017).

The recognition of multiple heterogeneous phenotypes of ALS not only facilitates an early diagnosis but also supports a finer prognosis, as demonstrated by several population registers (The Australian national motor neuron disease, the Italian Registries of Emilia Romagna

Region and Piedmont and Valle d'Aosta) (Goutman et al., 2022). Consistently, patients with bulbar onset have a higher risk of FTD compared to other phenotypes (Chio et al., 2020) and earlier placement of a feeding tube due to dysphagia (Moglia et al., 2019; Gianferrari et al., 2022a) if compared to spinal onset. Moreover, the multistep model of ALS suggests that the accumulation of genetic and environmental risk factors accelerates disease onset, while individuals carrying ALS risk variants may require fewer steps for the initiation of pathogenic processes (Al-Chalabi et al., 2014). Still, the prognosis for ALS patients remains suboptimal because the determinants of progression are not fully understood. To facilitate early diagnosis and improve prognosis, research is ongoing on new criteria and scoring systems for ALS, as well as emerging diagnostic and prognostic biofluid markers, imaging modalities, and electrophysiological measurements. Nevertheless, the pathophysiology of ALS is incompletely understood, a general assumption is that the disease results from a complex interplay between genetic and environmental factors, with involved genes having a major role in determining disease onset and progression, clustered into several categories, namely protein homeostasis, RNA homeostasis/processing, and cytoskeletal dynamics, and immune dysregulation (Brown and Al-Chalabi, 2017; Beers and Appel, 2019a), as represented in Figure 1.

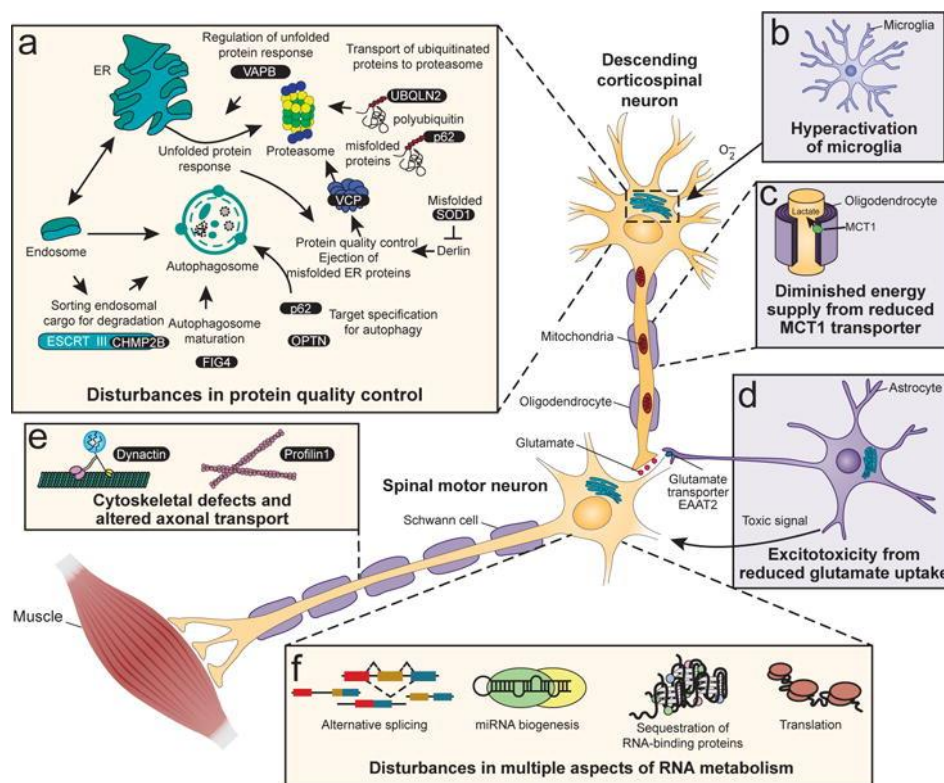


Figure 1. Schematic representation of the major cellular disease mechanisms implicated in ALS. (From Taylor et al., 2016)

1.2. Clinical indicators for Amyotrophic Lateral Sclerosis prognosis: state of art

Survival for patients with ALS varies greatly, ranging from several months to more than 10 years. The current body of epidemiological evidence identifies several factors as putative prognostic indicators in ALS. These include age at onset, phenotype, certain genetic variants, nutritional status and disease severity at diagnosis. So far, there is limited evidence regarding various other factors, such as mood and psychological distress, marital status, and physical exercise, as potential prognostic elements in ALS (Chiò et al., 2009). Consistently, all epidemiologic studies align in indicating a more unfavorable prognosis with an increasing age at onset in ALS. Patients who experience the onset of the disease before their forties tend to survive for over 10 years, while those aged 80 and above typically succumb within 2 years from the onset of symptoms (Chiò et al., 2009; Calvo et al., 2017; Gianferrari et al., 2022b).

Then, there is substantial agreement across studies indicating a reduced survival when ALS first manifests with bulbar symptoms (Calvo et al., 2017; Gianferrari et al., 2022b). This effect persists even after adjusting analyses for the age at onset, emphasizing that the site of onset should be considered an independent prognostic factor (Chiò et al., 2009; Gianferrari et al., 2022b). In cases of bulbar onset, dysphagia typically occurs earlier, leading to the anticipation of the need for artificial nutrition and an increased risk of aspiration pneumonia. Additionally, the earlier involvement of the phrenic nerve in bulbar onset patients shortens the time to respiratory failure. Among spinal onset, however, survival could still be highly variable and predominant or pure UMN as well as pure LMN phenotypes show the longest tracheostomy-free survival (Chio et al., 2011). Cognitive impairment in ALS can be viewed as an additional manifestation of the disease's phenotype, spreading across the prefrontal cortex (Chio et al., 2019). However, its impact on survival is more pronounced as cognitively impaired patients tend to have reduced autonomy in their daily activities and self-care. Moreover, their compliance with interventions like non-invasive ventilation and gastrostomy is typically diminished. Consequently, patients with ALS-FTD, where cognitive impairment is severe enough to cause a loss of autonomy, exhibit shorter survival.

Mutations in the four major ALS causative genes can have varying effects on disease progression, with mutations of *SOD1* gene having variable effects depending on the mutation type and effect on SOD1 protein (Berdyński et al., 2022). A5V mutations result in a faster disease decline rate and a median survival of 12 months. In contrast, patients carrying E22G, G38R, D91A, G94C, or I114T mutations typically exhibit prevalent lower motor neuron

damage and a slower disease course (Opie-Martin et al., 2022; Martinelli et al., 2023). Interestingly, *TARDBP*-positive patients are associated with a relatively prolonged survival, with a median of 5 years (95% CI 3.6 – 7.2). (49) In contrast, *FUS*-positive patients exhibit the shortest survival, with a median of 1.9 years (95% CI 1.7 – 2.1)(Chiò et al., 2012). *C9orf72* repeat expansions often lead to an earlier age at onset, a higher prevalence of bulbar onset, and cognitive dysfunctions, which could contribute to a potentially poorer prognosis (Byrne et al., 2012).

Nutritional status has been widely studied as a prognostic indicator, assuming weight as a mediator of muscle waste, dysphagia and hypermetabolism. A faster decline in body mass index (BMI) from its premorbid values has been proven to predict a poorer prognosis in ALS (Shimizu et al., 2011; Moglia et al., 2019).

Finally, for what concerns disease severity, several measures have been proposed such as functional scales and staging systems, along with the diagnostic delay have been proven to be prognostic in ALS . Nowadays, the most widely used outcome measure in clinical trials is the disease progression rate assessed through the ALS Functional Rating Scale Revised (ALSFRS-R). The ALSFRS-R has been designed to monitor the progression rate of the disease, but changes in ALSFRS-R scores may not necessarily signify an improvement in the disease itself, as in case of symptom management (e.g., addressing sialorrhea) or medical decisions (e.g., discontinuing non-invasive ventilation). The multi-dimensionality of ALSFRS-R limits its clinical utility, particularly in clinical trials (Van Eijk et al., 2021), and its lack of responsiveness during plateau periods makes it challenging to discern treatment effects in such trials (Bedlack et al., 2016). To overcome ALSFRS-R limitations and to best represent true changes in disease progression, the self-reported Rasch-Built Overall ALS Disability Scale (ROADS) (Fournier et al., 2020) captures functional changes, accounting for function at the upper and lower ranges of disability. Additionally, the scale has high test-retest reliability and is designed for a 1-point change to represent the same change in function across the whole score spectrum. However, whether ROADS will integrate or supply ALSFRS-R in clinical practice is still to be verified since its recent introduction with limited application (Johnson et al., 2022; Manera et al., 2022; Fortuna et al., 2023).

Respiratory tests, such as forced vital capacity (FVC) (Pirola et al., 2019), also contribute to clinicians' prognostic assessments, with FVC being included in predictive models like ENCALS. The ENCALS survival model represents a recent and comprehensive approach to

forecasting the survival of ALS patients, defined by the criteria of noninvasive ventilation for over 23 hours per day, tracheostomy, or death (Westeneng et al., 2018). This model drew upon data from an extensive cohort of 11,475 ALS patients across 14 ALS centers situated in various European locations. Incorporating sixteen clinical predictors, the model identified eight predictors that reached statistical significance ($p < 0.001$). These influential predictors encompassed age at onset, time to diagnosis, ALSFRS-R progression rate, FVC, bulbar onset, definite ALS by revised El Escorial criteria (rECC), FTD, and the presence of *C9orf72* repeat expansion. This innovative survival model holds significant promise in providing personalized prognoses for ALS patients, a crucial aspect given the considerable heterogeneity of the disease (Goutman et al., 2022).

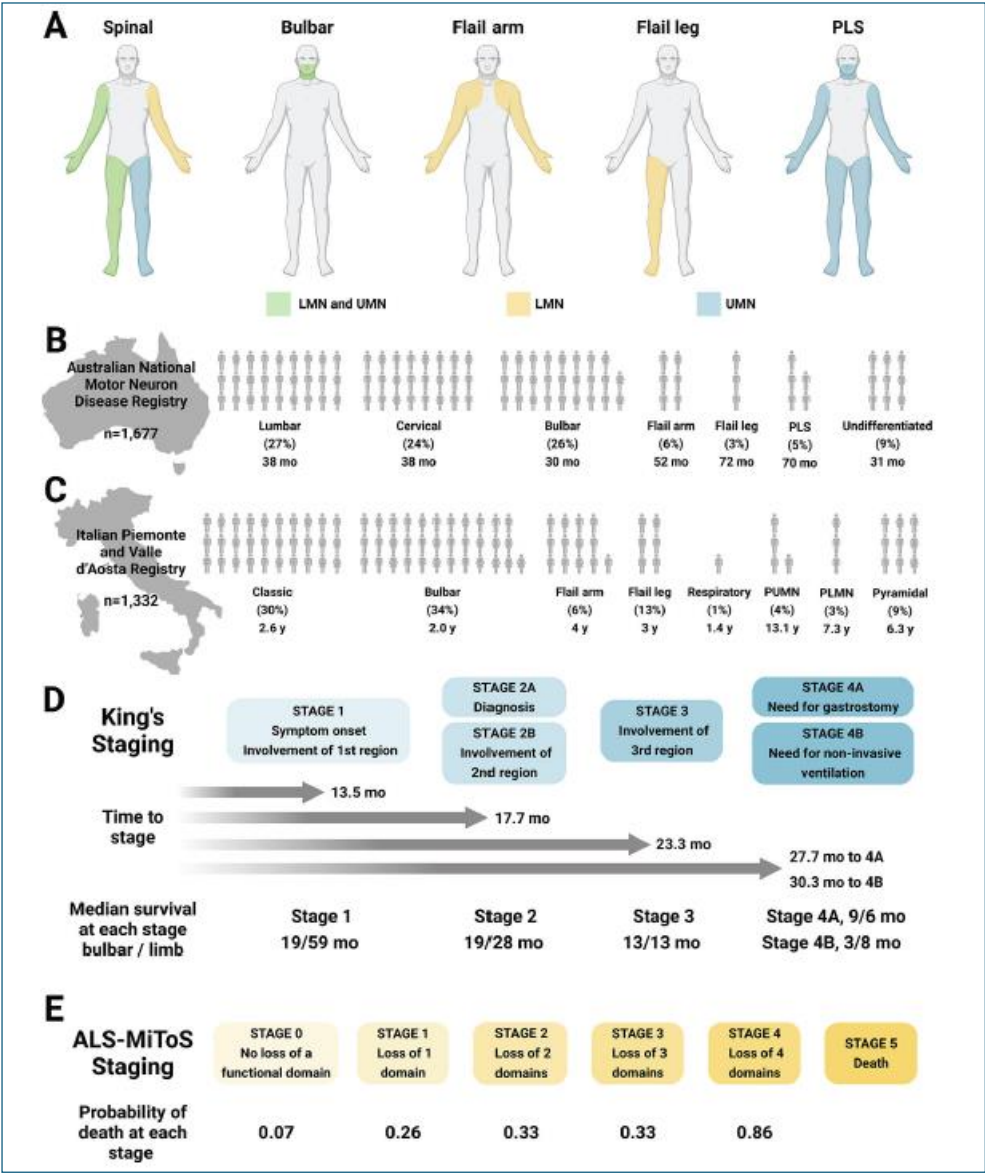


Figure 2. ALS phenotypic heterogeneity in initial presentation and staging (Goutman et al., 2022).

In clinical trials, staging systems play a crucial role in evaluating whether interventions can prevent the progression from less severe to more advanced disease stages. Two notable staging systems are the King's ALS staging and ALS Milano-Torino Staging (ALS-MiToS), which complement each other (Figure 2). The King's system excels in staging during the early stages of the disease, while ALS-MiToS demonstrates superior performance in the later stages (Fang et al., 2017). Despite neither system being currently in clinical use, both provide valuable insights into disease progression and survival, albeit with certain limitations (Luna et al., 2021) and hold potential utility in clinical trials (Al-Chalabi et al., 2021). The King's ALS staging (Roche et al., 2012) defines four progressive stages linked to survival, shedding light on the diverse progression patterns among patients. On the other hand, ALS-MiToS categorizes patients into six stages based on specific ALSFRS-R responses across four functional domains (Chiò et al., 2015). Progression through the stages in ALS-MiToS is expected to occur sequentially, with a rising likelihood of death at each subsequent stage rather than skipping stages.

1.3. Fluid biomarkers for Amyotrophic Lateral Sclerosis: state of art and current needs

In past years several candidate biomarkers for ALS have emerged, including biofluid, neurophysiological and neuroimaging measures that indicate various aspects of the disease, from network-level dysfunction to structural changes and basic cellular processes (Kiernan et al., 2021).

Investigating biomarkers in ALS may have several aims and applications, from a deeper understanding of the pathological basis of the disease to implications in the clinical practice, enabling an early diagnosis of ALS. Effective diagnostic biomarkers for ALS would allow patients to start receiving treatment or be enrolled in clinical trials at a stage when therapeutics might be more efficacious. Diagnostic biomarkers could also potentially reveal and differentiate ALS subtypes since ALS is a clinically and pathophysiologically heterogeneous condition (Bendotti et al., 2020).

Moreover, Since ALS can be defined as an “etiologically and biologically heterogeneous disease” (Taylor et al., 2016), the concept of therapy that is tailored to an individual’s genetic signature has started to emerge (Kiernan et al., 2021). The understanding of individual differences in response to a trial therapy, particularly in a longitudinal manner to assess

progression, requires reliable biomarkers. an appropriate stratification of patients according to biomarker values may implement trial design and favor research for disease treatments. For this reason, the search for biomarkers has been incorporated into Airlie House consensus guidelines for trial implementation (Van Den Berg et al., 2019). In these consensus criteria, particular attention was drawn to the necessity to include prognostic and predictive biomarkers as eligibility criteria, and pharmacodynamics biomarkers as proof of the adequacy of drug delivery, target engagement, or biological activity of the experimental therapeutic. An example of how this approach could be beneficial has come from a post hoc analysis of three trials of lithium for ALS (van Eijk et al., 2017), which showed that patients experienced different effects of the therapy according to their UNC13A genotype. Thus, the pathway for biomarker discovery and validation in ALS is tightly connected to that of therapeutic development and pathogenic characterization. Therefore, several biomarker types, as defined by BEST glossary (BEST), would have a wide range of applications in ALS.

Finally, biomarkers are also needed to enable the testing of preventive strategies for at-risk individuals, informing us on molecular and cellular alterations, and providing new therapeutic targets to be addressed in future clinical trials. Currently, the identification of individuals who are at risk of developing ALS can only be done by screening individuals from families that carry highly penetrant mutations. It is thus notable that a growing body of evidence suggests that neurofilaments show promise as susceptibility/risk biomarkers given their potential to track disease progression and possibly predict the timing of clinical phenoconversion for asymptomatic individuals with a known mutation in an ALS-associated gene, namely *SOD1*, *C9orf72*, *FUS* (Benatar et al., 2018).

1.4. Targetable players in Amyotrophic Lateral Sclerosis pathophysiology

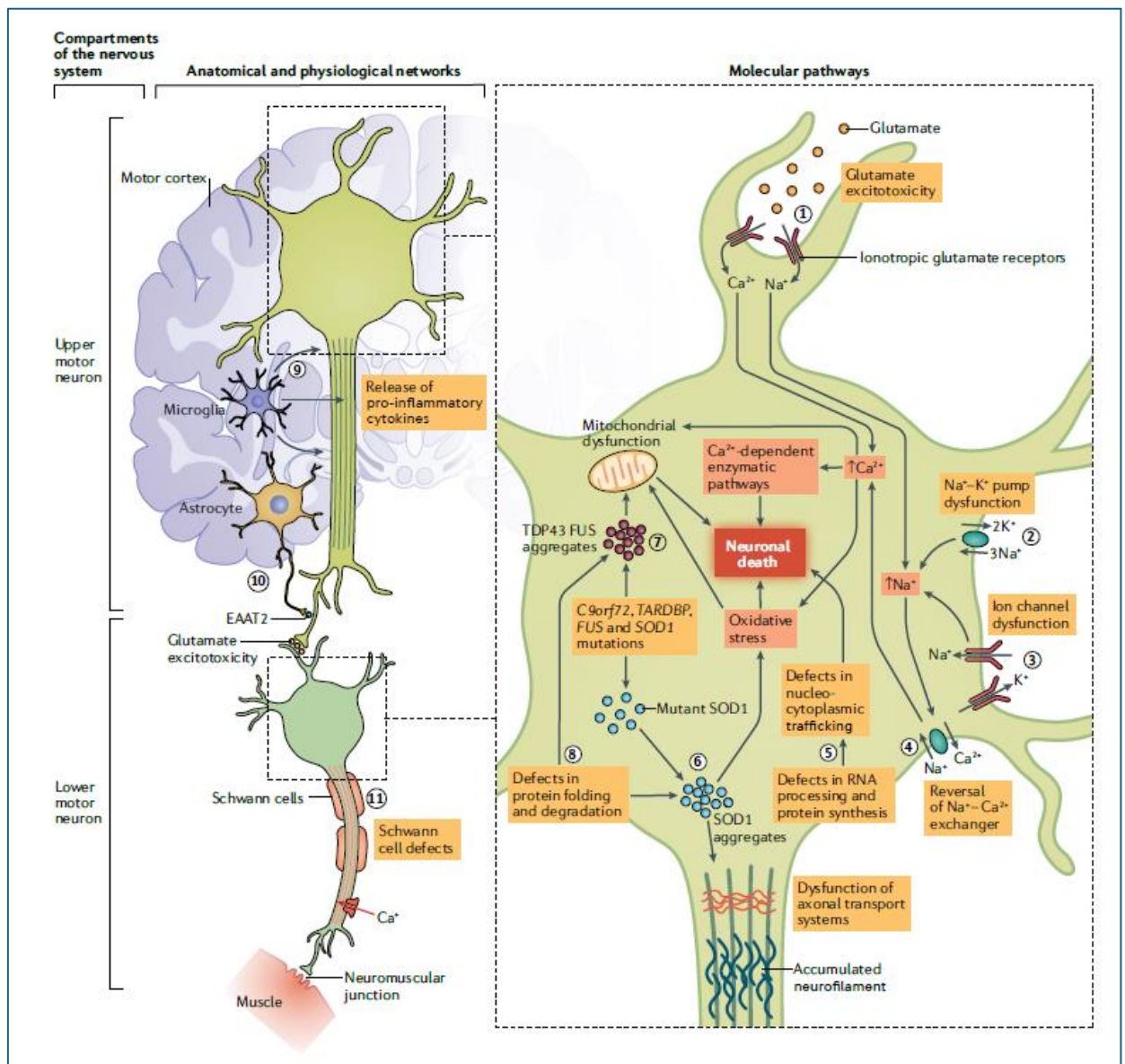


Figure 3. The pathophysiology of ALS From Kiernan et al., 2021

1.4.1 Neuroinflammation, microglia and astrocytes dysfunction

Although the full mechanisms of ALS is not yet fully understood, accumulating evidence suggests that neuroinflammation plays a key role in ALS pathogenesis (Figure 3), with several actors coming on stage in different times (Beers and Appel, 2019a). Indeed, dysfunctional microglia have been implicated in playing a role in ALS, with variable contributions to disease, since its complex pathomechanism (Clarke and Patani, 2020).

Microglia are brain-dwelling macrophages and major parts of the neuroimmune system that broadly contribute to brain development, homeostasis, aging and injury repair in the central nervous system (CNS). They act by providing neuroprotection in normal physiology with sentinel and housekeeping functions. When dysregulated, from a defensive role it becomes detrimental, initiating or exacerbating neurodegeneration (Azam et al., 2021) (Figure 3).

In ALS patients microglial activation has been caught by PET images (Turner et al., 2004; Corcia et al., 2012; Tondo et al., 2020)) and histopathological confirmation (Beers et al., 2017). Furthermore, in transgenic mSOD1^{G93A} mice overexpressing activation of microglia induces proinflammatory cytokine release and aggravates neuronal injury (Frakes et al., 2014). Microglia change their phenotype with disease progression with complex interactions with other immune components. The interplay between ALS and microglial aberrant activation is strengthened by the presence of several mutations that are associated with microglial host defense functions including mSOD1 (ROS production), C9orf72 (cytokines), and C9orf72 and TDP43 (phagocytosis) (Azam et al., 2021).

Theron, novel candidates biomarkers of neuroinflammation have recently emerged in ALS, as linked to microglial cell/macrophage activation and a faster disease progression, including YKL40 - a glycoprotein of the family of chitinase-like proteins - (Illán-Gala et al., 2018), macrophage chemotactic protein-1 (Guo et al., 2017), lipocaline-2 (Petrozziello et al., 2020), SerpinA1 (Wormser et al., 2016), TREM-2 (Cooper-Knock et al., 2017).

Astrocytes, as the largest glial cell components of the CNS, perform vital functions in the maintenance and support of neurons. They contribute to the modulation of inflammatory signaling by releasing proinflammatory or anti-inflammatory molecules and play a crucial role in upholding the integrity of the blood-brain barrier (BBB) (Sofroniew, 2015). In individuals with ALS, astrocytes exhibit a downregulation of the EAAT2 transporter, leading to a less efficient uptake of glutamate from the synaptic cleft. This contributes to excitotoxicity (Pardo et al., 2006). Moreover, several studies have provided evidence of dysfunction in peripheral immune cells, including lymphocytes, monocytes, and macrophages. The activation of monocytes in the peripheral blood of ALS patients, along with increased invasion of peripheral monocytes into the spinal cord, has been linked to motor neuron (MNs) loss. These activated monocytes display decreased phagocytosis, altered adhesion behavior, and impaired secretion of proinflammatory cytokines (Mantovani et al., 2009; Zondler et al., 2016).

CHI3L1 - YKL40

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. Mammalian chitinases have been reported in the context of adaptive T-cell response mediation (Hong et al., 2018), tissue remodelling and repair and oligodendrogenesis (Starossom et al., 2019). CHI3L1 is also produced by reactive astrocytes and is associated with chronic neuroinflammation (Bonneh-Barkay et al., 2012), showing a strong upregulation during the late stages of macrophage differentiation (Di Rosa et al., 2013). In vitro evidence suggests that, at least in ALS, they may act in a “feed-forward” loop that sustains neuroinflammation and exacerbates disease (Gaur et al., 2020). For instance, in a transgenic rat model, TDP-43 induced astrocytic CHI3L1 up-regulation; in turn, synthetic CHI3L1 caused neuronal death in a dose-dependent manner (Huang et al., 2014). Connolly et al. proposed the hypothesis that CHI3L1 may promote neurodegeneration and trigger degenerative diseases of the nervous system, acting as a signaling molecule in neuroinflammatory responses and functional impairments. As research advances, the association between ALS and CHI3L1 begins to emerge, underscoring the promising potential of investigating CHI3L1 as a biomarker for ALS (Connolly et al., 2023).

The first report on chitinases variation in the context of ALS (Varghese et al., 2013) used quantitative mass spectrometry and ELISA-based validation showing cerebrospinal (CSF) levels of CHIT1, CHI3L1, and CHI3L2 significantly elevated in ALS patients relative to healthy controls. This elevation has since been subsequently confirmed by several studies using a range of proteomic and transcriptomic methods (Pagliardini et al., 2015; Andrés-Benito et al., 2018; Illán-Gala et al., 2018; Steinacker et al., 2018; Thompson et al., 2018; Oeckl et al., 2019; Thompson et al., 2019; Abu-Rumeileh et al., 2020b; Gray et al., 2020; Vu et al., 2020; Costa et al., 2021). Globally, ALS patients demonstrated a highly significant elevation in CHI3L1 levels in CSF in comparison to healthy controls, as confirmed by a recent meta-analysis (Xu et al., 2024), and asymptomatic mutation carriers, while serum levels present a more variable behavior in ALS patients. Moreover, increased levels of YKL40 in CSF, expressed in inflammatory astrocytes correlated with disease progression (Andrés-Benito et al., 2018; Gille et al., 2019) and degree of cognitive dysfunction (Thompson et al., 2019). However, chitinases failed to surpass the diagnostic performance of pNfH and NfL (Steinacker et al., 2018; Thompson et al., 2019) and exhibit limited

effectiveness in distinguishing ALS from PLS (Thompson et al., 2018, 2019) or other neurodegenerative disorders (Steinacker et al., 2017; Gille et al., 2019).

SerpinA1

SerpinA1, also known as alpha-1 antitrypsin (AAT) acts as a serine protease inhibitor in the acute phase of inflammation by modulation of proinflammatory cytokine release (Pott et al., 2009), binding to complement C3 (O'Brien et al., 2020) and neutrophil functions (Bergin et al., 2014). From a neurological point of view, a first study in 1998 demonstrated that imbalance in serpins was crucial in neurofilaments conglomerates (a histopathological hallmark for early stages of ALS) and inclusion formation (Chou et al., 1998). More recently its immunomodulation role in neurodegenerative diseases was consolidated since AAT has been involved in microglia polarization, with a shift in microglial phenotype from pro-inflammatory to anti-inflammatory profile (Yang et al., 2018; Zhou et al., 2018), by attenuating microglial activation.

Nonetheless, several pieces of evidence suggest that SerpinA1 may play a role in neurodegeneration. In this regard, overexpression of the protein or its RNA has been described in the brains of patients with Alzheimer's disease (AD) (Gollin et al., 1992) and frontotemporal lobar degeneration (FTLD) (Ebbert et al., 2017). Previous studies documented a higher concentration of CSF SerpinA1 and/or different levels of its charge isoforms in AD, Parkinson's disease, Parkinson's disease dementia, and dementia with Lewy bodies, Creutzfeldt–Jakob disease (CJD) and FTLD in comparison with controls (Nielsen et al., 2007; Jesse et al., 2012; Halbgebauer et al., 2016a; Abu-Rumeileh et al., 2020a).

Notwithstanding these biological bases, only a few studies specifically focused on SerpinA1 determination in ALS patients. Specifically, Brettschneider et al. have included ATT among candidate markers of disease progression in a proteome analysis of CSF in ALS patients, since its overexpression in patients with a fast progression (Brettschneider et al., 2010). Subsequently, Wormser et al. analyzed AAT by ELISA in CSF samples from newly diagnosed ALS patients, finding a statistically significant reduction in mean AAT levels as compared to the age-matched control group (Wormser et al., 2016).

TREM2

TREM2 (triggering receptor expressed on myeloid cells 2) is a cell-surface receptor expressed selectively in myeloid cells. Recent studies have provided new insights about TREM2 biology and microglial activities in aging and neurodegenerative brains, with increasing knowledge mainly in AD pathogenesis. The impaired signalling of TREM2 has been associated with abnormalities in phagocytosis, cytokine production, cell proliferation, and cell survival (Mecca et al., 2018). Loss-of-function mutations in TREM2 have been linked not only to AD (Colonna, 2023) but also in ALS (Cady et al., 2014) where variant p.R47H is considered a significant risk. Furthermore, TREM2 expression was increased in spinal cords from ALS patients (Jauregui et al., 2023; Jericó et al., 2023) and SOD1^{G93A} mice with a negative correlation with survival (Cady et al., 2014). Moreover, up-regulation of microglial activation genes, such as TREM2, has been demonstrated in mice with knocked down *C9Orf72* expression (Lall and Baloh, 2017).

Soluble TREM2 (sTREM2) cleaved from the surface of microglia has been proposed as a biomarker in other neurological diseases including AD, Down Syndrome and multiple sclerosis (MS) (Piccio et al., 2008; Suarez-Calvet et al., 2014; Heslegrave et al., 2016; Weber et al., 2020). To our knowledge, only two studies explored CSF sTREM2 variation in ALS patients (Cooper-Knock et al., 2017; Jericó et al., 2023): a first study revealed significant elevated levels of sTREM2 in CSF, predicting survival when measured in late stages of disease (Cooper-Knock et al., 2017), while Jerico et al. have demonstrated a statistically significant elevation of sTREM2 not only in CSF, but also in the serum of ALS patients compared to controls (Jericó et al., 2023).

S100B

Another candidate indicator of neuroinflammation is S100B, a calcium-binding protein mainly concentrated in astrocytes in the nervous system glial protein and marker for astrogliosis in a variety of neurodegenerative diseases (Steiner et al., 2011), including ALS, where elevated levels of the S100B protein have been identified in patients' neural tissues, particularly in astrocytes (Migheli et al., 1999).

Interestingly, a subpopulation of “aberrant” astrocytes overexpressing S100B and its receptor RAGE has been delineated as potentially involved in neuron toxicity, at least in the

SOD1^{G93A} animal experimental model of ALS (Serrano et al., 2017). The suppression of S100B in astrocytes derived from the SOD1^{G93A} mouse model resulted in the inhibition of several genes that are commonly overexpressed in ALS astrocytes, including TNF- α , C-X-C motif chemokine, chemokine ligand 6, and Glial Fibrillary Acidic Protein. Collectively, these findings suggest a potential role for S100B in the pathogenic processes of ALS. However, the existing correlations between pathological parameters of ALS and S100B levels are currently confined to cultured astrocytic cells. To establish the putative pathogenic role of S100B in ALS definitively, further confirmation through in vivo experiments is essential.

Moreover, conflicting data exist regarding the reliability of S100B as a biomarker in biological fluids for ALS (Otto et al., 1998; Süßmuth et al., 2010; Cao et al., 2017; Steinacker et al., 2017). In particular, S100B was elevated in the CSF and associated with survival time and decreases during progression (Otto et al., 1998; Süßmuth et al., 2010). Serum levels do not differ in ALS and controls but, similar to CSF levels, are reported to decrease during progression (Otto et al., 1998). More recently, Steinacker et al. demonstrated a trend for a relation between reduced S100B serum levels with shorter survival of ALS patients, but the S100B serum levels at the consecutive examinations, did not differ significantly (Steinacker et al., 2017). Similarly, Cao et al. have shown that S100B levels in ALS sera were not different from control groups, nor was S100B altered with disease progression (Cao et al., 2017).

Osteopontin

Osteopontin (OPN) is a phosphoprotein encoded by the SPP1 gene, expressed in various tissues, including nervous tissue, and secreted by various cells such as microglia in the CNS, and macrophages and T lymphocytes in the periphery. Thrombin cleavage of OPN results in the generation of two fragments, N-terminal and C-terminal (Cappellano et al., 2021). The N-terminal fragment primarily interacts with integrins via the arginine-glycine-aspartic acid sequence, while the C-terminal binds to the CD44 receptor expressed by several myeloid cell types, including M1 microglia. The hypothesis of an interaction between OPN and microglia is supported by the expression of several OPN receptors on microglia. Through binding to CD44, OPN can induce the expression of pro-inflammatory genes, while binding to integrins promotes actin remodeling, cell migration, and increased phagocytic activity of

microglia (Rosmus et al., 2022). Accumulating evidence in ALS indicates that OPN may engage in alternative interactions with either harmful (M1) or beneficial (M2) microglia, and these interactions may vary in proportions during different stages of the disease. The overall impact of these interactions, however, remains undetermined (Mousavi et al., 2020; Cappellano et al., 2021). Furthermore, a thorough investigation is necessary to determine whether the expression of OPN in microglia exerts a neurotoxic or neuroprotective influence at different stages of ALS pathogenesis (Rosmus et al., 2022). High OPN expression has been noted in various pathological processes related to blood-brain barrier (BBB) damage, including vascular dementia (Ueno et al., 2016) and ischemic stroke (Spitzer et al., 2022), suggesting a role for OPN in vascular remodeling. However, it remains unclear whether OPN is protective against barrier damage or if it contributes to the damage itself by increasing BBB permeability (Rosmus et al., 2022).

1.4.2 Neuro-axonal loss - Neurofilaments

Neurofilaments, consisting of neurofilament heavy, medium and light chains (pNfH, NfM, and NfL, respectively) and α -internexin, are abundantly expressed in neurons and thought to function in axonal growth and maintenance. Although axonal degeneration is a pathogenetic mechanism not exclusive to ALS and is shared by various neurological diseases (Khalil et al., 2018), there is a consistent association between elevated neurofilaments (NFs) levels in biofluids and ALS, supporting their potential introduction as a promising biomarker for this neurodegenerative disorder (Falzone et al., 2021).

Numerous studies have delved into the prognostic role of NfL and pNfH, consistently highlighting the reliability of both biomarkers in predicting ALS survival when measured in either serum or CSF (Gaiottino et al., 2013; Lu et al., 2015; Gaiani et al., 2017; Steinacker et al., 2017; Rossi et al., 2018; Falzone et al., 2020; Gagliardi et al., 2021). A widely observed trend indicates that NFs levels in both biofluids correlate with the rate of disease progression (Gaiani et al., 2017; Steinacker et al., 2017; Gille et al., 2019; Verde et al., 2019; Falzone et al., 2020) and are most strongly associated with the involvement of the upper motor neuron system (Verde et al., 2019; Zucchi et al., 2020). Correlations between elevated levels of pNFH and NFL are observed in individuals with ALS compared to controls (Zucchi et al., 2020). This association is further highlighted by the increase in NFL levels in presymptomatic individuals carrying an ALS gene, occurring one year before

phenoconversion (Benatar et al., 2018). CSF and serum levels of the NfL correlate tightly with one another and serum assays have increased in sensitivity to offer a simple, non-invasive measure (Verde et al., 2019).

However, most of the prognostic studies on NFs have been monocentric, did not assess pNfH and NfL simultaneously, explored NFs concentration only in one biofluid and lacked a correlation between NFs and disease progression rate longitudinally. A recent study partially overcame these limitations demonstrating that NfL levels in either CSF or serum are the main predictor of the ALSFRS-R slope (Benatar et al., 2019). Moreover, elevated neurofilaments are a common feature in neurodegenerative diseases (Zucchi et al., 2020), raising uncertainty about their standalone diagnostic and prognostic utility (Halbgebauer et al., 2022). In the context of monitoring treatment responses in trials of therapies for ALS, failure of serum NfL levels to decrease during treatment could enable decisions regarding the efficacy of potential therapeutic agents to be made earlier. The impending integration of NFs into clinical practice necessitates the establishment of standardized cut-off values. This standardization is crucial for ensuring consistency and facilitating comparisons between measurements obtained from different laboratories.

1.4.3 Systemic inflammation

Dysregulated inflammatory pathways are a recurring theme in ALS (Beers and Appel, 2019a). Central and peripheral inflammation is present in *C9orf72*, *SOD1*, and *TARDBP* animal models, as well as in patients with familial ALS (fALS). This pathophysiology is characterized by the infiltration of immune cells into the central nervous system, dysregulated peripheral immune cell counts, induction of an activated immune phenotype, and altered cytokine production (Beers and Appel, 2019a). CD8 cytotoxic T cells infiltrate the central nervous system of *SOD1*^{G93A} mutant mice and selectively destroy motor neurons; genetic ablation of this immune cell population slows motor neurodegeneration (Coque et al., 2019). Additionally, mutant *SOD1*^{G93A} CD8 T cells express elevated levels of interferon-gamma, a cytokine linked to ALS progression (Coque et al., 2019). Patients with ALS exhibiting loss of normal *C9orf72* activity due to abnormal repeat expansions lose the ability to regulate interferon production through the innate immune system (cGAS/STING pathway), leading to systemic and central nervous system inflammation mediated by type I interferon (McCauley et al., 2020).

Similarly, elevated production of interferon is associated with TDP-43 pathology in cellular and animal models of ALS. Blocking innate immunity signaling in *TARDBP* mutant mice normalizes interferon levels, slows disease progression, and prolongs survival (Yu et al., 2020). Additionally, less frequent ALS mutations induce inflammation, including *OPTN*, *SQSTM1*, *TBKI*, and *VCP* (Beers and Appel, 2019b). In sporadic ALS (sALS) patients without any known genetic etiology, the mechanism of immune dysregulation in ALS remains uncertain, but include altered peripheral immunity, induction of an activated immune phenotype, and changes in peripheral cytokine levels (Beers and Appel, 2019b).

Concurrent with increased cytotoxic immune cells, several alterations in T lymphocyte populations have been described in ALS patients (De Marchi et al., 2021). Despite the differences among studies on lymphocytes subpopulations, changes in Tregs are more consistent and their levels inversely correlate with the rate of disease progression, supporting that Tregs actively contribute to neuroprotection through their interactions with microglia (Beers et al., 2011; Zhao et al., 2012).

Moreover, innate immunity encompasses diverse types of cells of the myeloid lineage, including dendritic cells, monocytes, macrophages, polymorphonuclear cells, mast cells, innate lymphoid cells, and natural killer (NK) cells (Vivier et al., 2011). On the other hand, adaptive immunity is characterized by the presence of T and B lymphocytes (Vivier et al., 2011). The most straightforward and widely applicable method to measure peripheral immunity is through blood cell counts of different leukocyte types. Additionally, derived ratios like the neutrophil-to-lymphocyte ratio (NLR), monocytes-to-lymphocyte ratio (MLR), the systemic inflammation response index (SIRI), the aggregate index of systemic inflammation (AISI) and systemic immune-inflammation index (SII) are believed to more accurately reflect the strength of peripheral immunity (Hu et al., 2014; Nøst et al., 2021). These new inflammation indexes that integrate neutrophils, monocytes, lymphocytes and platelets, have been proposed as novel systemic inflammation markers with prognostic relevance in patients with malignancy, cardiovascular and inflammatory diseases. Neutrophils, the most abundant type of leukocytes, are frontline cells that mediate the inflammatory response through migration, production of reactive oxygen species, and secretion of neutrophil serine proteases. Additionally, neutrophils can interact with lymphocytes and monocytes in a bidirectional and multicompartmental manner. Neutrophils recruit and activate lymphocytes and monocytes, while T cell and monocyte-derived products can, in turn, activate neutrophils. During systemic inflammation, neutrophil

activation can stimulate the production of certain mediators, such as IL-1 α , which subsequently induces megakaryocytes to produce platelets. Neutrophils and monocytes are involved in the persistent inflammatory response, while lymphocytes play a role in immune regulation. It is well established that platelets are crucial in vascular injury and in the prevention of bleeding. Recently, researchers have suggested that platelets are capable of participating in most stages of inflammation and are a key mediator in the response to circulating immune complexes. Collectively, these cells participate in both innate and adaptive immune responses; therefore, these indexes that include these populations may better reflect the inflammatory state and enhance predictive capabilities. Some studies have proposed that these ratios could serve as systemic biomarkers for ALS prognosis (Choi et al., 2020; Leone et al., 2022; Wei et al., 2022; Grassano et al., 2023) showing that an elevated innate immune response is associated with faster progression and reduced survival.

At the serum level, various immunological pathways can be explored, and disparities in the immune profile between ALS patients and healthy individuals, as well as among ALS patients in distinct disease stages, have been documented. Nonetheless, the concordance between blood and CSF biomarkers remains a subject of debate. In ALS, the dysregulation of plasma inflammatory biomarkers may encompass various interleukins, exhibiting both pro-inflammatory and anti-inflammatory functions.

Some studies have indicated a significant correlation between blood and CSF levels for certain biomarkers, including growth factors, IFN- γ (Guo et al., 2017). Disease progression may be linked to the immunological response to neuronal degeneration, which is reproduced by altered blood levels of cytokines (IL-6 and IL-13, among others), predominantly in ALS faster evolution (Lu et al., 2016; Jin et al., 2020) or by the correlation between a specific inflammatory profile and different ALS subtypes (Olesen et al., 2020). Along with cytokine levels, rising evidence emerges for the complement system to such an extent to be a potential therapy for ALS (e.g. trial with Ravulizumab or Pegcetacoplan): complement dysregulation or upregulation likely occurs early in ALS pathogenesis (Kjældgaard et al., 2018; Parker et al., 2019) and correlates with disease progression (Lee et al., 2018). To date, despite the large number of cytokines and complement that participate neuroinflammation mechanism in ALS, there is no definitive inflammatory biomarker that can be singularly used in clinical evaluation.

2 PURPOSE OF THE RESEARCH

Even if consistent findings in the literature have confirmed NfL as among the most dependable and promising biomarkers for ALS (Gaiottino et al., 2013; Wilke et al., 2016; Verde et al., 2019), studies investigating the utility of NfL in distinguishing ALS from its mimicking disorders have produced varied outcomes, potentially also due to the heterogeneity of the disease (Poesen et al., 2017; Simonini et al., 2021; Verde et al., 2021) along with the lack of specificity of NfL across neurodegenerative conditions (Rossi et al., 2018; Brodovitch et al., 2021).

Moreover, considering neuroinflammation role in ALS progression, even if several inflammatory markers tested have individually demonstrated their potential role as prognostic biomarkers, to date we cannot conclude that a unique CSF/serum biomarker can capture all the pathogenic processes involved in the disease. Accordingly, we embrace the idea that a panel of multiple markers representing different pathogenetic steps should be considered for use in both clinical practice and as a common outcome in clinical trials.

Due to the multitude of postulated mechanisms underlying ALS, we propose to implement a molecular panel longer representative the disease behavior, considering that each detectable biomolecule may act distinctly, reflecting the corresponding pathogenetic mechanism of ALS.

In particular, we aimed to comprehensively investigate the diagnostic value of well-established CSF/serum biomarkers of axonal injury (NfL and pNfH) and microglial and astrocytic activation (Serpina1, TREM2, CHI3L1, OPN and S100B, respectively) as prognostic biomarkers in our ALS population. Then, we evaluated the prognostic performance of neuroinflammation biomarkers in comparison with currently established indicators of neuroaxonal injury, namely neurofilaments.

In a subpopulation of patients, we describe the systemic immune role in ALS patients by analyzing complete blood count- derived parameters, including their behavior in our population and their association with other “neuro”-biomarkers . Finally, we evaluated whether CSF/serum biomarker levels are associated with clinical variables, and the severity of ALS, measured by clinical scores and scales.

3 SUBJECTS AND METHODS

Study population

ALS patients were recruited among residents of Emilia-Romagna region, Northern Italy, who presented to the ALS Center of the Modena University Neurological Department between January 1st, 2007 and December 1st, 2023. Lumbar puncture (LP) was performed as part of the diagnostic work-up and serum and CSF samples were stocked thereafter. Patients were followed up with multidisciplinary approach for a median of 31 months. Eligible participants were considered all those patients who received a diagnosis of definite or probable ALS according to El Escorial criteria-revised criteria (Brooks et al., 2000), who still had at least 0.5 mL of CSF and serum available for analysis, stocked in Neurobiobank of Modena. ALS diagnosis has been confirmed by subsequent application of the Gold Coast Criteria (Shefner et al., 2020), after reviewing the medical histories of each individual patient.

Standard Protocol Approvals, Registrations, and Patient Consents

This retrospective study was performed according to the Declaration of Helsinki and prior approval from the Ethical Committee of Area Vasta Emilia Nord (protocol number (RF-2021-12373036) was obtained. All participants signed written informed consent for LP procedure and the permission to use their stocked biological specimen for future research studies.

Clinical measures

We collected the following demographic and anthropometric variables for each patient: sex, age at onset and at sampling, height, healthy weight, weight and BMI at sampling, weight loss at sampling, civil status (single, common-in-law, married, widow(e), divorced, not reported).

Then, we considered the following clinical variables: site of onset (bulbar, upper limb or lower limb, respiratory), phenotype (flail, UMNp, bulbar, respiratory, classic) (Chio et al., 2011), spasticity in most affected limb (according to Ashworth's scale) (Ashworth, 1964), the presence of pseudobulbar affect, frontal release signs (palmomental reflex, glabellar

reflex, snout reflex, masseter reflex). Corticospinal involvement at brain MRI was assessed in 147 patients as the presence of corticospinal tract hyperintensity in FLAIR or diffusion tensor images. Motor Evoked Potentials (MEPs) were assessed in 151 patients and the presence of prolongation of central conduction time was considered as a sign of corticospinal involvement. All but one of the patients started Riluzole treatment after ALS diagnosis.

We dated the disease onset based on patients' referral of a distinct motor impairment in one district, excluding prodromal signs and symptoms as cramps, fasciculations or mild motor impairment (Benatar et al., 2022). Cognitive and behavioural impairment in the frontotemporal dementia disease spectrum (ALS with behavioural impairment – ALSbi - , ALS with combined cognitive and behavioural impairment – ALScbi - , ALS with cognitive impairment – ALS – ci, FTD) were evaluated according to Strong criteria (Strong et al., 2017).

Genetic analysis was available for 175 patients; 121 samples were analyzed by PCR Sanger sequencing for *SOD1*, *FUS*, *TARDBP* mutations and *C9ORF72* expansions (Chio et al., 2012), while for 54 patients a next generation sequencing (NGS) panel of 78 genes was performed as described elsewhere (Pensato et al., 2020).

The following clinical variables were considered as surrogates of disease progression: time to generalization, described as the time between onset and spreading of clinical signs from spinal or bulbar localization to both (Tortelli et al., 2016); diagnostic latency (time in months between referred onset of motor symptoms and date of diagnosis); respiratory function as assessed by FVC at sampling; time to non-invasive ventilation (NIV), time to invasive ventilation (IV), time to percutaneous endoscopic gastrostomy (PEG); King's ALS staging and ALS Milano-Torino Staging (ALS-MiToS) at sampling and at last observation; disease progression rate (DPR) calculated as monthly decline in ALSFRS-R score from onset to diagnosis assuming a total score of 48 at onset (Kimura et al., 2006) and at the time of the sampling and at the last observation, monthly decline of the ALSFRS-R from sampling to last observation (Decline FRS) (Mandrioli et al., 2015). Patients were categorized in different classes of progressors on the basis of DPR at sampling: slow progressors were defined as those patients with a Delta FS inferior to 0.3 points/month, while fast progressors had a DPR superior to 1 point/month; intermediate progressors had DPR values in between. Next, we identified a subcategory of participants crossing DPR at sampling and last observation: very slow progressors were defined as those patients with a Delta FS inferior to 0.3 points/month

at sampling and last observation, while very fast progressors had a DPR superior to 1 point/month not only at sampling but also at last observation; intermediate progressors had DPR values in between. Survival was defined as the timespan between disease onset and date of death/tracheostomy. Moreover, other comorbidities or conditions were considered and categorized as concomitant depression or psychosis, parkinsonism, hypertension, dyslipidemia, cardiopathies, chronic obstructive pulmonary disease (COPD) and other respiratory diseases, diabetes mellitus, autoimmune diseases, oncological history, previous trauma, past or actual tobacco use and concomitant statins.

Sample collection and laboratory assays

Serum and CSF samples were obtained by venipuncture and lumbar puncture, respectively, and processed following standard procedures. After samples centrifugation for 10 minutes at 1300 x g, the supernatant was divided into aliquots and stored in polypropylene tubes at -80°C until analysis.

Determination of pNfH, NfL, CHI3L1, SerpinA1, OPN, Cytokines, S100B levels and hematology parameters

SerpinA1, Neurofilament Light Chain (NfL), Neurofilament Heavy Chain (NfH), chitinase-3-like-1 (CHI3L1), Osteopontin (OPN), and a cytokines panel (IL-1b, IL-10, IL-2, IL-18, CCL-2, IFN γ , TNF α , and IL-6) were determined using an automated next generation ELISA, via Ella Simple Plex assay technology (BioTechne, ProteinSimple, San Jose, California, 95134 USA) following the manufacturers' instructions as previously described (Simonini et al., 2021; Martinelli et al., 2024). In this immunoassay samples run through a channel each composed of three Glass Nano Reactors coated with a capture antibody. In this way the sample is automatically read in triplicate. The samples were loaded into the cartridge with the following dilutions: 1:200.000 for serum and 1:10.000 for CSF for SerpinA1 cartridge; 1:2 for both serum and CSF for NfL cartridge; 1:2 for serum and 1:4 for CSF for pNfH cartridge; 1:10 for serum and 1:100 for CSF for CHI3L1 cartridge; 1:100 for both serum and CSF for OPN; 1:10 for both serum and CSF for TREM2. For the cytokines panel, a customized cartridge was designed with the eight cytokines described above in order to load the serum and CSF sample only once with a 1:2 dilution and thus reduce the variability of

the data. Intra-assay and inter-assay variability are evaluated by the manufacturer: for the first each control was tested 16 times in one assay, while for inter-assay the replicates of each control were tested in multiple assays performed by at least three technicians using two batches of reagents.

Protein S100B concentrations were measured with an automated chemiluminescence immunoassay for the quantitative determination of S-100B protein in human serum and CSF (LIAISON S100/DiaSorin S.p.A.). The LIAISON® S100 test is based on the use of paramagnetic particles coated with two monoclonal antibodies and a conjugated monoclonal antibody labeled with an isoluminol derivative. The test had a detection limit at serum concentrations of 0.02 µg/L. Intra-assay and inter-assay variability are evaluated by the manufacturer. The reported range in 95% of healthy subjects is <0.15 µg/L for serum and <2.7 µg/L for CSF.

Blood samples were analyzed at the CoreLab laboratory using DxH 900 (Beckman Coulter, Brea, CA, USA), a fully automated hematology analyzer for white blood cell (WBC) counts and five-part leucocyte differential counting, and red blood cell (RBC) counts. The analyzer uses volume, conductivity, and scatter parameters for controlled flow cytometric analysis of WBC differential. The analytical software is self-gating and separates WBC populations by automatic logic pathways. All analytical procedures were conducted in accordance with the manufacturer's manual. Further we derived from complete blood count five ratios, including Neutrophil-to-lymphocyte ratio (NLR) (neutrophils/ lymphocytes), Monocyte-to-lymphocyte ratio (MLR) (monocytes/lymphocytes), systemic inflammation response index (SIRI) (neutrophil count* monocyte count/lymphocyte count), Aggregate Systemic Inflammation Index (AISII) (neutrophils*monocytes*platelets/lymphocytes) and systemic-immune-inflammation index (SII) (neutrophils*platelets/lymphocytes).

Statistical methods

Continuous variables were reported as means (standard deviations, SD) or medians [interquartile ranges, IQR], according to the distribution, categorical variables as absolute numbers (%). We evaluated clinical and demographic features according to gender, phenotype and genotype performing 2-tailed t-tests and ANOVA for continuous variables between 2-groups or multiple groups, respectively, and chi-square test for comparisons between categorical variables. A p-value <0.05 was considered as statistically significant.

Comparisons between two or multiple groups were performed by Mann-Whitney U test and Kruskal-Wallis test with post-hoc Dunn correction for pairwise comparisons, respectively, with statistical significance set at $p < 0.05$ for each biomarker. The influence of age, gender, tobacco consumption, CSF conservation time on SerpinA1, NfL, pNfH, CHI3L1 within ALS patients and controls was priorly tested by one-way ANOVA and Spearman/Pearson correlation test with a 2-tailed significance level set at 0.05. Correlations between each biomarker and variables related to ALS severity and progression (diagnostic latency, ALSFRS-r at time of sampling, FVC, DPRsa, DPRlo, time to generalization) were assessed using Spearman's test.

Survival (recorded in months from the referred onset of disease to death/tracheostomy, and alive patients censored at last observation available/30th March 2024) was assessed first with univariate analysis using a Cox proportional hazards regression model and next multivariable analysis with stepwise backward selection and a retention criterion set at $p < 0.10$ with the variables priorly tested which reached a threshold significance level ($p < 0.1$). Hazard ratios (HR) were calculated for each variable with 95% CI. Since SerpinA1 in serum and CSF tended to have an opposite effect on survival, a SerpinA1 ratio was calculated dividing SerpinA1 concentrations in serum over CSF. When biomarkers were previously shown to be influenced by some demographic or anthropometric measures, these were included as covariates for adjusting the univariate Cox analysis.

Since sex-specific differences in prognosis according to the inflammatory status have been recently demonstrated (Grassano et al., 2023), the interaction between each single biomarker and sex was tested in a Cox regression model. Similarly, the effect of age on the prognostic value of each biomarker was tested by computing the interaction between each age class (younger than 65, between 65-75, and older than 75-years old, as in Grassano et al., 2023) and each biomarker. Kaplan-Meier curves and log-rank tests were next employed to graphically display survival analysis results and test cut-offs for each prognostic biomarker, respectively. Data analysis was performed using the STATA statistical package 18 (StataCorp. 2023. College Station, TX: StataCorp LLC).

Data availability statement

Anonymized data not published within this dissertation will be available upon request from any qualified investigator.

4. RESULTS

4.1 Participants' characteristics according to sex and type of onset

Based on the inclusion criteria, the CSF and serum aliquots of 182 ALS patients were available for analysis.

The principal clinical features of these patients according to sex and type of onset are reported in **Tables 1a** and **1b**, respectively.

Table 1a. Clinical characteristics according to sex.

Variable	Female (n=67), n (%), mean [SD]	Male (n=115), n (%), mean [SD]	p-value
Age at onset, years	59.59 [11.84]	57.16 [12.26]	0.192
Age at sampling, years	60.87 [11.86]	58.54 [12.41]	0.216
Diagnostic delay, months	11.29 [11.11]	10.40 [12.1]	0.620
Weight loss at sampling (%)	0.04 [0.09]	0.04 [0.07]	0.886
BMI at sampling, kg/m ²	24.62 [5.03]	24.44 [3.15]	0.774
ALSFRS-r total score at sampling, points	39.69 [5.99]	40.62 [6.71]	0.345
Time to generalization, months	16.25 [13.62]	19.71 [21.6]	0.244
DPR at sampling*, points/month	1.22 [2.03]	0.85 [1.01]	0.100
FVC at sampling, %	91.12 [23.88]	92.31 [22.83]	0.744
King's staging at sampling 1, 2, 3, 4	28 (41.8) / 21 (31.3) / 13 (19.4) / 5 (7.5)	51 (44.3) / 44 (38.3) / 13 (11.3) / 7 (6.1)	0.290
MiTos score at sampling 0, 1, 2, 3, 4	54 (80.6) / 11 (16.4) / 1 (1.5) / 1 (1.5) / 0 (0.0)	101 (87.8) / 12 (10.4) / 0 (0.0) / 0 (0.0) / 2 (1.7)	0.195

Civil status Single, common-in-law, married, widow(e), divorced, not reported	5 (7.5) / 5 (7.5) / 45 (67.2) / 2 (2.9) / 2 (2.9) / 8 (11.9)	14 (12.2) / 6 (5.2) / 79 (68.3) / 2 (2.6) / 5 (4.3) / 8 (6.9)	0.844
Mutational status WT/C9/SOD1/other /not performed	55 (82.1) / 7 (10.4) / 1 (1.5) / 2 (2.9) / 2 (2.9)	95 (82.6) / 6 (5.2) / 5 (4.3) / 4 (3.5) / 5 (4.3)	0.443
Site of onset Bulbar, Spinal,	19 (28.4) / 48 (71.6)	24 (20.9) / 91 (79.1)	0.251
Phenotype Flail, UMNp, Bulbar, Respiratory, Classic	9 (13.4) / 3 (4.5) / 16 (23.9) / 1 (1.5) / 38 (56.7)	20 (17.4) / 7 (6.1) / 19 (16.5) / 2 (1.7) / 67 (58.3)	0.765
FTD ALS-ci / ALS-bi	4 (5.9) 6 (8.9) / 6 (8.9)	12 (10.4) 16 (13.9) / 13 (11.3)	0.305 0.329 / 0.627
CS hyperintensity Present /absent/ not reported	12 (17.9) / 39 (58.2) / 16 (23.9)	20 (17.4) / 67 (58.3) / 19 (16.5)	0.706
MEPs prolongation Present /absent/ not reported	37 (55.2) / 14 (20.9) / 16 (23.9)	72 (62.6) / 28 (24.3) / 15 (13.1)	0.943
NIV	37 (55.2)	67 (28.3)	0.690
PEG	33 (49.3)	50 (43.5)	0.451
IV	19 (28.4)	40 (34.8)	0.372
Time to NIV, months	29.55 [3.75]	34.62 [3.39]	0.346
Time to PEG, months	27.41 [3.22]	36.87 [4.62]	0.135
Time to IV, months	36.08 [5.41]	47.45 [5.76]	0.220

Legend Data are represented as the median (IQR; range) or mean [SD]. **Abbreviations:** ALSFRS-r= Amyotrophic Lateral Sclerosis Functional Rating Scale- revised; BMI: Body Mass Index; CS: Corticospinal; MEPs = Motor Evoked Potentials, UMN-p: Upper Motor Neuron predominant; FTD: frontotemporal dementia; FVC: forced vital capacity; PEG: percutaneous endoscopic gastrostomy; NIV: non-invasive ventilation; IV: invasive ventilation; SD: standard deviation. *Progression rate at sampling is calculated as monthly decline of ALSFRS-R score assuming a total score of 48 at onset. Data on FVC at sampling was available for 65 female patients and 108 males. Data on time to generalization was available for 177 patients (66 women and 111 men). Genetic analysis was performed for 175 patients (65 females and 110 males). Data on MEP prolongation were collected for 51 females and 100 males, while brain MRI (for CS hyperintensity) was performed for 51 females and 96 males.

Demographic and clinical data from our patient cohort show no significant differences for any of the variables analysed, when considering sex. Moreover, data reveal some differences between women and men without reaching statistical significance, such as mean age at onset and at sampling (older in women, 59.59 ± 11.84 vs 57.16 ± 12.26 years, $p=0.192$; 60.87 ± 11.86 vs 58.54 ± 12.41 years, $p=0.216$), diagnostic delay (11.29 ± 11.1 vs 10.4 ± 12.1 months, $p=0.620$, slightly longer in women), time to generalization (16.25 ± 13.62 vs 19.7 ± 21.6 months, $p=0.244$, longer in men).

While the ALSFRS-r at sampling was higher in men (40.62 ± 6.71 vs 29.69 ± 5.99 points, $p=0.345$) and cognitive impairment was more frequently reported in men (FTD 10.4% vs 5.9%, $p=0.305$, ALS-ci 13.9% vs 8.9%, $p=0.329$, ALS-bi 11.3% vs 8.9%, $p=0.627$), indicating a more severe form of disease, the DPR at sampling was higher among women (1.22 ± 2.03 vs 0.85 ± 1.01 points/month, $p=0.100$). Consistently, *C9ORF72* expansion, was significantly more frequent in female patients analyzed (10.4% vs 5.2%) contrary to the distribution of *SOD1* mutations (more frequent in men, 4.3% vs 1.5%). The corticospinal involvement estimated by CS hyperintensity at MRI and by MEPs prolongation was similar for the two groups, as reported in **Table 1a**.

Then, data outline some differences between women and men in NIV and IV utilization, and PEG positioning. Specifically, 55.2% of women underwent NIV compared to only 28.3% of men ($p=0.690$) while forty men (43.8%) and nineteen women (28.4%) underwent IV through tracheostomy ($p=0.372$) with slightly shorter times for women (28.55 ± 3.75 vs 34.62 ± 3.39 months, $p=0.346$ for NIV; 36.08 ± 5.41 vs 47.45 ± 5.76 months, $p=0.220$, for IV). The percentage of patients undergoing PEG was similar among men and women (43.5% and 49.3% respectively, $p=0.451$) but the time to PEG was longer for men (36.87 ± 4.62 vs 27.41 ± 3.22 months, $p=0.135$).

Regarding co-pathologies, the frequency in the two groups is equivalent except for oncological diseases, which were more frequent in women (62.5 % vs 37.5%, $p=0.027$). Phenotypes were distributed uniformly among men and women, but the bulbar phenotype was more frequent in women (24% vs 17%) (**Figure 4**).

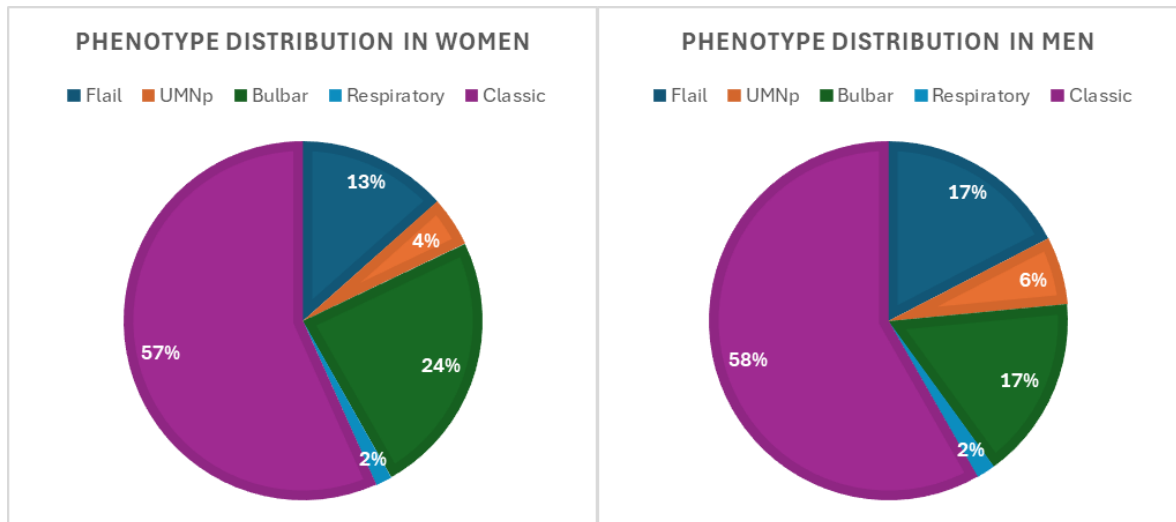


Figure 4. Phenotypic distribution in our ALS population, according to sex.

King's staging at sampling were similar between women and men, as represented in **Figure 5**, although we notice a tendentially higher percentage of female patients in stages 3 and 4 (27% vs 17%), which indicates a higher level of disease severity.

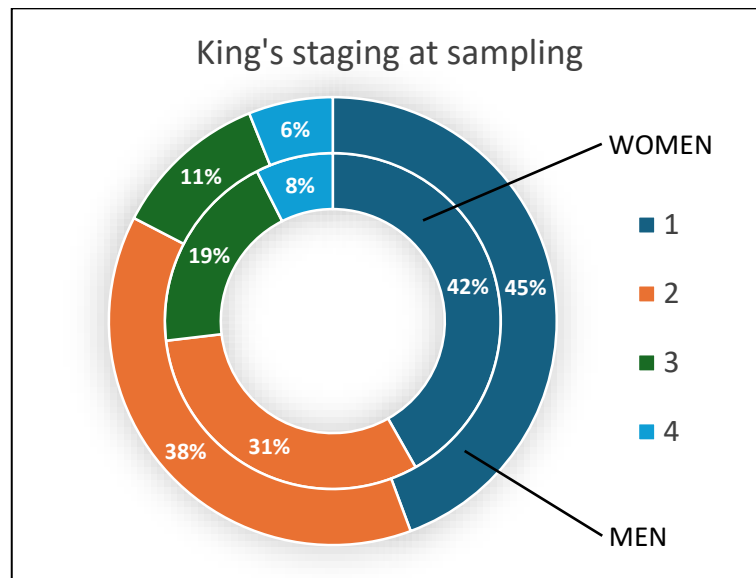


Figure 5. Distribution of King's stages at sampling according to sex

Similarly, the MiToS score at sampling represented in **Figure 6**, showed a tendentially higher percentage of female patients in stages 2 and 3 (19% vs 10%), while for two male patients already undergoing tracheostomy, corresponding to stage 4, only serum specimens were available.

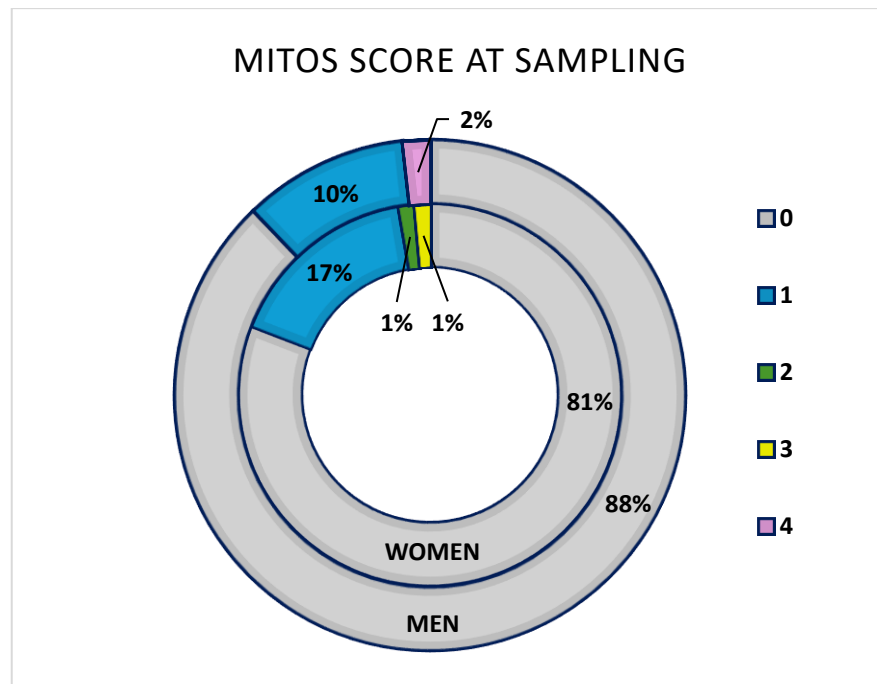


Figure 6. Distribution of MiToS scores at sampling according to sex.

Patients' features according to site of onset (spinal vs bulbar) are summarized in **Table 1b**.

Table 1b. Clinical characteristics according to onset.

Variable	Bulbar Onset (n=43), n (%), mean [SD]	Spinal Onset (n=139), n (%), mean [SD]	p-value
Age at onset, years	60.02 [11.72]	57.44 [12.26]	0.225
Age at sampling, years	60.89 [11.62]	58.93 [12.41]	0.361
Diagnostic delay, months	8.53 [7.93]	11.40 [12.61]	0.161
Onset to sampling, months	10.43 [8.12]	17.87 [26.78]	0.075
Height, m	1.70 [0.96]	1.70 [0.85]	0.812
Weight at sampling, kg	70.05 [12.27]	71.18 [13.29]	0.621
Healthy weight, kg	74.23 [10.31]	73.53 [10.51]	0.769
Weight loss at sampling (%)	0.06 [0.08]	0.04 [0.07]	0.102
BMI at sampling, kg/m ²	24.16 [3.57]	24.62 [4.05]	0.502
ALSFRS-r total score at sampling, points	42.74 [4.19]	39.52 [6.84]	0.004
Time to generalization, months	13.74 [8.93]	19.87 [21.09]	0.068
DPR at sampling, points/month	0.69 [0.69]	1.07 [1.63]	0.136
FVC at sampling, %	87.3 [19.21]	93.24 [24.13]	0.156
King's staging at sampling	1.58 [0.88]	1.92 [0.90]	0.031
MiTos score at sampling	0.02 [0.15]	0.25 [0.65]	0.024
Civil status Single, common-in-law, married, widow(e), divorced, not reported	3 (6.9) / 4 (9.3) / 28 (65.1) / 2 (4.6) / 2 (4.6) / 4 (9.3)	16 (11.5) / 7 (5.1) / 96 (69.1) / 3 (2.1) / 5 (3.6) / 12 (8.6)	0.634
Mutational status † WT/C9/SOD1/other /not performed	36 (83.7) / 4 (9.3) / 0 (0.0) / 2 (4.6) / 1 (2.3)	114 (82.0) / 9 (6.5) / 6 (4.3) / 4 (2.9) / 6 (4.3)	0.474
FTD	6 (13.9)	10 (7.2)	0.171

ALS-ci / ALS-bi	10 (23.3) / 8 (18.6)	9 (6.5) / 14 (10.1)	0.01 / 0.123
CS hyperintensity Present /absent/ not reported	4 (9.3) / 30 (69.8) / 9 (20.9)	28 (20.1) / 85 (61.2) / 26 (18.7)	0.107
MEPs prolongation Present /absent/ not reported	19 (44.2) / 13 (30.2) / 11 (25.6)	90 (64.7) / 29 (20.9) / 20 (14.4)	0.068
NIV	18 (41.86)	86 (61.88)	0.020
PEG	28 (65.12)	55 (39.57)	0.003
IV	14 (32.56)	45 (32.37)	0.982
Time to NIV ‡, months	28.04 [3.45]	33.88 [3.03]	0.380
Time to PEG §, months	24.29 [2.44]	37.60 [4.39]	0.041
Time to IV ¶, months	38.38 [4.71]	45.47 [5.45]	0.488

Legend Data are represented as the median (IQR; range) or mean [SD]. **Abbreviations:** ALSFRS-r= Amyotrophic Lateral Sclerosis Functional Rating Scale- revised; BMI: Body Mass Index; CS: Corticospinal; MEPs = Motor Evoked Potentials, UMN-p: Upper Motor Neuron predominant; FTD: frontotemporal dementia; FVC: forced vital capacity; PEG: percutaneous endoscopic gastrostomy; NIV: non-invasive ventilation; IV: invasive ventilation; SD: standard deviation. *Progression rate at sampling is calculated as monthly decline of ALSFRS-R score assuming a total score of 48 at onset. Data on FVC at sampling was available for 40 patients with bulbar onset and 133 patients with spinal onset. Data on time to generalization was available for 177 patients (42 with bulbar onset and 135 with spinal onset). Genetic analysis was performed for 175 patients (42 with bulbar onset and 133 with spinal onset). Data on MEPs prolongation were collected for 32 patients with bulbar onset and 119 with spinal onset, while brain MRI (for CS hyperintensity) was performed for 34 patients with bulbar onset and 113 with spinal onset.

Bulbar onset was associate with a shorter time from onset to sampling (10.43 ± 8.12 months vs 17.87 ± 26.78 months, $p=0.075$) and to generalization (13.74 ± 8.93 vs 19.87 ± 21.09 , $p=0.068$). Significantly, ALSFRS-r total score at sampling in women was higher than in men (42.74 ± 4.19 vs 39.52 ± 6.84 points, $p=0.004$), along with a tendentially lower DPR (0.69 ± 0.69 vs 1.07 ± 1.63 points/month, $p=0.136$).

Considering the site of onset, data show some differences, which are mean age at onset and at sampling (older in bulbar form, 60.02 ± 11.72 vs 57.44 ± 12.26 years, $p=0.225$; $60.89 \pm$

11.62 vs 58.93 $\pm$ 12.41 years, $p=0.361$), diagnostic delay (longer for patient with spinal onset, 11.4 $\pm$ 12.61 vs 8.53 $\pm$ 7.93 months, $p=0.161$), time to generalization (shorter for bulbar onset, 13.74 $\pm$ 8.93 vs 19.87 $\pm$ 21.09 months, $p=0.068$). Interestingly, patients with bulbar onset do not show a more drastic weight loss at sampling (0.06 $\pm$ 0.08 vs 0.04 $\pm$ 0.07 in %, $p=0.102$) neither a lower BMI (24.16 $\pm$ 3.57 vs 24.62 $\pm$ 4.05 Kg/m², $p=0.502$).

As expected, PEG positioning was more frequent for patients with bulbar onset (65.12% vs 39.57%, $p=0.003$) with shorter times (24.49 $\pm$ 2.44 vs 37.60 $\pm$ 4.39 months, $p=0.041$). Regarding respiratory function, although FVC at sampling was higher in spinal form (93.24% $\pm$ 24.13 vs 87.3 % $\pm$ 19.21, $p=0.156$), NIV positioning was more frequent for this group (61.88% vs 41.86%, $p=0.020$) with longer time for NIV (33.88 $\pm$ 3.03 months vs 28.04 $\pm$ 3.45, $p=0.380$) and IV (45.47 $\pm$ 5.45 vs 38.38 $\pm$ 4.71 months, $p=0.488$).

Then, the comorbidities were similarly distributed between the two groups (data not shown). CS hyperintensity at MRI was more frequently reported in patients with spinal onset (20.1% vs 9.3%), as well as MEPs prolongation (64.7% vs 44.2%).

Patients with cognitive impairment were more common in bulbar onset group (FTD 10.4% vs 5.9%, $p=0.305$, ALS-ci 13.9% vs 8.9%, $p=0.0329$, ALS-bi 11.3% vs 8.9%, $p=0.627$). Furthermore, *SOD1* mutations have been found only in six patients with spinal onset.

Finally, King's stage and MiToS score at sampling were higher among patients with spinal onset (1.92 $\pm$ 0.90 vs 1.58 $\pm$ 0.88, $p=0.031$; 0.25 $\pm$ 0.65 vs 0.02 $\pm$ 0.15, $p=0.024$), indicating a more widespread involvement and a more severe functional impairment for patient with spinal onset.

4.2 Biomarkers distribution according to demographic and clinical features

When accounting sex, neither CSF/serum pNfH, CSF NfL, serum SerpinA1, nor CSF or serum CHI3L1 were different among ALS patients, as shown in **Table 2a**.

On the other hand, CSF levels of SerpinA1 were significantly lower in female patients [4.43 ug/ml (IQR 2.76-6.33 ng/ml) vs 5.61 ug/ml (IQR 3.39-9.16 ng/ml), $p=0.032$]. Further, serum NfL levels were slightly lower among males [11 pg/ml (IQR 70.4-166 pg/ml) vs 131.8 pg/ml (IQR 93.9-185 pg/ml), $p=0.091$]. Interestingly, SII index was significantly different between the two classes, being higher in female [540.67 (IQR 363.05-807.24) vs 384.89 (307.5-578.52), $p=0.015$).

Then, TREM2 levels in CSF and serum specimens revealed that CSF TREM2 were slightly lower in women [20.22 ng/ml (IQR 15.38-17.8 ng/ml) vs 21.15 ng/ml (IQR 16.35-29.85 ng/ml), $p=0.088$] while serum TREM2 were tendentially higher in females [24.13 ng/ml (IQR 16.36-30.4 ng/ml) vs 18.8 ng/ml (IQR 14.8-26.7 ng/ml), $p=0.054$]. Conversely, neither serum/CSF OPN or S100 were differently distributed between the two groups.

Table 2a. Biomarkers distribution according to sex

Biomarker	N	Female median (IQR)	N	Male median (IQR)	p- value
SerpinA1_{CSF} ug/ml	49	4.43 (2.76 - 6.33)	90	5.61 (3.39 - 9.16)	0.032
SerpinA1_{serum} ug/ml	65	1065.54 (765.71 - 1311.83)	113	1043.76 (665.18 - 1597.31)	0.946
NfL_{CSF} pg/ml	50	5620.5 (3991-10987)	88	6561 (3302-11780.5)	0.845
NfL_{serum} pg/mL	66	131.8 (93.9 - 185)	109	111 (70.4 - 166)	0.091
pNfH_{CSF} pg/mL	50	5350 (3097 - 7307)	92	4601 (2741-9305)	0.723
pNfH_{serum} pg/mL	67	1534 (689 - 2596)	114	1454 (680-1841)	0.729
CHIT3L1_{CSF} ng/mL	50	104.46 (54.45 - 175.09)	89	106.11 (64.59 - 183.15)	0.527
CHIT3L1_{serum} ng/ml	67	48.46 (32.89 - 86.76)	114	41.35 (30.24 - 66.34)	0.210
TREM2_{CSF} ng/ml	33	20.22 (15.38 - 27.8)	56	21.15 (16.35-29.85)	0.088
TREM2_{serum} ng/ml	37	24.13 (16.36 - 30.4)	61	18.8 (14.8 - 26.7)	0.054
OPN_{CSF} ng/ml	22	177.01 (130.25 - 280.02)	36	165.40 (106.77 - 283.09)	0.328
OPN_{serum} ng/ml	22	46.68 (290.3 - 60.31)	36	37.06 (28.54 - 63.02)	0.369
S100_{CSF} ug/ml	24	1.09 (0.81-1.53)	44	1.32 (0.81-1.98)	0.359
S100_{serum} ug/ml	24	0.06 (0.04-0.07)	47	0.05 (0.04 - 0.06)	0.148
Leucocytes x 10⁹/L	42	6.62 (5.81 - 7.9)	71	6.2 (5.3 - 7.2)	0.260
PLT x 10⁹/L	42	256.5 (201 - 304)	71	212 (178 - 260)	0.005
CRP mg/L	36	0.5 (0.5-0.5)	48	0.5 (0.5-0.5)	0.554
Neutrophils x 10⁹/L	41	4.14 (3.33-4.94)	71	3.53 (3.16-4.64)	0.132
Lymphocytes x 10⁹/L	40	1.91 (1.5-2.29)	66	1.9 (1.51-2.25)	0.848
Monocytes x 10⁹/L	40	0.52 (0.42-0.61)	66	0.54 (0.45-0.61)	0.562
Eosinophils x 10⁹/L	40	0.14 (0.09-0.24)	66	0.16 (0.09-0.22)	0.715
Basophils x 10⁹/L	40	0.03 (0.025-0.05)	66	0.03 (0.03-0.04)	0.867
NLR	40	2.1 (1.65-2.95)	66	2.02 (1.57-2.47)	0.295
MLR	40	0.27 (0.21-0.34)	66	0.28 (0.23-0.34)	0.516
SIRI	40	1.13 (0.78-1.61)	66	1 (0.76-1.39)	0.456
AISI	40	300.83 (173.56-470.56)	66	218.94 (148.36-325.75)	0.075
SII	40	540.67 (363.05-807.24)	66	384.89 (307.5-578.52)	0.015

Abbreviations: AISI : aggregate index of systemic inflammation; CRP : C-reactive protein; Hb: haemoglobin; NLR : neutrophil to lymphocyte ratio, MLR : monocyte to lymphocyte ratio; PLT : platelets; RBC : Red Blood Cells; SIRI : systemic inflammation response index; SII : systemic immune-inflammation index; WBC : White Blood Cells

Next, as regards the type of onset (**Table 2b**), the distribution of all biomarkers was comparable between the two groups, except for the pNfH value in the CSF, which was significantly lower in patients with bulbar onset [3638.5 pg/mL (IQR 2545.5-6318.5 pg/mL) vs 5931.5 (IQR 3097-9228 pg/mL), $p=0.022$]. Moreover, we observed that several subpopulations of lymphocytes were differently distributed between patients with bulbar or spinal onset: monocytes were lower in patients with bulbar onset [$0.43 \times 10^9/L$ (IQR 0.36-0.5) vs $0.55 \times 10^9/L$ (IQR 0.46-0.64), $p=0.001$] as well as eosinophil [$0.1 \times 10^9/L$ (IQR 0.08-0.15) vs $0.16 \times 10^9/L$ (IQR 0.1-0.28), $p=.046$] and basophils [$0.03 \times 10^9/L$ (IQR 0.02-0.03) vs 0.03-0.05), $p=0.007$]. Likewise, SIRI and AISI indexes of systemic inflammation were statistically lower among patients with bulbar onset respect to patients with spinal onset [For SIRI: median 0.88 (IQR 0.64-1.08) vs 1.13 (IQR 0.84-1.49), $p=0.02$; for AISI: median 177.3 (IQR 131.3-263.7) vs 248.67 (IQR 156.5-369.4), $p=0.04$].

Table 2b. Biomarkers distribution according to onset.

Biomarker	N	Bulbar onset median (IQR)	N	Spinal onset median (IQR)	p-value
SerpinA1 _{CSF} ug/ml	31	4.49 (3.39-7.14)	108	5.22 (2.89-8.29)	0.678
SerpinA1 _{serum} ug/ml	40	827.19 (681.59-1256.76)	138	1090.43 (683.10-1525.75)	0.163
NfL _{CSF} pg/ml	32	4924.5 (3540.01-7152.09)	106	6680 (3526-12398)	0.141
NfL _{serum} pg/mL	41	127 (70.4-173)	134	119.19 (80.3-183)	0.875
pNfH _{CSF} pg/mL	32	3638.5 (2545.5-6318.5)	110	5931.5 (3097-9228)	0.022
pNfH _{serum} pg/mL	42	1626 (611-2841)	139	1454 (710-2725)	0.712
CHIT3L1 _{CSF} ng/mL	31	86.45 (48.87-147.84)	108	114.58 (65.26-189.59)	0.105
CHIT3L1 _{serum} ng/ml	42	42.04 (30.89-64.26)	139	46.07 (31.57-82.14)	0.319
TREM2 _{CSF} ng/ml	23	23.57 (16.14 – 33.86)	66	20.82 (16.47 – 29.63)	0.708
TREM2 _{serum} ng/ml	24	20.39 (16.48-28.40)	74	20.22 (15.23-27.36)	0.626
OPN _{CSF} ng/ml	14	243.46 (125.08-341.49)	44	162.81 (119.44-239.19)	0.173
OPN _{serum} ng/ml	14	49.74 (26.59-67.08)	44	40.18 (28.92-56.09)	0.537
S100 _{CSF} ug/ml	18	1.05 (0.81-1.99)	50	1.23 (0.81-1.85)	0.983
S100 _{serum} ug/ml	18	0.05 (0.04-0.06)	53	0.05 (0.04-0.07)	0.792
WBC x 10 ⁹ /L	25	6.1 (4.9-6.9)	88	6.45 (5.6-7.61)	0.095
RBC million/mm ³	25	4.65 (4.3 - 4.85)	88	4.69 (4.39- 4.94)	0.961
Hb g/dL	25	14 (12.9 - 14.9)	88	13.9 (13 – 14.8)	0.789
PLT x 10 ⁹ /L	25	212 (190 - 243)	88	229 (182.5 - 278)	0.382

CRP mg/L	19	0.5 (0.5-0.5)	65	0.5 (0.5-0.5)	0.604
Neutrophils x 10⁹/L	25	3.35 (2.96-4.38)	87	3.72 (3.19-4.84)	0.191
Lymphocytes x 10⁹/L	23	1.86 (1.47-2.15)	83	1.91 (1.51-2.28)	0.359
Monocytes x 10⁹/L	23	0.43 (0.36-0.5)	83	0.55 (0.46-0.64)	0.001
Eosinophils x 10⁹/L	23	0.1 (0.08-0.15)	83	0.16 (0.1-0.28)	0.046
Basophils x 10⁹/L	23	0.03 (0.02-0.03)	83	0.03 (0.03-0.05)	0.007
NLR	23	1.84 (1.56-2.51)	83	2.11 (1.63-2.62)	0.432
MLR	23	0.23 (0.19-0.32)	83	0.28 (0.23-0.33)	0.069
SIRI	23	0.88 (0.64-1.08)	83	1.13 (0.84-1.49)	0.022
AISI	23	177.33 (131.3-263.73)	83	248.67 (156.46-369.4)	0.044
SII	23	379.49 (295.91-635.6)	83	462.57 (326.56-717.97)	0.443

Abbreviations: AISI : aggregate index of systemic inflammation; CRP : C-reactive protein; Hb: haemoglobin; NLR : neutrophil to lymphocyte ratio, MLR : monocyte to lymphocyte ratio; PLT : platelets; RBC : Red Blood Cells; SIRI : systemic inflammation response index; SII : systemic immune-inflammation index; WBC : White Blood Cells

Looking at the presence of FTD, data as reported in **Table 2c** show a significant difference in serum NfL levels between FTD group [155 pg/ml (IQR 107-334)] and cognitively spared ALS patients [115 pg/ml (IQR74.35-166.5), $p=0.036$], while we notice a similar tendency for CSF NfL and serum pNfH levels not reaching the statistical significance [6340 pg/ml (IQR 3937-9725) vs 6201 pg/ml (IQR 3496.01-11503), $p=0.750$; 1933 pg/ml (IQR 762-3008) vs 1454 pg/ml (IQR 683-2725), $p=0.529$, respectively]. For all the other biomarkers no different distribution was noticed, apart from CSF S100B levels which were slightly lower in the subgroup of FTD patients [0.80 ug/ml (IQR 0.45-1.73) vs 1.28 ug/ml (IQR 0.95-1.87), $p=0.073$].

Table 2c. Biomarkers distribution according to FTD.

Biomarker	N	Cognitively spared median (IQR)	N	FTD median (IQR)	p-value
SerpinA1 _{CSF} ug/ml	127	5.12 (3.02-7.84)	12	5.89 (2.52-8.39)	1.00
SerpinA1 _{serum} ug/ml	162	1067.13 (683.1-1517.81)	16	988.04 (594.81-1561.43)	0.629
NfL _{CSF} pg/ml	127	6201 (3496.01-11503)	11	6340 (3937-9725)	0.750
NfL _{serum} pg/mL	160	115 (74.35-166.5)	15	155 (107-334)	0.036
pNfH _{CSF} pg/mL	130	5309 (2784-8353)	12	3945 (2817.5-5706)	0.344
pNfH _{serum} pg/mL	165	1454 (683-2725)	16	1933 (762-3008)	0.529
CHIT3L1 _{CSF} ng/mL	128	108.13 (61.29-179.21)	11	100.9 (66.21-185.94)	0.852
CHIT3L1 _{serum} ng/ml	165	43.95 (31.57-74.43)	16	46.62 (26.32-85.42)	0.885
TREM2 _{CSF} ng/ml	81	22.02 (16.35-29.63)	8	19.29 (17.07-35.67)	0.920
TREM2 _{serum} ng/ml	89	21.23 (15.26-28.05)	9	16.61 (15.76-16.69)	0.287
OPN _{CSF} ng/ml	51	167.79 (117.77-272.92)	7	207.57 (122.93-341.49)	0.559
OPN _{serum} ng/ml	51	42.53 (28.81-64.63)	7	35.61 (24.33-42.34)	0.267
S100 _{CSF} ug/ml	61	1.28 (0.95-1.87)	7	0.80 (0.45-1.73)	0.073
S100 _{serum} ug/ml	64	0.05 (0.042-0.067)	7	0.043 (0.03-0.051)	0.128
WBC x 10 ⁹ /L	102	6.4 (5.5-7.4)	11	5.6 (4.6-8)	0.574
RBC million/mm3	102	4.66 (4.36 - 4.92)	11	4.72 (4.24- 4.99)	0.915
Hb g/dL	102	13.95 (13 - 14.8)	11	14 (13 – 15.5)	0.535
PLT x 10 ⁹ /L	102	231.5 (18k4 - 277)	11	212 (135 - 231)	0.104
CRP mg/L	77	0.5 (0.5-0.5)	7	0.5 (0.5-2)	0.212
Neutrophils x 10 ⁹ /L	101	3.69 (3.18-4.66)	11	3.6 (2.7-4.84)	0.601
Lymphocytes x 10 ⁹ /L	96	1.92 (1.53-2.27)	10	1.68 (1.41-2.03)	0.196
Monocytes x 10 ⁹ /L	96	0.53 (0.44-0.61)	10	0.56 (0.37-0.74)	0.638
Eosinophils x 10 ⁹ /L	96	0.15 (0.09-0.24)	10	0.12 (0.09-0.22)	0.841
Basophils x 10 ⁹ /L	96	0.03 (0.03-0.05)	10	0.03 (0.03-0.03)	0.614
NLR	96	2.05 (1.63-2.62)	10	2.34 (1.35-2.51)	0.918
MLR	96	0.27 (0.22-0.34)	10	0.34 (0.2-0.44)	0.499
SIRI	96	1.04 (0.78-1.45)	10	1.06 (0.73-2.08)	0.833
AISI	96	239.76 (153.82-358.45)	10	194.69 (144.53-408.48)	0.503
SII	96	454.76 (324.54-683.84)	10	342.15 (294.31-552)	0.331

Abbreviations: AISI : aggregate index of systemic inflammation; CRP : C-reactive protein; Hb: haemoglobin; NLR : neutrophil to lymphocyte ratio, MLR : monocyte to lymphocyte ratio; PLT : platelets; RBC : Red Blood Cells; SIRI : systemic inflammation response index; SII : systemic immune-inflammation index; WBC : White Blood Cells

Finally, we considered the behavior of biomarkers according to clinical scales at sampling. In this direction, we observed that biomarkers did not differentiate among King's staging at sampling scores in a significative manner (**Table 2d**). In particular, we noted that CSF and serum NfL and CSF pNfH showed a tendency to differently express among patients with involvement of different regions as identified by each score but they did not reach the statistical significance ($p=0.083$, $p=0.081$, $p=0.084$, respectively), neither demonstrated a demonstrate an upward or downward trend across the stages. Interestingly, serum S100B levels were significantly different along the severity of ALS measured by King's staging score (KSS) [34.99 ug/ml (27.33-50.01) for KSS 1, 43.96 ug/ml (28.81-67.19) for KSS 2, 52.47 ug/ml (55.79-71.46) for KSS 3 and 28.81 ug/ml (16.01-41.6) for KSS 4, $p=0.026$]. Conversely, all the other biomarkers failed to be differently expressed across King's stages. With a similar trend, biomarkers do not distributed differently considering the values of MiToS scores at sampling as reported in **Table 2e**, which shows the scarcity of patients represented in MiToS score 2-3-4.

Table 2d. Biomarkers distribution according to KSS at sampling

Biomarker	N	KSS 1 median (IQR)	N	KSS 2 median (IQR)	N	KSS 3 median (IQR)	N	KSS 4 median (IQR)	p-value
SerpinA1_{CSF} ug/ml	62	4.95 (3.39-7.12)	52	5.36 (2.51-8.03)	21	5.88 (4.13-8.78)	4	4.61 (2.57-8.58)	0.547
SerpinA1_{serum} ug/ml	77	1084.27 (801.27-1502.93)	64	1030.485 (654.04-1645.45)	25	791.55 (616.79-1226.37)	12	1045.21 (831.94-1765.07)	0.390
NfL_{CSF} pg/ml	62	4788.5 (2997-9440)	51	8549.09 (3937-14838)	21	7138.84 (4120-8145)	4	7824.5 (3587.5-45800)	0.083
NfL_{serum} pg/mL	75	102 (66.3-162)	64	128 (77.96-189.5)	25	141 (119-206)	11	115 (94.2-184)	0.081
pNfH_{CSF} pg/mL	65	4027 (2620-7307)	52	6472.5 (2789-9577)	21	5817 (3402-7601)	4	13182 (4662.5-22786)	0.084
pNfH_{serum} pg/mL	79	1291 (691-2538)	65	1454 (581-3105)	25	2037 (1031-2889)	12	1286.5 (807-2140)	0.744
TREM2_{CSF} ng/mL	45	19.89 (15.29-28.11)	31	22.96 (18.31-32.84)	11	24.34 (17.92-23.97)	2	21.67 (19.22-24.11)	0.601
TREM2_{serum} ng/mL	47	19.5 (15.23-26.73)	36	20.22 (15.39-29.13)	10	24.45 (13.58-37.48)	5	24.13 (15.78-27.8)	0.824
CHIT3L1_{CSF} ng/mL	62	103.26 (65.49-155.56)	52	109.71 (52.91-197.27)	21	121.45 (98.17-183.15)	4	67.43 (45.91-320.97)	0.638
CHIT3L1_{serum} ng/ml	79	41.07 (29.79-62.83)	65	40.64 (29.88-86.76)	25	55.33 (34.06-89.79)	12	77.35 (51.09-106.45)	0.059
OPN_{CSF} ng/mL	28	167.74 (117.24-234.88)	22	194.99 (128.87-317.76)	6	238.56 (140.51-455.58)	2	138.16 (112.72-163.6)	0.457
OPN_{serum} ng/mL	28	34.99 (27.33-50.01)	22	43.96 (28.81-67.19)	6	52.47 (55.79-71.46)	2	28.81 (16.01-41.6)	0.026
S100_{CSF} ug/ml	34	1.07 (0.81-1.73)	25	1.28 (0.97-1.85)	7	0.95 (0.73-1.74)	2	2.33 (2.17-2.49)	0.189
S100_{serum} ug/ml	34	0.05 (0.042-0.062)	28	0.05 (0.036-0.072)	7	0.06 (0.046-0.083)	2	0.03 (0.034-0.036)	0.117

Table 2e. Biomarkers distribution according to MiTos scale at sampling

Biomarker	N	MiTos 0 median (IQR)	N	MiTos 1 median (IQR)	N	MiTos 2 median (IQR)	N	MiTos 3 median (IQR)	N	MiTos 4 median (IQR)	p-value
SerpinA1_{CSF} ug/ml	121	5.15 (3.18-7.84)	17	3.99 (2.79-6.11)	0	-	1	11.18	0	-	0.349
SerpinA1_{serum} ug/ml	151	1043.76 (668.05-1502.93)	23	1096.6 (791.34-1565.65)	1	1159.82	1	390.59	2	1059 (1965.48-2415.93)	0.293
NfL_{CSF} pg/ml	120	6004.5 (3511.01-10642.5)	17	6454 (3584-12058)	0	-	1	79542	0	-	0.228
NfL_{serum} pg/mL	148	148 (119.19-74.41-167)	23	115 (80.3-191)	1	94.2	1	911	2	152 (120-184)	0.447
pNfH_{CSF} pg/mL	124	4773.5 (2783.5-8207)	17	5845 (3480-8319)	0	-	1	25053	0	-	0.234
pNfH_{serum} pg/mL	154	1462.5 (680-2715)	23	1638 (683-3579)	1	938	1	8242	2	954 (525-1383)	0.439
TREM2_{CSF} ng/mL	79	20.68 (16.22-29.85)	10	23.62 (18.31-31.68)	0	-	0	-	0	-	0.785
TREM2_{serum} ng/mL	85	19.98 (14.99-27.36)	11	24.45(15.76-29.11)	0	-	0	-	2	21.79 (15.78-27.8)	0.606
CHIT3L1_{CSF} ng/mL	121	107.45 (64.59-178.47)	17	88.60 (54.45-175.09)	0	-	1	556.42	0	-	0.214
CHIT3L1_{serum} ng/ml	154	43.82 (30.24-68.53)	23	40.95 (34.45-11.18)	1	88.55	1	191.70	2	71.15 (56.13-86.17)	0.165
OPN_{CSF} ng/mL	49	167.19 (117.77-293.25)	9	177.93 (162.02-217.26)	0	-	0	-	0	-	0.584
OPN_{serum} ng/mL	49	40.36 (28.81-60.31)	9	41.60 (29.03-81.43)	0	-	0	-	0	-	0.644
S100_{CSF} ug/ml	59	1.13 (0.81-1.76)	9	1.54 (1.09-1.98)	0	-	0	-	0	-	0.475
S100_{serum} ug/ml	62	0.05 (0.42-0.66)	9	0.42 (0.35-0.48)	0	-	0	-	0	-	0.189

4.3 Influence of demographic variables on biomarkers' concentrations

Firstly, we tested the influence of demographic variables on all the biomarkers in our battery. While sex had no effect on pNFH, NfL, serpinA1, CHI3L1, OPN, S100 β concentrations both in serum and CSF, we found significant associations between sex and serum TREM2 levels $p=0.0398$ for serum at one-way ANOVA analysis, while CSF TREM2 concentrations showed a tendency to correlate with sex, but not reaching a statistical significance ($p=0.094$). Peripheral inflammatory indexes including IL1 β , leucocytes, NLR, MLR, SIRI were not influenced by sex, AISI index and SII showed a significant correlation with sex ($p=0.042$ and $p=0.014$, respectively).

Moreover, we found an association between age at sampling and TREM2 levels, both on serum ($p<0.001$) and CSF ($p=0.014$). Similarly, CHI3L1 concentrations were associated with age of sampling ($p<0.001$ for serum and $p=0.001$ for CSF), as well as CSF SerpinA1 values ($p=0.022$). In an opposite direction, we did not reveal an effect of age at sampling on OPN, S100, NfL and pNFH concentrations both in serum and CSF, serum SerpinA1 and peripheral markers of inflammation.

Next, studying the effect of BMI on biomarkers' concentrations by ANOVA analysis, our data reveal that both serum TREM2 and CHI3L1 levels were correlated with BMI ($p=0.005$ and $p=0.017$ respectively). Conversely, BMI did not demonstrate an effect on neurofilaments, SerpinA1, OPN, S100 β and peripheral inflammatory indexes, neither on CSF TREM2 and CHI3L1 concentrations.

4.4 Influence of clinical variables on biomarkers' concentrations

Overall, as reported in **Table 3**, Nf levels, especially in CSF, correlated with clinical and disease progression variables of ALS (diagnostic latency, DeltaFS at sampling and last observation, time to generalization, ALSFRS-r at sampling), while a correlation was not found for FVC at sampling and age of onset. CHI3L1 in CSF correlated strongly with age at onset ($\rho=0.26$, $p=0.002$) and DPR at last observation ($\rho=0.2$, $p\text{-value}=0.018$). CHI3L1 serum levels showed a similar tight correlation with age at onset ($\rho=0.48$, $p<0.001$), as well as with time to generalization ($\rho=0.19$, $p=0.009$) and diagnostic delay ($\rho=0.19$, $p=0.009$).

Table 3. Correlations between biomarkers and clinical variables of disease progression in ALS.

Biomarkers	NfL _{CSF}	NfL _{serum}	pNfH _{CSF}	pNfH _{serum}	TRE _{M2CSF}	TRE _{M2serum}	Serpi _{nA1CSF}	Serpi _{nA1serum}	CHI3 _{L1CSF}	CHI3 _{L1serum}	OPN _{CSF}	OPN _{serum}	S100 β _{CSF}	S100 β _{serum}	NLR	MLR	SIRI	AISI	SII
Diagnostic delay	rho	-0.25	-0.23	-0.12	0.02	0.26	-0.16	0.14	-0.10	0.19	-0.01	0.04	0.12	-0.03	-0.01	0.01	0.04	-0.01	-0.04
	p-value	<0.001	0.004	0.06	0.86	0.008	0.06	0.05	0.24	0.009	0.92	0.75	0.34	0.882	0.90	0.045	0.65	0.88	0.65
Delta FS at last observation	rho	0.55	0.48	0.35	0.08	-0.09	0.15	-0.22	0.20	-0.02	0.21	0.12	0.03	0.11	0.21	0.08	0.13	0.19	0.26
	p-value	<0.001	<0.001	<0.001	0.47	0.33	0.07	0.003	0.018	0.77	0.12	0.37	0.64	0.78	0.029	0.4	0.18	0.045	0.006
ALSFRS-r at sampling	rho	-0.24	-0.26	-0.15	-0.05	-0.005	-0.07	0.04	-0.11	-0.07	-0.16	-0.32	-0.14	0.01	-0.26	-0.17	-0.24	-0.19	-0.19
	p-value	0.003	0.001	0.017	0.62	0.96	0.44	0.60	0.18	0.33	0.22	0.014	0.23	0.91	0.006	0.09	0.02	0.046	0.045
FVC	rho	-0.01	0.04	-0.04	-0.07	-0.02	0.08	-0.03	0.10	-0.14	-0.17	-0.008	-0.03	-0.002	-0.05	-0.03	-0.06	-0.08	-0.04
	p-value	0.88	0.67	0.59	0.52	0.82	0.39	0.68	0.24	0.06	0.19	0.96	0.82	0.99	0.64	0.74	0.54	0.44	0.65
Time to generalization	rho	-0.32	-0.15	-0.28	0.09	0.28	-0.11	0.11	0.06	0.19	-0.02	-0.12	-0.05	-0.21	-0.09	-0.06	-0.09	-0.12	-0.08
	p-value	<0.001	0.003	<0.001	0.38	0.005	0.22	0.14	0.46	0.009	0.86	0.36	0.66	0.08	0.32	0.56	0.34	0.23	0.40
Delta FS at sampling	rho	0.32	0.29	0.19	0.13	-0.19	0.07	-0.12	0.07	0.001	0.03	0.12	-0.10	0.003	0.10	0.05	0.09	0.12	0.11
	p-value	<0.001	<0.001	0.002	0.21	0.06	0.44	0.12	0.41	0.99	0.81	0.39	0.40	0.98	0.29	0.59	0.33	0.22	0.28
Decline FRS	rho	0.51	0.10	0.27	0.16	-0.08	0.09	-0.15	0.19	-0.03	0.19	0.11	0.05	0.12	0.18	0.06	0.07	0.14	0.26
	p-value	<0.001	<0.001	0.001	0.18	0.46	0.37	0.08	0.05	0.75	0.18	0.45	0.67	0.36	0.11	0.56	0.53	0.19	0.01
Age at onset	rho	-0.14	-0.06	-0.01	0.21	0.54	0.28	-0.17	0.26	0.48	0.14	0.01	-0.07	-0.05	-0.52	0.02	-0.09	-0.21	-0.20
	p-value	0.08	0.42	0.89	0.05	<0.001	0.001	0.03	0.002	<0.001	0.29	0.94	0.59	0.67	0.86	0.86	0.37	0.03	0.04

Abbreviations: AISI : aggregate index of systemic inflammation; FVC: Forced Vital Capacity. NfL: neurofilament light chain. pNfH: neurofilament heavy chain. NLR : neutrophil to lymphocyte ratio, MLR : monocyte to lymphocyte ratio; SIRI : systemic inflammation response index; SII : systemic immune-inflammation index;. Please refer to Methods section for calculation of clinical variables.

We therefore looked whether any correlation existed between yet unexplored biomarkers as SerpinA1, TREM2, OPN, S100 β and peripheral indicators of inflammation, by Spearman correlation analysis.

Among all the biomarkers, diagnostic delay only correlated with TREM2 on serum ($\rho=0.26$, $p=0.008$) and MLR ($\rho=0.01$, $p=0.045$), while TREM2 was the sole biomarker correlated with time to generalization ($\rho=0.28$, $p=0.005$).

ALSFRS-r at baseline inversely correlated with OPN serum levels ($\rho=-0.32$, $p=0.014$) and with several peripheral biomarkers of inflammation (for NLR $\rho=-0.26$, $p=0.006$; for SII $\rho=-0.24$, $p=0.02$; for AISI $\rho=-0.19$, $p=0.046$, for SII $\rho=-0.19$, $p=0.045$), while no correlations were found between each biomarker and FVC at sampling or DPR at sampling. Concerning DPR at last observation, only peripheral inflammatory markers were correlated, namely NLR ($\rho=0.21$, $p=0.029$), AISI ($\rho=0.19$, $p=0.045$) and SII ($\rho=0.26$, $p=0.006$). Age at onset strongly correlated with TREM2 levels on serum ($\rho=0.54$, $p<0.001$) as well with SerpinA1 levels, both on CSF ($\rho=0.28$, $p=0.001$) and on serum with an inverse direction ($\rho=-0.17$, $p=0.03$). Interestingly, SerpinA1 serum levels demonstrate an inverse correlation with DPR at last observation ($\rho=-0.22$, $p=0.003$), in a opposite direction relative to NFs and CHI3L1 on CSF, while for SerpinA1 CSF concentrations we observed only a tendency to directly influence DPR at last observation ($\rho=0.15$, $p=0.07$).

Finally, considering the monthly decline in ALSFRS-r, the only biomarker that demonstrated a correlation is SII index ($\rho=0.26$, $p=0.01$), while all the other failed to be associated with this measure of progression.

4.5 Correlations among biomarkers

We evaluated by Spearman correlation test whether CSF and serum concentrations correlated for each biomarker: NfL and NfH confirmed significant associations between the two compartments [for NfL, $\rho(150)=0.74$, $p\text{-value}<0.0001$; for NfH, $\rho(155)=0.52$, $p\text{-value}<0.0001$], as well as for CHI3L1 concentrations [$\rho(138)=0.40$, $p\text{-value}<0.0001$], while TREM2 and OPN levels have a mild but significant correlation [$\rho(88)=0.25$, $p\text{-value}=0.021$ and $\rho(58)=0.42$, $p=0.001$ respectively], which was not found between SerpinA1 and S100 β concentrations in serum and CSF [$\rho(137)=-0.02$, $p\text{-value}=0.85$ and $\rho(68)=-0.05$, $p\text{-value}=0.68$].

In order to investigate whether correlations exist between these biomarkers, Spearman correlation test was run between each couple of biomarker at the time, revealing that TREM2 levels, both on serum and CSF, correlate significantly with several biomarkers, as shown in **Table 4**, as well as CHI3L1_{CSF} concentrations.

Table 4. Correlations among CHI3L1_{CSF}, TREM2_{CSF} and TREM2_{serum} concentrations with other biomarkers.

Biomarker		CHI3L1 _{CSF}	TREM2 _{CSF}	TREM2 _{serum}
pNfH _{serum}	Spearman rho correlation	0.18	-0.10	-0.01
	p-value	0.04	0.33	0.89
pNfH _{CSF}	Spearman rho correlation	0.41	0.29	-0.05
	p-value	<0.001	0.007	0.64
NfL _{CSF}	Spearman rho correlation	0.31	0.21	-0.03
	p-value	0.003	0.05	0.55
NfL _{serum}	Spearman rho correlation	0.29	0.11	0.02
	p-value	0.005	0.31	0.87
SerpA1 _{serum}	Spearman rho correlation	0.030	-0.21	0.24
	p-value	0.74	0.05	0.02
SerpA1 _{CSF}	Spearman rho correlation	0.48	0.29	0.29
	p-value	<0.001	0.005	0.005
CHIT3L1 _{serum}	Spearman rho correlation	0.40	0.20	0.49
	p-value	<0.001	0.06	<0.001
CHIT3L1 _{CSF}	Spearman rho correlation	-	0.39	0.20
	p-value	-	0.002	0.06
OPN _{serum}	Spearman rho correlation	0.21	0.40	0.15
	p-value	0.12	0.002	0.27
OPN _{CSF}	Spearman rho correlation	-0.01	0.38	0.20
	p-value	0.96	0.005	0.13
S100 _{serum}	Spearman rho correlation	-0.15	0.14	-0.04
	p-value	0.22	0.25	0.76
S100 _{CSF}	Spearman rho correlation	0.003	0.34	-0.07
	p-value	0.98	0.005	0.56
NLR	Spearman rho correlation	0.26	0.19	-0.05
	p-value	0.01	0.13	0.68
MLR	Spearman rho correlation	0.13	-0.23	-0.20
	p-value	0.23	0.06	0.08
SIRI	Spearman rho correlation	0.20	-0.05	-0.04
	p-value	0.05	0.67	0.74
AISI	Spearman rho correlation	0.21	-0.03	-0.17
	p-value	0.05	0.83	0.14
SII	Spearman rho correlation	0.22	0.19	-0.19
	p-value	0.03	0.13	0.09

As far as serum concentrations concerns, neither CHI3L1_{serum} or S100 β _{serum} correlated with other biomarkers levels, while OPN_{serum} concentrations mildly relate only with NFL_{CSF} and S100 β _{CSF} [$\rho(58)=0.29$, $p\text{-value}=0.02$; $\rho(55)=0.28$, $p\text{-value}=0.04$), and SerpinA1_{serum} just showed a weak correlation with OPN_{CSF} [$\rho(58)= -0.27$, $p=0.04$].

SerpinA1_{CSF} strongly correlates with pNFH_{CSF} [$\rho(139)=0.24$, $p\text{-value}=0.004$] and with CHI3L1_{CSF} [$\rho(138)=0.48$, $p\text{-value}<0.0001$], while SerpinA1_{CSF} mildly correlates with NLR [$\rho(90)=0.23$, $p\text{-value}=0.033$]. Then, S100 β _{CSF} strongly relates with NfL_{CSF} [$\rho(68)=0.40$, $p\text{-value}<0.001$], while our data reveal a weak correlation with pNFH levels both on serum and CSF [$\rho(68)=0.26$, $p\text{-value}=0.03$ and $\rho(68)=0.30$, $p\text{-value}=0.01$, respectively].

4.3 Clinical status in ALS with different progression

The principal clinical features of ALS patients according to DPR in **Tables 5a** and **5b**.

In accordance with a more aggressive disease, several variables are differently distributed among the groups with different progression calculated at sampling as reported in **Table 5a**, including diagnostic delay (shorter with a slow progression: in months 20.29 ± 19.91 vs 9.29 ± 4.85 vs 5.21 ± 3.13 , $p<0.001$), ALSFRS-r at sampling (lower score for patients with a faster disease course, in points 31.23 ± 7.54 vs 40.62 ± 5.48 vs 43.28 ± 5.31 , $p<0.001$), time to generalization (shorter for patients with hasty evolution, in months 9.04 ± 5.74 vs 15.61 ± 12.82 vs 36.42 ± 27.89 , $p<0.001$), FVC at sampling (higher in case of patient with a moderate progression, in %, 98.98 ± 21.23 vs 92.88 ± 22.34 vs 84.02 ± 24.29 , $p=0.008$), time to procedures (shorter for faster forms of disease with $p<0.001$ for NIV, IV and PEG) and King's staging at sampling (lower for patients with a course less severe, 1.39 ± 0.73 vs 1.88 ± 0.89 , 2.13 ± 0.93 , $p<0.001$) while we observe only a tendency to higher levels of MiTos scores for ALS less aggressive, without reaching a statistical significance (0.09 ± 0.29 vs 0.16 ± 0.53 vs 0.35 ± 0.79 , $p=0.08$).

Table 5a. Clinical characteristics according to disease progression at sampling.

Variable	Slow Progressors (n=43) n (%), mean [SD]	Intermediate Progressors (n=87) n (%), mean [SD]	Fast Progressors (n=52) n (%), mean [SD]	p value
Age at onset, years	57.52 [10.17]	57.66 [12.45]	59.15 [13.20]	0.745
Age at sampling, years	60.43 [10.53]	58.73 [12.45]	59.64 [13.28]	0.753
Diagnostic delay, months	20.29 [19.91]	9.29 [4.85]	5.21 [3.13]	<0.001
Weight at sampling (Kg)	72.30 [13.12]	70.89 [12.47]	69.79 [14.00]	0.648
Weight loss at sampling (%)	0.02 [0.07]	0.05 [0.07]	0.05 [0.08]	0.182
BMI at sampling, kg/m2	24.71 [3.58]	24.59 [4.22]	24.21 [3.77]	0.798
ALSFRS-r total score at sampling, points	43.28 [5.31]	40.62 [5.48]	37.23 [7.54]	<0.001
Time to generalization [#] , months	36.42 [27.89]	15.61 [12.82]	9.04 [5.74]	<0.001
FVC at sampling [§] , %	98.98 [21.23]	92.88 [22.34]	84.02 [24.29]	0.008
King's staging at sampling	1.39 [0.73]	1.88 [0.89]	2.13 [0.93]	<0.001
MiTos at sampling	0.09 [0.29]	0.16 [0.53]	0.35 [0.79]	0.08
Civil status Single, common-in-law, married, widow(e), divorced, not reported	5 (11.63) / 2 (4.65) / 27 (52.79) / 2 (4.65) / 2 (4.65)	9 (10.34) / 6 (6.89) / 58 (66.67) / 2 (2.29) / 4 (4.59)	5 (9.62) / 3 (5.77) / 39 (75) / 1 (1.92) / 4 (7.77)	0.971
Mutational status* WT/C9/SOD1/other	33 (76.74) / 2 (4.65) / 2 (4.65) / 3 (6.98)	75 (86.21) / 7 (8.05) / 1 (1.15) / 3 (3.45)	42 (80.77) / 4 (7.69) / 3 (5.77) / 3 (5.77)	0.348
Site of onset Bulbar, Spinal, Respiratory	11 (25.58) / 32 (74.42) / 0	23 (26.44) / 63 (72.41) / 1 (1.15)	9 (17.31) / 41 (78.85) / 2 (3.85)	0.436
Phenotype Flail, UMNp, Bulbar, Respiratory, Classic	12 (27.91) / 4 (9.3) / 8 (18.6) / 0 / 19 (44.19)	12 (13.79) / 3 (3.45) / 21 (24.14) / 1 (1.15) / 50 (57.47)	5 (9.62) / 3 (5.77) / 6 (11.54) / 2 (3.85) / 36 (69.23)	0.062
FTD ALS-ci / ALS-bi	4 (9.3) 5 (11.63) / 5 (11.63)	7 (8.05) 7 (8.05) / 10 (11.49)	5 (9.62) 7 (13.46) / 7 (13.46)	0.943 0.569 / 0.928
CS hyperintensity Present /absent/ not reporter	6 (13.95) / 26 (60.47) / 11 (25.58)	54 (62.07) / 17 (19.54) / 16 (18.39)	9 (17.31) / 35 (67.31) / 8 (15.38)	0.813
MEPs prolongation Present /absent/ not reported	18 (41.86) / 14 (32.56) / 11 (25.58)	60 (69.97) / 15 (17.24) / 12 (13.79)	31 (59.62) / 13 (25) / 8 (15.38)	0.041
Cramps	22 (51.16)	46 (52.87)	16 (30.77)	0.015
Pseudobulbar affect	7 (16.28)	23 (26.44)	6 (11.54)	0.087
Palmomental reflex	8 (18.6)	37 (42.53)	12 (23.08)	0.026
Glabellar reflex	3 (6.98)	18 (20.69)	11 (21.15)	0.080
Snout reflex	6 (13.95)	27 (31.03)	14 (26.92)	0.095

Masseter reflex	9 (20.93)	31 (35.63)	17 (32.69)	0.163
NIV	25 (58.14)	45 (51.72)	34 (65.38)	0.286
PEG	16 (37.21)	42 (48.28)	25 (48.08)	0.449
IV	14 (32.56)	26 (29.89)	19 (36.54)	0.72
Diabetes	6 (13.95)	2 (2.29)	6 (11.54)	0.03
Statins	8 (18.6)	6 (6.19)	2 (3.85)	0.047

Legend Data are represented as the median (IQR; range) or mean [SD]. [§] Data on FVC at sampling were available for 41 slow progressors, 84 intermediate progressors and 48 fast progressors. [#] Data on time to generalization at sampling was available for 40 slow progressors, 86 intermediate progressors and 51 fast progressors. *Genetic analysis was performed for 40 slow progressors, 86 intermediate progressors and 49 fast progressors. **Abbreviations:** ALSFRS-r= Amyotrophic Lateral Sclerosis Functional Rating Scale- revised; BMI: Body Mass Index; CS: Corticospinal; MEPs = Motor Evoked Potentials, UMN-p: Upper Motor Neuron predominant; FTD: frontotemporal dementia; FVC: forced vital capacity; PEG: percutaneous endoscopic gastrostomy; NIV: non-invasive ventilation; IV: invasive ventilation; SD: standard deviation.

The other demographic data from our patient cohort show no significant differences for any of the variables analysed, when considering disease progression, namely age at onset or sampling, weight, and weight loss at sampling, as well as BMI at sampling and civil status. As regards clinical variables, neither the site of onset nor cognitive impairment were differently distributed among the three groups, while the phenotype was differently represented in each group, although not reaching the statistical significance.

Interestingly, we did not find a different representation of genetic mutations among the three groups, in particular for *C9orf72* expansion or *SOD1* mutation ($p=0.436$). While CS hyperintensity did not indicate a more severe involvement, patients with intermediate or fast DPR had more frequently MEPs prolongation (69.97% and 59.62%), in comparison with patients with slower disease (41.86%, $p=0.041$).

Next, cramps were more frequently reported in patients with slow or intermediate ALS course (51.16% and 52.87% vs 30.77%, $p=0.015$), while among frontal release signs we noted only a difference in palmomental reflex presence, although it was more frequently reported in intermediate progressors (42.53% vs 18.6% in slow vs 23.08% in fast, $p=0.026$). Finally, regarding co-pathologies, the frequency in the three groups was equivalent except for diabetes mellitus, which was less frequent in patient with intermediate progression (2.23% vs 13.95% in slow and 11.54% in fast progressors, $p=0.02$) and for the concomitant use of statins, clearly more common in patients with slower disease (18.6% vs 6.19 % vs 3.85%, $p=0.047$).

Table 5b. Clinical characteristics according to disease progression at sampling and last observation.

Variable	Very Slow Progressors (n=20) n (%), mean [SD]	Intermediate Progressors (n=97) n (%), mean [SD]	Very Fast Progressors (n=65) n (%), mean [SD]	p value
Age at onset, years	53.11 [11.18]	58.82 [12.07]	58.43 [12.33]	0.153
Age at sampling, years	57.61 [12.29]	60.02 [12.22]	59.01 [12.33]	0.690
Diagnostic delay, months	24.67 [16.92]	6.74 [9.01]	3.29 [2.03]	<0.001
Weight at sampling (Kg)	75.45 [13.57]	70.75 [12.91]	69.75 [12.97]	0.229
BMI at sampling, kg/m ²	24.82 [3.54]	24.65 [4.18]	24.21 [3.69]	0.733
ALSFRS-r total score at sampling, points	42.35 [5.68]	41.51 [5.69]	37.8 [7.05]	<0.001
Time to generalization #, months	46.79 [33.84]	19.10 [14.88]	9 [5.44]	<0.001
FVC at sampling \$, %	98.52 [22.11]	95.34 [23.31]	84.49 [21.69]	0.007
Site of onset Bulbar, Spinal, Respiratory	2 (10) / 18 (90) / 0	29 (29.89) / 67 (69.07) / 1 (1.03)	12 (18.46) / 51 (78.46) / 2 (3.08)	0.169
Phenotype Flail, UMNp, Bulbar, Respiratory, Classic	6 (30) / 4 (20) / 2 (10) / 0 / 8 (40)	16 (16.49) / 3 (3.09) / 24 (24.74) / 1 (1.03) / 53 (54.64)	7 (10.77) / 3 (4.2) / 9 (13.85) / 2 (3.08) / 44 (67.69)	0.012
Cramps	9 (45)	51 (52.58)	24 (36.92)	0.119
Pseudobulbar affect	3 (15)	25 (25.77)	8 (12.31)	0.086
Palmomental reflex	3 (15)	34 (35.05)	20 (30.77)	0.031
Glabellar reflex	1 (5)	16 (16.49)	15 (23.08)	0.102
Snout reflex	2 (10)	29 (29.89)	16 (24.62)	0.177
Masseter reflex	3 (15)	32 (32.99)	22 (33.85)	0.217
NIV	11 (55)	53 (54.64)	40 (61.54)	0.671
PEG	5 (25)	42 (43.29)	36 (55.38)	0.046
IV	6 (30)	28 (28.87)	25 (38.46)	0.428
Diabetes	1 (5)	7 (7.22)	6 (9.23)	0.798
Statins	1 (5)	12 (12.37)	3 (4.2)	0.380

Legend Data are represented as the median (IQR; range) or mean [SD]. **Abbreviations:** ALSFRS-r= Amyotrophic Lateral Sclerosis Functional Rating Scale- revised; BMI: Body Mass Index; CS: Corticospinal; MEPs = Motor Evoked Potentials, UMN-p: Upper Motor Neuron predominant; FTD: frontotemporal dementia; FVC: forced vital capacity; PEG: percutaneous endoscopic gastrostomy; NIV: non-invasive ventilation; IV: invasive ventilation; SD: standard deviation.

When considering disease progression rate not only at sampling but also at last observation, as shown in **Table 5b**, we noted similar significant differences for clinical and demographic variables, including diagnostic delay, ALS-FRS-r at sampling, time to generalization, FVC at sampling, and palmomental reflex. Interestingly, phenotype distribution was significantly

different among the three groups, as represented in **Figure 7**, reaching the statistical significance ($p=0.012$). Conversely, cramps were not differently distributed in very slow progressors in comparison with intermediate and very fast progressors (45% vs 52.58% vs 36.92%, $p=0.019$), as well as all the comorbidities including diabetes mellitus (5% vs 7.22% vs 9.23%, $p=0.798$) and statins (5% vs 12.3% vs 4.2%, $p=0.380$). Lastly, very fast progressors more commonly underwent PEG (55.38%) in comparison with very slow progressors (25%) and intermediate progressors (43.29%, $p=0.046$), whereas respiratory procedures were more equally adopted among the three groups.

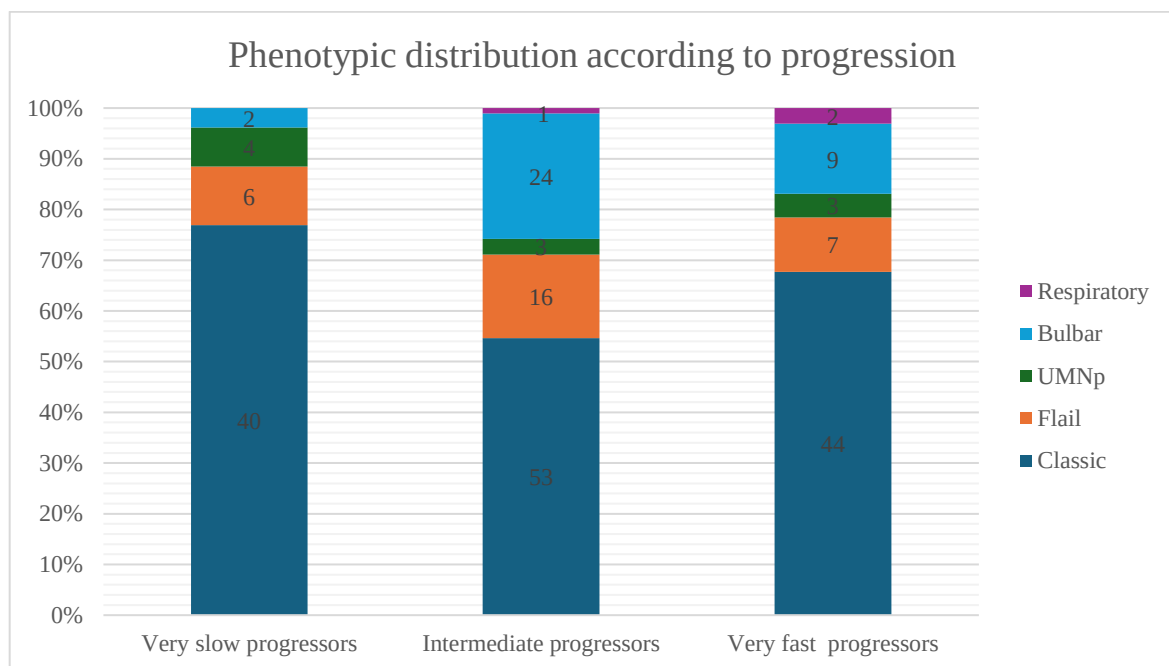


Figure 7 Phenotypic distribution according to progression rate calculated at sampling and last observation.

4.4 Biomarker status in ALS with different progression

We assessed whether differences existed between slow, intermediate and fast progressors according to each biomarker levels (**Table 6a**).

Table 6a. Comparison of biomarker levels among slow, intermediate and fast progressors.

Biomarkers	N	Slow Progressors (n=43)	N	Intermediate Progressors (n=87)	N	Fast Progressors (n=52)	p value
NfL _{CSF} pg/mL	29	4435 (2997-9055)	71	5533 (3526-9725)	38	8444.5 (4294-14838)	0.027
NfL _{serum} pg/mL	41	102 (55.6 – 157)	84	119.5 (75.69-166)	50	128 (96-231)	0.008
NfH _{CSF} pg/mL	31	3863 (2206-7077)	71	5114 (2637-8082)	40	6393 (3208-9787.5)	0.052
NfH _{serum} pg/mL	43	1249 (574-1954)	86	1939.5 (755-2949)	52	1315 (504.5 -2584)	0.090
SerpinA1 _{CSF} ug/mL	30	4.84 (2.96-8.31)	69	5.15 (3.02-7.28)	40	5.24 (3.22-8.58)	0.816
SerpinA1 _{serum} ug/mL	43	1130.93 (850-14-1597.31)	85	1043.76 (677.78-1523.09)	50	1030.49 (610.31-1384.73)	0.240
CHIT3L1 _{CSF} ng/ml	30	106.78 (65.97-150.61)	86	42.29 (32.82-61.09)	40	116.53 (66.16-197.97)	0.712
CHIT3L1 _{serum} ng/ml	43	48.28 (29.76-86.77)	86	42.29 (32.82-61.09)	52	48.08 (29.18-87.09)	0.676
TREM2 _{CSF} ng/ml	22	24.42 (19.37-36.74)	41	19.83 (16.22-28.11)	26	20.29 (16.11-27.2)	0.203
TREM2 _{serum} ng/ml	22	22.92 (15.92-31.44)	46	19.91 (15.63-28.05)	30	18.17 (13.14-26.75)	0.307
OPN _{CSF} ng/ml	15	167.79 (122.93-329.93)	26	169.26 (129.31-325.93)	17	176.29 (117.77-217.26)	0.882
OPN _{serum} ng/ml	15	32.81 (24.33-73.61)	26	45.38 (30.37864.63)	17	40.00 (32.97-54.83)	0.591
S100 _{CSF} ug/ml	16	1.41 (0.99-1.80)	31	1.4 (0.81-1.76)	21	1.06 (0.78-1.92)	0.625
S100 _{serum} ug/ml	17	0.05 (0.04-0.06)	33	0.05 (0.04-0.07)	21	0.05 (0.04-0.07)	0.868
Leucocytes x 10 ⁹ /L	25	5.81 (5.26-6.7)	55	6.8 (5.81-7.7)	33	6.1 (5.2-7.8)	0.067
RBC million/mm ³	25	4.74 (4.36-5.01)	55	4.65 (4.41-5.09)	33	4.69 (4.25-4.8)	0.599
Hb g/dL	25	13.9 (13-14.9)	55	13.9 (13-14.9)	33	14 (13-14.8)	0.958
PLT x 10 ⁹ /L	25	205 (161-275)	55	229 (190-267)	33	234 (183-279)	0.589
Neutrophils x 10 ⁹ /L	25	3.42 (3.09-4.13)	55	4.01 (3.18-4.94)	32	3.58 (3.13-4.64)	0.291
Lymphocytes x 10 ⁹ /L	24	1.72 (1.44-1.99)	53	1.98 (1.76-2.3)	29	1.61 (1.43-2.05)	0.047
Monocytes x 10 ⁹ /L	24	0.47 (0.41-0.55)	53	0.57 (0.46-0.64)	29	0.54 (0.43-0.6)	0.165
Eosinophils x 10 ⁹ /L	24	0.18 (0.09-0.26)	53	0.13 (0.09-0.27)	29	0.15 (0.09-0.19)	0.572
Basophils x 10 ⁹ /L	24	0.03 (0.02-0.4)	53	0.03 (0.03-0.05)	29	0.03 (0.03-0.05)	0.154
NLR	24	2.03 (1.63-2.73)	53	2.06 (1.58-2.47)	29	2.15 (1.7-2.62)	0.658
MLR	24	0.27 (0.22-0.33)	53	0.26 (0.23-0.34)	29	0.3 (0.22-0.36)	0.617
SIRI	24	0.97 (0.71-1.46)	53	1.03 (0.83-1.44)	29	1.1 (0.83-1.57)	0.724
AISI	24	183.95 (145.12-357.29)	53	241.34 (168.44-355.86)	29	248.67 (148.36-369.4)	0.544
SII	24	397.59 (323.81-600.53)	53	445.77 (321.26-649.71)	29	497.22 (322.51-637.62)	0.763

Legend: Data are presented as medians (IQR). **Abbreviations:** AISI : aggregate index of systemic inflammation; NfL: neurofilament light chain. pNfH: neurofilament heavy chain; NLR : neutrophil to lymphocyte ratio, MLR : monocyte to lymphocyte ratio; SIRI : systemic inflammation response index; SII : systemic immune-inflammation index. Please refer to Methods section for calculation of clinical variables.

If NfL both on serum and CSF were increasingly elevated according to the category of progression, pNFH on CSF showed a tendency to higher levels with faster progression ($p=0.052$) while pNFH on serum failed to be significantly associated with progression ($p=0.09$) being more elevated in intermediate progressors group.

All the other neuroinflammation biomarkers included, namely SerpinA1, CHI3L1, TREM2, OPN, S100B were not differently represented among the three classes. As regards systemic inflammation, only lymphocytes were sensibly higher in intermediate group in comparison with the two others ($p=0.047$) but similarly distributed in slow and fast progressors.

Next, when considering the progression rate calculated also at last observation, the discriminative power of NfL both on serum and CSF increases ($p<0.001$), as well as pNFH CSF levels ($p<0.001$) (**Figure 8 and Figure 9**, respectively) (**Table 6b**).

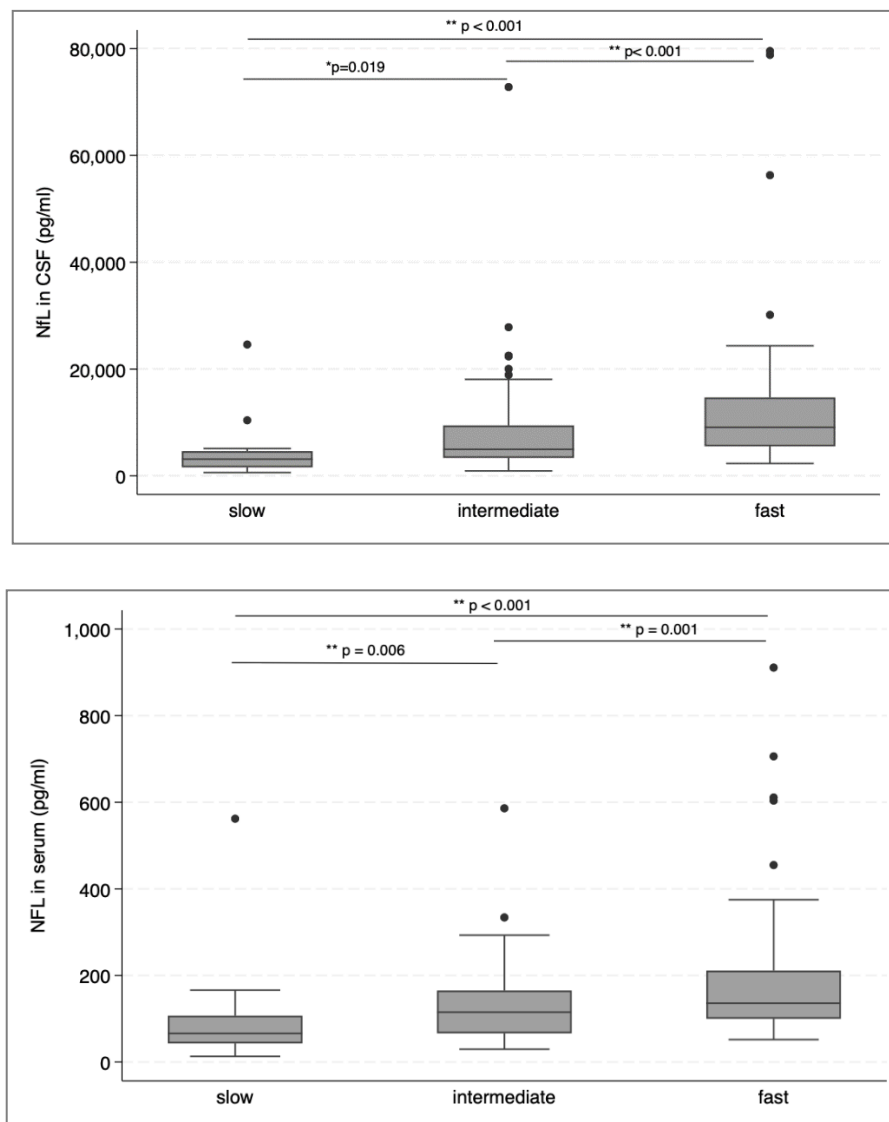


Figure 8. NfL distribution according to disease progression rate at sampling and LO.

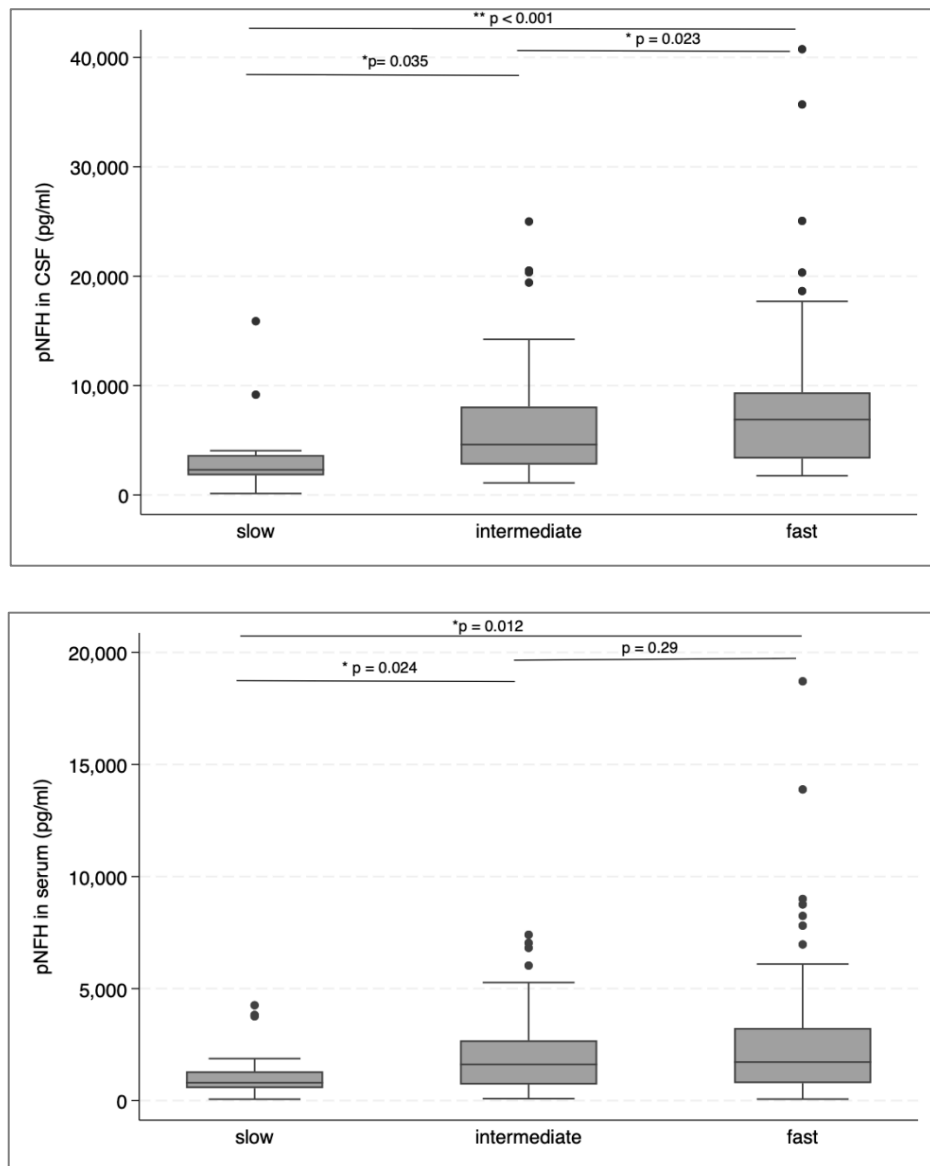


Figure 9. pNFH distribution according to disease progression rate at sampling and LO.

Table 6b. Comparison of biomarker levels among very slow, intermediate and very fast progressors.

Biomarkers	N	Very Slow Progressors (n=20)	N	Intermediate Progressors (n=97)	N	Very Fast Progressors (n=65)	p value
NfL _{CSF} pg/mL	12	3123 (1607.5-4614.5)	78	4974 (3368-9440)	48	9110 (5536.5-14679)	<0.001
NfL _{serum} pg/mL	18	66 (43.1-107)	95	115 (66.3-165)	62	135.86 (100-211)	<0.001
NfH _{CSF} pg/mL	14	2304.5 (1800-3658)	78	4610 (2783-8082)	50	6891 (3336-9382)	<0.001
NfH _{serum} pg/mL	20	799 (560-1306.5)	97	1614 (716-2691)	64	1720.5 (781.5-3242)	0.08
SerpinA1 _{CSF} ug/mL	13	5.39 (3.93-8.31)	76	5.24 (2.89-7.81)	50	5.11 (3.21-7.51)	0.946
SerpinA1 _{serum} ug/mL	20	1243.54 (999.91-1565.82)	96	1046.07 (688.50-1590.47)	62	906.31 (610.31-1315.50)	0.043
CHIT3L1 _{CSF} ng/ml	13	69.78 (43.79-114.62)	76	103.17 (61.72-177.29)	50	130.16 (65.79-189.99)	0.093
CHIT3L1 _{serum} ng/ml	20	40.16 (31.39-84.06)	97	46.07 (32.06-69.39)	64	43.35 (30.69-71.30)	0.975
TREM2 _{CSF} ng/ml	9	21.15 (12.69-27.82)	49	20.31 (17.97-30.16)	31	22.02 (14.69-28.01)	0.559
TREM2 _{serum} ng/ml	9	18.81 (12.91-26.8)	53	21.39 (15.63-28.8)	36	18.34 (14.26-25.72)	0.289
OPN _{CSF} ng/ml	4	126.97 (88-204.45)	33	167.19 (116.72-272.92)	21	177.83 (140.51-293.25)	0.769
OPN _{serum} ng/ml	4	30.81 (27.7-57.12)	33	42.34 (24.33-64.63)	21	40 (32.87-55.79)	0.486
S100 _{CSF} ug/ml	6	1.15 (0.97-2.04)	37	1.4 (0.91-1.73)	25	0.68 (1.09-0.79)	0.796
S100 _{serum} ug/ml	6	0.05 (0.04-0.6)	40	0.05 (0.04-0.65)	25	0.05 (0.04-0.07)	0.945
Leucocytes x 10 ⁹ /L	13	5.9 (5.5-6.7)	56	6.4 (5.5-7.55)	44	6.45 (5.28-7.66)	0.748
RBC million/mm ³	13	4.65 (4.38-4.74)	56	4.67 (4.34-4.99)	44	4.71 (4.28-4.86)	0.999
Hb g/dL	13	13.9 (13.4-14.1)	56	13.95 (12.9-14.9)	44	14 (13.15-14.95)	0.832
PLT x 10 ⁹ /L	13	215 (165-282)	56	228 (186.5-264)	44	230 (183-298)	0.731
Neutrophils x 10 ⁹ /L	13	3.69 (3.26-3.91)	56	3.67 (3.16-4.89)	43	3.71 (3.19-4.77)	0.871
Lymphocytes x 10 ⁹ /L	12	1.66 (1.38-2.07)	54	1.97 (1.62-2.3)	40	1.85 (1.48-2.08)	0.164
Monocytes x 10 ⁹ /L	12	0.53 (0.44-0.64)	54	0.53 (0.44-0.61)	40	0.54 (0.44-0.61)	0.988
Eosinophils x 10 ⁹ /L	12	0.22 (0.14-0.33)	21	0.15 (0.09-0.27)	40	0.14 (0.08-0.18)	0.142
Basophils x 10 ⁹ /L	12	0.03 (0.02-0.04)	54	0.03 (0.03-0.04)	40	0.03 (0.03-0.05)	0.676
NLR	12	2.27 (1.7-2.78)	54	1.98 (1.57-2.41)	40	2.12 (1.37-2.63)	0.372
MLR	12	0.29 (0.25-0.39)	54	0.27 (0.22-0.32)	40	0.28 (0.22-0.37)	0.412
SIRI	12	1.12 (0.91-1.46)	54	0.98 (0.73-1.39)	40	1.08 (0.83-1.61)	0.673
AISI	12	231.87 (169.1-357.29)	54	236.88 (147.68-355.86)	40	237.52 (150.63-404.2)	0.825
SII	12	441.61 (301.88-680.39)	54	430.86 (326.56-592.74)	40	512.85 (315.01-735.36)	0.648

Legend: Data are presented as medians (IQR). **Abbreviations:** AISI : aggregate index of systemic inflammation; NfL: neurofilament light chain. pNfH: neurofilament heavy chain; NLR : neutrophil to lymphocyte ratio, MLR : monocyte to lymphocyte ratio; SIRI : systemic inflammation response index; SII : systemic immune-inflammation index;. Please refer to Methods section for calculation of clinical variables.

Interestingly, we observed that serum SerpinA1 levels act differently across the three classes, being more elevated in patients with very slow progression, and decreasing with faster progression in a significant manner (for very slow progressors median 1243.54 $\mu\text{g/ml}$ (IQR 999.91-1565.82), for intermediate progressors median 1046.07 (IQR 688.50-1590.47), for very fast progressors median 906.31 $\mu\text{g/ml}$ (IQR 610.31-1315.50, $p=0.043$) (**Figure 10**).

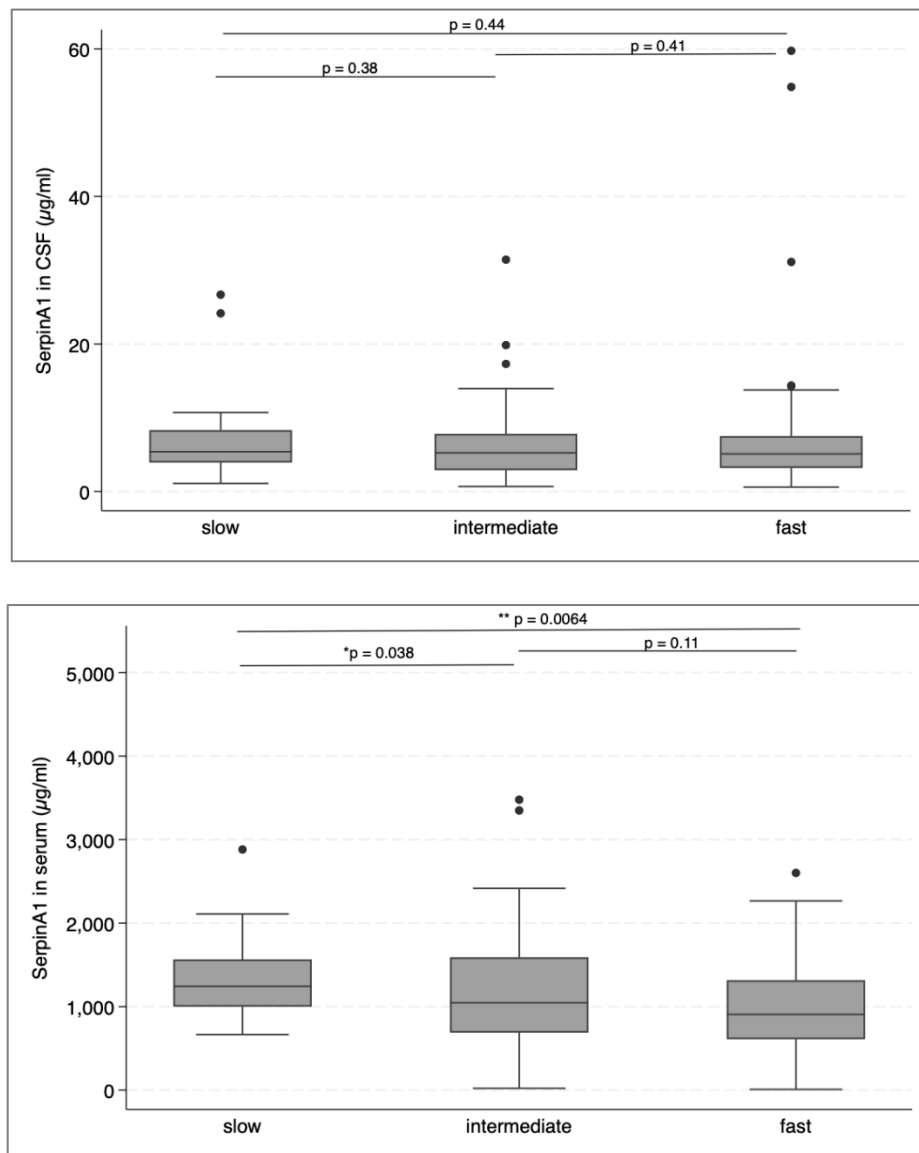


Figure 10. SerpinA1 distribution according to disease progression rate at sampling and LO.

Then, in an opposite direction, CHI3L1 concentrations on CSF differently behave in respect of ALS progression, with lower levels in patients with very slow progression, and increasing with a high rate of disease course, although not reaching a statistical significance ($p=0.093$) (**Figure 11**). Lastly, considering these classes of progressors, lymphocytes distribution still

shows a trend to be higher in intermediate progressors, but losing the statistical power ($p=0.164$) respect to disease progressors calculated at sampling (compared to values reported in **Table 6a**).

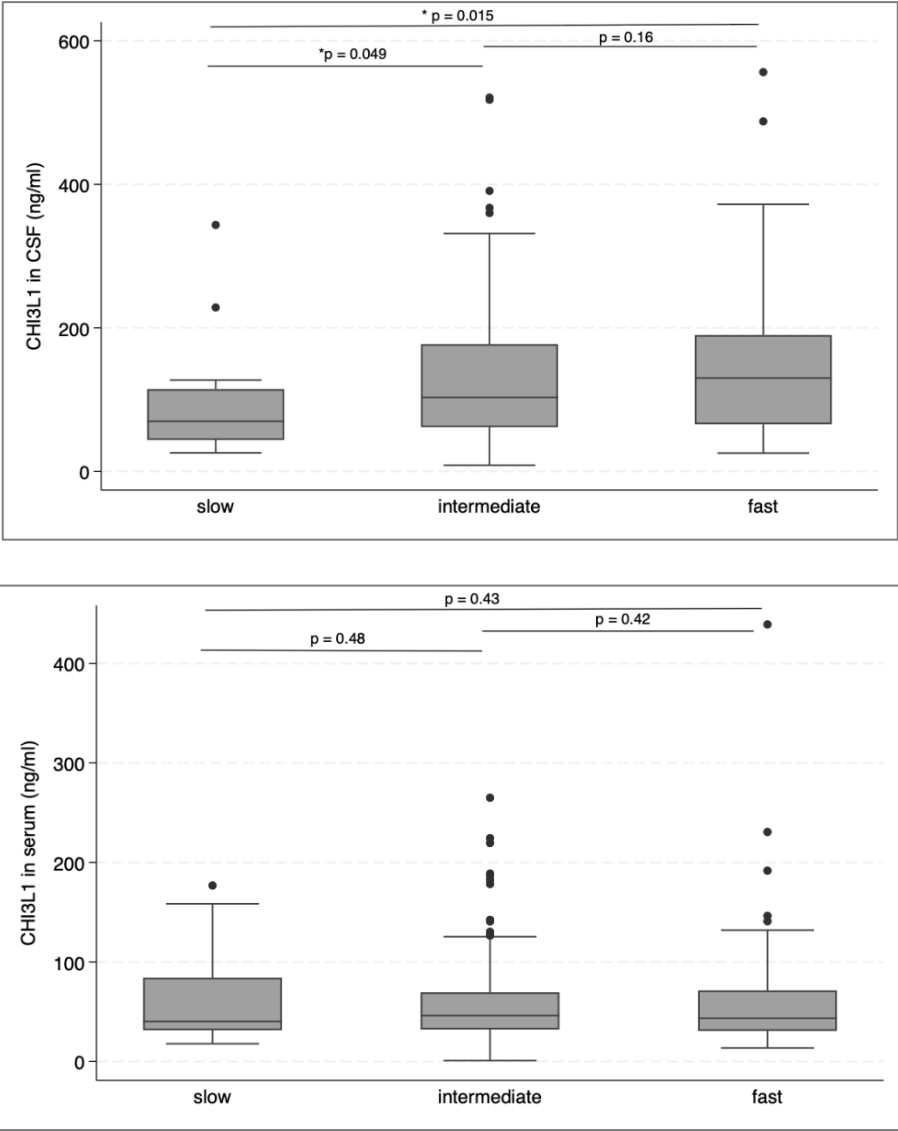


Figure 11. CHI3L1 distribution according to disease progression rate at sampling and LO.

4.5 Biomarkers and survival

The results of the univariate analysis of factors influencing tracheostomy-free survival in our patients' cohort are shown in **Table 7**.

Table 7. Cox proportional hazard regression at univariate analysis for clinical variables.

Variable	Observations (n)	Univariate analysis	
		HR (95% CI)	p-value
Sex (male as reference)	182	0.8 (0.65-1.25)	0.53
Age at onset	182	1.03 (1.01- 1.04)	<0.001
Diagnostic delay	182	0.96 (0.95 -0.98)	<0.001
Weight at sampling	182	0.99 (0.98-1)	0.19
BMI at sampling	182	0.98 (0.94- 1.02)	0.31
Mutation (WT as reference)	175	0.87 (0.54-1.41)	0.58
<i>c9orf72</i>		1.47 (0.79-2.74)	0.22
<i>SOD1</i>		0.37 (0.12-1.17)	0.09
other		0.79 (0.322-1.94)	0.61
Site of onset (spinal as reference)	182	1.48 (1.02-2.15)	0.04
Phenotype (classic as reference)	182		
Flail		0.43 (0.26-0.71)	0.001
UMNp		0.17 (0.07-0.43)	<0.001
Bulbar		1.12 (0.75-1.68)	0.56
Respiratory		0.93 (0.29-2.99)	0.90
Spasticity	177	0.96 (0.84-1.09)	0.53
Pseudobulbar Affect	179	1.35 (0.91-1.99)	0.13
<i>Frontal release signs</i>			
Palmomental reflex	164	1.02 (0.73-1.43)	0.89
Glabellar reflex	164	1.38 (0.89-2.12)	0.15
Snout reflex	169	1.22 (0.84-1.76)	0.29
Masseter reflex	163	1.27 (0.89-1.81)	0.19
CS hyperintensity	147	0.65 (0.41-1.03)	0.07
MEP prolongation	151	0.93 (0.64-1.38)	0.75
FTD	182	2.15 (1.28-3.63)	0.004
ALS-ci	180	2.29 (1.41-3.73)	0.001
ALS-bi	180	2.15 (1.36-3.4)	0.001
FVC at sampling	173	0.99 (0.98 -99)	<0.001
DeltaFS at sampling	182	1.03 (0.97-1.1)	0.31
ALSFRS-r at sampling	182	0.98 (0.96 - 1.00)	0.10
King's at sampling	182	1.16 (0.98-1.37)	0.08
MiTos at sampling	182	1.14 (0.87-1.49)	0.35
Concomitant depression	182	1.06 (0.76-1.48)	0.73
Concomitant psychosis	182	0.78 (0.19-3.12)	0.72

Parkinsonism	182	0.34 (0.08-1.37)	0.13
BPCO	182	2.38 (1.31-4.32)	0.004
Diabetes	182	1.25 (0.71-2.22)	0.44
Hypertension	182	1.44 (1.04-1.99)	0.027
Cardiopathies	182	1.03 (0.63-1.68)	0.92
Dyslipidemia	182	1.4 (0.99-1.98)	0.06
Autoimmune diseases	182	0.48 (0.21-1.08)	0.08
Oncological history	181	0.87 (0.49-1.55)	0.65
Concomitant statins	76	1.08 (0.59-1.97)	0.81

Legend. Hazard ratios (HR) are presented with 95% confidence interval (95%CI) and relative p-value. CI: confidence interval. HR: hazard ratio. BMI: body mass index. CSF: cerebrospinal fluid. WT: wild-type. FTD: frontotemporal dementia. FVC: forced vital capacity. ALSFRS-r: Amyotrophic Lateral Sclerosis Functional Rating Scale-revised. Please refer to Methods section for calculation of clinical variables.

Univariate analysis showed that factors impacting on tracheostomy free survival in our cohort were diagnostic delay (months, HR 0.96, 95% CI 0.95-0.98, $p<0.001$), age at onset (years; HR 1.03, 95% CI 1.01-1.04, $p<0.001$), cognitive impairment (FTD HR 2.15, 95% CI 1.28-3.63, $p=0.004$, ALSci HR 2.29, 95% CI 1.41-3.73, $p=0.001$, ALSbi HR 2.15, 95% CI 1.36-3.4, $p=0.001$), FVC at sampling (%; HR 0.99, 95% CI 0.98-0.99, $p<0.001$), phenotype (flail with respect to classic HR 0.43, 95% CI 0.26-0.71, $p=0.001$; UMNp with respect to classic HR 0.17, 95% CI 0.07-0.43, $p<0.001$), site of onset (spinal as reference HR 1.48, 95% CI 1.02-2.15, $p=0.04$). Interestingly, MiTos score and King's staging at sampling fail to predict survival alone (MiTos HR 0.35, 95% CI 0.87-1.49, $p=0.35$; King's HR 1.16, 95% CI 0.98-1.37, $p=0.08$), as well as ALSFRS-r and DPR at sampling (for ALSFRS-r HR 0.98, 95% CI 0.96-1, $p=0.10$; for DPR HR 1.03, 95% CI 0.97-1.1) . Among comorbidities, in our population the presence of COPD and hypertension leads to shorter survival (HR 2.38, 95% CI 1.31-4.32, $p=0.004$; HR 1.44, 95% CI 1.04-1.99, $p=0.027$ respectively), while we observed a mild influence of dyslipidemia (HR 1.4, 95% CI 0.99-1.98, $p=0.06$) and protective action by the presence of concomitant autoimmune diseases (HR 0.48, 95% CI 0.20-1.08, $p=0.08$) without reaching a statistical significance. Conversely, weight and BMI at diagnosis as long as weight loss at sampling did not influence survival, neither genetic status with the sole exception of *SOD1* mutation that trend to predict a relentless course (HR 0.37, 95% CI 0.12-1.17, $p=0.09$).

Besides demographic and clinical features associated with tracheostomy-free survival, both NfL and pNFH confirmed as a good predictor of survival, as shown in **Table 8**, as well as

CHI3L1 levels (HR 1.003, 95% CI 1.001-1.004, p=0.001) among all the neuroinflammatory biomarkers.

Table 8. Cox proportional hazard regression at univariate and multivariate analysis for biological markers.

Variable	Observations (n)	Univariate analysis		Multivariate analysis (n=86)	
		HR (95% CI)	p-value	HR (95% CI)	p-value
NfL _{serum}	175	1.003 (1.002 - 1.004)	<0.001	1.004 (1.0018-1.0062)	<0.001
NfL _{CSF}	138	1 (1-1.00004)	<0.001	0.99997 (0.99993-1)	0.14
pNfH _{serum}	181	1 (1-1.0002)	<0.001	0.9999 (0.9997- 1)	0.39
pNfH _{CSF}	142	1 (1 -1.0002)	<0.001	0.999997 (0.99993-1)	0.94
SerpinA1 _{serum}	178	0.9998 (0.9996-1)	0.18		
SerpinA _{CSF}	139	1.014 (0.99-1.04)	0.18		
TREM _{serum}	98	1.01 (0.98-1.03)	0.55		
TREM _{CSF}	89	1.008 (0.98-1.03)	0.45		
CHI3L1 _{serum}	181	1 (0.9977-1.003)	0.69		
CHI3L1 _{CSF}	139	1.003 (1.001-1.004)	0.001	1.002 (0.9997- 1.0049)	0.08
OPN _{serum}	58	1.002 (0.99-1.01)	0.71		
OPN _{CSF}	58	1.001 (0.9988-1.004)	0.31		
Leucocytes	113	1.016 (0.9-1.15)	0.79		
Platelets	113	1.003 (0.999-1.006)	0.053		
Neutrophils	112	1.086 (0.942-1.25)	0.25		
Linfociti	106	0.758 (0.50-1.14)	0.18		
Monocytes	106	1.18 (0.267-5.18)	0.83		
NLR	106	1.15 (0.99-1.34)	0.064		
MLR	106	3.89 (0.54-27.99)	0.18		
SIRI	106	1.43 (1.03-1.966)	0.03	0.6089 (0.0217-17.065)	0.77
AISI	106	1.002 (1.001-1.003)	0.002	1.002 (1.0004- 1.0031)	0.012
SII	106	1.001 (1.0003-1.001)	0.003	0.9996 (0.9923-1.007)	0.92

Abbreviations: AISI : aggregate index of systemic inflammation; HR: Hazard Ratio; NfL: neurofilament light chain. pNfH: neurofilament heavy chain; NLR : neutrophil to lymphocyte ratio, MLR : monocyte to lymphocyte ratio; SIRI : systemic inflammation response index; SII : systemic immune-inflammation index;. Please refer to Methods section for calculation of clinical variables.

For NfL and CHI3L1 on CSF we built up Kaplan-Meier survival estimates grouping ALS patients according to median (**Figure 12, 13**).

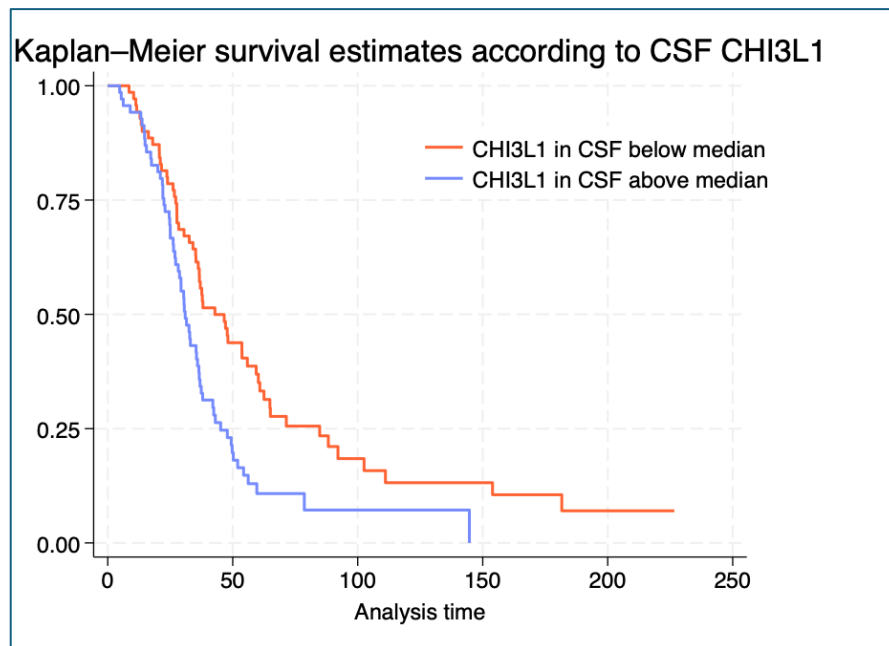


Figure 12. Kaplan-Meier survival curves grouping ALS patients according to CSF CHI3L1 values below and above median.

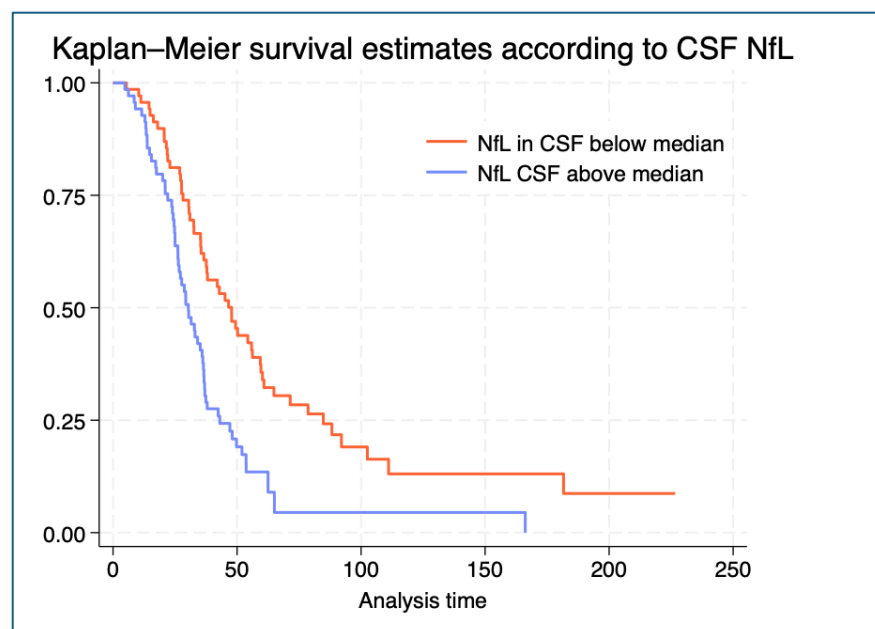


Figure 13. Kaplan-Meier survival curves grouping ALS patients according to CSF NfL values below and above median.

For what concern systemic indexes, it's noteworthy that several indicators of inflammation influence survival, namely SIRI (HR 1.43, 95% CI 1.03-1.966, $p=0.03$), AISI (HR 1.002, 95%CI 1.001-1.003, $p=0.002$) and SII (HR 1.001, 95% CI 1.0003-1.001, $p=0.003$), while NLR only tend to be impacting on survival (HR 1.15, 95% CI 0.99-1.34, $p=0.064$). Interestingly, among them AISI retained its power also at multivariate analysis (HR 1.002, 95% CI 1.0004-1.001, $p=0.012$).

Accordingly, after grouping ALS patients according to quartile AISI values, we noted that AISI has an unfavorable prognostic value for survival at extreme values (lowest and highest quartile) compared to intermediate values (interquartile range) (**Figure 14**)

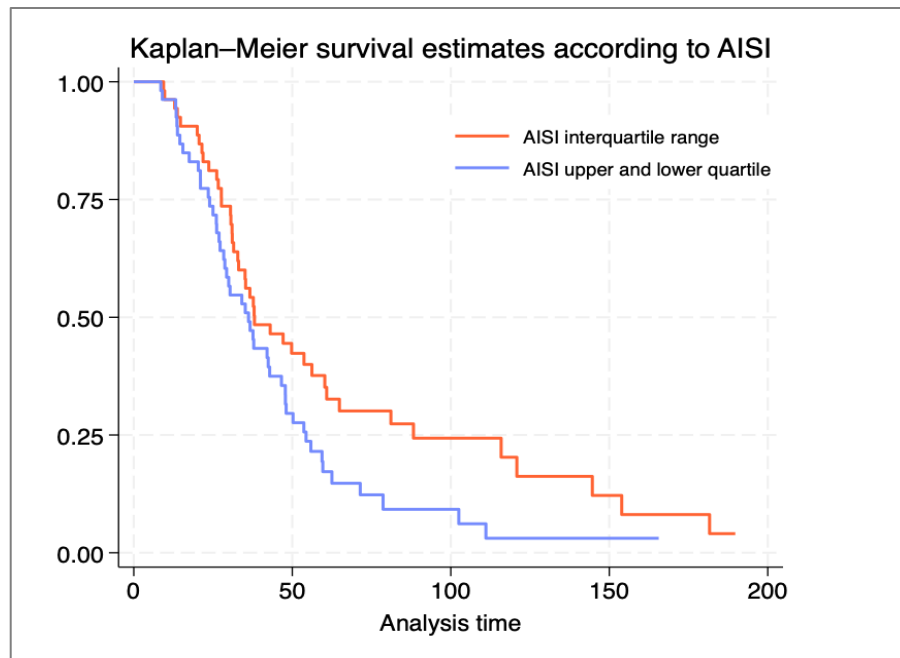


Figure 14 Kaplan-Meier survival estimates according to AISI values, grouping ALS patients according to interquartile range vs extremity quartiles.

Rather, for SII index we built up Kaplan-Meier survival estimates grouping ALS patients according to upper and lower quartile (**Figure 15**)

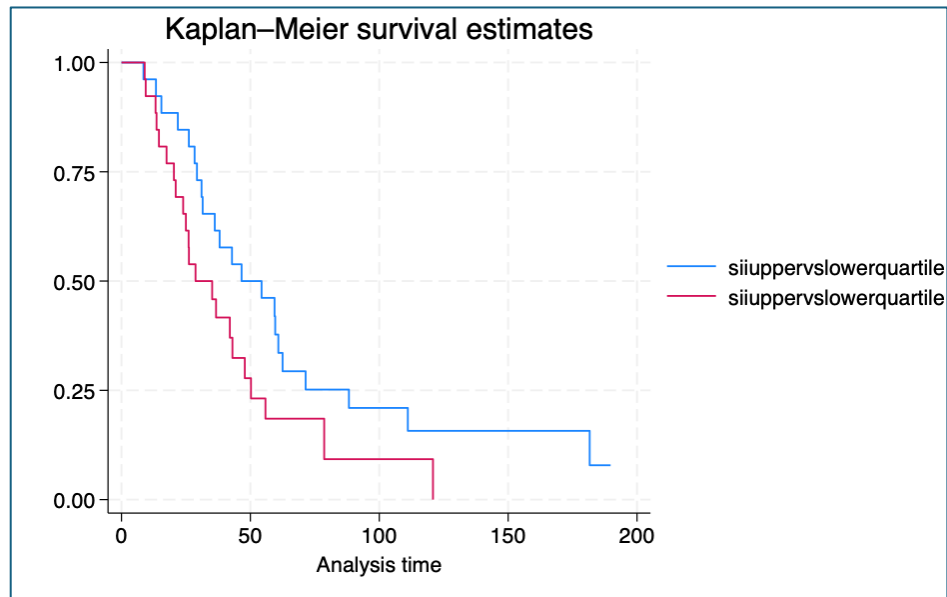


Figure 15 Kaplan-Meier survival estimates according to SII indexes, grouping ALS patients according to quartiles (upper quartile vs lower quartile).

Finally, we've taken into consideration sex and age in univariate analysis of survival, showing several differences between females and males on biomarkers impacting on tracheostomy free survival (**Table 9**).

Indeed, only for females SerpinA1 levels in CSF influenced survival (HR 1.042, 95%CI 1.0065-1.078, $p=0.02$), in a similar manner of other inflammatory biomarkers, namely NLR (1.154, 95% 0.099-1.333, $p=0.05$), MLR (HR 8.572, 95%CI 1.046-70.23, $p=0.045$), SIRI (HR 1.483, 95% 1.082-2.0334, $p=0.014$) and SII (HR 1.00099, 95% 1.0008-1.003, $p=0.001$). Conversely, for pNFH, NfL, CHI3L1 on CSF and AISI, the influence on survival did not differ between females and males, although a stronger statistical significance emerged for female group.

Table 9. Cox proportional hazard regression at univariate for biological markers considering sex.

Variable	Observations (n)	Univariate analysis			
		Female		Male	
		HR (95% CI)	p-value	HR (95% CI)	p-value
pNFH serum	181	1.0001 (1.00006-1.0002)	0.001	1.0001 (1.00002-1.0002)	0.014
pNFH CSF	142	1.00009 (1.00003 -1.0001)	0.001	1.00005 (1.00002 - 1.00008)	0.008
NfL serum	165	1.005 (1.004- 1.007)	<0.001	1.003 (1.0014 - 1.0039)	<0.001
NfL CSF	138	1.00006 (1.00003 -1.00008)	<0.001	1.00003 (1.00001 -1.00004)	<0.001
TREM2 serum	98	1.007 (0.98258 - 1.0327)	0.56	1.007 (0.98136 - 1.0335)	0.59
TREM2 CSF	89	1.006 (0.9847- 1.028)	0.57	1.0107 (0.9882 - 1.0337)	0.35
Serpina1 serum	178	0.99989 (0.9995-1.0002)	0.58	0.9998 (0.9995 - 1.00007)	0.16
Serpina1 CSF	139	1.042 (1.0065-1.078)	0.02	1.0104 (0.98805-1.0332)	0.37
CHI3L1 serum	181	1.0014 (0.99805 -1.00476)	0.41	0.99988 (0.9965 -1.0033)	0.95
CHI3L1 CSF	131	1.003 (1.0009 -1.005)	0.005	1.0024 (1.0007 -1.0042)	0.006
OPN serum	58	1.0019 (0.9906 1.01338)	0.74	1.00234 (0.98926 - 1.0156)	0.73
OPN CSF	58	1.0012 (0.99848 - 1.0040)	0.38	1.00137 (0.998507 -1.0042)	0.35
Neutrophils	112	1.094 (0.951-1.2577)	0.21	1.052 (0.893-1.2399)	0.54
Platelets	113	1.0034 (1.0001-1.00665)	0.043	1.0025 (0.9989-1.006)	0.17
Lymphocytes	106	0.82 (0.5389- 1.2527)	0.36	0.707 (.04619-1.0817)	0.11
Monocytes	106	1.688 (0.3666 - 0.7695)	0.50	0.93 (0.2055-4.2507)	0.92
NLR	106	1.153 (0.9986 - 1.333)	0.05	1.06 (0.8399 - 1.346)	0.61
MLR	106	8.57 (1.0462 -70.228)	0.045	2.528 (0.32 -19.905)	0.378
SIRI	106	1.48 (1.08- 2.03)	0.014	1.26 (0.84-1.889)	0.26
AISI	106	1.002 (1.0008-1.003)	0.001	1.0015 (1.0001 -1.003)	0.036
SII	106	1.0009 (1.0004- 1.0016)	0.002	1.0008 (0.9999-1.0016)	0.07

Abbreviations: AISI : aggregate index of systemic inflammation; HR: Hazard Ratio; NfL: neurofilament light chain. pNFH: neurofilament heavy chain; NLR : neutrophil to lymphocyte ratio, MLR : monocyte to lymphocyte ratio; SIRI : systemic inflammation response index; SII : systemic immune-inflammation index;. Please refer to Methods section for calculation of laboratory variables.

When considering age for stratifying ALS patients, we observed CSF SerpinA1 acted as a negative prognostic factor in patients older than 65 years old (HR for age class 65-75 years old: 1.030, 95% CI 1.0005-1.059, p=0.046; HR for patients older than 75 years old: 1.087, 95% CI 1.016-1.16, p=0.015) while AISI and SII influenced more patients younger than 75 years old [for patients < 65 years HR 1.002 (95% CI 1.0005-1.003) for AISI and HR 1.0008 (95% 1.0001-1.0015) for SII, p=0.02; for patients 65-75 years, HR 1.002 (95% 1.0006-1.003), p=0.005) for AISI and HR 1.001 (1.0003-1.002), p=0.005) for SII] (**Table 10**).

Table 10. Cox proportional hazard regression at univariate for neuroinflammatory and systemic inflammation markers considering age.

Variables	Observations	Univariate Analysis					
		<65 years		65-75 years		>75 years	
		HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
TREM2 serum	98	0.999 (0.9706-1.0299)	0.99	1.007 (0.9807-1.0336)	0.615	1.009 (0.9823 - 1.038)	0.49
TREM2 CSF	89	1.004 (0.9817 - 1.027)	0.735	1.015 (0.988-1.0435)	0.273	1.0147 (0.988-1.0421)	0.284
SerpinA1 serum	187	0.999 (0.9995-1.00006)	0.119	0.9998 (0.9995-1.0002)	0.43	1.00008 (0.9996- 1.0005)	0.735
SerpinA1 CSF	139	1.003 (0.9731-1.0331)	0.86	1.0296 (1.0005-1.0595)	0.046	1.0868 (1.016-1.1627)	0.015
CHI3L1 serum	181	0.999 (0.9939-1.0033)	0.562	1.001 (0.9973 - 1.0048)	0.596	1.0014 (0.9974-1.0054)	0.487
CHI3L1 CSF	139	1.002 (1.0003 - 1.004)	0.023	1.003 (1.0006-1.0059)	0.016	1.004 (1.00114-1.006)	0.004
OPN serum	58	0.998 (0.9856-1.0107)	0.764	1.0046 (0.9937-1.0156)	0.408	1.004 (0.984-1.025)	0.689
OPN CSF	58	1.001 (0.9982 - 1.0038)	0.466	1.0017 (0.998-1.005)	0.297	1.0016 (0.9976 - 1.0056)	0.438
Neutrophils	112	1.059 (0.9125-1.2289)	0.451	1.1187 (0.9518 - 1.3149)	0.174	0.9571 (0.6715-1.364)	0.808
Lymphocytes	106	0.723 (0.4796-1.0897)	0.121	0.858 (0.5502-1.337)	0.498	0.5845 (0.286-1.154)	0.122
NLR	106	1.125 (0.9532-1.3267)	0.164	1.213 (0.996-1.477)	0.55	0.8145 (5146-1.625)	0.761
MLR	106	2.996 (0.358-25.054)	0.311	4.843 (0.0585-40.085)	0.143	1.2806 (0.0262-62.686)	0.901
SIRI	106	1.356 (0.952-1.932)	0.091	1.503 (1.030-2.192)	0.035	0.994 (0.298-3.311)	0.992
AISI	106	1.002 (1.0005-1.003)	0.009	1.002 (1.0006-1.003)	0.005	1.001 (0.995-1.008)	0.678
SII	106	1.0008 (1.0001-1.0015)	0.02	1.001 (1.0003-1.002)	0.005	1.0004 (0.997-1.004)	0.804

Abbreviations: AISI : aggregate index of systemic inflammation; HR: Hazard Ratio;NLR : neutrophil to lymphocyte ratio, MLR : monocyte to lymphocyte ratio; SIRI : systemic inflammation response index; SII : systemic immune-inflammation index;. Please refer to Methods section for calculation of laboratory variables.

5 DISCUSSION

Over the past decade, significant progress has been made in identifying NFs as the most promising biomarkers for ALS in clinical practice, crucial for diagnostic purpose and better prognostic accuracy. Traditional approaches to assessing disease dynamics typically focus on neuronal axon-derived markers like NFs, general epidemiological factors such as age at onset and diagnostic delay, or clinical indicators like bulbar onset and functional rating scales. These methods provide a limited perspective because they do not fully account for the interdependent roles of various cell types in the nervous system and do not represent faithfully ALS heterogeneity in terms of neurological involvement and survival. Since ALS neurodegeneration dynamics are influenced by support mechanisms from multiple cell types including astrocytes and microglia, the neuron-centric view does not adequately explain the diverse sporadic ALS phenotypes (Tam et al., 2019) and the disease's progression dynamic (Westeneng et al., 2018). Most current molecular indicators used in clinical settings to predict survival variability are based on neuron-derived proteins, with only recent consideration of microglia-derived proteins (Gille et al., 2019).

In this context, in a deeply phenotyped ALS cohort we explored if SerpinA1, CHI3L1, S100B and OPN may have an independent value respect to neurofilaments in ALS prognosis.

One of the novel findings is the sex-mediated action of CSF SerpinA1 and CHI3L1 on survival, with a more severe impact in survival analysis for females. CSF SerpinA1 levels were significantly higher in male patients, while serum levels of SerpinA1 were not differently expressed in our cohort in relation with sex. The reason behind the distinct expression of SerpinA1 in the two group is not univocally explainable. Indeed, in literature only few papers have analyzed the impact of sex in SerpinA1 distribution: studies in PD did not found that SerpinA1 levels were not influenced by sex (Halbgebauer et al., 2016a), as well as in AD (Nielsen et al., 2007), while for ALS patients no reports on sex influence of SerpinA1 CSF levels have been published.

Even more interesting, after stratification of ALS patients by age, we noted that CSF SerpinA1 acted as a negative prognostic factor only in older patients, in a similar way to CSF CHI3L1. While for SerpinA1 we did not find in literature data on these age-dependent action on survival, for CHI3L1 the effect of age has been already considered (Sanfilippo et al., 2019; Gray et al., 2020; Gaur et al., 2021). Our results, along with literature reports, seem

to suggest that the increase of gliosis biomarkers with age could indicate a wider “inflammaging” process. Another potential explanation may stem from similar findings on CHI3L1 in AD, indicating an age-related pattern (Sanfilippo et al., 2019) potentially influenced by the loss of sex hormones, especially in females (Nissen, 2017). Increasing evidence suggests that not only do the numbers of glial cells differ between males and females, but so do their activities and signaling. For instance, 75% of adult male mice microglia display an inflammatory phenotype (globular morphology) compared to the protective phenotype (ramified branches) common in adult female mice, where only 50% of microglia are activated (Villa et al., 2018). This suggests that as women age, microglia may undergo transcriptome changes, shifting from a protective to an inflammatory state. Therefore, CHI3L1 and SerpinA1 expression levels in the aged patients could reflect not just the imbalance in glial cell numbers but also their different activity (Sanfilippo et al., 2019).

Serum NfL levels tended to be higher among females than males, an observation already reported in literature (Lu et al., 2015; Benatar et al., 2020; Verde et al., 2021). Although the biological reasons for the sex differences observed in ALS are not well understood, our data suggest that the higher sNFL levels seen in female patients might be due to a more aggressive disease among females, as indicated by the higher DPR at sampling and a greater prevalence of bulbar onset. Further, the demographic characterization of our population counting for sex revealed that *C9ORF72* expansion, known to be associated with a faster disease course (Millecamps et al., 2012; Gianferrari et al., 2022b; Grassano et al., 2022; Mandrioli et al., 2023), and bulbar onset were more frequent among females, in accordance with literature data (Chio et al., 2020; Gianferrari et al., 2022b).

Conversely, we observed that TREM2 serum levels were higher in females than in males, while an opposite behavior emerged from TREM2 CSF levels, with lower concentrations for females. In literature, we did not find reports on sex influence in ALS cases (Cooper-Knock et al., 2017; Jericó et al., 2023), while in AD higher levels had been reported in males (Suárez-Calvet et al., 2016). Moreover, serum levels of TREM2 correlates with increasing age, while its levels on CSF only showed a trend to increase, a notion already reported in AD and healthy controls (Suárez-Calvet et al., 2016).

When considering the site of onset, not a single neuroinflammatory biomarker demonstrated a difference in behavior between spinal and bulbar onset, suggesting that the type of initial

involvement impact limitedly on biomarkers' concentration. Clinical features according to the site of onset show longer diagnostic delay and time to generalization, in accordance with previous reports (Chio et al., 2011; Gianferrari et al., 2022b). Patients with bulbar onset more frequently presented with concomitant cognitive impairment, as already reported (Chio et al., 2020; Carbayo et al., 2024), and did not show a more drastic weight loss at sampling neither a lower BMI confirming literature data (Moglia et al., 2019). The genetic assessment in the two groups revealed a sole presence of *SOD1* mutations in patients with spinal onset, a distribution in line with the typical phenotype associated with *SOD1* (Chio et al., 2020; Opie-Martin et al., 2022; Martinelli et al., 2023). In an opposite way, we observed that pNfH concentrations in CSF were clearly higher among spinal forms compared with bulbar ones. This trend was not valid for NFs on serum, which were not differently distributed among the two different onset, being also characterized by relatively high levels in bulbar forms. A reasonable explanation behind the higher CSF concentrations of NFs could reside in the lower ALSFRS-R and higher DPR, MiTos and King's staging scores at sampling, reflecting a more severe involvement of spinal forms in our cohort. Similar to previous evidence (Verde et al., 2023b), also in our study population there was no association between NFs levels and respiratory impairment, as measured by FVC at sampling.

Quite surprisingly, one of the clearest findings of our study is the association of increased serum NfL levels with the presence of FTD in ALS, indicating a pathological contribution of cognitive dysfunction in increasing serum NfL, in contrast with previous literature reports (Steinacker et al., 2018; Falzone et al., 2022; Verde et al., 2023a, 2023b) where Nfs more accurately represent MNs degeneration. Furthermore, if serum NFs did not correlate significantly with age at onset, NfL on CSF which revealed a weak negative correlation with age of onset, in disagreement with literature reports (Verde et al., 2019, 2023a). The reason behind this discrepancy could derive from a high proportion of younger patients among males, with presented higher disease severity scales at sampling (DPR and ALSFRS-R).

Another key-point of our explorative study concerns biomarkers' association. In our population NFs confirmed the already reported strong association between CSF and serum (Gaiottino et al., 2013). With a similar trend, we observed that also CHI3L1, TREM2 and OPN levels reveal an association between the two compartments, indicating that the production of this molecules is mainly localized in the central nervous system and

subsequently diffuse in venous blood. The findings on CHI3L1 are in contrast to literature data, which indicate a poor correlation between CSF and serum levels of the chitinase proteins (Thompson et al., 2019), along with to their low utility as an ALS biomarker (Steinacker et al., 2017; Oeckl et al., 2019; Bombaci et al., 2023).

Interestingly, in contrast to the other biomarkers, SerpinA1 and S100B concentrations in CSF and serum did not correlate. Although SerpinA1 can be easily quantified in the CSF, the source of CSF serpinA1 is still not clear (Barba et al., 2022), as well as what cell types express SerpinA1 in the CNS, and which isoforms of SerpinA1 are contained in the CSF. The protein might diffuse out of venous blood and be released from the brain tissue into the CSF (Halbgebauer et al., 2016b). Alternatively, but not mutually exclusive, they may result from an intrathecal production of SerpinA1, as already reported for AD patients (Jesse et al., 2012). Future investigations are required to understand the pathological significance of the lack of correlation between serum and CSF SerpinA1.

In our population CHI3L1 levels on CSF are also correlated with both NfL and pNFH, a strong marker of axonal neurodegeneration, confirming the link between neuroinflammation and neurodegeneration, in agreement with previous works (Illán-Gala et al., 2018; Oeckl et al., 2019; Thompson et al., 2019; Vu et al., 2020). Importantly, we've observed that CHI3L1, TREM2, SerpinA1 levels on CSF correlate each other, indicating a common pathway in neuroinflammation. Indeed, CHI3L1 is also produced by reactive astrocytes and elevated levels of this protein in the CSF are indicative of glial activation that may potentiate neuroinflammation and MN death in ALS (Gaur et al., 2020).

Quite surprisingly, CSF TREM2 represents the more frequently associated biomarker with the others, namely both CSF neurofilaments and SerpinA1 levels in serum (negative correlation) and CSF, OPN levels in serum and CSF and S100B in CSF. Serum TREM2 concentrations were linked with SerpinA1 measures both on serum (positive correlation) and CSF. A mild yet significant correlation between CSF and serum sTREM2 levels has been already reported in ALS population (Jericó et al., 2023), while to the best of our knowledge our work firstly reveal this widespread association with other biomarkers, indicating a common pathway between neurodegeneration and neuroinflammation. In line with this hypothesis, Jericó et al. revealed that pTDP43 levels in CSF increased the predictive value of sTREM2, suggesting that the two pTDP43 and sTREM2 may share a common pathogenesis. Actually, TREM2 is a key component of the immune module and has been

linked to ALS pathogenesis (Cady et al., 2014) and microglial activation (Cantoni et al., 2015). This protein, when cleaved from microglial surfaces, becomes soluble TREM2, which has been proposed as a biomarker for neurological diseases such as AD and multiple sclerosis (Piccio et al., 2008; Suarez-Calvet et al., 2014; Heslegrave et al., 2016). Loss-of-function mutations in the *TREM2* gene, associated with increased risks of ALS (Cady et al., 2014), result in reduced phagocytosis of aggregated proteins by microglia (Kleinberger et al., 2014). The decreased phagocytic function may harm stressed neurons, and TREM2 activity has been linked to a neuroprotective microglial phenotype (Raha et al., 2017).

Subsequently, we analyzed neuroinflammatory biomarkers' distribution among ALS patients according to several measures of progression. Firstly, neurofilaments demonstrated a strong correlation with the principal surrogates of disease progression, but failed to be associated with MiToS scores and King's staging, disagreeing with previous works (Verde et al., 2023b). In particular, NFs has a negative correlation with both ALSFRS-R and diagnostic delay/time to generalization and a stronger positive correlation with DPR calculated at sampling and last observation, in line with literature (Gaiani et al., 2017; Steinacker et al., 2017; Verde et al., 2019; Abu-Rumeileh et al., 2020b; Verde et al., 2023b). The rapid progression prompts patients to seek medical attention earlier, by which time the disease has already caused significant functional deterioration. This interpretation aligns with the negative association between neurofilaments and diagnostic delay, time to generalization and survival, both at univariate and multivariate analysis.

While no other biomarker related with MiTos score, we noted that CHI3L1 serum concentrations tended to be higher with higher King's staging score, being similar in stages 1 and 2 and increasing progressively from stage 2 to stage 4. To the best of our knowledge, no one had never correlated CHI3L1 with the King's and MiTos disease stages, while several papers have revealed an intriguing correlation with UMN/LMN burden (Thompson et al., 2019; Abu-Rumeileh et al., 2020b), indicating a significant upregulation of CHI3L1 in corticospinal tract (Steinacker et al., 2017; Oeckl et al., 2019). Then, both CHI3L1 in CSF and serum directly correlate with age at onset, according to literature reports on healthy population (Bojesen et al., 2011). The absence of correlation between CHI3L1 and respiratory measures has been already reported (Costa et al., 2021; Bombaci et al., 2023). Although previous reports revealed CHI3L1 is increased in ALS-FTD continuum (Alcolea

et al., 2014; Thompson et al., 2019; Bombaci et al., 2023), we failed to demonstrate a different distribution according to cognitive impairment. Instead, when considering FTD, only CSF S100B concentrations were tendentially lower in FTD subgroup in comparison with cognitively spared patients, a result not reported in literature where only serum levels of S100B have been measured (Steinacker et al., 2017).

In our cohort, CHI3L1 in CSF correlates with DPR at last observation and significantly impacts on survival both at univariate and multivariate analysis. The role of chitinases in ALS prognosis has been investigated with various clinical outcomes, resulting in conflicting findings (Gaur et al., 2020). While Thompson et al. found no correlation between CHI3L1 levels in CSF and the rate of disease progression after accounting for known confounders (Thompson et al., 2019), other studies indicated that "fast" progressors had significantly higher levels of CHI3L1 compared to "slow" progressors (Illán-Gala et al., 2018; Gille et al., 2019; Oeckl et al., 2019; Abu-Rumeileh et al., 2020b; Vu et al., 2020; Costa et al., 2021). Interpreting these results is difficult because classifying progression rates based on individual cohorts limits inter-study comparability (Gaur et al., 2020). Furthermore, studies on survival have not consistently determined the extent to which chitinases influence survival: some studies found that higher chitinase levels were associated with shorter survival (Di Rosa et al., 2013; Illán-Gala et al., 2018; Gille et al., 2019), whereas Thompson et al. reported opposite findings (Thompson et al., 2018, 2019).

Interestingly, SerpinA1 in serum correlates with diagnostic delay and DPR at last observation in a sensitive manner, while CSF levels reveal an opposite tendency, without reaching a statistical significance. Therefore, serum SerpinA1 levels differently distributed along the classes of progressors, being higher for very slow progressor and decreasing gradually in intermediate and very fast progressors. Accordingly, sustained levels of circulating SerpinA1 have been correlated with increased Treg cells (Subramanian et al., 2011), that likely contribute to disease progression in ALS where high frequency of activated Tregs and high ratio between activated and resting Tregs in blood are associated with better survival (Yazdani et al., 2022). Decreased serum levels of SerpinA1 may mirror the already described Tregs reduction and dysfunction in ALS patients (Henkel et al., 2013). In this hypothetic explanation, SerpinA1 may exert a protective immune action also by inhibiting neutrophil-released elastase and proteinase-3 inside and outside the cell, in this way controlling inflammation deleterious effects, such as activation of IL-1 β . SerpinA1 has been found to have independent antiinflammatory/ immunomodulatory properties, protecting

against cell apoptosis, inhibiting superoxide production, and inducing IL-1 receptor antagonist expression (Aggarwal et al., 2016). Moreover, in vitro and in vivo studies on SerpinA1 expression might be important to understand whether SerpinA1 really mitigates the pathological hallmarks through the modulation of neuroinflammation in some patients or whether its dysregulation is only one of several downstream effects of the pathological process characterizing ALS. To this extent, besides similar observations in other neurodegenerative conditions (Puchades et al., 2003; Nielsen et al., 2007; Halbgebauer et al., 2016b; Abu-Rumeileh et al., 2020a), more recently, different SerpinA1 isoforms were found prevalent in different dementia groups, underlying the complexity of these proteases and the importance of further studies focused on possible ALS-related isoforms. Lastly, SerpinA1 was not related to any demographic variable, nor with the inflammatory markers, or “pro-inflammatory” habits or comorbidity (i.e. COPD, autoimmunity diseases) (Kelly-Robinson et al., 2021) measured in our study population. Further larger studies should assess also these interesting and possibly clinically useful points.

In our population, we noted that TREM2 on serum (not on CSF) correlated directly with longer time to generalization and delay, in contrast to neurofilaments, a finding concordant with previous reports (Cooper-Knock et al., 2017). Soluble TREM2 levels are elevated in the early stages of ALS compared to the later stages (Cooper-Knock et al., 2017), a pattern also observed in AD (Suarez-Calvet et al., 2014). Although significant elevated levels were found to predict survival when measured in late stages of disease (Cooper-Knock et al., 2017), we did not reveal a correlation between CSF or serum sTREM2 levels in ALSFRS-R score, neither survival, similarly to Jericó et al., (Jericó et al., 2023).

Finally, although neither OPN in CSF neither S100B demonstrate a correlation with ALS clinical indicators of progression, OPN in serum correlates with ALSFRS-r score at sampling, similarly to a recent report (de Luna et al., 2023). Rather, OPN serum levels differently expressed along King’s staging but without a direct correspondence with increasing severity. OPN concentrations are reported to increase in microglia during the ALS progression, playing a role in the pathogenesis, but it is unclear whether OPN expression in microglia plays a neurotoxic or neurotropic role at the different stages of ALS disease (Rosmus et al., 2022). Coherently, osteopontin (also named Spp1) had been reported to be differentially expressed in the CSF and the motor cortex transcriptome of patients with ALS compared with that in HCs and serum levels were significantly higher in sera from patients with ALS than in HCs (de Luna et al., 2023; Lumi et al., 2024). More recently, Månberg et

al. demonstrated that increased levels of plasmatic SPP1 at disease diagnosis predicted shorter survival with stronger effect than the established risk factors of bulbar onset or neurofilament levels in cerebrospinal fluid (Månberg et al., 2021). The authors interpreted these results considering perivascular fibroblast activity which preceded the microglial response and neurodegeneration, rediscussing the importance of blood vessel-derived mechanisms (Månberg et al., 2021).

In a separate part of our research, we comprehensively evaluated multiple blood-formula derived inflammatory indexes in a subgroup of ALS patients.

Interestingly, in contrast to the other neuroinflammatory and axonal biomarkers, in our cohort we noted a different distribution of several peripheral biomarkers according to the type of onset. In this regard, monocytes were more sharply reduced among patient with bulbar onset, as well as other components of leucocytes formula (eosinophils and basophils) along with monocytes' derivative indexes SIRI and AISI (SIRI: $neutrophils \times monocytes/lymphocytes$; AISI: $neutrophils \times platelets \times monocytes/lymphocytes$). Our data align coherently with McGille et al. research (McGill et al., 2020), where an association between monocyte frequencies and the degree of functional impairment of bulbar muscles in patients with ALS has been reported. The interpretation of these data could take into account that a reduction in peripheral blood monocytes could be due to their recruitment and infiltration into the CNS (Mantovani et al., 2009; Butovsky et al., 2012). Research on patients with greater bulbar involvement has reported atypical neurofibrillary tangles, basophilic inclusions, distinct metabolic states, more significant microstructural damage to cerebral white matter, and impaired cognition (Schreiber et al., 2005; Shellikeri et al., 2017; Trojsi et al., 2017). Future studies should investigate monocyte infiltration in post-mortem brain and spinal cord tissue from ALS patients and correlate it with bulbar symptoms and associated pathology.

Next, NLR demonstrates a correlation with disease progression rate at last observation, as well as with ALSFRS_r at sampling, similar to AISI and SIRI indexes. These data are consistent with previous studies that have linked neutrophil levels/NLR with disease progression and NLR with reduced survival (Choi et al., 2020; Murdock et al., 2021; Leone et al., 2022; Grassano et al., 2023). When considering our patients grouped by disease progression rate, we've only noticed that lymphocytes were higher among intermediate

progressors, suggesting a connection between progression and the adaptive immune response in ALS. However, the nature of our study prevented us from detecting changes in lymphocyte subpopulations, which have been extensively reported in ALS research (Mantovani et al., 2009; Sundal et al., 2015; Gustafson et al., 2017).

Overall, SII index (calculated as *neutrophils* $\times$ *platelets/lymphocytes*) acted in a peculiar way, deserving a special attention. Indeed, relative to demographic features, SII was remarkably higher in females and demonstrated a significant correlation with age of onset (with a negative direction). More interestingly, SII linked with DPR at last observation, ALSFRS_r at sampling and uniquely correlated with the decline FRS and CHI3L1 levels on CSF. In addition, at univariate analysis SIRI, AISI and SII were associated with survival, mainly in younger patients, while only AISI retained its value at multivariate analysis.

The Systemic Immune-Inflammation Index (SII) is a multi-marker index that provides a comprehensive measurement of the systemic immune-inflammatory response (Hu et al., 2014), studied in hepatocellular carcinoma and other malignant tumors (Chen et al., 2017), serving as a prognostic indicator in carcinoma research (Lu et al., 2016; Fu et al., 2018). More recently, in literature have emerged papers on SII levels in cardiovascular disease (Fani et al., 2020) and stroke (Wang et al., 2023), along with intracerebral hemorrhage and aneurysmal subarachnoid hemorrhage (Cheng et al., 2023), where it predicts poor outcomes with higher risk of cerebral vasospasm. To the best of our knowledge, in neurodegenerative disorders SII has been investigating in AD (Li et al., 2023), being associated with brain atrophy and A β deposition, PD (Li et al., 2021) where elevated SII was negatively associated with the ADL score, and SCA2 (Vázquez-Mojena et al., 2023) where it correlates with cognitive impairment.

Our stratified data by sex in survival analysis reveal a different behaviour of peripheral markers: NLR, MLR SIRI and AISI impacted on survival only in females, while AISI demonstrated to influence survival both in females and in males. This findings may suggest that in our ALS patients the response of immune system varies according to sex. While the reason for this discrepancy remains unclear, previous reports have highlighted sex-based differences in peripheral immunity in ALS (Zondler et al., 2016; Murdock et al., 2021; Grassano et al., 2023). Further research is needed to determine whether these immunological differences contribute to the variations in clinical manifestations between male and female ALS patients.

Taken together, the results of this part of our research reinforced that the peripheral immune system plays a significant role in the progression of ALS. These data add to the growing evidence suggesting that dysregulation of systemic immunity is a common feature among individuals with ALS, although only several indicators from systemic immunity related with patients' characteristics and survival. Although our study is far to fully consider all the complexes immunological mechanisms (i.d. the lymphocytes subpopulations noted to have a crucial role in ALS pathogenesis), our data showed that the measure of these easily available indicators could help clinicians in patients' evaluation and identification of well-responders to immunomodulating treatment. Not secondary, our results on sex and age differences emphasize the importance of considering these factors as variables when studying phenotypes and biomarkers in MND. This notion is reinforced by literature data on sex-differences in ALS pathobiology, not limited to CBC formula. For example, one study found that knocking out the fractalkine receptor in the microglia of ALS mice accelerated disease progression only in males (Cardona et al., 2006). Another study observed variations in axonal sprouting between male and female SOD1^{G37R} mice (Martineau et al., 2020). Furthermore, inherent differences in neutrophil activity have been described between males and females (Miller et al., 2004; Marin et al., 2010). Globally, all these data seem to suggest that separate immune mechanisms may exist in men and women and that future immune-based ALS studies should account for sex.

The present study has some strengths and limitations. All ALS patients were deeply clinically and genetically characterized and followed up, and a large set of comorbidities possibly affecting the levels of inflammatory markers and their ability to predict survival were taken into account. The association with survival data, the simultaneous determination on serum and CSF, the correlation with systemic inflammation indicators and more solid ALS biomarkers constitute a significant added value to our work. However, when ALS patients were stratified based on clinical variables, sample size for each subgroup became smaller with decreased power. Furthermore, we were not able to draw a more robust conclusion on the significance of the observed behavior of neuroinflammatory levels in ALS patients, since the diagnostic power of biomarkers was not addressed.

Another critical point is the cross-sectional nature of this study, that does not allow to obtain information on the longitudinal trajectory of biomarkers during ALS course, as well as the

correlative approach that did not assess if the biomarkers' behaviour in the CNS or peripheral blood could be a byproduct of neuronal damage, rather than a contributing factor.

Nevertheless, the present investigation contributes to deepen our knowledge of the clinico-biological significance of multimodal biomarker in ALS, which is of critical relevance in order to fully understand its potential and limitations. The development of biomarker panels and of multimodal biomarkers, where different modalities of investigations are combined to obtain more sensitive measurements, has been challenging.

By understanding how different biomarkers correlate with disease progression and severity, treatments could be tailored based on a patient's unique biomarker profile (Das et al., 2022). This individualized approach could potentially lead to improved outcomes, mitigating disease progression and enhancing the quality of life for patients. Additionally, the study of distinct biomarkers could contribute to drug development and clinical trials. Serving as objective measures of disease state or response to therapy, these biomarkers can provide critical insights into a drug's mechanism of action or efficacy (Shefner et al., 2022).

Exploring how these biomarkers change in response to disease progression and their interrelationships can help scientists build a more complete picture of the molecular and cellular changes driving neurodegenerative diseases. Understanding how biomarker levels change over the course of the disease and correlating these changes with clinical progression is a complex task (Shefner et al., 2022). Since many biomarker studies are based on small sample sizes, limiting their generalizability to larger, more diverse patient populations, larger, multi-center studies are urgently needed to validate these biomarkers.

6 CONCLUSIONS

In conclusion, one of the novel findings in our work is the sex-dependent influence of several neuroinflammatory and systemic inflammatory biomarkers on survival. Despite the studied-established differences in ALS rates between males and females, only few studies take sex into consideration as an independent variable in biomarkers' evaluation. Our current data indicate that differences in immunity exist between males and females affected by ALS, being linked to different disease outcomes. Moreover, SerpinA1 impact negatively on survival in older patients, while AISI and SII acted mainly in case of younger patients.

However, both neurofilament or CHI3L1 CSF levels outperform the inflammatory biomarkers at predicting rate of disease progression in our entire cohort of patients, so the prognostic value of these measure alone remains inconclusive.

Quite surprisingly, we've found a potential prognostic role for several indexes of peripheral immunity, mainly SII and AISI, which could easily derived from CBC formula and include mainly neutrophils and platelets compared to lymphocytes.

Should our findings be confirmed, they would be useful in clinical practice. On the one hand, predicting a different evolution is valuable for patients' management, counselling, and treatment choices. Fast progressors ALS are an even more fragile subgroup of people affected by ALS, as they are frequently excluded from research studies and clinical trials due to their devastating conditions, requiring the greatest efforts from ALS multidisciplinary teams that try to face in time the complex and various needs claimed by the fast disease progression. On the other hand, our observations suggest that immunomodulatory therapies for the treatment of ALS should be assessed separately by sex and age in both preclinical and early phase studies to better account for patient-specific immune signatures.

Altogether, our findings warrant further confirmatory studies in larger cohorts, where exploring candidate biomarkers could be a fascinating field of knowledge. Larger collaborative longitudinal studies will be necessary to explore how gliosis surrogate markers change across different disease stages. The differential behavior of inflammatory markers in ALS patients remains puzzling. Further research is needed to explore the origins of these markers and the activation patterns of various glial cells over the course of the disease. Unravelling these aspects will shed light on the role of systemic and neuro-inflammation in ALS, clarifying its impact on patient survival.

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