PhD program in Neuroscience
PhD School in Life and Health Sciences
University of Turin

CYCLE: XXXV

DOCTORAL THESIS

New insights in early diagnostic and prognostic clinical, instrumental, and fluids biomarkers in Amyotrophic Lateral Sclerosis

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ACADEMIC YEAR: 2022-2023

Alla mia famiglia

che mi ha sempre sostenuto

“La vera saggezza sta in colui che sa di non sapere”

(Socrate)

Summary

Section 2: New fluid diagnostic and prognostic biomarkers in ALS

Rationale of my PhD Thesis

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disease that provoke huge disability and shows a fatal exitus. No real cure exists, despite sizeable efforts have been done during the last two decades. Moreover, the onset of bulbar and respiratory impairments represents a frightful event since it is associated with a shorter survival and a worst quality of life.

The need to identify easily accessible, reproducible, and non-invasive biomarkers is crucial for enabling early disease diagnosis and, consequently, initiating available therapies as early as possible to slow down its progression. Simultaneously, there is a requirement for biomarkers capable of stratifying patients based on the risk of developing bulbar and/or respiratory impairments. This stratification allows for the early implementation of potential prophylactic therapies to slow the onset of these symptoms and provide accurate behavioral guidelines to patients. Furthermore, it is essential to identify prognostic biomarkers that enable both clinicians and patients to understand the disease's progression in the subsequent months/years and better select patients for enrollment in clinical trials. Predictive biomarkers of treatment response are also necessary, complementing the clinical scales currently used in clinical trials.

Building upon these premises, in the course of my doctoral research, I have sought to identify novel diagnostic and prognostic clinical, instrumental and fluids biomarkers.

I'm going to show you results obtained during these four years of PhD course.

My thesis is divided in 2 sections: in the first I'm presenting the results of studies I led in which I wanted to find out new clinical, instrumental and bio-humoral biomarkers with the aim of early identification of bulbar and respiratory impairment, while in the second part I'm presenting results of novel fluid biomarkers, some of them are still preliminary data.

Chapter 1: Overview of Amyotrophic Lateral Sclerosis

In this chapter, I provide a general overview of ALS, on the current diagnostic criteria, phenotypic heterogeneity, itemizing the different features of motor, bulbar, respiratory and sleep impairment. I summarize the most relevant knowledges related to bulbar, respiratory and sleep impairment and their main known biomarkers. Then, I summarize and review the existing evidences on fluid biomarkers, their relation to clinical features, and perspectives.

1.1 Epidemiology

ALS is the most frequent and aggressive motor neuron disease (MND), a heterogeneous and progressive neurodegenerative syndrome. ALS is characterized by the degeneration of both upper and lower motor neurons, leading to motor and non-motor symptoms [1]. MND also include other diseases, mainly primary lateral sclerosis (PLS), which involve only upper motor neuron, and progressive muscular atrophy (PMA).

MND have significant social, economic, and quality-of-life impacts. Global epidemiological data, primarily derived from meta-analyses and systematic reviews, highlight Europe as the most extensively studied geographical region.

1.1.1 Incidence and Prevalence

Xu et al. [2] reported a global incidence rate of 1.59 (95% CI 1.39–1.81) per 100,000 person-years and a global prevalence of 4.42 (95% CI 3.92–4.96) per 100,000. The Global Burden of Disease Study 2016 estimated an incidence of 0.78 (95% UI 0.71–0.86) and a prevalence of 4.5 (4.1–5.0) per 100,000 person-years for all ages [3]. European data [4], the most comprehensive, showed consistent incidence (around 2.16 per 100,000 person-years), prevalence (7-9 per 100,000 persons), and a median survival of 30 months from symptom onset.

1.1.2 Age

Age stands as the only established risk factor for ALS, along with genetics. Incidence peaks between 60 and 75 years, plateauing after 75 and decreasing beyond 80. This challenges the notion of ALS as

strictly age-related compared to other neurodegenerative diseases [5]. Median onset age ranges from 51 to 66 years, slightly higher in Europe than in non-European regions.

1.1.3 Male-to-Female Ratio

ALS is slightly more common in males (M:F ratio 1.2-1.5:1), with a lifetime risk of 1:350 for men and 1:400 for women [1,5]. The ratio appears to have decreased over time, possibly due to study variations, increased medical consultations by women, or an actual rise in female incidence [6]. Recent studies call for caution in interpreting this trend, urging further investigation [7].

1.1.4 Geographic Heterogeneity

A significant aspect in epidemiological studies is the diversity in incidence, prevalence, and mortality across different regions and populations. Europe and Caucasian populations exhibit higher ALS rates, suggesting multifactorial causation involving demographic, genetic, environmental, and lifestyle factors [5]. Clusters observed in specific locations, such as Guam and Kii peninsula in Japan, emphasize the need for broader epidemiological research [8].

1.1.5 Temporal Trends

The question of increasing ALS incidence and prevalence over time is a subject of debate. The Global Burden of Disease Study 2016 indicates a substantial rise in disease burden from 1990 to 2016 [3]. While some studies show an increase in incidence [2], others attribute it to improved diagnosis, aging populations, and heightened awareness rather than a genuine surge [7]. Future trends may see a more pronounced increase in developing countries due population aging and lifestyle changes [6].

1.1.6 Economic Implications

Despite its low incidence and prevalence, ALS exerts a substantial social, economic, and quality-of-life impact. Economic estimates, such as those by Berry et al. projecting national costs in the United States, emphasize the urgent need for increased resources for ALS research [9].

1.2: Etiopathogenesis

Efforts to identify a specific cause for ALS, a multifactorial disease, have been extensive. The disease is divided into sporadic (sALS, 90-95% of cases) and familial forms (fALS, 5-10%) [1].

1.2.1 Genetic Factors

1. **Familial ALS (FALS):** Familial ALS, representing a minority of cases, is characterized by a clear familial inheritance pattern. Approximately 30 genes are associated with familial ALS, with prominent players such as *C9orf72*, *TARDBP*, *SOD1*, and *FUS* accounting for nearly 70% of cases. These genes exhibit Mendelian inheritance patterns, predominantly autosomal dominant, though recessive and X-linked forms have also been reported. The phenotypic and genetic heterogeneity of familial ALS complicates clinical differentiation from sporadic forms, emphasizing the need for detailed genetic analysis. Factors indicating a suspicion of familial ALS include a family history of ALS or other neurodegenerative diseases, atypical features like early onset or sensory loss. However, the absence of familial history does not definitively rule out a familial form, as discussed earlier.
2. **Genetic Risk Factors in Sporadic ALS (SALS):** Genetic factors implicated in sporadic ALS partly overlap with those in familial cases, with 15% of sporadic cases exhibiting mutations in known ALS-associated genes. Unlike familial ALS, these mutations act as low-penetrance susceptibility factors. Genes such as *SOD1*, *C9orf72*, *TARDBP*, *FUS*, and others contribute to sporadic ALS, indicating a shift from the classic familial and sporadic ALS dichotomy towards a genetically confirmed and unconfirmed ALS classification.

Genome-wide association studies reveal that ALS's genetic architecture primarily involves rare variants with intermediate to large effects, following a monogenic inheritance pattern. Unlike other diseases such as schizophrenia, which rely on additive effects of common variants, ALS exhibits oligogenic and polygenic inheritance, along with pleiotropy. Notably, ALS-associated genes like *C9orf72* and *ATXN2* are implicated in diverse neurodegenerative conditions beyond ALS, emphasizing the complex genetic landscape.

Key Genes in ALS Pathogenesis:

1. ***SOD1* Gene:** Located on chromosome 21q22.1-22.2, the *SOD1* gene encodes the ubiquitous cytoplasmic enzyme Cu-Zn SOD. Mutations in *SOD1*, representing 15-20% of familial ALS cases and 1% of apparently sporadic cases, lead to toxic gain-of-function effects. The high penetrance and autosomal dominant transmission characterize *SOD1* mutations.
2. ***FUS/TLS* Gene:** Situated on chromosome 16q12, the *FUS/TLS* gene is associated with ALS6. Mutations in *FUS*, found in approximately 3% of European familial ALS cases and <1% of

sporadic cases, lead to cytoplasmic localization of the protein, impacting RNA processing. The mutations exhibit autosomal dominant transmission, with an early onset and rapid progression phenotype.

3. ***TARDBP* Gene:** Found on chromosome 1p36, *TARDBP* is responsible for ALS10. *TARDBP* gene codes for the TDP43 protein. Mutations in *TARDBP*, identified in 2-5% of familial ALS and 1% of sporadic ALS cases, lead to the accumulation of TDP-43 in the cytoplasm. TDP-43 pathology is a hallmark of ALS, regardless of *TARDBP* mutational status, suggesting a broader role for TDP-43 in ALS pathogenesis.
4. ***C9orf72* Gene:** The *C9orf72* gene, located on chromosome 9p21.2, is the most frequently mutated gene in ALS, accounting for 40% of European familial cases and 5% of sporadic cases. The gene's expansion (G4C2) results in an autosomal dominant transmission pattern. The pathogenic mechanisms involve alterations in splicing, RNA-mediated toxic gain-of-function, and repeated-associated non-AUG translation. *C9orf72* mutations are often associated with frontotemporal dementia, highlighting the phenotypic heterogeneity of ALS.

The physiological functions of these genes and their loss in mutation cases are intricately linked to proposed pathogenic mechanisms in ALS.

In conclusion, the genetic landscape of ALS is characterized by a myriad of factors influencing both familial and sporadic cases. Advances in genetic research, especially through genome-wide association studies, have provided valuable insights into the intricate genetic architecture of ALS. Unravelling the complexities of ALS genetics is pivotal for advancing diagnostic precision and developing targeted therapeutic strategies for this devastating neurodegenerative disorder.

1.2.2 Environmental Risk Factors

In contrast to the genome, identifying environmental factors remains challenging due to the vast research space, changing exposomes, and potential confounding variables. In fact, the ‘exposome’ is infinite, not only in space but also in time [4]. Another difference from the genome, which is essentially fixed at birth, the exposome changes over time and can only be assayed using prospective studies or retrospective cohort or case-control studies. The only way to replicate a sort of genome-wide association study for the exposome is to create very detailed prospective longitudinal observation over a lifetime in large numbers of people with similar genetic backgrounds and, until now, it has been impossible to perform. Moreover, retrospective environmental research required

time-consuming interviews with participants or detailed examination of records and the research that has been done has often had important methodological limitations, principally due to sample size, recall bias and other issues. Sampling from clinics rather than populations introduces bias such as the overrepresentation of patients with slowly progressive disease and younger age at onset. For this reason, population-based cohorts are preferable to establish environmental risks in a rare disease. Besides the lack of clear evidence on risk factors, also the determinants of both cognitive and motor phenotypic heterogeneity are quite unknown. Even in this case, population-based registries are the most important tools able to define correlations between clinical features and other preclinical conditions, such as age, sex, genetic background and environmental exposures (*Chiò et al., 2020*).

Some of the studied factors include:

- **Cigarette Smoking:** Inconclusive evidence with a weak association [10], particularly in females [11].
- **Physical Exercise:** Contrasting findings with potential associations in leisure-time physical activity [12], suggesting it as a risk marker rather than a direct cause [13].
- **Military Service:** Increased mortality risk among veterans, possibly linked to battlefield exposures [14].
- **Agricultural Work and Pesticides:** Limited evidence suggests an association, requiring further research [4,15].
- **Heavy Metals:** Varied studies on metals like lead, aluminium, mercury, cadmium, manganese, and selenium show inconclusive results [4,15].
- **Occupation:** Conflicting data on certain occupations like hairdressers, veterinarians, athletes, and military personnel [16].
- **Electromagnetic Fields:** Limited evidence with inconclusive findings [17,18].

1.3 Neuropathology

ALS manifests histologically with the degeneration and loss of motor neurons, accompanied by astrocytic gliosis in both grey and white matter as a reparative response. The affliction extends from cortical motor neurons to lower motor neurons in the spinal cord and bulbar-pontine motor nuclei.

These alterations lead to spinal atrophy and denervation-induced muscle atrophy, featuring fibre type grouping and clusters of angular atrophic fibres [19].

Notably, diverse neuron types, including frontal or temporal cortical neurons, may succumb, particularly in ALS cases associated with Frontotemporal Dementia (FTD). Additionally, non-motor neurons contributing to the descending fronto-ponto-cerebellar tract may be lost. Subtle sensory system involvement in the posterior columns of the spinal cord, primarily histologically evident, further accentuates the neuropathological spectrum [20].

A distinctive feature of ALS pathology is the presence of intracellular inclusions in degenerating neurons, surviving neurons, and glial cells. These inclusions encompass phosphorylated and non-phosphorylated neurofilaments, Bunina bodies positive for cystatin C specific to ALS, and ubiquitinated inclusions, with the prominent inclusion being TDP-43. While SOD1 and FUS may also contribute, TDP-43 stands out as a specific marker for sporadic ALS, certain familial ALS forms, and cases of ALS associated with FTD.

The pathogenic toxicity is attributed to high-molecular-weight complexes preceding aggregate formation rather than the aggregates themselves. A comprehensive understanding of the etiological mechanisms remains elusive, but ongoing research has delineated various processes implicated in ALS genesis, particularly building upon evolving genetic insights [21].

1.3.1 Aetiology

One major avenue of investigation involves approximately 30 genes associated with familial ALS, classified based on predominant pathophysiological mechanisms—RNA biology, protein turnover, and axonal transport [1]. This categorization underscores the heterogeneous nature of ALS, suggesting convergence of multiple mechanisms contributing to a common outcome:

1. Altered RNA Metabolism: Mutations in RNA-binding proteins such as TDP-43 and FUS, crucial in transcription, splicing, and transport regulation, characterize certain ALS forms. Their mutated variants, located in the cytoplasm instead of the nucleus, possess prion-like domains, potentially promoting stress granule formation or protein aggregation, leading to cellular damage.

2. Proteostasis Disruption: Mutations in various genes result in misfolded proteins that interfere with proteasomal or autophagic mechanisms. SOD1 mutations, for instance, associate with reduced ubiquitin-proteasome system components, fostering misfolded protein accumulation. TDP-43 misfolding inhibits proteasomal clearance, while C9ORF72 mutations impact autophagy regulation.

3. Axonal Transport Aberrations: Mutations in TDP-43, ALS2, and UNC13A contribute to dysregulated endosomal and vesicular transport processes, affecting the proper functioning of neurons.

4. Oxidative Stress: Accumulation of reactive oxygen species (ROS) is linked to ALS, notably in SOD1 mutations. However, this association is characterized more by gain-of-toxic-function rather than loss of the enzyme's antioxidant role. Oxidation-induced post-translational modifications in wild-type SOD1 may contribute to pathology in non-SOD1-mutated cases.

5. Non-Neuronal Cells and Microenvironment: Studies in SOD1-mutated mice emphasize the crucial role of abnormal astroglia surrounding neurons, suggesting a microenvironmental contribution to disease pathogenesis.

6. Excitotoxicity: Excessive glutamate neurotransmitter stimulation, particularly through AMPA and NMDA receptors, may induce neuronal damage and degeneration. Elevated glutamate levels in ALS patients' cerebrospinal fluid support this hypothesis, with potential therapeutic interventions targeting glutamate release.

7. Inflammatory Response: Neuroinflammatory processes, involving microglial activation and release of pro-inflammatory cytokines, might play a more significant role in disease progression rather than initiation. Various immune cells infiltrate the central nervous system, contributing to neuronal damage.

8. Mitochondrial Dysfunction: Implicated particularly in SOD1 mutations, this mechanism involves SOD1 deposition on mitochondrial membranes, causing respiratory chain complex dysfunction and ATP level reduction. Stress-induced oxidative damage exacerbates mitochondrial abnormalities.

9. Cytoskeletal Alterations: Neurofilaments, part of the neuronal cytoskeleton, are found in degenerated neuron inclusions and could serve as potential biomarkers for diagnosis and prognosis. Disruptions in axonal transport, crucial for motoneuron health, contribute to neurodegeneration.

10. DNA Repair Impairment: Associated with mutations in FUS, TARDBP, NEK1, and C21ORF2, this mechanism remains to be fully elucidated.

11. Growth Factor Deficiency: Reduced levels of trophic factors, including CTNF, BDNF, GDNF, IGF-1, and VEGF, have been observed in ALS patients. Whether these reductions are primary or secondary to the disease remains uncertain.

12. Viral Infections: While the role of viruses like poliovirus, enterovirus, or retrovirus has been speculated, clear causative associations are yet to be established.

13. Oligodendrocyte Degeneration: Although not conclusively proven as a causal factor, animal models indicate that oligodendrocyte precursor dysfunction might contribute to ALS pathogenesis by depriving neurons of metabolic support.

14. Apoptosis: Markers of apoptosis are evident in degenerating cells, suggesting potential therapeutic avenues that interfere with this pathway.

In summary, ALS likely results from an intricate interplay of various mechanisms, converging on common pathways, leading to the retraction and loss of motoneuron axonal projections. This culminates in denervation of target cells, affecting both upper and lower motoneurons. Understanding the progression and spread of the disease involves considering prion-like transfer of TDP-43 and SOD1 aggregates between cells. Ongoing research endeavours continue to unravel the complex tapestry of ALS neuropathology, providing hope for targeted therapeutic interventions.

1.4: Clinical Manifestations

ALS primarily affects upper and lower motor neurons, causing progressive muscle weakness and atrophy. The disease's clinical heterogeneity, involving various phenotypes, adds complexity to diagnosis and management.

1.4.1 Onset and progression

Around 30% of ALS patients show a bulbar-onset of disease, causing dysarthria and dysphagia, while 70% experience a spinal-onset disease, with limb muscles involvement, and only 1% have a respiratory onset [22]. The onset is usually insidious but the damage spreads relentlessly to involve most muscles, including the diaphragm, leading to respiratory insufficiency. Typically, death due to respiratory paralysis or to infective diseases occurs in 3 to 5 years [22].

1.4.2 Clinical Phenotypes

Patients were systematically classified into eight recognized phenotypes of Amyotrophic Lateral Sclerosis (ALS): classic, bulbar, flail arm, flail leg, pyramidal, respiratory, Pure Lower Motor Neuron (PLMN), and Pure Upper Motor Neuron (PUMN). [23], including:

- **Classic (Charcot's) Phenotype**: Classic ALS is characterized by symptom onset in the upper or lower limbs with evident but not predominant pyramidal signs.

- **Bulbar Phenotype**: Patients with bulbar onset exhibit dysarthria, dysphagia, tongue wasting, and fasciculation, without peripheral spinal involvement for the first 6 months. Pyramidal signs become mandatory after the initial 6 months.
- **Flail Arm Phenotype**: This phenotype features progressive, predominantly proximal weakness and wasting in the upper limbs. Functional impairment must be confined to the flail limbs for at least 12 months post-symptom onset.
- **Flail Leg Phenotype**: Patients in this category display a progressive distal onset of weakness and wasting in the lower limbs, with specific criteria for pathological reflexes. Proximal leg involvement at presentation is classified as classic ALS.
- **Pyramidal Phenotype (Predominant Upper Motor Neuron ALS)**: This group manifests severe spastic para/tetraparesis with prominent pyramidal signs and concurrent clear-cut lower motor neuron impairment from onset.
- **Respiratory Phenotype**: Individuals in this category exhibit predominant respiratory impairment at onset, with mild spinal or bulbar signs in the first 6 months.
- **Pure Lower Motor Neuron**: These patients show clinical and electrophysiological evidence of progressive Lower Motor Neuron (LMN) involvement, excluding specific conditions and genetic mutations.
- **Pure Upper Motor Neuron**: This category displays clinical signs of Upper Motor Neuron (UMN) involvement, excluding LMN signs during follow-up, history of diseases mimicking motor neuron disease, family history of spastic paraparesis/tetraparesis, and mutations in specified genes.

1.4.3 Extra-motor Symptoms

Although the primary symptoms of MND are associated with motor dysfunction, up to 50% of patients develop cognitive and/or behavioural impairment during the course of disease and around 14% of patients present with concomitant behavioural variant of frontotemporal dementia (bv-FTD)[1]. The presence of cognitive impairment in ALS was not fully recognized in literature until the end of the 20th century [24]. Later, two centre-based and population-based studies [25,26] confirmed the real prevalence of cognitive disturbances in ALS. Both studies confirmed that half of the patients showed various degrees of cognitive impairment, involving language, memory and executive domains. The capability to predict who will develop these impairments is fundamental, although nowadays there are no established biomarkers [27].

Beyond the cognitive impairment, in ALS there are many other non-motor signs and symptoms, such that now ALS is recognized as a systemic disease. Recent studies draw attention to the non-motor symptoms in ALS that significantly reduce the quality of life and can be related to the diffusion of the pathological process to structures such as frontal and temporal cortices, hypothalamus, basal ganglia, and autonomic nervous system [28]. These symptoms include, but are not limited to sleep problems, pain, fatigue, sialorrhea, dysautonomia, and sensory symptoms [29,30].

1.4.4 Diagnostic criteria

The criteria for defining amyotrophic lateral sclerosis (ALS) diagnosis have evolved over time in an effort to identify the disease with maximum sensitivity and specificity. Since 1998, the El Escorial revised criteria (or Airlie House criteria) have been employed [31]. They require simultaneous presence of: (A) signs of LMN degeneration via clinical, electrophysiological, or neuropathological examination, signs of UMN degeneration in clinical examination, and progressive spreading of signs or symptoms in regions determined by history or objective examination; and (B) absence of electrophysiological or pathological signs of other pathological processes explaining I or II MN degeneration, and absence of neuroradiological or biopsy evidence of other pathological processes explaining observed clinical and electrophysiological signs.

Diagnostic certainty levels (clinically defined) include clinically definite ALS, clinically probable ALS, clinically probable ALS with laboratory confirmation, clinically possible ALS, clinically defined familial ALS with laboratory confirmation, and clinically suspected ALS (pure II MN syndrome), excluded from the revised El Escorial criteria. The term "region" refers to cranial, cervical, thoracic, and lumbosacral areas.

These criteria have been adapted with Awaji criteria [32], considering electro-physiological involvement equivalent to clinical involvement, eliminating the category of clinically probable ALS with laboratory confirmation. Additionally, fasciculation potentials are deemed as important as fibrillation potentials or positive sharp waves.

In 2019, the Gold Coast criteria [33] were proposed to simplify diagnosis and reduce inter-observer variability in applying El Escorial criteria. These criteria consider three conditions: documented progressive motor impairment, progressive symptoms and signs of I and II MN, and absence of signs explaining I and/or II MN degeneration. The new criteria increase the sensibility in diagnosis and improve the recognise of atypical ALS.

In situations not meeting ALS diagnosis criteria, consideration should be given to various forms within the motor neuron disease spectrum, as described in the preceding chapter.

1.4.5 Clinical Scales: ALSFRSr and ROADS

The Revised ALS Functional Rating Scale (ALSFRSr)[31], a 48-point clinical scale that stratifies the severity of ALS by analyzing 12 items related to 4 domains: bulbar functions (language, salivation, swallowing), general motor capacity (or related to the lower limbs: turning in bed and arranging blankets, walking, climbing stairs), fine movements (or related to the upper limbs: handwriting, cutting food and using utensils, dressing, and personal hygiene), and respiration (shortness of breath, orthopnea, respiratory failure). A score from 0 to 4 is given to each function based on the level of impairment, with a total maximum score of forty-eight.
In research the ALSFRSr sub-scales can be used to analyse specific domains. In particular, is used the bulbar sub-scale, given by the sum of items 1, 2, and 3, the motor sub-scale, given by the sum of items 4, 5, 6, 7, 8, and 9, and the ALSFRSr without the three respiratory items.

Another relevant clinical scale is the Rasch-Weighted Overall ALS Disability Scale (ROADS)[34] that has been successfully developed and validated to address limitations of the current ALSFRSr. Utilizing Rasch methodology brings mathematical precision to subjective self-reported outcome measures, overcoming shortcomings of standard ordinal scales. ROADS exhibits a broader item targeting range than ALSFRSr, allowing better differentiation of overall disability levels. Linear weighting in ROADS ensures consistent measurement, unlike ALSFRSr's non-quantifiable 1-point changes. Dimensionality analyses confirm uniform disability domain measurement in ROADS, contrasting with ALSFRSr's unidimensionality violation. The careful selection of ROADS questions by clinical experts mitigates biases associated with disease reversal or non-ALS factors. Given drug approval reliance on small ALSFRSr differences, the introduction of ROADS provides a crucial improvement.

1.4.6 Staging Systems

Various disease staging systems have been developed, including:

- The *King's College ALS Staging System*, which categorizes progression based on the number of involved body regions (three main regions: bulbar, upper limbs, and lower limbs) and the need for nutritional or respiratory support [35].

- The *MiToS* (Milano-Torino System), which defines different levels based on the progressive loss of independence in fundamental functional domains (walking/self-care, swallowing, communication, and breathing) [36].

Both systems utilize information gathered from ALSFRS-r.

1.5: Management

ALS management aims at enhancing quality of life, managing symptoms, and providing multidisciplinary care. While no cure exists, various interventions address specific aspects of the disease.

1.5.1 Pharmacological Interventions

- **Riluzole:** The first FDA-approved medication, riluzole, slightly extends survival by modulating glutamate release.
- **Edaravone:** An antioxidant approved for ALS treatment by FDA in May 2017, slowing functional decline in some patients.
- **Tofersen:** FDA has approved in April 2023 Tofersen, in treating ALS caused by *SOD1* gene mutation. Approval is based on Tofersen's reduction of neurofilaments, proteins released during neurodegeneration. Acting as a surrogate biomarker, this reduction is indicative of potential clinical benefits. To confirm approval, Biogen company must validate efficacy in the ongoing ATLAS study. This study evaluates whether Tofersen delays ALS symptoms in presymptomatic patients with SOD1 gene mutation and elevated plasma neurofilaments.

Various experimental treatments, including other gene therapies, stem cell therapies, and neuroprotective agents, are under investigation all over the world. However, none have yet shown definitive efficacy in large-scale clinical trials.

1.5.2 Non-Pharmacological Approaches

- **Physical Therapy:** Essential for maintaining mobility, managing spasticity, and preventing complications like contractures.
- **Occupational Therapy:** Focuses on activities of daily living to enhance independence.

- **Speech and Swallowing Therapy:** Crucial in bulbar-onset ALS to address communication and nutrition issues.
- **Respiratory Support:** As respiratory muscles weaken, non-invasive ventilation (NIV) or invasive ventilation may be necessary to manage respiratory insufficiency.
- **Nutritional Support:** Dysphagia and weight loss are common. Nutritional interventions, including enteral feeding, address these challenges.
- **Palliative and End-of-Life Care:** Palliative care plays a central role in managing symptoms, addressing psychological aspects, and supporting patients and families throughout the disease course.

1.6 Bulbar impairment in ALS and its biomarkers

1.6.1 Overview on bulbar impairment in ALS

Bulbar involvement in ALS is often characterized by the presence of dysarthria, dysphagia, and sialorrhea [1]. Dysarthria manifests as slurred speech, reduced articulation precision, and changes in voice quality. Dysphagia, a common and challenging symptom, leads to difficulties in swallowing, most of all liquids, contributing to malnutrition and respiratory complications.

Bulbar impairment is present in around 1/3 of ALS at onset and approximately 85% of people with ALS experience bulbar dysfunction all along the disease progress [14].

Diagnosing bulbar-onset ALS poses unique challenges. The insidious onset of symptoms and variability in their presentation may lead to delayed or misdiagnosis. Differential diagnoses, including bulbar myasthenia gravis and primary lateral sclerosis, require careful consideration.

Research show that patients with bulbar-onset ALS may experience a more rapid disease progression and exhibit poorer survival rates compared to those with limb-onset [37]. The rate of bulbar decline is identified as a critical prognostic factor impacting overall survival and quality of life.

Moreover, it is established that ALS-FTD is more frequent in ALS patients with bulbar onset and in those with bulbar involvement at time of cognitive testing [38].

1.6.2 Marker of bulbar impairment in ALS

As reported in a review by Green et al. [39], “no standardized diagnostic procedure for assessing bulbar function in ALS exists” and “adequate markers of bulbar dysfunction have yet to be identified”. There are a lot of primary studies concerning evaluation of bulbar functions, and many exams were

proposed as markers for longitudinal evaluation of bulbar impairment. A great importance would be fulfilled by the identification of biomarkers able to detect an early bulbar involvement, although none of those proposed has been established in clinical routine.

Some studies focused on research of biomarkers of speech impairment, others of swallowing impairment, others on direct or indirect early predictive biomarkers of general bulbar disfunction [40]. All these possible markers could be gathered in 6 sub-groups, based on approach utilised: clinical bulbar scales, neurophysiology, imaging, swallowing tests, speech evaluation and bulbar muscle strength evaluation.

1) ***clinical bulbar scales*** → many scales were developed for early bulbar disfunction recognition and its monitorization beyond ALSFRSr scale.

   The *Center for Neurologic Study-Bulbar Function Scale (CNS-BFS)* is a self-administered 21-questions scale which interrogate speech, salivation and swallowing domain [41]; it demonstrated a good relationship with clinician diagnosis and it seem to identify patients with early bulbar impairment not inferior to clinical evaluation.

   The *Eating Assessment Tool (EAT-10) survey* is another validated, self-administered scale specific for dysphagia which demonstrated, in comparison with video-fluoroscopy, a good capability of discrimination safe versus unsafe ALS swallowers and, in this group, non-aspirators versus aspirators [42].
2) ***neurophysiological evaluation*** → *electromyography (EMG)* is important in ALS to find signs of acute and chronic denervation. Tankisi et al. [43] demonstrated a role of spontaneous activity detected in tongue EMG in assessing both clinical and subclinical bulbar involvement. Another important examination is the *electrical impedance myography (EIM)* of tongue: it seems to be a promising biomarker of progression in ALS [44]. In a recent paper [45] they developed a "novel EIM device capable of multi-dimensional electrode configuration" that demonstrated to be a good marker of early bulbar impairment, even in inexperienced hands.
3) ***swallowing tests*** → Fattori et al. [46] reported that *fibreoptic endoscopy evaluation of swallowing (FEES)* is a good indicator of the dysphagia severity and a useful test for the follow-up of dysphagia in patients with ALS. Also, *video-fluoroscopy* has been largely studied in swallowing dysfunction in ALS patients. Murono et al. [47] proposed a possible standard for radiographic interpretations of dysphagia in those patients. In particular, they observed that

impairment in bolus transport from the oral cavity to pharynx, pharyngeal constriction as well as both oral and pharyngeal residue are early signs of pre-clinical bulbar involvement.

4) ***speech evaluation*** → since '90s many tests were studied in order to best analyse speech impairment and to identify it in a very early phase of disease, when it is not subjectively recognizable. During the last decay electromagnetic tracking devices had been used to evaluate three dimensional movements of tongue and jaw.

   Rong et al. [48] assessed *multiple speech tasks* (acoustic, kinematic, and aerodynamic instruments were used) in order to evaluate functions of the articulatory, resonatory, phonatory, and respiratory subsystems. They observed that the first subsystem to get altered is the articulatory one, as indicated by reductions in lip and jaw movement velocities, and the second is resonatory function, as indicated by increases in nasal airflow leakage.

   Shellikeri et al. [49] demonstrated that tongue movement measures may be more suitable for tracking early changes in bulbar function than the speaking rate. They used the *Speech Intelligibility Test* and concluded that decrease in tongue movement size with disease progression may serve as a potential diagnostic marker for early detection of bulbar involvement.

   Allison et al. [50] reported that "patients' self-report and clinicians' perceptual judgments were inadequate for detecting early bulbar involvement" and they observed a better ability of instrumentation-based speech measures (in particular of measures of pausing and rate of speech movement) in recognising pre-symptomatic bulbar impairment in spinal-onset ALS.

   Perry et al. [51] used a *3D electromagnetic articulography* (composed of two electrodes on tongue, one on jaw, one on lips and one reference on the head) to demonstrate the ability of that technique to identify pre-clinical speech and swallowing impairments after drinking a cup of water. Authors reported that "this technology is well suited to serve as a gold standard'', although is time consuming and is not easy to apply in a clinical routine setting.

5) ***MRI and ultrasound (US) of tongue*** → US morphological evaluation of tongue and MRI muscle intensity seem to be a good biomarker of bulbar involvement [52]. In particular, low T1 MRI intensity of tongue is associated with bulbar impairment; small US sagittal and coronal area, small US coronal height and width and US rounded sagittal tongue shape predict more rapid bulbar functional impairment.

   Moreover, many works concerning functional MRI (fMRI) in ALS patients have been published in literature [44], but only few concern with bulbar impairments. Mohammadi et al. [53]

highlighted a specific and significant reduction of brain activity in pre- and postcentral areas as well as the thalamus during vertical movement of the tongue in ALS patients with bulbar involvement compared with ALS with no bulbar signs. No differences were observed between ALS patients with bulbar onset of disease and ALS patients who developed bulbar signs only later.

6) ***bulbar muscle strength evaluation*** → Hiraoka et al. [54] compared maximum tongue pressure (MTP) with ALSFRSr bulbar sub-score and with impairment at videofluoroscopy and they observed a good relationship between these parameters. Beyond, they showed reduction of MTP in patients without refered bulbar impairments, so MTP could be used as a pre-clinical bulbar marker.

All these studies highlight the heterogeneity of works on bulbar impairment, although none of the markers identified have been introduced in clinical routine. Most of them are time consuming and/or difficult to set up in a clinical routine and/or need a specialised staff and/or are lacking in evidence. The easier to apply and less expensive in terms of time and money are the bulbar clinical scales. Moreover, it's not established the time of follow-up both for ALS with and without bulbar impairment.

Nowadays, none of these exams could substitute the complete clinical evaluation, consistent in research of bulbar signs of upper motor neurons damage (including pathological reflexes e.g., brisk jaw jerk, gag, and other facial reflexes) and lower motor neurons damage (consisting of muscle weakness, atrophy and fasciculations in the jaw, face, tongue, and palate).

In conclusion, there is no consensus on which exam is the best one to use for early detection and monitoring of bulbar impairment in ALS patients without bulbar sign or symptoms. Further studies on ALS patients without bulbar involvement, maybe comparing longitudinally all exams and clinical scales previously described, are needed.

1.7 Respiratory impairment in ALS and its biomarkers

1.7.1 Overview on respiratory impairment in ALS

In the context of Amyotrophic Lateral Sclerosis (ALS), respiratory insufficiency (RI) typically manifests in the advanced stages of the disease, although it can occasionally be an initial symptom. Respiratory

complications, particularly hypoventilation, decreased bronchial clearance, and lung infections, are major contributors to mortality in ALS [55,56]. Even mild respiratory involvement leads to fatigue in daily activities, sleep disturbances, and a decline in quality of life. Additionally, hypoxemia can negatively impact cognitive function, which is particularly significant in a population with lower cognitive reserve [57,58].

Both inspiratory and expiratory muscles, together with upper airway muscles, affect ALS patients. The impairment of cough reflex, crucial for airway protection, is associated with bulbar muscle dysfunction. Weakness in expiratory muscles, particularly pharyngeal and laryngeal muscles, increases the risk of aspiration and lung infections, especially when coupled with significant cough deficiency. In ALS, diaphragm involvement is a primary cause of respiratory failure, with dyspnea closely linked to diaphragmatic dysfunction. The assessment of diaphragm physiology is thus pertinent in individuals with ALS.

1.7.1.1 The role of diaphragm in breath

The diaphragm, a vital respiratory muscle, forms a dome-shaped structure dividing thoracic and abdominal cavities. Comprising a central non-contractile fibrous region and contractile muscle fibers, it has a musculo-fibrous composition rich in capillaries, resisting aging. Unlike expiratory muscles, it contains equal proportions of type I and type II fibers, with relatively scarce muscle spindles influencing phrenic neuronal excitability [59]. Motor innervation, primarily from the phrenic nerve (C3–5), extends throughout the muscle. Diaphragm contraction induces axial descent, lowering intrapleural pressure, facilitating inspiration. It flattens during full inspiration and rises during forced expiration, with robust diaphragmatic contraction needed for coughing and sneezing [60]. Calm breathing involves passive expiration, while forced expiration engages expiratory muscles. Effective coughing requires strong inspiration, glottis closure, and increased intra-abdominal and intra-thoracic pressures. Parameters like peak cough flow are crucial for mucus expectoration and preventing aspiration pneumonia in neuromuscular disorders. The inspiratory pace-maker in the pre-Bötzinger complex is vital for rhythmic ventilation [61]. Corticospinal inputs, crucial for voluntary breathing control and speech, can be explored through magnetic stimulation, while various neural elements contribute to respiratory modulation in response to factors like hypercapnia, hypoxemia, and sleep-wake states. Spinal interneurons play a role in modulating phrenic motoneuronal activity [62].

1.7.2 Marker of respiratory impairment in ALS

The assessment of respiratory dysfunction in ALS involves the progressive involvement of inspiratory and expiratory muscles, alongside upper airway muscles. While studies on dysfunctional central respiratory drive in ALS are limited, there is a likelihood of its impact on certain patients [63]. Consequently, a variety of tests are essential to gain a comprehensive understanding of respiratory function in individuals affected by ALS. Both American and European guidelines advocate for an initial respiratory evaluation during baseline clinical assessments and subsequent periodic evaluations [64]. However, the frequency of assessments should be adjusted based on the individual progression of the disease and the occurrence of intercurrent events, such as infections, that could influence respiratory function. I will present a comprehensive overview of available tests, their utility, and limitations, subdividing in those useful for a global respiratory evaluation, those specific for inspiratory or expiratory evaluation and those specific for looking at central respiratory drive.

In the global respiratory evaluation, Forced Vital Capacity (FVC), a well-established non-invasive test, has been extensively employed in ALS. It assesses both inspiratory and expiratory loops, demonstrating sensitivity to change and predictive value for hypoventilation and survival in ALS [65]. Despite its utility, FVC can be unreliable in patients with bulbar involvement due to orofacial weakness, leading to air leakage around the mouthpiece [55]. Slow Vital Capacity (SVC) is an alternative, particularly suitable for patients with bulbar involvement, exhibiting a strong correlation with FVC and serving as a predictor of disease progression, positive pressure ventilation needs, and survival [66].

Maximal Voluntary Ventilation (MVV), assessing respiratory function during sustained efforts, is demanding for ALS patients due to respiratory fatigue [65]. Although it can be a sensitive measure of disease progression in the early stages, MVV is rarely performed in daily practice. Nocturnal Pulse Oximetry (NPO) emerges as a valuable, non-invasive, and cost-effective method, providing insights into respiratory function during the demanding state of lying and sleeping. NPO is predictive of survival, identifies central drive dysfunction, and is essential for monitoring non-invasive ventilation adaptation [67].

In evaluating inspiration, tests such as Maximal Inspiratory Pressure (MIP) and Nasal Inspiratory Pressure during a maximal sniff (SNIP) offer non-invasive insights into maximal inspiratory muscular strength. These tests, complemented by phrenic nerve stimulation and ultrasound studies, contribute to understanding diaphragmatic dynamics and detecting early deterioration [68,69].

Transcutaneous Capnometry (PtcCO2) is a modern approach to evaluating respiratory function, showing strong correlation with arterial measurements. It is particularly useful for detecting nocturnal hypoventilation in ALS patients [70]. Blood gas measurements, providing information on CO2 retention and hypoxemia in severe respiratory failure, are not extensively used in ALS due to the focus on early changes. Sleep studies reveal alterations in REM sleep stages, with a connection between respiratory function impairment, poor sleep quality, and daytime somnolence in ALS [71].

Expiratory evaluation focuses on peak expiratory flow (PEF), peak cough flow (PCF), and maximal expiratory pressure (MEP) to assess expiratory muscle function. Abnormally reduced values indicate an increased risk of respiratory infections and mortality [72].

Assessing the central respiratory drive involves tests like Inspiratory Mouth Occlusion Pressure, revealing indicators of respiratory drive, especially in spastic patients [73].

In conclusion, multiple tests are available to evaluate different aspects of respiratory function in ALS. While many centres conventionally assess SVC and FVC, additional tests, including maximal pressure measurements, expiratory peak flows, and nocturnal oximetry, may be necessary to comprehensively evaluate respiratory function in ALS patients, considering disparities in patient tolerability and technical limitations.

1.8 Sleep impairment in ALS and its biomarkers

1.8.1 Overview on sleep structure

Sleep is a coordinated neurochemical process that involves the collaboration of brain regions responsible for both arousal and sleep. This physiological phenomenon facilitates the recovery of brain energy, fosters plasticity in brain changes associated with learning, memory consolidation, and extinction, and activates the glymphatic system, responsible for eliminating waste products from the brain. These intricate processes play a vital role in brain growth, overall physical and mental health, as well as the maintenance of cognitive abilities, ultimately contributing to feelings of well-being and daytime alertness [74,75].

At the macrostructural level, sleep is categorised into two distinct states:

- Rapid Eye Movement (REM)
- Non-Rapid Eye Movement (NREM), further subdivided into:
    - NREM Stage 1 (N1)
    - NREM Stage 2 (N2)

- NREM Stage 3 (N3)

During REM sleep, the electroencephalogram (EEG) signal closely resembles wakefulness in terms of frequency (high) and amplitude (low), characterized by predominantly beta (>14 Hz, 30 μV) and alpha waves (8-12 Hz, 30-50 μV). In contrast, NREM sleep exhibits a decrease in frequency and an increase in amplitude, progressing from theta waves in N1 to delta waves in N3, with enhanced synchronization. Typically, individuals go through 4 to 5 sleep cycles during the night, following the order of N1, N2, N3, N2, and REM. Each complete sleep cycle lasts 90 to 110 minutes, featuring shorter initial REM periods that gradually extend as the night progresses, accompanied by reduced time spent in NREM [75].

Various components of the central nervous system regulate the onset and expression of REM, NREM, and wakefulness. The suprachiasmatic nucleus (SCN), the hypothalamic circadian clock, operates on a 24-hour cycle and relies on a core transcription-translation feedback loop (TTFL) to control the expression of key clock transcription factors (TFs). Disruption of this 24-hour cycle can lead to non-physiological changes and potential neurological disorders.

The SCN, through excitatory glutamatergic inputs, regulates melatonin secretion by the pineal gland. Strong inputs occur during the night when melatonin concentrations peak between 3 a.m. and 5 a.m. In contrast, during the light phase, the paraventricular nucleus of the hypothalamus (PVN) receives inhibitory signals from the SCN, blocking PVN projections to the pineal gland and resulting in decreased melatonin levels [76].

The SCN receives innervation from retinal ganglion cells, which, through melanopsin-secreting cells, respond to light and contribute to the photic entrainment of circadian rhythms [77]. The sleep-wake balance hinges on the activity of two essential circuitries: the arousal-producing circuit and the sleep-producing circuit. During sleep, ventrolateral preoptic nucleus (VLPO) neurons inhibit brain structures associated with the ascending reticular activating system, while orexin neurons remain active during wakefulness, projecting to the cerebral cortex and arousal system [78].

The precise role of sleep remains unknown, but it is evidently restorative for the brain. Three types of sleep regulation are identified [78]:

- Homeostatic sleep regulation or process S, driven by adenosine accumulation and activation of VLPO neurons.
- Process C, influenced by circadian rhythms controlled by the SCN.
- Allostatic sleep regulation, considering modifications by external factors, with limited understanding of their impact on homeostatic and circadian systems.

Several sleep disorders are recognized, including [79]:

- Obstructive Sleep Apnea Syndrome (OSAS): characterized by upper airway collapse, leading to oxygen level decline, frequent awakenings, and hypertension.
- Narcolepsy: marked by chronic excessive daytime sleepiness, sleep attacks, cataplexy, sleep paralysis, and hallucinations.
- Restless Legs Syndrome (RLS): involves painful leg sensations during inactivity, particularly in the evening, leading to insomnia or disrupted sleep.
- Idiopathic Hypersomnia: a disorder featuring excessive daytime sleepiness without specific accompanying symptoms.
- REM Behavior Disorder (RBD): a parasomnia where individuals act out their dreams during REM sleep, lacking the muscle atonia typical in this stage.
- Sleep-Wake Cycle Disturbances

1.8.1.1 Melatonin

Melatonin, the primary hormone from the pineal gland, stems from the amino acid L-tryptophan. This conversion involves tryptophan hydroxylase, leading to 5-hydroxytryptophan, which is then decarboxylated to serotonin and acetylated to N-acetyl-5-methoxytryptamine. Neural pathways crucial for melatonin production include the retinohypothalamic tract connecting retinal ganglion cells to the suprachiasmatic nucleus (SCN) and the paraventricular nucleus (PVN), with efferent fibers extending to the pineal gland [77].

Characterized by high water and lipid solubility, melatonin easily traverses cell membranes, released into the third ventricle and then into circulation. It's found in various bodily fluids, tissues, and cellular compartments.

Melatonin serves as an endogenous synchronizer, stabilizing and reinforcing circadian rhythms, regulating the light/dark cycle. Receptors are widely distributed, influencing blood pressure, immune response, cell regulation, and circadian-influenced pathways [77].

A reliable indicator of the central circadian clock in humans is the onset of evening melatonin production measured in dim light (DLMO). It accurately represents SCN timing, with levels rising 2–3 hours before sleep, peaking in the early morning, and returning to daytime levels upon waking. DLMO is a convenient, noninvasive marker measurable through saliva in a short 6–8-hour window, valuable for assessing circadian phase [80].

1.8.2 Marker of Sleep impairment in ALS

In ALS sleep and wakefulness disturbances, are common and believed to have multifactorial origins [81]. Respiratory symptoms become prevalent in almost all ALS patients at some point in the course of the disease [82].

The sleep disruptions observed in ALS encompass challenges in initiating and maintaining sleep, reduced sleep efficiency, heightened N1 sleep, and irregularities in both REM and non-REM sleep durations [81]. Sleep disturbances in ALS patients arise from various factors such as respiratory issues (e.g., nocturnal hypoventilation, obstructive sleep apnea), insomnia, motor problems including RLS, parasomnias like RBD, and sleep-wake cycle disturbances. Notably, the use of NIV has been demonstrated to be effective, enhancing patients' overall quality of life [83].

A comprehensive understanding of both sleep and respiratory symptoms, along with the implicated neural pathways, is imperative for the effective management of ALS patients. It is increasingly evident that sleep symptoms, such as nocturnal hypoventilation and hypoxia, may manifest independently of respiratory dysfunction [82].

Research indicates that circadian rhythms and sleep abnormalities may manifest early in the development of neurodegenerative diseases [84]. These disruptions not only serve as symptoms but may also play a direct role in disease pathogenesis. ALS patients frequently report symptoms like fatigue, daytime sleepiness, and sleep disturbances. While respiratory and muscular factors contribute, circadian rhythm disorders are also plausible. Brain regions regulating circadian rhythms, notably the thalamus and hypothalamus, have been implicated in ALS and other neurodegenerative diseases [82].

In a 2018 study by Diaz-Abad et al., the presence of poor sleep quality in newly diagnosed ALS patients was investigated [83]. The study demonstrated poorer sleep quality in ALS patients at the time of diagnosis compared to controls, with associations noted between sleep disturbances, symptoms of depression, and limited mobility in bed. However, melatonin levels were not addressed.

Existing studies have not definitively determined if sleep difficulties in ALS arise from a progressive loss of pyramidal system cells or if the disease process extends to circadian rhythm structures. Limitations in previous studies include the inclusion of patients in mid- to advanced disease stages, those with respiratory compromise, NIV, or parenteral nutrition. Additionally, the absence of investigations into endogenous melatonin levels in ALS patients remains a notable gap in the literature, despite evidence suggesting that Obstructive Sleep Apnea Syndrome (OSAS), with or without NIV, can impact melatonin secretion [85].

1.9 Fluid Biomarkers in ALS

1.9.1 Introduction to biomarkers in ALS

ALS diagnosis is a meticulous process, generally leaving little ambiguity for proficient neurologists. Nevertheless, some atypical cases pose challenges in deciphering clinical signs at onset. The diagnosis relies on recognising the progressive extension of lower and upper motor neuron involvement signs across various regions. Recent focus on early disease recognition has driven revised diagnostic criteria, incorporating biomarkers to facilitate early diagnosis, reduce diagnostic uncertainties, minimize non-specialist consultations, and expedite referrals to neurologists or motor neuron disease clinics. Biomarkers may also assist in identifying pre-symptomatic or prodromal phases, enhancing therapeutic interventions targeting a smaller motor cell population. Gold Coast diagnostic criteria recommend utilizing nerve conduction studies, electromyography (EMG), magnetic resonance imaging (MRI), and blood or cerebrospinal fluid (CSF) biomarker studies to exclude other diseases, enhance diagnostic accuracy, timeliness, and select homogenous ALS patient cohorts for clinical trials.

Despite the potential benefits, ALS biomarker discovery, especially in blood, faces challenges due to the matrix's complexity. CSF emerges as a promising source, reflecting neuro-axonal loss and subtle neuronal metabolism changes. However, invasive procedures, such as lumbar puncture for CSF collection, become impractical in advanced ALS stages. Blood, urine, and CSF exhibit non-interchangeable biomarker characteristics. Blood, rich in neuro-axonal and muscle degeneration biomarkers, may better represent ALS pathobiology and disease biotype comprehensively. Skeletal muscle's role in ALS progression makes blood the primary reservoir for molecules linked to neuro-muscular destruction.

The complex and poorly understood bioavailability of ALS blood biomarkers poses analytical challenges. Peptide analysis encounters hindrance from abundant proteins, impacting sensitivity. Biomarkers' concentration may be influenced by blood volume, body mass index, and late-stage ALS effects on fluid intake and nutrition. While blood accessibility is advantageous, its complexity remains a hurdle.

1.9.2 Most common fluid biomarkers in ALS

During the last twenty years in MND and others neurodegenerative diseases a lot of molecules have been studied as possible biomarkers of disease activity and progression, but only few were

associated with disease course. Notable results include: CHIT1, YKL-40 and MCP-1 [86], adipsin, MIP-1 and IL-8 [87], creatinine and albumin [88], although most of interest was put on neurofilaments (Nfs) as important diagnostic and prognostic biomarker [89]. Neurofilaments (Nfs), particularly Nf light-chain (NfL), are now becoming predictors of disease progression in ALS, while their role as pharmacodynamic markers is not yet clear. Unfortunately, the increase of Nfs is not specific for ALS [90].

Therefore, there is an important need of identification of easily accessible and reproducible biomarkers able to do early diagnosis and to help in prognosis definition, in patients' stratification and in selection of patients to be enrolled in clinical trials.

1.9.3 Pitfall in the evaluation of circulating proteins

Detecting protein biomarkers in biofluids, particularly blood, presents challenges. Low-abundance brain-derived proteins, potential biomarkers, rely on molecular processes, such as post-translational modifications [91]. Proteins like Nfs, tau, and alpha-synuclein, released into peripheral biofluids, follow distinct routes. The stoichiometry of Nfs subunits may adapt in ALS, potentially serving as a disease-specific biomarker, reflecting a shift towards energy-efficient NfL.

ALS patients exhibit altered Nfs subunit stoichiometry, possibly conserving energy and preventing neuronal death. This adaptive stoichiometry hypothesis suggests that high NfL levels correlate negatively with survival. Nfs isoforms' differential recruitment into aggregates complicates blood measurement, exemplified by the "hook-effect" in NfH measurements [92]. Therefore, the difficulties in measuring circulating proteins released by neurons are due to protein aggregation, clearance, and humoral immune response [91]. Brain protein content in circulating protein aggregates offers insights, potentially serving as an alternative ALS biomarker. Dysfunctional molecular chaperones may impact protein clearance, affecting blood biomarker detection. Changes in autoantibody levels and innate immune responses further complicate protein clearance and detection, influencing the sensitivity of immunodetection methods.

In conclusion, ALS biomarker discovery faces hurdles, particularly in blood, due to its complex matrix. Utilizing CSF for early diagnosis and prognostication seems viable, but blood or urine-based biomarkers may offer practical monitoring options in late-stage disease. Understanding the blood matrix effect, protein aggregation, and the humoral immune response is crucial for advancing ALS biomarker research. Overcoming these challenges will enhance diagnostic accuracy, prognostic capabilities, and enable the development of targeted therapeutic interventions for ALS patients.

1.9.4 The role of Blood Biomarkers in ALS

Biomarkers play a pivotal role in ALS aiding in early diagnosis, reducing diagnostic uncertainties, and expediting referrals to neurologists or motor neuron disease clinics. The Gold Coast diagnostic criteria propose a comprehensive approach, integrating nerve conduction studies, electromyography, magnetic resonance imaging, and blood/cerebrospinal fluid biomarkers to enhance accuracy and timeliness of ALS diagnosis. However, the quest for easily accessible blood biomarkers faces complexities. Interesting findings in fluid biomarkers concern:

- **Early Diagnosis:** Early ALS diagnosis remains paramount for effective interventions. Diagnostic latency, averaging 12 months, underscores the need for expedited diagnoses. Genetic mutations aid identification in familial ALS, but sporadic cases demand molecular biomarkers. Retrospective studies show elevated phosphorylated NfH (pNfH) levels up to 18 months before diagnosis, with plasma NfL rising 12–24 months pre-diagnosis [93,94]. Serum NfL levels precede symptom onset in some mutations, suggesting varied biomarker initiation times.
- **Differentiating ALS from Mimics:** Blood biomarkers play a crucial role in distinguishing ALS from mimics [91]. Studies confirm elevated CSF NfL and NfH in ALS, demonstrating high sensitivity and specificity. Combining these markers differentiates ALS from mimics, even beyond six months from symptom onset. Serum NfL effectively discerns ALS from mimics, with few exceptions. Additional biomarkers, like phosphorylated tau and chitinase proteins, show promise in CSF studies.
- **Prognostic Blood Biomarkers:** Blood biomarkers aid in predicting ALS prognosis. NfL emerges as a significant predictor, correlating with disease progression and survival. Higher plasma NfL levels indicate faster disease progression [95]. Micro-RNA-181 and blood TDP-43 also exhibit prognostic potential [96,97]. Immunological markers, Tregs and cytokines, suggest immune dysregulation affecting survival. Metabolic markers, lipid, and glucose levels indicate prognosis. Non-neurocentric markers like creatine kinase, perivascular fibroblasts' phosphoprotein 1, and high-sensitivity cardiac troponin also show prognostic relevance [91].
- **Monitoring Disease Progression:** Monitoring ALS progression through blood biomarkers is crucial for clinic-based assessment. Nfs biomarkers exhibit stable expression during the initial 12 months after symptom onset. Immune response markers like IL-6 and myeloid cells show an upward trend. Autoantibodies against Nfs and other proteins present alternative biomarkers.

Additionally, biomarkers differentiate patient phenotypes, such as cognitive impairment, aiding tailored treatment strategies [91].

In conclusion, the evolving landscape of ALS biomarkers in blood holds promise for early diagnosis, differentiation from mimics, and accurate prognosis. Understanding the dynamics of these biomarkers, their initiation times, and their relevance in disease progression provides a foundation for future research. As blood-based biomarkers continue to be explored, their integration into clinical practice could revolutionize ALS management, offering timely interventions and personalized treatment strategies for improved patient outcomes.

Section 1: New clinical, instrumental and bio-humoral biomarkers of bulbar and respiratory impairment in ALS

Chapter 2. Stapedial reflex: a possible novel biomarker of early bulbar involvement in Amyotrophic Lateral Sclerosis patients

In this chapter I'm presenting the result of a study already published in Audiology and Neurotology Journal [98], focused on the evaluation of stapedial reflex in ALS patients as precocious biomarker of bulbar impairment in patients which are not still symptomatic. This longitudinal study is the upgrade of a previous cross-sectional study done by our teamwork [99].

2.1 Introduction

In order to conduct effective clinical trials, it is paramount to have quantitative and reliable measures of disease progression. Nowadays, one of the most important primary outcome measures in ALS is the ALSFRSr. Several prognostic factors have been identified in ALS (*Chiò and Traynor, 2015*), but there is still a lack of clinical prognostic markers useful in care management and in clinical trials. Moreover, a marker of early bulbar impairment could be important in prevention of life-threatening events, such as ab ingestis pneumonia.

Stapedial reflex (SR), also known as acoustic reflex, could play this part. It is a pontine multi-synaptic involuntary reflex characterized by stapedial muscle activation after exposure to high-intensity sounds (*Borg, 1973*) and vocalization (*Borg and Zakrisson, 1975*). The afferent stimulus runs through the VIII cranial nerve and, after multiple synapsis with other inter-neurons, the efferent stimulus runs through the VII facial nerve, directed to stapedial muscle. SR could be absent or could have morphological abnormalities in all diseases in which there is a damage of afferent or of efferent pathways or of descending motor cortical pathways directed to facial nucleus that give synapsis on

the trunk. It is established that its alteration is present in many neurological diseases involving both the central *(Yamane and Nomura, 1984; Jerger et al., 1986; Murofushi et al., 1992; Lew et al., 1992*) and the peripheral (*RALLI et al., 1986*) nervous system. Two cross-sectional studies, one performed in Japan (*Shimizu et al., 1996*) and one in Italy (*Canale et al., 2017*), highlighted abnormalities in SR in patients affected by ALS; in particular, alterations were observed in patients presenting bulbar involvement.

Based on this evidence, we wanted to find out if alterations of stapedial reflex could be considered a biomarker of preclinical bulbar impairment in ALS patients without bulbar signs or symptoms.

2.2 Material and methods

2.2.1 Study design

This is a longitudinal study in which we enrolled 36 ALS patients, visited in the motor neuron clinic at the Molinette Hospital in Turin, Italy. The overall duration of the study was between January 2016 and August 2019. ALS patients underwent neurological evaluations and SR measurements every 3-4 months for a total of 4 visits. If a patient had not developed bulbar impairments by the fourth visit, the study would be extended for up to 15 months with a series of follow-ups until onset of bulbar disfunction or death.

Exclusion criteria at baseline: presence of bulbar involvement, history of brain stem disease, acute or chronic diseases of middle ear, severe hearing loss, presence of a tympanogram type B (flat, without a peak pressure; it suggests a middle ear disease, e.g. otitis media with effusion) type As (low compliance of the middle ear system, e.g. otosclerosis) type D (shows a small notch in the peak, e.g. scarred eardrums) type E (shows a deep notch in the peak; e.g complete ossicular discontinuity).

2.2.2 Clinical assessment

All ALS patients recruited had a diagnosis of definite or probable ALS according to the revised El-Escorial criteria (*Brooks et al., 2000*); disease severity was assessed by using the ALSFRSr score. Besides, we created an FRS sub-score excluding all three respiratory items and we named it NoResp-ALSFRSr.

Bulbar impairment was defined using the first 3 items of the ALSFRSr score and the neurological examination.

We gathered in **Table 1** demographic information (age, gender) and clinical information (time between disease onset and Decay alteration [ΔDecay], time between disease onset and bulbar impairment [Δsymptoms], ALSFRSr and NoResp-ALSFRSr at diagnosis, progression rate to last visit and No-respiratory-progression rate to last visit [NoResp-progression rate]). Progression rate and NoResp-progression rate was calculated as follow:

$$Progression\ rate = \frac{48 - ALSFRSr_{Last\ Visit}}{\text{Date Last Visit } - \text{Date symptom onset}}$$

$$NoResp - progression\ rate = \frac{36 - NoRespALSFRSr_{Last\ Visit}}{\text{Date Last Visit } - \text{Date symptom onset}}$$

2.2.3 Audiological evaluation

All patients underwent otoscopy, pure tone audiometry and tympanometry in order to exclude middle ear diseases; auditory thresholds and SR thresholds are listed in **Table 2**. Then we studied the SR, evaluating its presence (or absence), the intensity of appearance, the Acoustic Reflex Latency Test (ARLT) and the Decay Test. All the tests were performed at the pressure corresponding to the peak of the tympanogram obtained at 226 Hz and by using contralateral stimuli. Regarding normative tone thresholds we used the EN ISO 7029: 2017 that describes statistical distribution of tone thresholds related on age and gender in the normal population.

We used an impedance audiometer (Amplaid A728, Amplifon) to administer pure-tone stimuli at 0,5–1–2–4 KHz with a starting stimulation intensity of 80 dB HL, up to 115 dB HL with 5 dB steps, Broadband Noise (BBN, 0,25-4KHz) and Low-Pass Noise (LPN, 0,25-1,8KHz) with a starting stimulation intensity of 80 dB HL, up to 100 dB HL with 5 dB steps (on-time=1s, on/off-ratio=1s).

SR parameters were studied with the ARLT and the Decay test, using only the 0,5 KHz and 1 KHz frequencies, which are the most suitable ones in the analysis of this kind of reflex.

The ARLT analyzed the maximum amplitude (the highest value of impedance, expressed in cm^3), the latency (the time from the presentation of the stimulus to the achievement of 5% of the maximum amplitude, expressed in ms) and the Rise Time (the time that elapses between reaching 10% and 90% of the maximum amplitude, expressed in ms). Stimuli were presented 5 dB above the SR threshold, on-time=1s.

The Decay test analyzed the maximum amplitude, expressed in cm^3, at the beginning of the test and the amplitude 10s after the stimulus started. Stimuli were presented 10 dB above the SR threshold, on-time=10s. Due to many artefacts created by the instrument during Decay test evaluation, we analysed data by using a manual qualitative evaluation of it and we considered it positive if a reduction of more than 50% within 10s was observed.
In order to better evaluate the progression of impairment of motor neuron damage we decided to distinguish unilateral from bilateral positivity of the Decay test.

2.2.4 Statistical analysis

Shapiro-Wilk tests showed that data were not normally distributed.
In order to study the changes of quantitative and qualitative measurements of SR parameters during the study, we applied the Friedman test. Mann-Whitney U test was performed to analyse differences in progression rate between patients who did not develop bulbar signs until the end of the study and the rest of the cohort. Correlation between parameters was calculated by Spearman rank correlation r_s. The level of significance for all statistical tests was set at 0.05.
We used the SPSS Statistic V26 program (Chicago, IL, USA) to perform statistical calculations.
The study was approved by the Ethical Committees of the Turin ALS Center (Comitato Etico Azienda Ospedaliero-Universitaria Città della Salute e della Scienza, Torino) protocol number 0043607/2019. All patients provided a written informed consent. The databases were anonymized according to the Italian law for the protection of privacy.

2.3 Results

We enrolled 36 patients affected by probable or definite diagnosis of ALS, according to the revised El-Escorial criteria. All of them did not present bulbar signs or symptoms at baseline. 4 of them were excluded from the study after the baseline visit in which they showed a tympanogram type B. 2 of them died before the end of the study, so they were censored. Of the remaining 30 patients who completed the study, 28 developed SR's Decay alteration by the end, while 2 patients did not develop Decay alteration nor bulbar symptoms. They were excluded from the data analysis.
Focusing on these 28 patients, 16 developed bulbar symptoms, always after Decay alteration, before the final visit, while 12 did not. The latter continued the extension phase for fifteen months and we

observed the following: 8 of them developed bulbar involvement over the course of the succeeding fifteen months, while 4 did not develop bulbar impairment.

Participant demographics and clinical characteristics of the remaining 28 patients considered for data analysis are summarized in **Table 1**.

Table 1 – Cohort demographic and scales values of 28 ALS patients included in study analysis

Sex M/F	Age at diagnosis ± SD (y)	Diagnostic delay ± SEM (months)	ALSFRSr at diagnosis ± SEM	PR at diagnosis ± SEM	Δdecay ± SEM (months)	Δsymptoms ± SEM (months)
20/8	64.1 ± 10.5	16.0 ± 2.4	41.6 ± 0.6	0.65 ± 0.15	24.4 ± 2.5	30.2 ± 2.6

Abbreviations: standard deviation (SD), standard error of the mean (SEM); years (y); female (F); male (M); amyotrophic lateral sclerosis function rating revised scale (ALSFRSr); progression rate to last visit (PR); time between disease onset and decay alteration (Δdecay); time between disease onset and bulbar impairment (Δsymptoms).

2.3.1 Pure Tone Average and Tympanogram

All patients included in the data analysis showed a tympanogram type A or Ad or C. According to the EN ISO 7029 we observed that the mean initial pure tone thresholds were within the 75th percentile, so in the normal range for the age group (see **Table 2**). The longitudinal analysis highlighted no changes of these thresholds along the study (Friedman test; $p>0.05$).

Table 2 – Pure tone thresholds and SR thresholds in all 28 patients

Ear	auditory thresholds (dB)		SR threshold pure tone stimulus (dB)		SR threshold BBN stimulus (dB)	SR threshold LNP stimulus (dB)
	500Hz	1KHz	500Hz	1KHz		
RIGHT	20.7 ± 8.4	17.3 ± 8.8	91.6 ± 7.9	90.5 ± 7.2	88.5 ± 8.3	88.9 ± 7.1
LEFT	17.5 ± 8.6	15.4 ± 8.7	90.2 ± 6.5	91.1 ± 7.8	88.5 ± 7.3	88.9 ± 7.2

Abbreviations: Stapedial reflex (SR); Broadband Noise (BBN); Low-Pass Noise (LPN).

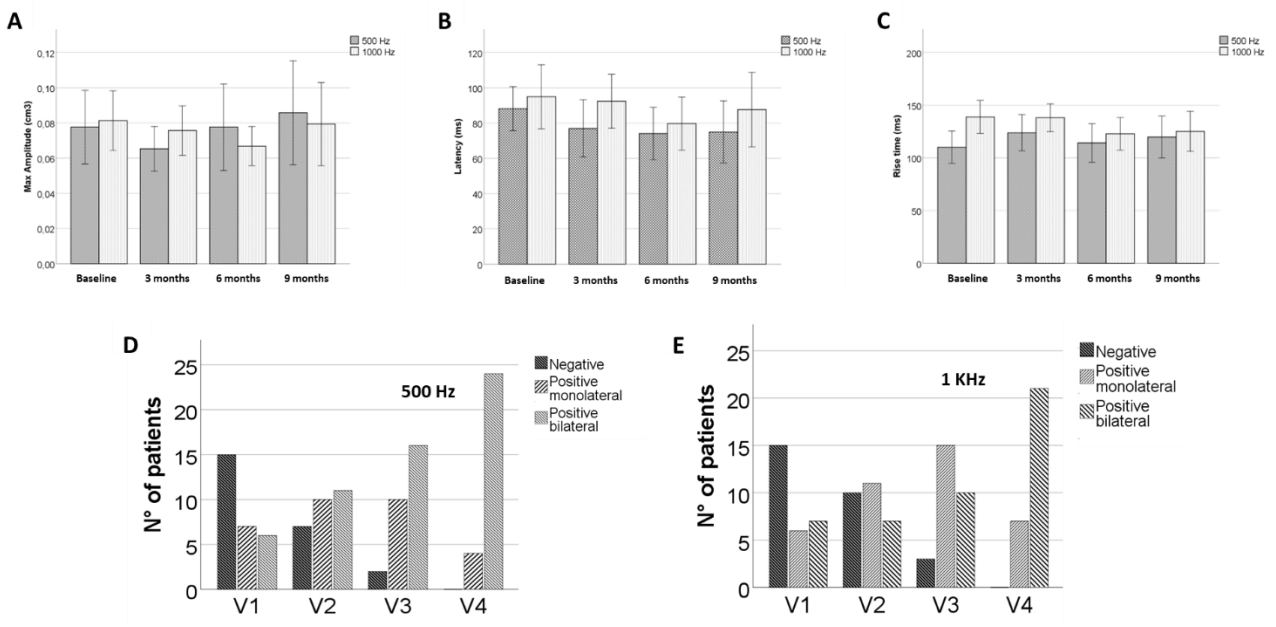


Figure 1 – Longitudinal modifications of ARLT parameters and of Decay

(***A***) Mean acoustic reflex amplitudes, (***B***) mean latencies and (***C***) mean rise times were observed across four measurement intervals. Contrarily, we showed statistically significant changes of Decay from one visit to another (from V1 to V4) at 500Hz (***D***) and at 1KHz (***E***), evaluated manually. We considered 'normal' a reflex if no reduction in amplitude or a reduction lower than 50% was observed over the 10s recording interval; 'positive monolateral' if reduction in amplitude was more than 50% after 10s in one ear; 'positive bilateral' if reduction in amplitude was more than 50% after 10s in both ears.

Error bars: 95% Confidential Intervals.

Abbreviations: First Visit (V1); Second Visit (V2); Third Visit (V3); fourth Visit (V4).

2.3.2 Analysis of Stapedial Reflex

The longitudinal analysis showed an absence of statistically significant changes (Friedman test; $p>0.05$) in all of the evaluated parameters (SR threshold with pure tone and BBN - LPN stimulus, amplitude, latency and rise time of SR evaluated at 500 Hz and 1000 Hz through ALRT), as shown in **Figure 1**. These analyses were led separately for the right and the left ears.

Looking at longitudinal variation of Decay both at 500 and 1000 Hz (**Table 3**), we found statistically significant variation between each visit (Friedman test; $p<0.0001$), considering as progression of impairment changes of Decay test from negative to positive unilateral or bilateral and from positive unilateral to positive bilateral.

Besides, Decay at 500 and 1000 Hz seemed to have a good temporal relationship with the development of bulbar impairment. In all patients included in the study it got altered or absent

before the onset of bulbar signs or symptoms. So, we created two variables: ΔDecay (time between disease onset and alteration of Decay expressed in days) and Δsymptoms (time between disease onset and onset of bulbar impairment expressed in days). We showed a good correlation between ΔDecay and Δsymptoms (r_S= 0.764; p<0.0001, **Fig. 2E**).

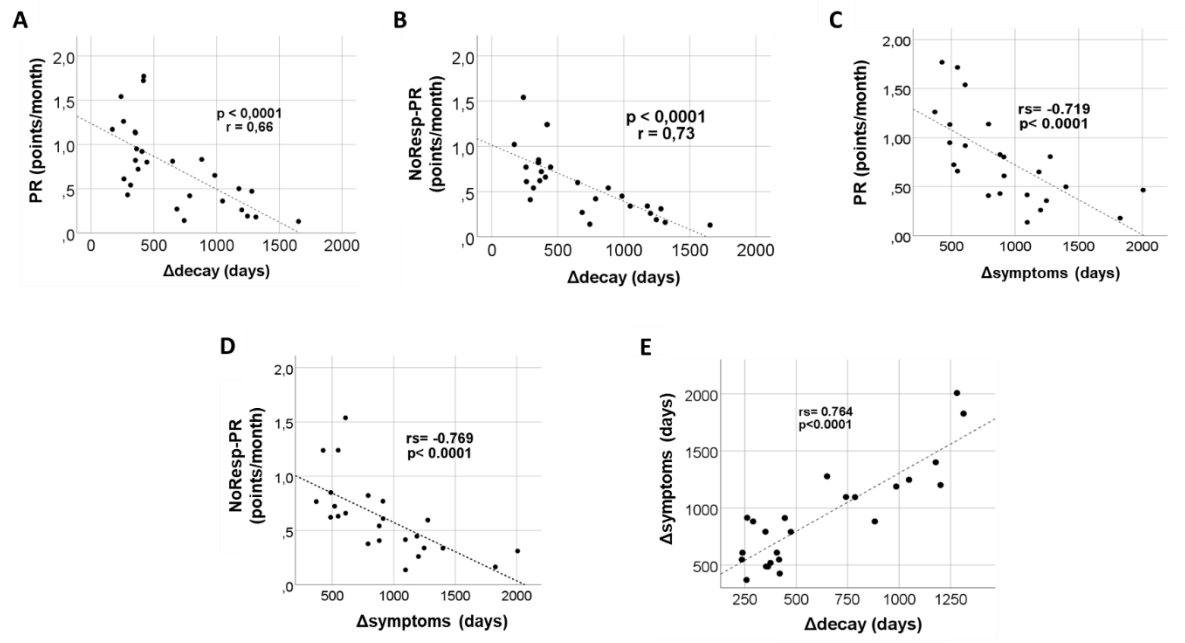


Figure 2 – Correlation between ΔDecay, Δsymptoms and progression rate

We observed a good correlation between progression rate to last visit and both ΔDecay (***A***) and Δsymptoms (***C***). This correlation is more evident if we use a sub-score of ALSFRSr scale obtained excluding respiratory items (NoResp-progression rate to last visit) (***B,D***). We also found a good relationship between Δsymptoms and Δdecay (***E***).

Abbreviations: progression rate to last visit (PR); progression rate to last visit excluding respiratory items (NoResp-PR); time between disease onset and Decay alteration (ΔDecay); time between disease onset and bulbar impairment (Δsymptoms); Spearman coefficient (r_S), p-value (p).

Table 3 – Changes in Decay test evaluation at 500 Hz and 1 KHz during disease progression in ALS patients

The first 24 patients developed bulbar impairment until the end of the study, while the last 4 did not.

Patients	Sex	DECAY 500HZ V1	DECAY 500HZ V2	DECAY 500HZ V3	DECAY 500HZ V4	DECAY 1KHZ V1	DECAY 1KHZ V2	DECAY 1KHZ V3	DECAY 1KHZ V4
1	F	N	N	N	PU	N	N	N	PU
2	M	PB	PB	PB	PB	PB	PB	PB	PB
3	M	N	N	PU	PB	N	N	N	PB
4	M	N	PU	PB	PB	N	PU	PB	PB
5	F	N	N	PU	PB	PU	PB	PB	PB
6	M	PB	PB	PB	PB	PB	PB	PB	PB
7	M	PB	PB	PB	PB	PB	PB	PB	PB
8	M	PU	PU	PB	PB	N	N	PU	PU
9	M	PU	PU	PB	PB	N	PU	PU	PU
10	M	N	PU	PU	PB	N	PU	PB	PB
11	M	N	PU	PU	PB	PU	PU	PU	PB
12	M	PB	PB	PB	PB	PB	PB	PB	PB
13	M	N	N	PU	PU	N	N	PU	PB
14	F	PB	PB	PB	PB	PB	PB	PB	PB
15	F	PU	PB	PB	PB	PU	PB	PB	PB
16	M	N	PU	PB	PB	PB	PB	PB	PB
17	F	PB	PB	PB	PB	PB	PB	PB	PB
18	M	PU	PB	PB	PB	PU	PU	PB	PB
19	F	PU	PB	PB	PB	PU	PB	PB	PB
20	M	N	PU	PB	PB	N	PU	PB	PB
21	M	PU	PB	PB	PB	PU	PB	PB	PB
22	M	PU	PB	PB	PB	N	PU	PU	PU
23	M	N	PU	PU	PB	N	N	N	PB
24	F	N	PU	PU	PB	N	N	PU	PU
25	F	N	N	N	PU	N	N	PU	PU
26	M	N	N	PU	PU	N	N	PU	PU
27	M	N	N	PU	PB	N	N	PU	PB
28	M	N	PU	PU	PB	N	N	PU	PB

Abbreviations: Decay test normal (N); Decay test positive unilateral (PU); Decay test positive bilateral (PB).

We also calculated time between Decay alteration and development of bulbar impairment: it is variable with a mean of around 10 months (9.8 ± 7). In order to better understand the great variability between time of Decay alteration and onset of symptoms and to explain the reason of absence of bulbar symptoms in those 4 patients until the end of the study, we decided to look at progression rate of disease. We highlighted a good correlation between ΔDecay and both progression rate and NoResp-progression rate (respectively, r_s= -0.584, p=0.03, **Fig. 2A**; r_s= -0.712; p< 0.0001, **Fig. 2B**) and between Δsymptoms and both progression rate and NoResp-progression rate (respectively, r_s= -0.719, p< 0.0001, **Fig. 2C**; r_s= -0.769, p< 0.0001, **Fig. 2D**).

Besides, we noticed that the progression rate in those 4 patients who did not develop bulbar impairment until the end of the study was statistically lower than the others (Mann-Whitney U test; p=0.024, **Fig. 3**).

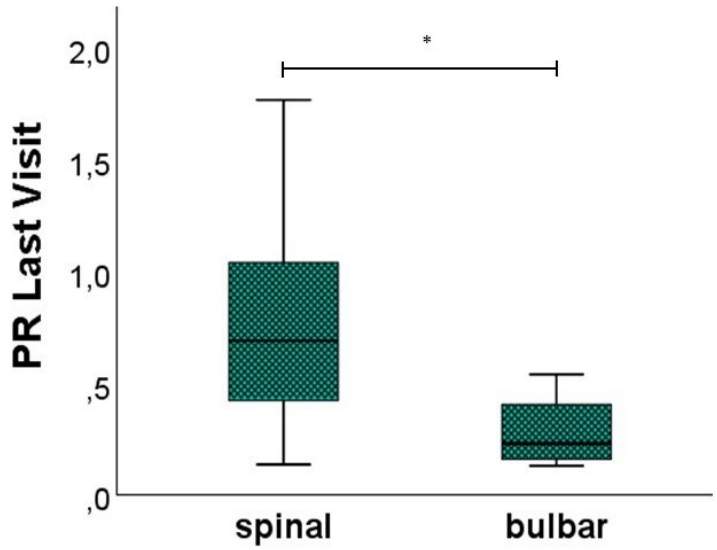


Figure 3 – Comparison between progression rate in patients who didn't develop bulbar impairment until the end of the study and the others

4 patients (box-plot on the right) who did not develop bulbar impairment before the end of the study present a progression rate to last visit (PR-Last Visit) significantly lower than other patients (box-plot on the left). Statistical analysis: U-Mann-Whitney, p= 0,018. Error bars indicate maximum and minimum.

Discussion

This is the first longitudinal study, led on ALS patients, in which the role of stapedial reflex was evaluated as a predictive factor for future bulbar involvement during the course of the disease.
In line with our previous paper [Canale et al., 2017], the 28 patients included in the current data analysis showed normal values in the baseline evaluation of initial pure tone thresholds and SR thresholds with pure tone and BBN-LPN stimuli (**Table 2**). In the longitudinal analysis we did not observe any alteration of those thresholds, suggesting that auditory and SR thresholds did not play a role in the evaluation of disease progression in ALS patients.
In contrast to previous evidence [Shimizu et al., 1996], we did not observe any variation of ARLT parameters (amplitude, latency and rise time) in predicting bulbar involvement.

Moreover, we showed the important role of SR's Decay test as a possible predictive biomarker of bulbar impairment in ALS patients and as its use as an effective tool in monitoring the progression of disease and in clinical trials recruitment. In all patients included in final analysis Decay test got positive until the end of the study, always before development of bulbar signs or symptoms. We observed a progressive impairment of the Decay test in a continuum from negative to positive unilateral or bilateral and from positive unilateral to positive bilateral; we never actually observed the inverse. That seems to reflect the gradual relentless damage, which occurs in ALS patients, of both upper and lower motor neurons connected to facial nuclei.

We highlighted a good direct correlation between ΔDecay and Δsymptoms (**Fig. 2E**): the later Decay gets altered the slower patients develop bulbar signs or symptoms. We found out that, in our cohort, Decay test became positive around ten months before the development of bulbar impairment, although we observed an important variability. Unexpectedly, 4 patients, who presented alteration of Decay, had not developed bulbar symptoms at the end of the study (after the fifteen months of the extension phase).
Looking at the disease's progression rate, we observed a tight relationship between progression rate and both ΔDecay and Δsymptoms (**Fig. 2A, C**). This correlation is even more evident if we consider the progression rate without respiratory items (NoResp- progression rate, **Fig. 2B, D**). Besides, we discovered that progression rate in those 4 patients without bulbar symptoms at the end of the study was statistically significantly lower than the other 24 patients included in the study (**Fig. 3**). All these

data let us to infer that the faster is the progression of disease the shorter is the period between the time when SR's Decay test get positive and the time when patients develop bulbar impairment.

Stratification based on the region of onset and cognitive profile was impossible due to the small number of patients. Besides, we were not able to obtain reliable sensibility and sensitivity values of Decay test because we enrolled an insufficient number of patients. Nevertheless, we suppose most likely that they are high. In fact, as highlighted by Ünsal et al. in 2016 [Unsal et al., 2016], in elderly people without previous ear diseases and hearing loss Decay test should be negative, although it could show morphological changing. Contrarily, in all our ALS patients we observed a clear alteration of Decay test as the disease was progressing. So, our data suggest the importance to perform Decay test regularly during follow-up of ALS patients without bulbar symptoms.

Strengths of our work lie in progression of Decay alteration (test negative – test positive unilateral – test positive bilateral) throughout the course of the disease and in correlation between Decay alteration and bulbar involvement.

Limitations of this study include the small number of patients and the absence of research of swallowing impairment through in-depth otolaryngologic examination.

Further multi-centre studies, involving more patients and controls, are needed to define more precisely the time between Decay alteration and the onset of bulbar impairment.

Conclusions

This study demonstrated a potentially important role of SR's Decay test as a useful non-invasive and standardized biomarker of early pre-clinical bulbar impairment in ALS patients. SR's Decay test can be considered positive if its reduction in amplitude is more than 50% within 10 seconds of a stimulus. We recommend the evaluation of SR's Decay test in monitoring progression of disease and in clinical trials recruitment of ALS patients.

Chapter 3. Novel Polysomnographic biomarkers of early respiratory and sleep disturbances: results of an ongoing longitudinal study

This longitudinal observational study is still ongoing and I'm presenting the unpublished partial results obtained from patients enrolled between July 2021 and August 2023.

3.1 Introduction

There is limited knowledge of sleep disturbances in the context of ALS and the still limited understanding of the precise causes and relationships of these disturbances with the pathology, a study assessing qualitatively and quantitatively the sleep patterns in this patient category, longitudinally and prospectively, was deemed valuable.

Sleep disturbances in individuals with ALS can be attributed to both the progressive impairment of the pyramidal system, primarily affecting the respiratory aspect but also influencing cramps, immobilization, and pain, and the extension of the neurodegenerative process to structures responsible for sleep regulation and circadian rhythm [111]. This mechanism could explain the association of sleep disorders such as Restless Legs Syndrome (RLS), Rapid Eye Movement Sleep Behavior Disorder (RBD), Periodic Limb Movement Disorder (PLMD), insomnia, and circadian rhythm disorders with ALS, as supported by the neuro-physio-pathological correlates analyzed in sections related to these pathologies.

A meta-analysis has observed alterations in the microstructure of white matter in ALS patients compared to healthy controls. This involves, in particular, the left and right corona radiata, the body and splenium of the corpus callosum, the posterior limb of the internal capsule, the left superior longitudinal fasciculus, and the bilateral cingulate gyrus, in addition to the right corticospinal tract and right cerebral peduncle [112]. These structures play a crucial role in regulating sleep-wake systems, providing evidence that supports the correlation of sleep disturbances in individuals with ALS.

Another crucial aspect to consider is the lack of markers capable of early identification of respiratory impairment [113], enabling the initiation of nocturnal NIV as soon as possible, since it improve the life expectancy [114]. In fact, respiratory impairment in ALS is a prognostic factor crucial for survival and its

complications (such as acute respiratory insufficiency and pulmonary infections) are the most important causes of death.

Since when we sleep our respiratory muscles, mainly diaphragm, are more relaxed and respiratory efficiency is physiologically reduced due to chest position and to less reactivity of central activation [113], it's possible that the first signs of respiratory impairment can manifest at night.

Therefore, to study better early signs of respiratory impairment is fundamental to explore breath during night.

Previous studies, led on topics above mentioned have often exhibited limitations, such as the use of a cross-sectional design and questionnaires as the primary assessment tool (at the expense of more comprehensive and objective laboratory or instrumental examinations, such as polysomnography), small sample sizes, lack of enrollment of subjects in early phase of disease and presence of patients with respiratory compromise and the need for ventilation or parenteral nutrition.

Therefore, we wanted to evaluate longitudinally sleep and respiratory impairment in a cohort of ALS patients enrolled at the beginning of the disease.

This study assesses the trajectory of parameters related to sleep and respiratory function over time in a cohort of ALS patients, using both questionnaires and instrumental investigations (polysomnography, spirometry) as well as laboratory tests (venous blood and arterial blood gas analysis). These assessments are compared with a healthy control population age and sex matched. The project's objectives include validating existing literature data on the incidence of sleep disorders in ALS and observing their evolution over time, comparing polysomnography with current clinical practices for early respiratory assessment, and correlating polysomnographic parameters with clinical ones.

3.2 Materials and methods

We enrolled 59 ALS patients meeting the revised El Escorial Criteria for defined, probable and probable-laboratory supported ALS (*Brooks et al., 2000*) diagnosed in the period July 2021- August 2023 in our specialized Centre CRESLA (Centro Regionale Esperto sulla SLA), Turin, Italy.

We also enrolled 59 healthy controls (HC), age and sex-matched with ALS, visited in the Sleep Centre of Molinette Hospital between 2020 and 2022.

3.2.1 Inclusion and exclusion criteria

Inclusion criteria for ALS: disease duration < 36 months, sum of item 1 and item 3 of the ALSFRSr > 6, ALSFRSr score > 39, capability to understand tasks and to give consent to take part in the study.
Exclusion criteria for ALS: known sleep disorders before the onset of ALS, patients in NIV, presence of dementia and of COPD, and FVC<60%.
Exclusion criteria for healthy controls: presence of neurodegenerative diseases, presence of known respiratory impairment or sleep diseases, presence of ISI scale positive for insomnia, assumption of benzodiazepines, antidepressant, antipsychotic, and β-blockers drugs.

3.2.2 Study protocol

The study design is a prospective observational longitudinal study, with patients undergoing follow-up every three months for a maximal duration of 18 months. In case of necessity to start NIV the patient will discontinue the study after the first follow-up visit in course of NIV. Moreover, there is the possibility of discontinuation in case of excessive deterioration, refusal to continue, tracheostomy or death.
The protocol involved detailed neurological examinations every three months for patients with ALS (V0: baseline; V1: month 3; V2: month 6; V3: month 9; V4: month 12; V5 month 15; V6: month 18). Polysomnography, spirometry, and arterial blood gas analysis were performed every 6 months, in particular at the visit of enrolment (V0), at month six (V2), month twelve (V4), and, potentially, at month 18 (V6). Salivary collection was performed every 3 months.

Neurological Evaluation
During scheduled visits, patients underwent a thorough neurological examination to assess disease progression. ALSFRSr and MRC scales were completed to evaluate various muscle groups.
Demographic and clinical data were collected at each visit, including age, gender, marital status, occupation, years of education, and various clinical details.
The medications taken as home therapy at the time of each visit were also documented, with specific attention given to serotoninergic and noradrenergic antidepressants, antipsychotics, benzodiazepines, and beta-blockers. These medications are known to potentially affect the quality and/or architecture of sleep, particularly influencing the aspects of Rapid Eye Movement Behaviour Disorder (RBD) and/or Restless Legs Syndrome with Arousal (RSWA), as analysed in polysomnographic examinations.

Questionnaires

Several questionnaires (STOP-BANG, ISI, ESS, PSQI, MEQ, PIRS, RRS, STAY, BDI) were administered to investigate sleep and assess neuropsychological aspects such as anxiety and depression. Two additional questionnaires (STAI and BDI) were specifically dedicated to investigating psychological aspects in ALS patients.

Instrumental Measurements

Ambulatory polysomnography was conducted to capture various sleep and respiratory parameters. Spirometry, including forced vital capacity (FVC), forced expiratory volume in the first second (FEV1), forced expiratory flow (FEF), maximum expiratory flow (MEF), and peak expiratory flow (PEF), was performed to monitor respiratory function.

Arterial blood gas analysis included pH, pO2, pCO2, bicarbonates (HCO3-std), and base excess (BE).

3.2.3 Statistical Analysis

IBM SPSS 28 was used for statistical analyses.

Descriptive statistics included mean and standard deviation for continuous variables and absolute frequency and relative percentage for categorical variables.

Comparison between controls and patients was performed using the Mann-Whitney U test and Kruskal Wallis test with post-hoc Dunn's test.

Longitudinal analyses in ALS patients were conducted using the Friedman two-way ANOVA.

Chi-square test was used to analyse qualitative variables.

Spearman's coefficient was calculated to assess correlations between different variables.

Significance was set at $p<0.05$.

3.3 Results

3.3.1 Patient Recruitment

Using the previously listed criteria, 59 patients were selected and agreed to participate in the study. Among them, 4 subjects died during the study, 9 subjects deteriorated and exited the study, and 5 patients refused to continue. As the study is still ongoing, many subjects have not completed the 24-month follow-up and data collection for this thesis was closed in September 2023. At the 6-month follow-up 32 patients had at least one of the predefined investigations (polysomnographic recording,

spirometry, blood gas analysis, psychological and sleep questionnaires), at 12-months follow-up 17 patients completed at least one of the investigations, and at 18 months, only 3.
Additionally, the initiation of NIV in ALS subjects was indicated when an AHI>15 was observed in PSG, leading them to undergo a pneumological evaluation. Four subjects underwent PEG placement: one between recruitment and 6 months of follow-up, two between 6 and 12 months, and one after 18 months. One patient underwent tracheostomy between 6 and 12 months of follow-up.

3.3.2 Demographic and Clinical Data

Out of the total sample of 59 patients enrolled in the study, 38 subjects were male (64.4%), and 21 were female (35.6%). The average height was 1.68±0.09 m; the healthy body weight (HBW) was 70.4±12.4 kg, and the weight at diagnosis was 67.64±13.05 kg, resulting in a healthy-BMI of 24.8±3.6 and a BMI at diagnosis of 23.9±3.6.
Regarding marital status, which is relevant for potential caregiver presence, 11 subjects were single (18%), 41 were married (70%), 3 were separated (5%), and 3 were widowed (5%).
The age at the time of ALS diagnosis ranged from 43 to 82 years, with an average of approximately 65 years (64.43±9.05).
The onset site of the disease was bulbar in 24% of cases (14 patients), lower limbs in 37% of cases (22 patients), with 24% distal and 15% proximal, and upper limbs in 39% of cases (23 patients), with 25% distal and 12% proximal. Overall, 76% of patients had spinal onset. No patient experienced symptom onset in respiratory muscles. The side of symptom onset was left in 36% of cases, right in 27% of cases, and bilateral in 13% of cases; the remaining percentage corresponds to patients with bulbar onset.
During the neurological examination, family history of ALS was investigated through history, with only 3 cases (5%) reporting familial cases. Genetic analysis for the most common mutations recognized as pathogenic for ALS (in *SOD1*, *C9orf72*, *FUS*, and *TARDBP* genes) was conducted in all patients and resulted negative in 52 patients (88%). Among the 7 positive cases (12%), two had the *SOD1* mutation (3.8%), and five had the *C9orf72* gene expansion (9.4%). Among the three subjects with familial history, one tested positive for the *C9orf72* expansion.
In **Table 1** we inserted the main demographic and clinical data analysed at V0, subdividing them based on the type of onset (spinal or bulbar) and the presence or absence of respiratory compromise at V0 (for this criterion, items 10, 11 of ALSFRSr at V0 were evaluated, considering subjects with respiratory symptoms having a score below 4 in at least one of the two items).

Table 1 - Clinical and demographic characteristic of ALS patients

	Sex (M/F)	p-value	Age at diagnosis	p-value	ALSFRSr score at V0	p-value	PR	p-value
Spinal (n=45)	64% M 36% F	>0.05	63.1 ± 9.5	0.042	38.5 ± 5.4	0.007	0.89 ± 0.78	0.043
Bulbar (n=14)	64% M 36% F		68.7 ± 5.7		42.0 ± 3.7		0.47 ± 0.24	

Looking at ALSFRS-r score, we observed an actual reduction over time, both in patients who underwent NIV before V2 follow-up visit and the others. Moreover, ALS patients who started NIV before V2 follow-up visit showed a significantly lower ALSFRS-r score both at V0 and at V2, as reported in **Table 2**.

Table 2 – Longitudinal evaluation of ALSFRSr score in ALS patients with and without NIV at V2

	V0	V2	p-value
ALSFRSr in ALS patients NOT IN NIV at V2	40.6 ± 3.9	35.8 ± 6.3	<0.001
ALSFRSr in ALS patients IN NIV at V2	37.3 ± 4.2	26.1 ± 8.4	<0.001
p-value	<0.001	<0.001	

Regarding the occupational status of patients, professions were grouped into ISTAT170 categories for easier differentiation. The classification is visible in **Table 3**.

Table 3 – Occupancy of ALS patient included in this study

Occupancy	Absolute frequency	(%)
Legislators, entrepreneurs, and senior executives	0	0
Intellectual, scientific, and highly specialized professions	8	13.6
Technical professions	4	6.7
Executive roles in office work	4	6.7
Qualified professions in business activities and services	9	15
Craftsmen, skilled workers, and farmers	22	36.7
Plant operators, operators of fixed and mobile machinery, and vehicle drivers	3	5
Unskilled professions	3	5
Unskilled professions	3	5
Unemployment	3	5

The most represented category is that of artisans, skilled workers, and farmers (36.7%), followed by qualified professions in trade and services, and intellectual, scientific, and highly specialized professions. These represent both current and past occupations: at the time of the first neurological visit, 25 subjects were retired (42.37%).

The average years of education amounts to 10.5 ± 3.8. Analysing potential risk factors, 55.9% of the patients report never having smoked, 23.3% are former smokers, while 21.7% are active smokers. Those who smoke or have smoked did so for an average of 28 years, consuming an average of 18 cigarettes per day.

38.3% of the subjects claim to be abstinent, while 55% use alcoholic beverages; most of them report consuming alcohol occasionally, usually with meals.

Some possible comorbidities were also taken into consideration: 36.7% of the subjects are hypertensive, 6.7% have diabetes or prediabetes, and 3.3% have COPD. The prevalence of thyroid disorders in the sample is 20%, with nine cases of hypothyroidism and only one of hyperthyroidism. Five patients underwent cholecystectomy.

Furthermore, during the diagnostic process, a cervical spine MRI was performed in almost all subjects to exclude possible differential diagnoses. It was found that 84.6% of patients undergoing

this examination had cervical discopathy (43 out of 52 cases), but spinal cord damage due to the compression was evident in only 2 subjects. The prevalences are summarized in **Table 4**.

Table 4 – Comorbidities in our ALS patients

	Absolute frequency	(%)
Blood Hypertension	21	36.7
Diabetes type II	4	6.7
COBP	2	3.3
Thyroid diseases	12	20
- **hyperthyroidism**	1	1.7
- **hypothyroidism**	9	15
- **Hashimoto thyroiditis**	2	3.3
Cholecystectomy	5	8.3
Cervical discopathy	43	73.3
Cervicale myelopathy	2	3.3

3.3.3 Psychological Assessment (STAI and BDI Questionnaires)

At baseline we can observe a moderate level of anxiety symptoms (STAY>40) in 22 ALS patients (45%) and the presence of clinically significant depressive symptoms in 14 ALS patients (30%). Looking at longitudinal evaluation (**Table 5**), we can observe that until 12-months visit (V4) after the enrolment there is not a statistically significant changing of these scores (p-value = 0.58).

Table 5 – Longitudinal evaluation of STAI and BDI Questionnaires scores

	V0	V2	p-value
STAI-Y1	40.71 ± 11.07	41.31 ± 12.47	>0.05
STAI-Y2	38.22 ± 10.46	38.38 ± 8.49	>0.05
BDI	11.12 ± 7.43	11.75 ± 9.34	>0.05

3.3.4 Subjective Sleep Assessment through Questionnaires

RLS (Restless Legs Syndrome)

The presence of restless legs syndrome was investigated with a preliminary screening using the four diagnostic criteria. Only 3 patients satisfied them at the time of enrolment and completed the RLS scale, resulting in one case of mild and two cases of severe forms.

OSAS (Obstructive Sleep Apnoea Syndrome) Risk

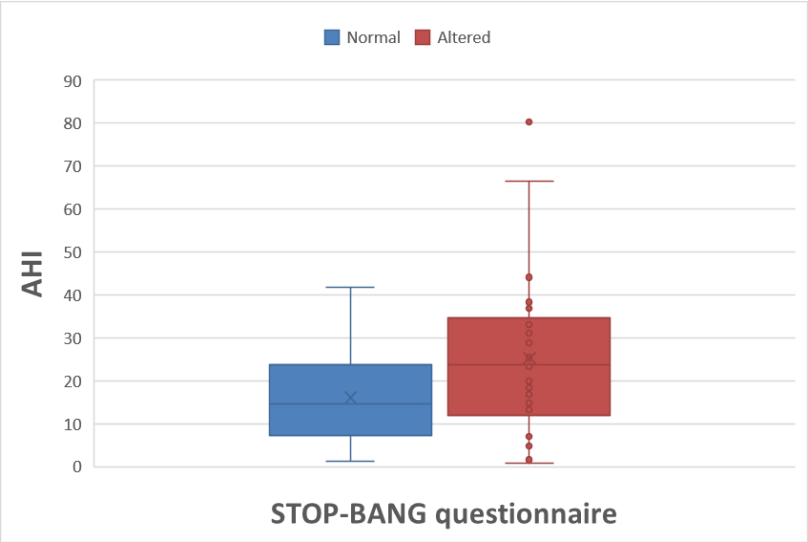
Around 60% of patients show a STOP-BANG questionnaire score greater than or equal to 3, indicating an increased risk of obstructive sleep apnoea. This data is confirmed, also, at V2 visit (**Table 6**).

Moreover, comparing levels of AHI and row values of the STOP-BANG questionnaire (SBQ) we observe a trend of direct correlation, although it is not statistically significant ($p>0.05$). If we consider the AHI of patients with a SBQ altered with that of patients with SBQ normal we observe a statistically significant difference ($p= 0.015$; **Figure1**).

Table 6 - Longitudinal evaluation of sleep scales

	V0	V2	p-value
SBQ	2.9 ± 1.2	3.0 ± 1.3	>0.05
ISI	5.0 ± 3.8	3.9 ± 4.5	>0.05
ESS	6.0 ± 4.0	6.2 ± 4.9	>0.05
PSQI	5.1 ± 3.3	4.2 ± 3.7	>0.05
MEQ	23 ± 5	23 ± 5	>0.05
PIRS	40 ± 24	39 ± 32	>0.05

Figure 1 – Comparison of levels of AHI among ALS patients with and without alteration of STOP-BANG questionnaire



Insomnia

Degree of insomnia was measured using the Insomnia Severity Scale (ISI), considering a clinically significant insomnia a score greater than or equal to 15 (moderate to severe insomnia). At baseline we do not observe a significant increase of ISI score in our cohort od patients. Looking at longitudinal evaluations, no statistically significant differences are observed (p=0.27).

Daytime Sleepiness

The Epworth Sleepiness Scale (ESS), considered positive for excessive daytime sleepiness if the score is >10, result still normal at baseline in our ALS patients. No variation at V2 are observed (**Table 6**), also analysing separately ALS patients with and without NIV at V2, as shown in **Table 7**.

Table 7 – Longitudinal analysis of ESS in patients with and without NIV at V2

	V0	**V2**	**p-*value***
ESS in ALS patients NOT IN NIV at V2	6.1 ± 4.7	5.5 ± 4.5	0.27
ESS in ALS patients IN NIV at V2	7.1 ± 2.9	7.0 ± 5.3	0.93

Sleep Quality

Poor sleep quality was defined if the PSQI questionnaire score was greater than 5. Around 36% of ALS patients report a poor sleep quality at baseline and the data seems to remain fairly stable over time.

Longitudinally obtained data comparing the total PSQI score of patients who completed the questionnaire at V0 and V2, divided based on NIV initiation, are summarized in **Table 8**.

Table 8 – Longitudinal analysis of PSQI in patients with and without NIV at V2

	V0	V2	p-value
PSQI in ALS patients NOT IN NIV at V2	6.1 ± 4.7	5.5 ± 4.5	0.18
PSQI in ALS patients IN NIV at V2	7.1 ± 2.9	7.0 ± 5.3	0.87

Chronotype

Patients' chronotypes over time, based on the Morningness-Eveningness Questionnaire (MEQ), show a more represented morning type that evening type at the moment of the enrolment. Longitudinal evaluation until month 6 do not show not statistically significant changes (p= 0.27).

3.3.5 Instrumental evaluations

Spirometry

At baseline we observe normal levels of spirometry parameters (considered in a normal range above the 80% of predict) in 47 ALS patients, while a mild respiratory impairment (60%< FVC <80%) is observed in 12 patients. Among them 4 patients do not refer any respiratory symptoms.

If we compare spirometry parameters at baseline of patients who will undergo or not NIV before V2 (the 6-month visit) we can observe that there is a general trend of reduction of all of them, although it is not still statistically significant (**Table 9**).

Longitudinal analysis from V0 to V2 shows a statistically significant reduction of FVC and FEV1 both on total patients (p= 0.001) and separating patients NIV-free from patients who undergo NIV before month 6 (**Table 10**). The other spirometry parameters do not reach the statistically significance.

Table 9 – Spirometry parameters at baseline in ALS patients

	Total (59)	Not NIV at V2 (37)	NIV at V2 (22)	p-value
FVC	94 ± 19	104 ± 14	89 ± 20	0.09
FEV1	97 ± 21	104 ± 15	92 ± 24	0.15
MEF25	85 ± 30	92 ± 26	85 ± 38	0.44
MEF50	85 ± 29	93 ± 26	82 ± 36	0.25
MEF75	79 ± 21	84 ± 21	74 ± 21	0.37
PEF	75 ± 19	78 ± 17	73 ± 18	0.56

Table 10 - Longitudinal analysis of spirometry parameters in the 32 ALS patients who underwent both V0 and V2 examinations

	Not NIV at V2 (18)			NIV at V2 (14)		
	V0	**V2**	**p-value**	**V0**	**V2**	**p-value**
FVC	104 ± 14	93 ± 21	0.021	89 ± 20	61 ± 30	0.018
FEV1	104 ± 18	96 ± 24	0.048	92 ± 24	68 ± 35	0.018
MEF25	89 ± 33	110 ± 48	0.40	85 ± 38	68 ± 45	0.11
MEF50	84 ± 30	85 ± 31	0.29	82 ± 36	67 ± 45	0.068
MEF75	80 ± 24	76 ± 27	0.18	74 ± 21	63 ± 31	0.78
PEF	76 ± 18	73 ± 25	0.31	73 ± 18	48 ± 30	0.068

Arterial Blood Gas Analysis

At baseline we observe normal levels of ABG parameters (using our laboratory range of normality), except for a very slight increase of HCO3- levels (in the range 26.1-28.9) in 39 ALS patients. Among them 14 patients refer respiratory symptoms and 37 show significant alteration of the main PSG parameters (AHI total and/or AHI supine and/or AHI in REM).

If we compare ABG parameters at baseline of patients who will undergo or not NIV before V2 (the 6-month visit) we do not observe any difference (**Table 11**).

Longitudinal evaluation of ABG analysis do not show any statistically significant alteration ($p>0.05$) both considering all the patients together, both sub-dividing NIV-free patients from patients who undergo NIV before V2 (**Table 12**).

Table 11 – ABG parameters at baseline in ALS patients

	Total (59)	**Not NIV at V2 (37)**	**NIV at V2 (22)**	**p-value**
pH	7.44 ± 0.03	7.43 ± 0.03	7.44 ± 0.02	0.16
pO_2	91.9 ± 13.2	89.5 ± 6.1	85.43 ± 14.8	0.32
pCO_2	39.9 ± 3.9	40.2 ± 4.0	41.43 ± 3.31	0.69
HCO_3^-	27.2 ± 1.6	26.84 ± 1.87	27.64 ± 0.48	0.13
BE	3.2 ± 2.0	2.74 ± 2.24	3.54 ± 0.50	0.81

Table 12 – Longitudinal evaluation of ABG analysis in the 32 ALS patients who underwent both V0 and V2 examinations

	Not NIV (18)			**NIV (14)**		
	V0	**V2**	**p-value**	**V0**	**V2**	**p-value**
pH	7.43 ± 0.03	7.44 ± 0.04	0.076	7.44 ± 0.02	7.43 ± 0.02	0.58
pO_2	89.5 ± 6.1	91.4 ± 12.2	0.78	85.4 ± 14.8	76.3 ± 22.0	0.08
pCO_2	40.2 ± 4.0	37.5 ± 3.3	0.062	41.4 ± 3.3	47.7 ± 10.6	0.08
HCO_3^-	26.8 ± 1.9	26.5 ± 2.0	0.46	27.6 ± 0.5	27.8 ± 1.7	1.00
BE	2.7 ± 2.2	2.0 ± 2.5	0.46	3.5 ± 0.5	3.9 ± 2.3	0.68

Polysomnography

Respiratory parameters at baseline

We compared respiratory PSG parameters of ALS patients at baseline with healthy controls and we observed that most of them resulted to be altered. In particular, we observed that in 54 out of 59 ALS patients (92%) there is an increased number of apnoea/hypopnoea events, most of all in supine position and during REM sleep, as showed in **Table 13**. Moreover, comparing total AHI and AHI in REM is evident a clear increase of this parameter in REM sleep (p= 0.001); this data is still significant if we repeat the analysis separately for ALS without respiratory symptoms and with a ISI scale normal at V0 and for the others.

To avoid potential bias due to the fact that observed differences could be attributed to patients with respiratory and sleep disorders clinically evident at V0, and consequently altered respiratory parameters in PSG, these patients were excluded. Only ALS without respiratory symptoms and with a ISI Scale normal at V0 were compared with healthy controls. The results adding this filter are superimposable to those reported previously **Table 13**.

Both ALS groups (the one including patients with respiratory symptoms and the other without respiratory symptoms) show a statistically significant difference in respiratory parameters compared to HC ($p<0.001$; **Figure 2**). No statistically significant difference between the two ALS groups can be demonstrated, although mean and median are more altered in the group including ALS patients with respiratory impairment compared to the other.

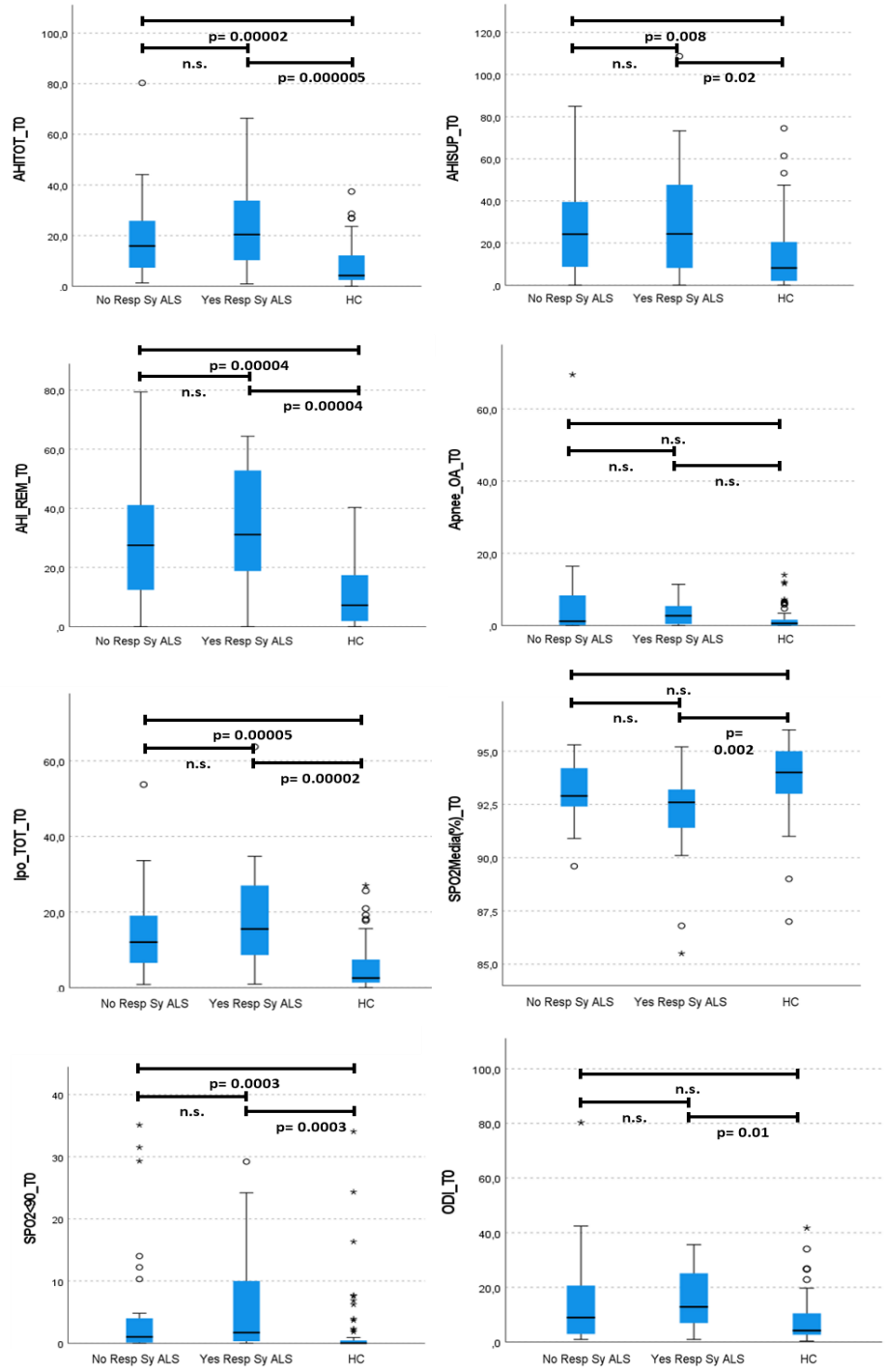
Table 13 – Respiratory PSG parameters in ALS and HC at baseline

In this table there is the comparison at baseline of respiratory PSG parameters between all the ALS included in the study and HC and between ALS without respiratory impairment and HC.

	ALS patients V0_tot (59)	ALS patients V0_noresp (43)	HC (59)	p-value (ALS_tot vs HC)	p-value (ALS_noresp vs HC)
TST (min)	367 ± 64	372 ± 64	385 ± 86	0.176	0.40
AHI total (ep/h)	20.5 ± 15.1	20.2 ± 15.3	7.8 ± 7.9	<0.001	<0.001
AHI supine (ep/h)	29.0 ± 24.8	28.5 ± 22.4	14.4 ± 16.6	<0.001	<0.001
AHI not supine (ep/h)	6.7 ± 7.6	6.2 ± 6.5	4.5 ± 6.2	0.662	0.46
AHI REM (ep/h)	30.1 ± 19.7	28.9 ± 18.5	11.5 ± 11.1	<0.001	<0.001
Obstructive apnoea (ep/h)	4.9 ± 9.8	5.8 ± 9.9	1.8 ± 3.1	0.01	0.023
Mixed apnoea (ep/h)	0.22 ± 0.54	0.20 ± 0.60	0.3 ± 1.0	0.459	0.48
Central Apnoea (ep/h)	0.44 ± 0.85	0.52 ± 0.91	0.29 ± 0.67	0.181	0.043
Hypopnoea total (ep/h)	15.7 ± 12.2	14.8 ± 10.4	5.3 ± 6.4	<0.001	<0.001
RDI	21.5 ± 15.0	21.1 ± 14.8	9.7 ± 8.6	<0.001	<0.001
ODI	14.1 ± 13.5	13.9 ± 14.4	7.7 ± 8.3	<0.001	0.009
SpO_2 mean (%)	92.7 ± 1.8	93.1 ± 1.3	93.7 ± 1.8	<0.001	0.009
SpO_2 minimum (%)	84 ± 5	84 ± 5	88.3 ± 3.7	<0.001	<0.001
SpO_2 <90% (%)	8.02 ± 19	4.6 ± 8.4	1.9 ± 5.6	<0.001	<0.001

Legend: ALS patients V0_noresp= ALS patients without respiratory symptoms and with a ISI Scale normal at V0; TST= Total Sleep Time; RDI= respiratory disturbance index; ODI= Oxygen Desaturation Index

Figure 2 – Comparison of the most relevant PSG parameters between ALS patient without respiratory impairment, ALS patients with respiratory impairment and HC.



<u>Legend</u>: AHI_TOT_T0= Total AHI at baseline; AHI_REM_T0= AHI in REM at baseline; AHISUP_T0= AHI in supine position at baseline; Ipo_TOT_T0= Total hypopnea at baseline; Apnoea_OA_T0= number of obstructive apnoea at baseline; ODI= Oxygen Desaturation Index; SPO2<90_T0= Time spent below 90% of saturation at baseline; SPO2Media_T0= Mean levels of SpO2 at baseline.

Black line inside blue boxes represents the median value.

Macro and microstructure of sleep

Looking at macrostructure of sleep (**Table 14**), ALS patients sleep a number of hour similar to HC (p=0.18), although they spend more time asleep (sleep efficiency of ALS 80.1 vs 84.2 of HC; p=0.026). An interesting data is the evident increase of Deep Sleep (N3) and a contextual reduction of REM Sleep in ALS compared to HC (p<0.001).

Table 14 – Sleep micro and macrostructural PSG parameters in ALS and HC at baseline

	ALS pazients V0 (59)	ALS pazients V0_nodrug (43)	HC (59)	p-value (ALT_tot vs HC)	p-value (ALS_no drug vs HC)
TST (min)	367 ± 64	357 ± 61	385 ± 86	0.176	0.060
Sleep efficiency (%)	80.1 ± 9.4	79.5 ± 9.2	84.2 ± 8.5	0.026	0.021
WASO (min)	74.0 ± 42.4	79.8 ± 43.2	61.7 ± 46.3	0.057	0.026
Sleep onset (min)	14.5 ± 22.6	14.8 ± 23.6	17.5 ± 21.3	0.881	0.856
REM onset (min)	120 ± 74	106 ± 68	93.3 ± 67.1	0.005	0.115
Microarousal total (episodi/h)	26.2 ± 12.2	26.3 ± 12.9	18.6 ± 9.9	<0.001	<0.001
Microarousal for limb movements (ep/h)	3.7 ± 6	3.8 ± 6.5	2.0 ± 2.8	0.685	0.874
Microarousal for respiratory events (ep/h)	13.8 ± 10.2	14.7 ± 10.7	3.4 ± 4.1	<0.001	<0.001
Microarousal spontaneous (ep/h)	7.8 ± 5.2	7.1 ± 3.5	10.3 ± 8.9	0.334	<0.001
Arousal in REM (ep/h)	27.5 ± 17.5	26.6 ± 15.4	15.6 ± 12.8	<0.001	<0.001
N1 (%)	10.6 ± 5.1	10.5 ± 4.6	11.1 ± 6.5	0.901	0.777
N2 (%)	47.9 ± 9.8	48.3 ± 10.4	49.9 ± 9.7	0.215	0.364
N3 (%)	27.1 ± 11.2	27.1 ± 11.7	20.0 ± 8.2	<0.001	0.006
REM (%)	14.4 ± 5.2	14.2 ± 4.9	19.1 ± 4.4	<0.001	0.005
Supine Time (%)	43.4 ± 36.9	45.6 ± 38.8	35.2 ± 26.0	0.363	0.331
Not Supine Time (%)	56.6 ± 34.0	26.4 ± 36.0	64.8 ± 26.4	0.289	0.264
Bruxism (ep/h)	3.0 ± 7.1	3.3 ± 8.3	1.9 ± 3.4	0.240	0.668

In order to reduce possible bias due to the assumption of medicaments that could interfere with the macrostructure of sleep (benzodiazepines, antidepressant, antipsychotic, β-blockers), we repeated the analysis removing from ALS this subgroup of patients. Statistically significant differences between ALS and controls are still evident (**Table 14**).

We also observed alteration of microstructure in ALS patients. Is evident that the arousal index of our patients (26.2 ± 12.2) is above the normal value (below 15 episodes per hour). Comparing the number of microarousal in ALS with those in HC we show an evident increase of microarousal due to respiratory events and of arousal in REM (**Table 14**; $p<0.001$). This data is in line with lite

In order to reduce possible bias due to the assumption of medicaments that could interfere with the microstructure of sleep (benzodiazepines, antidepressant, antipsychotic, β-blockers), we repeated the analysis removing from ALS this subgroup of patients. The results adding this filter are superimposable to those reported previously (**Table 14**).

Additionally, polysomnographic data were analysed by categorizing patients based on the onset type of the disease. No statistically significant differences were found between parameters of subjects with spinal onset compared to those with bulbar onset at V0 ($p>0.05$), even in the subgroup of patients without respiratory symptoms.

Longitudinal evaluations of PSG parameters

Focusing on variations of PSG parameters all along the study we decided to compare values at V0 with values at V2 in ALS patients not yet initiated on NIV at T1. Data from patients who reached the 6-month follow-up were selected with a total of 16 patients. The only parameters that show a statistically significant worsening are AHI_total ($p= 0.026$) and total hypopneas ($p= 0.008$), as reported in **Table 15**. Also, the other parameters show a general worsening although they are not statistically significant, maybe due to the limited number of cases included in this ad interim analysis.

Table 15 – PSG respiratory parameters significantly different from T0 to T1 in ALS patients not in NIV

	V0	V2	p-value
AHI total (ep/h)	6.8 ± 4.2	12.3 ± 4.6	0.026
Hypopnoea total (ep/h)	6.2 ± 3.7	11.0 ± 3.9	0.008

On the other side, in ALS patients who underwent NIV we observe a general improvement of all PSG respiratory parameters, but no changing in macro and microstructure parameters (**Table 16**).

Table 16 – PSG respiratory parameters significantly improved from T0 to T1 in ALS patients in NIV

	V0	V2	p-value
AHI totale (ep/h)	18 ± 11	11 ± 9	0.011
AHI supino (ep/h)	29 ± 26	14 ± 12	0.042
AHI REM (ep/h)	35 ± 25	8 ± 9	0.022

Periodic limb movements during sleep (PLM)

PLM index is increased in ALS patients: 30 out of 59 (51%) show a PLM index above 5 episodes/hour (limit of normality). In order to avoid mistakes due to medications that affect this parameter (pramipexole and dopamine agonists, benzodiazepines, gabapentin, and pregabalin), patients taking them have been excluded (13 out of 59). Among the remaining 46 subjects, 22 (47.8%) have a pathological PLM index at V0. At V2, 9 out of 20 subjects had PLM index >5 (45%). Also the 59 HC have been skimmed based on medication and the remaining 38 have been used for comparison with ALS. Both comparing the severity of PLM between ALS patients at V0 and HC and ALS patients at V2 and HC we observe a statistically significant increase of PLM among ALS patients (respectively p=0.015 and p= 0.004 at χ^2 test).

Table 17 – PLM index among ALS patients at V0 and at V2 compared to HC

	Mild PLM (5<PLMI≤25)	Moderate PLM (25<PLMI≤50)	Severe PLM (>50)	Total
V0 (n=46)	9 (19.56%)	5 (10.9%)	8 (17.39%)	22 (47.8%)
V2 (n=20)	2 (10%)	2 (10%)	5 (25%)	9 (45%)
HC (n=38)	13 (36.11%)	0 (0%)	1 (2.78%)	14 (38.89%)

Discussion

Regarding the demographic and clinical data, the male-to-female ratio in the analysed sample is 1.8:1, consistent with literature data. The average age at diagnosis, 65±9 years, and the median age, 64 years, reflect studies reporting an incidence peak between 60 and 75 years and an average or median age at diagnosis between 54 and 69 years [1].

The onset of ALS is described as spinal in approximately 65% of cases and bulbar in 30% of cases [1]. The study results align with these findings, indicating a bulbar onset in 24% of cases and spinal onset in 76%. There are no reported cases of respiratory onset, which is documented in literature between 1-3%.

Familial ALS cases constitute 5% of the sample, with the most frequently observed mutations being *C9orf72* (9.43%) and *SOD1* (3.77%). These genes are commonly mutated in sporadic ALS forms. *C9orf72* is also reported as mutated in 40% of familial cases in the European population, with 1 in 3 cases of familial familiarity presenting this mutation.

Demographic and clinical data were compared based on the type of onset and the presence or absence of respiratory symptoms at V0. At first glance, the percentage of males and females appears consistent in all categories. The age at diagnosis seems slightly higher in subjects with bulbar onset compared to those with spinal onset, and in those with respiratory symptoms compared to those without. The ALSFRSr score at V0 is higher in subjects with bulbar onset compared to spinal onset and in those without respiratory symptoms compared to those with symptoms. The longitudinal trend of the mean total ALSFRSr score from V0 to V4 shows a progressive significant decrease in the overall score, corresponding to increased functional impairment due to disease progression. This data is observed also subdividing patients that undergo NIV starting from the others.

Questionnaires

Regarding the psychological aspect, mood disorders are known to be important in ALS, attributable to various causes (primary or secondary to the disease, medications, or diagnostic uncertainty), and are related to sleep disorders, contributing to the alteration of sleep quality and insomnia. Apathetic and depressive features are described in 30% and 80% of patients, especially those with cognitive dysfunction [82], and the prevalence of confirmed depression is estimated between 27% and 41% in ALS patients, particularly at 50% when using the BDI as a detection tool [115]. In this study, the prevalence of patients with clinically significant depressive symptoms at T0 was 30%, a figure aligned with the literature. Longitudinally comparing BDI scores between V0 and V2 in patients who completed the questionnaire at both time points did not reveal a significant difference: depressive symptoms seem to remain stable over time. This result may be due to the small sample size or the use of antidepressant medications following neurological care, consistent with longitudinal studies in the literature showing stability or a decrease in the rate of depression with disease progression [116].

The prevalence of anxiety in ALS is estimated between 0% and 30% in the literature; almost 75% of patients reportedly experience moderate/severe state anxiety at baseline, but only 20% of cases exceed the clinical cutoff [116]. Anxiety appears to be particularly high at the time of diagnosis, decreasing over the course of the disease. In this study, the percentage of patients with clinically significant state anxiety symptoms (STAI-Y1>40) at T0 was 45%, and at the same time, 41% had clinically significant trait anxiety symptoms (STAI-Y2>40). Similarly, comparing scores of patients at T0 and T1 did not reveal statistically significant variations. However, as before, the result might be influenced by the low number of subjects analysed.

Sleep questionnaires investigated various aspects of sleep disorders in this study. The PSQI, providing an "overall" view of sleep quality, indicated poor quality in approximately 40% of patients at V0, a figure that did not seem to change over time, even when distinguishing between patients entering NIV and those not entering NIV. This finding may suggest that sleep quality is not solely related to respiratory disorders, as previously discussed [117]. This data is slightly lower than described in the literature, where 50-63% of ALS patients reportedly have poor sleep quality at diagnosis [81], with the average PSQI score significantly higher than that of controls [83].

Insomnia is a widely described symptom in ALS, complex because it can be due to various causes, not detailed in many studies [83,117], including mood disorders, as mentioned earlier. In this study,

moderate and severe insomnia, described for ISI scores greater than or equal to 15, is not present at V0, with a prevalence of about 4% at V2; the difference in mean scores over time, 4.29 ±3.52 at V0 and 3.29 ±3.91 at V2, is not statistically significant (p=0.27), and both values fall within the range corresponding to the absence of clinically significant insomnia.

To date, it has only been hypothesized that circadian rhythm disturbances may contribute to sleep disturbances in ALS patients. The MEQ questionnaire analysis revealed a prevalent moderate morning chronotype, contrary to the general population where it is neutral. This could be partly attributed to the high average age of the patients in the sample, as older individuals tend to have a moderate morning chronotype [118]. However, no significant changes over time were observed in the longitudinal comparison.

A high risk of obstructive sleep apnoea syndrome (OSAS), defined by a STOP-BANG score greater than or equal to 3, was found in about 60% of patients at V0. The prevalence of this disorder is reported to be increased in ALS patients in the literature [119]. Comparing data from patients who completed the questionnaire at both V0 and V2, with further discrimination based on NIV initiation, no significant changes over time were observed. The correlation of an altered STOP-BANG questionnaire with an increased AHI, and thus the risk of OSAS, was confirmed. Excessive daytime sleepiness in the sample is about 16% of patients at V0, with no statistically significant changes over time, even when dividing the data into those entering NIV and those not entering (this difference was made as nocturnal ventilation should improve sleep quality and, therefore, positively influence daytime sleepiness).

Regarding movement disorders related to sleep, the prevalence of restless legs syndrome (RLS) has been described between 14.6% and 25% in ALS patients, significantly higher than the general population (5-10%) [120]. In this study, only 3 patients met the criteria for the syndrome at enrolment (approximately 5%); this figure is lower than reported in the literature.
In conclusion, questionnaires in some cases confirmed data present in the literature, while in others, the prevalence of the disorder was lower than described. They seem to be a less sensitive tool in assessing sleep disorders compared to polysomnographic analyses.

Spirometry and ABG

Spirometry and ABG parameters do not seem to be useful in the detection of early respiratory impairment in ALS patients. In fact, their levels are in the normal range at V0, also considering separately ALS patients with and without NIV, except for carbonates.

The carbonate values, although without significant variations between V0 and V2, show a slight increase in ALS patients compared to normal values (between 22-26 mmol/L): this may suggest their role in indicating hypoventilation and/or nocturnal apnoeas (as their levels would increase to compensate for chronic respiratory acidosis) even before diurnal hypoventilation develops, as previously suggested by Manera et al [121]. On the other hand, carbon dioxide is not useful index, since it is a parameter that undergoes rapid changes. In fact, unlike bicarbonates, carbon dioxide might even be decreased compared to normal if the subject hyperventilates during the examination.

Performing a longitudinal comparison of spirometry and ABG parameters in both patients not undergoing NIV and those undergoing NIV, no statistically significant differences emerged.

Polysomnographic Findings

PSG represent the most important investigation in this study, aided by the availability of controls for comparisons.

Firstly, comparing the parameters of all patients at V0 with controls, we revealed significant differences in respiratory parameters, such as total AHI, supine AHI, AHI in REM, obstructive apneas, total hypopneas, RDI, ODI, average SpO2, minimum SpO2, and <90% SpO2, as well as respiratory-related microarousals. Therefore, a significant finding in this study is the presence of a high percentage of OSAS (92%) in recruited ALS subjects already at V0, while less than 30% of them show a reduction in spirometry or a clear alteration of ABG values (namely $pCO2 > 45$ mmHg or $HCO3- \geq 28$ mEq/l). Focusing on AHI, which revealed to be one of the best indices to identify respiratory impairment during sleep, an interesting data is the higher number of apnoea-hypopnoea events in REM sleep compared to the rest of the sleep. This is in line with the higher tendency of upper airway to collapse during REM sleep, due to reduced genioglossus activity [122]. Therefore, apnoea and hypopnea events are usually deeper, longer, and more frequent during REM sleep than events during non-REM sleep [123].

Another important finding is the alteration of sleep structure. In particular, REM sleep has a longer latency and shorter duration in ALS patients than in controls, and N3 sleep appears increased in ALS patients. Microarousals, spontaneous microarousals, and those in REM sleep are increased, along

with the time spent in a non-supine position, which is higher in healthy controls. Similar data are described in the literature, with the exception of the increase in N3 sleep, which is reported as reduced in ALS patients in some studies [111]. It is hypothesized that this finding may be attributed to chronic sleep deprivation experienced by ALS patients due to various factors affecting sleep quality, as reported in Chapter 1. The homeostatic system would compensate by increasing slow-wave sleep in subsequent nights. To avoid possible bias, i.e., that the observed differences were primarily due to patients with evident respiratory and/or sleep disorders and, consequently, altered PSG respiratory parameters, these patients were excluded. A comparison was made with healthy controls only for those without respiratory symptoms at V0 (based on items 10, 11, and 12 of the ALSFRSr). Even in this scenario (n=43 ALS), respiratory parameter differences remained significant, such as total AHI, supine AHI, REM AHI, total hypopneas, RDI, ODI, average SpO2, minimum SpO2, and <90% SpO2. Considerations on REM and N3 sleep stages are the same as in the previous analysis. Total microarousals, especially those related to respiratory events and those in REM sleep, and time spent in a non-supine position continue to differ. Additionally, a new difference in wake after sleep onset (WASO) emerges, appearing longer in ALS patients, in line with other data.

All these differences can be also confirmed dividing ALS patients in two groups: the one with respiratory symptoms and the other one without respiratory symptoms at V0. Comparing these two ALS groups, we observe worse parameters in the one including ALS patients with respiratory symptoms, in line with expectation, although results are not still statistically significant, maybe due to limited numerousness of the patients. Perhaps, with a larger number of patients, a difference hidden by the small sample size would be revealed.

Regarding the longitudinal analysis, if we focus on ALS patients that do not undergo NIV before V2, AHI index and total hypopneas result to be significantly increased in 6-months follow-up. That support the important role of PSG not only in detecting respiratory impairment but also in its longitudinal evaluation.

In contrast, in the longitudinal comparison between V0 and V2 of patients who undergo NIV, a significant reduction can be observed in total AHI, AHI in the supine position, and AHI in REM. This confirms the effectiveness of NIV in improving nocturnal respiration.

PLMs have been investigated in few studies in the ALS population, and their prevalence compared to the general population is unclear: it is reported at 4-11% in the general population, correlated

with age, with PLMs present in 20-30% of healthy individuals over 50 years of age [111]. They are also present in about 80% of RLS cases and correlate with other sleep disorders. In this study, evaluating polysomnographic data after excluding patients taking medications that interfere with PLM, it emerged that at V0 the percentage of ALS patients with any type of PLM was importantly higher than expected from literature data. Moreover, they are increased compared to our HC (p=0.015). In particular, we observed 10.8% of moderate form and 17.4% of severe form of PLM. These data get worst all along the disease if not treated.

In summary, all data derived from questionnaires, spirometry, ABG analysis and PSG could indicate that early respiratory disturbances in ALS patients can be detected more sensitively, and thus earlier, by PSG compared to the other instruments. Hence, the importance of conducting polysomnographic examination even when respiratory symptoms are not yet evident, to detect any respiratory alterations with greater sensitivity and initiate the patient on NIV earlier, as it is a procedure that improves the prognosis of the disease [114].

Conclusions

The study confirms the presence of multiple sleep disturbances in the clinical picture of Amyotrophic Lateral Sclerosis. Firstly, there is evident alteration of respiratory parameters (particularly total AHI, supine AHI and REM AHI) even in the early stages of the disease, even in patients without overt respiratory symptoms. This underscores the clinical importance of polysomnography in the early monitoring of ALS patients, especially in this subgroup, to potentially facilitate early initiation of NIV. Secondly, a modification of sleep stages has been observed in ALS patients compared to controls, not entirely consistent with the literature. The increase in N3 sleep and the reduction in REM sleep may represent homeostatic adaptation to chronic sleep deprivation in these individuals.

Furthermore, a high percentage of subjects with a PLM index exceeding normal limits was noted among ALS patients. This disturbance has not been thoroughly studied in this population and is clinically relevant as appropriate therapy may significantly contribute to improving sleep quality in these individuals.

Further multicentre longitudinal studies with a greater number of patients are mandatory for a better definition of respiratory and sleep disorders in ALS and a clinical trial focused on the early starting of NIV in patients with mild PSG alterations is fundamental to define the real effectiveness

of a very precocious starting of NIV in getting lower the progression of disease and increasing survival.

Section 2: New fluid diagnostic and prognostic biomarkers in ALS

Chapter 4. The role of CHI3L1 plasmatic levels in Amyotrophic Lateral Sclerosis

In this chapter I'm presenting the result of a study already published in Journal of Clinical Medicine [124], focused on the evaluation of Chitinase-3-like protein-1 (CHI3L1), a protein belonging to the family of chitinases, whose role in ALS is under investigation. In this study I measured levels of CHI3L1 in plasma of motor neuron diseases (MND)' patients, MND mimics and healthy controls (HCs): we observed that it could be a useful biomarker in the differential diagnosis process of MND and that it could be associated with inflammatory processes.

4.1 Introduction

There is a strong need of prognostic and diagnostic biomarkers able to early differentiate ALS from ALS-mimics, to stratify patients and to predict disease outcome, in order to improve patients' inclusion in clinical trials. Neurofilaments (Nfs) are now becoming a widely accepted prognostic biomarker for ALS and other neurodegenerative diseases [125],[126].

More recently, chitinases, a family of hydrolytic enzymes, have been studied in the cerebrospinal fluid (CSF) of ALS patients as possible liquid biomarkers. They include CHIT1, CHI3L1, and CHI3L2, and correlate with neuroinflammatory status [127,128]. CHIT has been extensively studied in the CSF of ALS patients [127–131] and investigated in plasma [127], showing a promising role in differential diagnosis. Conversely, CHI3L1 has been less studied. While lacking chitin hydrolase activity due to a mutation, CHI3L1 binds chitin oligomers and heparin/heparan sulphate with high affinities. Mutational analysis revealed distinct heparin-binding domains, suggesting a role in modulating cytokines concentrations[132]. In line with this, CHI3L1 is also involved in processes of systemic inflammation,

such as autoimmune diseases [133], cancers [134], liver disease [135], and diabetes [136]. A recent study showed that CHI3L1 is highly expressed in the activated astrocyte in the motor cortex of ALS patients [127]. Additionally, Thompson and colleagues observed that the CSF levels of CHI3L1 correlate with cognitive impairment in ALS [128]. This is an interesting result, since a validated biomarker that is related to cognitive impairment does not exist, and needs to be confirmed in plasma. Based on these premises, we decided to carry out a pilot study in order to compare the levels of the CHI3L1 in the plasma samples gathered during the first visits to our centre, which were obtained from MND patients, MND mimics, and healthy controls (HCs). We decided to focus on plasma, because blood sampling is an easier and less invasive procedure compared to cerebrospinal puncture.

4.2 Materials and Methods

4.2.1 Clinical assessment

MND patients received their diagnosis following the Gold Coast criteria[109] and the PLS criteria [137]. Disease severity was assessed using Amyotrophic Lateral Sclerosis revised Functional Rating Scale (ALSFRSr)[138]. We excluded patients with active cancer, infectious diseases, and ongoing immunosuppressive therapies.

All patients underwent blood sampling at diagnosis. Moreover, all MND patients underwent: a clinical evaluation (neurological examination, including the Medical Research Council scale [MRC][139] measurement, and ALSFRSr scale), an instrumental evaluation (electromyography, cerebral MRI with tractography and functional-MRI, spirometry), a neuropsychological evaluation [140] (including MMSE, ECAS, TMT-A, TMT-B, RAVLT, ROCF, FAB, Digit Span, FRSBE), and a genetic test for the most common mutation (*C9orf72, SOD1, TARDBP and FUS genes*). Patients' phenotypes have been classified as follows: classic ALS, bulbar ALS, flail arm, flail leg, prevalent upper motor neuron ALS [23].

Progression rate (PR) was calculated as (48 - ALSFRSr at blood sampling)/ time interval in months between symptoms onset and blood sampling. Fast- and slow-progressor ALS were divided using median value of PR (0.54 points/month).

4.2.2 Laboratory evaluation

From each patient and control, we collected 4 mL of fresh blood in an EDTA tube. The blood samples were centrifuged at 20 °C at 1500× *g* for 15 min within 1 h from collection, and the obtained plasma was stored at −80 °C.

The samples were kept at 4 °C overnight before the ELISA test to allow for slow thawing; then they were left at room temperature for 1 h and used to run the test.

We used the Human Chitinase-3-Like Protein 1 (CHI3L1) ELISA commercial Kit (Abbexa, Cambridge, UK), following the manufacturer's instructions. All the ELISA assays were performed in duplicate. The plates were read using a CLARIOstar Plus plate reader (BMG LABTECH, Ortenberg, Germany). The standard curves were fitted with 4-parameter logistic regression, using MARS data analysis software (BMG LABTECH). The samples were diluted 1:40 in order to achieve a concentration within the linear range of the standard curve measurements. The median intra-assay and inter-assay coefficients of variation were below 15% for all the assays.

General blood tests were performed by our hospital laboratory; the red blood cell and haemoglobin levels were measured using the IDEXX VetAutoread™ Hematology Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

4.2.3 Statistical analysis

A Shapiro–Wilk test showed that the data were not normally distributed.

A Kruskal–Wallis test was used to compare the groups. A Bonferroni' comparisons test was performed following the Kruskal–Wallis test, in case of any significant differences. Receiver operating characteristic (ROC) curves were not performed due to the relatively small number of patients that were involved. The correlations between the CHI3L1 levels, the motor and cognitive symptoms, and the laboratory and instrumental parameters were calculated by the Spearman rank correlation (rs). The level of significance for all the statistical tests was set at 0.05.

We used the program SPSS Statistic V26 (Chicago, IL, USA) to perform the statistical calculations.

All the patients signed a written informed consent form. The study design was approved by the Ethical Committees of the Turin ALS Center (Comitato Etico Azienda Ospedaliero-Universitaria Città della Salute e della Scienza, Torino) (n° 0011613, 03/02/2020).

4.3 Results

We performed a cross sectional study in which we enrolled 19 HCs, 44 MNDs, 7 hereditary spastic paraplegia (HSP), and 9 MND mimics (including myelopathy, radiculopathy, axonal neuropathies, and post-poliomyelitis syndrome), seen at the ALS Center, Molinette Hospital between Jan 2019 and Aug

2022. Our 44 MNDs include 25 ALS, 12 ALS with cognitive impairment (ALS/FTD or ALSci, using Strong classification [141]), 5 PLS and 2 PMA.

Participant demographic and clinical characteristics are summarized in **Table 1**.

None of the patients or the controls included in the study suffered from active cancer, infectious diseases, autoimmune diseases, diabetes, liver diseases, or had ongoing immunosuppressive therapies.

Table 1. Participant demographic and clinical information at blood sampling

Patients group	**n°**	**Sex M/F**	**Age ± SD (y)**	**Genetic mutation**	**ALSFRSr ± SD**	**FVC ± SD**
MND	44	20/24	63 ± 13	7/44	39,5 ± 4,8	98 ± 19
MND mimics	9	5/4	62 ± 15	N/A	N/A	N/A
HSP	7	5/2	64 ± 8	7/7	N/A	N/A
HC	19	10/9	60 ± 9	N/A	N/A	N/A

* N/A: not assessed

4.3.1. Plasmatic CHI3L1 in differential diagnosis

CHI3L1 plasma levels result to be different between groups (p= 0.005; **Figure 1**) Of note, Bonferroni's correction shows that MND mimics have significantly higher levels of CHI3L1 compared to MNDs (p=0.002) and HCs (p=0.023) and that HSP have higher levels of CHI3L1 compared to MNDs (p=0.03). We found no difference between HSP and MNDs versus HCs (p>0.05). Differences are confirmed co-variating for age and sex (p= 0.004). A sub-group analysis of MND patients (divided in PLS, ALS, ALS+FTD and PMA, **Figure 1**) do not show any difference in CHI3L1 levels (p>0.05). No differences in CHI3L1 levels between genetic and non-genetic forms of ALS nor between fast- and slow-progressor ASL (p>0.05).

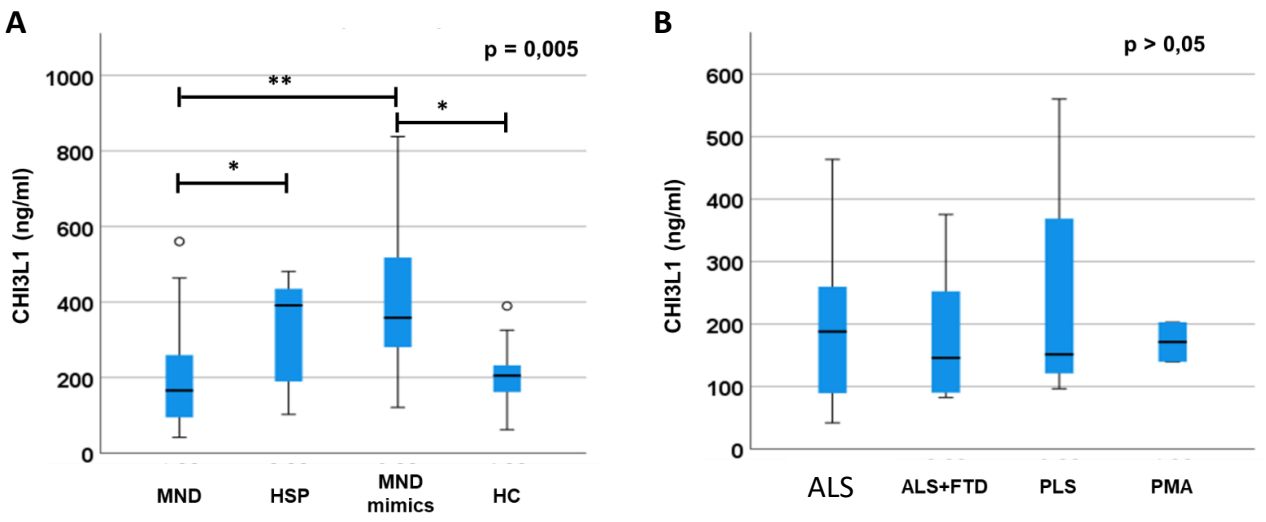


Figure 1 - CHI3L1 plasmatic levels in MND, MND mimics, HSP and HC

CHI3L1 plasmatic levels in MND, MND mimic, HSP, and HC. In MND mimics, we highlighted an important increase in CHI3L1 plasmatic levels compared to MND and HC (**A**). Moreover, we observed a slight difference between the MND and the HSP group. We also tried to search for differences in levels of CHI3L1 among the MND sub-groups (PLS, ALS/FTD, ALS, and PMA), but unsuccessfully (**B**).

*Error bars (whiskers) showed 95% confident intervals. * $p < 0.05$; and ** $p < 0.005$.*

Legend: MND: motor neuron diseases; HSP: hereditary spastic paraplegia, HC: healthy controls; ALS: amyotrophic lateral sclerosis; FTD: frontotemporal dementia; PLS: primary lateral sclerosis; and PMA: progressive muscular atrophy.

4.3.2 Correlation of plasmatic CHI3L1 with motor and cognitive symptoms and with laboratory and instrumental parameters

CHI3L1 does not correlate with weight, BMI, ALSFRSr, the MRC total score and sub-scores, forced vital capacity (FVC), forced expiratory volume in the 1st second (FEV1), the PR of the disease at the time of blood sampling, and blood examination, except for with red blood cells (RBC) and haemoglobin (respectively, $p < 0.001$, r = 0.63 and $p = 0.022$, r = 0.52, **Figure 2N,O**). Since the levels of RBC and haemoglobin are influenced by the BMI, we performed a multiple linear regression that confirmed the significance of the correlation of CHI3L1 with RBC and haemoglobin (respectively, $p < 0.0001$, r = 0.96 and $p = 0.016$, r = 0.73). None of the neuropsychological tests correlated with the CHI3L1 plasma levels ($p > 0.05$).

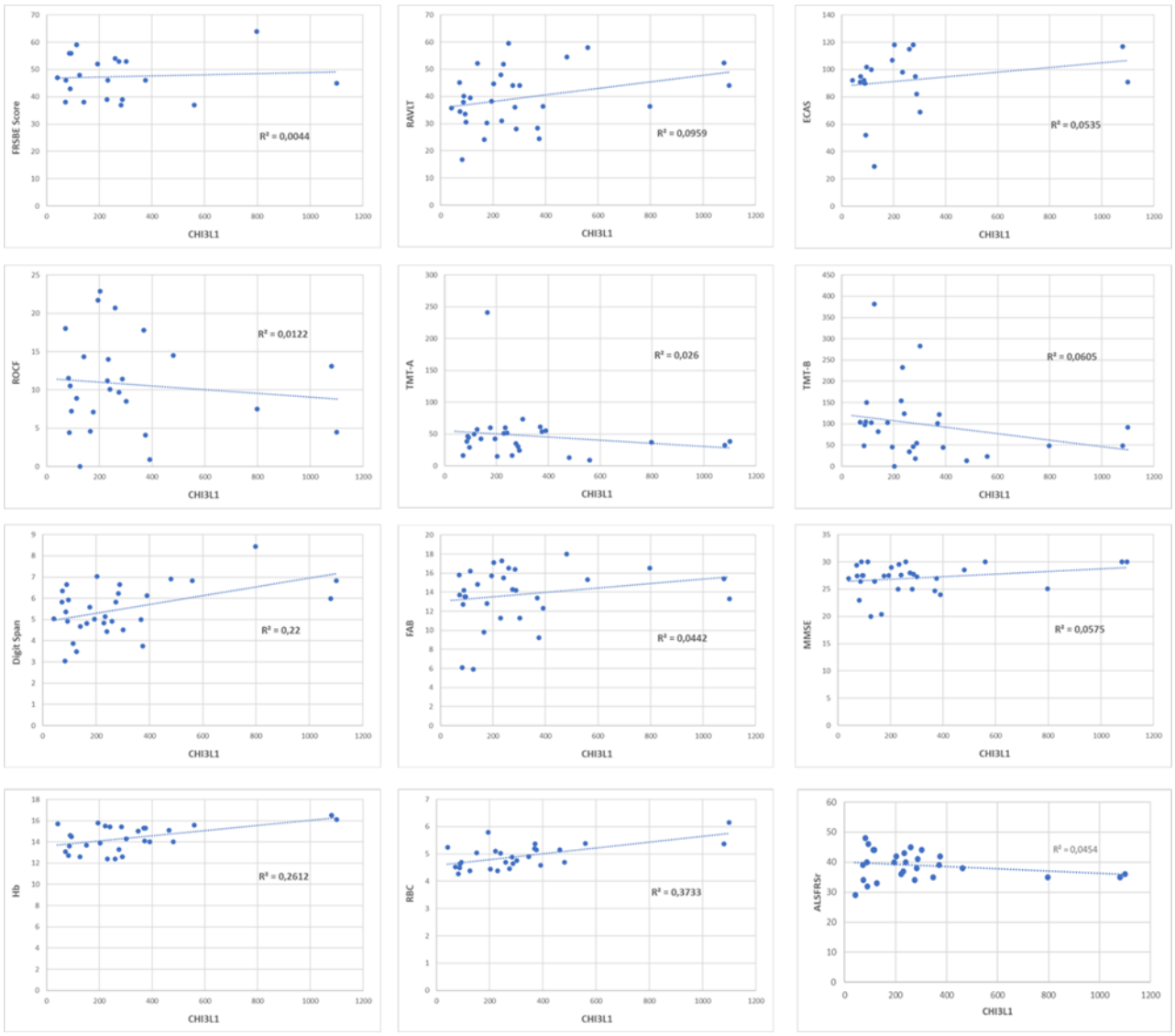


Figure 2 - Correlation between CHI3L1 plasmatic levels, clinical, neuropsychological, and laboratory data in MND patients

Correlation between CHI3L1 plasmatic levels and clinical, neuropsychological, and laboratory data in MND patients. We did not observe any statistical correlation ($p > 0,05$) between CHI3L1 plasmatic levels and any neuropsychological test (**A**–**J**), ALSFRSr scale (**K**), weight, or BMI (**L, M**). The only correlation we identified is between CHI3L1 plasmatic levels and haemoglobin (**N**), and CHI3L1 plasmatic levels and red blood cells (**O**).

Legend: ALSFRSr: ALS Functional Rating Scale revised; Hb: haemoglobin; RBC: red blood cells; FRSBE: Frontal Systems Behavior Scale; RAVLT: Rey Auditory Verbal Learning Test; ECAS: Edinburgh Cognitive and Behavioural ALS Screen; ROCF: Rey–Osterrieth complex figure; TMT-A: Trail Making Test A; TMT-B: Trail Making Test B; FAB: Frontal Assessment Battery; MMSE: mini–mental state examination;; BMI: body mass index; R: Pearson coefficient.

4.4 Discussion

This preliminary cross-sectional study highlights the possible benefits of dosing the CHI3L1 plasma levels in patients with suspected MNDs to differentiate them from MND mimics. In fact, the CHI3L1 plasma levels were increased in the evaluated MND mimics (including acute myelopathy, radiculopathies, and neuropathies) compared with the MND patients and HCs. These data are consistent with the known increase in CHI3L1 in diseases in which inflammation plays a crucial role, such as cancers or autoimmune/dysimmune diseases [134,143]. Therefore, the increase in CHI3L1 in the MND mimics group could be explained by the presence of an active peripheral inflammatory component.

Moreover, the CHI3L1 levels were statistically significantly higher in the HSP patients compared with the MND patients, likely due to the high frequency of neuropathies in HSP, in which there is usually an inflammatory component.

Contrarily to other studies performed on CSF, in which CHI3L1 allowed for differentiation between MND patients and HCs [127,128], the plasmatic levels of CHI3L1 do not permit the identification of these two groups. This could be due to the low ability of CHI3L1 to pass through the blood–brain barrier, and to the plasmatic release of CHI3L1 from other tissues, which masks its increase from the central nervous system.

In the MNDs cohort, we also correlated the CHI3L1 levels with several clinical, laboratory, and neuropsychological parameters that were collected within one month of the plasma collection, but we did not observe any association. In particular, in contrast with previous evidence from CSF [128], the plasmatic levels of CHI3L1 did not correlate with the score of the executive and visuo–spatial neuropsychological tests. The only correlation that was observed was with haemoglobin and RBC; this is consistent with previous evidence, since CHI3L1 seems to be more concentrated in RBCs [144].

This study has some limitations. First, the number of patients included was relatively small, in particular in the MND mimics group. Second, we have not included all the types of MND mimics that should be considered in a more exhaustive future study (i.e., myasthenia gravis, syringomyelia, adult polyglucosan body disease, Kennedy's disease, and inclusion body myositis). Moreover, we have not performed a comparison of the plasma levels of the CHI3L1 with the CSF ones, and we have not included other fluid biomarkers, since it was a pilot study.

To validate our data and observe the variation of CHI3L1 and other biomarkers throughout the disease course, further longitudinal and multicentre studies with a higher number of patients both

in the MND and MND mimics groups are needed. Finally, a comparison between the plasmatic and CSF levels of the CHI3L1 protein is mandatory, in order to confirm that the plasmatic levels of CHI3L1 do not satisfactorily reflect the CSF ones.

4.5 Conclusions

The measurement of plasmatic levels of CHI3L1 could be useful in the differential diagnosis between MNDs and MND mimics. This is an important issue since the early diagnosis of an MND is determinant in the early starting of a neuroprotective therapy and in clinical trials recruitment.
Further multicentre studies, including a huge number of patients, and testing together other fluid biomarkers, are needed to better explain the role of CHI3L1 in diagnosis and prognosis in MNDs and, also, in MND mimics.

Chapter 5. Alpha-internexin: the first description of a novel fluid biomarker in Amyotrophic Lateral Sclerosis

In this chapter I'm presenting the unpublished results of the measurement of a novel protein in human fluids affected by ALS, other neurological diseases and HCs. In particular, I measured in human CSF and in plasma alpha-internexin, a type of neurofilament mainly expressed in central nervous system neurons (CNS) and still poorly investigated. The interesting results are that it is measurable in CSF, but not in plasma, using standard ELISA Kit, that it seems to be useful in discriminating between ALS patients, HCs, and other neurological conditions, and that it seems to be an expression of the motor neuron attempt of regeneration in early phase of disease.

5.1 Introduction

There is a strong need of prognostic and diagnostic biomarkers able to early differentiate ALS from ALS-mimics, to stratify patients and to predict disease outcome, in order to improve patients' inclusion in clinical trials. Neurofilaments (Nfs) are now becoming a widely accepted prognostic biomarker for ALS and other neurodegenerative diseases [125,126].

The most known Nfs is Nfs light-chain (NfL), although Nf medium-chain (NfM) and phosphorylated-Nf high-chain (pNfH) exist also as essential components of axonal neuronal intermediate filament. They are considered the neurofilament triplet proteins (NFTPs). Moreover, in CNS neurons another protein, the α-internexin, is often expressed together with the NFTPs and has been proposed as the fourth subunit of the Nfs [145]. All these Nfs interact together, maintaining a specific stoichiometry that is fundamental for an adequate axonal transport and for an appropriate cell structure [146]. Alterations to this stoichiometric balance of intermediate filament is associated with the formation of cytoplasmatic inclusions in motor neurons of ALS-models [147]. This phenomenon seems to be the result of the loss of proper regulation of intermediate filament mRNA metabolism [146,148].

It has been demonstrated that miR-105 and miR-9 are fundamental in the regulation of NETPs and of INA mRNA. In sporadic ALS, thus, loss of these miRNAs is associated with intermediate filament dysregulation [149].

Starting from these evidences and due to the important role of INA in the health of motor neurons and in their capability to react to dangerous stimuli, I decided to try to measure, for the first time, this Nfs in human biofluids. Later, since I obtained encouraging results, I to set up a pilot study focused on the measurement of INA in CSF and in plasma of patients affected by ALS and by other non-neurodegenerative disorders.

5.2 Materials and Methods

5.2.1 Design of the study and clinical assessment

Firstly, we set up a preliminary test in order to evaluate the capability of the Human Internexin Neuronal Intermediate Filament Protein Alpha (INA) ELISA commercial Kit (Abbexa, Cambridge, UK) to measure INA in human biofluids and we found the best dilution factor for ALS patients.
Secondly, we realised a retrospective, longitudinal study on a cohort of 77 neurological patients referring to the Department of Neurosciences of the University of Turin (Italy), from March 2017 to December 2022, who underwent lumbar puncture and blood collection for diagnostic purposes.
Demographic and clinical characteristics of all patients were obtained from medical records and are listed in **Table 2**.
ALS patients or individuals in the comparative neurological control groups that exhibited active cancer, infectious diseases, or autoimmune disorders were excluded from the study.
ALS patients received their diagnosis following the Gold Coast criteria [109]. Disease severity was assessed using Amyotrophic Lateral Sclerosis revised Functional Rating Scale (ALSFRSr)[138]. ALSFRSr without respiratory items (ALSFRSr_noresp) was calculated.
Moreover, all ALS patients underwent: a clinical evaluation (neurological examination, including the Medical Research Council scale [MRC][139] measurement, and ALSFRSr scale) at the moment of diagnosis, at the moment of CSF sampling (T0), and after 6 months from sampling (T6), an instrumental evaluation (electromyography, cerebral MRI with tractography and functional-MRI, spirometry), a neuropsychological evaluation [140], and a genetic test for the most common mutation (*C9orf72, SOD1, TARDBP and FUS genes*). Patients' phenotypes have been classified as follows: classic ALS, bulbar ALS, flail arm, flail leg, prevalent upper motor neuron ALS [23].

Progression rate of disease (PR) and progression rate without respiratory items (PR_noresp) were calculated as follow:

$$Progression\ rate = \frac{48 - ALSFRSr_{Last\ Visit}}{\text{Date Last Visit } - \text{Date symptom onset}}$$

$$PR\_noresp = \frac{36 - ALSFRSr\_noresp_{Last\ Visit}}{\text{Date Last Visit } - \text{Date symptom onset}}$$

Moreover, we calculated in ALS patients an index of upper motor neuron (UMN) impairment, called UMN burden score (UMNBS), ranging from 0 to 24, collecting retrospectively data on burden and atrophy distribution from medical records of our Centre (**Table 1**). This is a readaptation of the Penn Upper Motor Neuron Score (PUMNS) [150], based on the availability of our data. We rated limb spasticity using the Modified Ashworth Scale (MAS) [151].

Table 1 – Upper Motor Neuron Burden Score (UMNBS)

Limb reflexes (*bicipital, brachioradialis, patellar, ankle*)	0 if absent, reduced or normal in a normotrophic area 1 if brisk or retained in an atrophic area
Pathological reflexes • *Palmomental reflex (bilateral), Hoffmann's sign (bilateral), Babinski's sign (bilateral)* • Jaw jerk	 0 if absent 1 if present 0 if absent 1 if present 2 if brisk
Spasticity at four limbs (as PUMNS)	0 if normal muscle tone 1 if MAS 2-3 2 if MAS 4-5

5.2.2 Laboratory evaluation

From each patient who underwent lumbar puncture, we collected 4 mL of CSF in glass tubes. The CSF samples were centrifuged at 4 °C at 1500× *g* for 15 min within 1 h from collection, and then stored at −80 °C.

To uphold participant privacy, an immediate anonymization process is implemented through the assignment of a unique code. Only authorized members of the research group possess the capability to correlate these codes with participants' identities.

Plasma samples used for preliminary evaluation of the kit were collected in EDTA tube, centrifuged at 18 °C at 1500× *g* for 15 min within 1 h from collection, and then stored at −80 °C.

The samples were kept at 4 °C overnight before the ELISA test to allow for slow thawing; then they were left at room temperature for 1 h and used to run the test.

5.2.2.1 Assay

We used the Human Internexin Neuronal Intermediate Filament Protein Alpha (INA) ELISA commercial Kit (Abbexa, Cambridge, UK), following the manufacturer's instructions.

Each plate contained calibrators (0.16–10 ng/mL) in duplicate and two samples with known concentration of INA, provided in each kit.

Manufacturer declared a Sample recovery range after spiking of 91–105% and a linearity range of 90–116% in dilutions up to 1:8 both in serum. For the preliminary analyses and validation of the kit in human CSF and plasma we conducted spike recovery test and dilution linearity test complying with protocols adapted from Andreasson et al. [152].

After preliminary analysis, for the pilot study led in CSF, all samples were distributed on the plate diluted 1:3 and measured in duplicate. The inter-assay and intra- assay coefficients of variance were all below 10%. According to manufacturer's instructions, analytical sensitivity was set <0.156 ng/mL.

The plates were read using a CLARIOstar Plus plate reader (BMG LABTECH, Ortenberg, Germany). The standard curves were fitted with 4-parameter logistic regression, using MARS data analysis software (BMG LABTECH). The samples were diluted 1:3 in order to achieve a concentration within the linear range of the standard curve measurements. The median intra-assay and inter-assay coefficients of variation were below 15% for all the assays.

General blood tests were performed by our hospital laboratory.

5.2.3 Statistical analysis

A Shapiro–Wilk test showed that the data were not normally distributed.

A Kruskal–Wallis test was used to compare the groups. A Bonferroni' comparisons test was performed following the Kruskal–Wallis test, in case of any significant differences. Receiver operating

characteristic (ROC) curves were not performed due to the relatively small number of patients that were involved. The correlations between INA levels and clinical, laboratory and instrumental parameters were calculated by the Spearman rank correlation (r_s). Multiple regression analysis was led. The level of significance for all the statistical tests was set at 0.05.

We used the program SPSS Statistic V28 (Chicago, IL, USA) to perform the statistical calculations.

All the patients signed a written informed consent form. The study design was approved by the Ethical Committees of the Turin ALS Center (Comitato Etico Azienda Ospedaliero-Universitaria Città della Salute e della Scienza, Torino) (n° 0011613, 03/02/2020).

5.3 Results

5.3.1 Preliminary evaluations in biofluids

This is the first attempt to measure out INA protein in human biofluids.

Using a standard human INA ELISA Kit we observed that this Nfs is not measurable both in plasma and in serum, since its levels are under the lower detectable threshold of the kit. We led different dilution (from no dilution to 1:8 dilution) of plasma and serum of 10 HCs and of 10 MND patients in all the different stages of disease, conforming to the MiToS system. Contrarily, in line with INA physiological distribution in CNS [145], we were able to measure it in CSF of patients with ALS and other neurological conditions. We observed that, using the Human Internexin Neuronal Intermediate Filament Protein Alpha (INA) ELISA Kit the best dilution for CSF samples both for ALS, other non-neurodegenerative diseases and HCs is 1:3-1:4.

5.3.2 Cross sectional evaluation of INA in ALS and neurological controls

We conducted a preliminary retrospective, longitudinal investigation involving 52 patients diagnosed with ALS (among which 6 ALS/FTD patients), 10 individuals with Normal Pressure Hydrocephalus (NPH), 10 cases of ALS-mimics (including 4 axonal neuropathies, 2 myasthenia gravis with bulbar onset, 1 cervical myelopathy, 2 primary progressive multiple sclerosis, 1 functional disorder), and 8 mild cognitive impairment or dementia. The study participants were sourced from the ALS Center at Molinette Hospital. Among ALS 32 were male (57.1%) and 17 had a bulbar onset of disease (30.4%). 6 had genetic mutation in *C9orf72* gene (9.25%)..

Demographic and clinical characteristics of the participants are summarized in **Table 2**.

Table 2. Participant demographic and clinical information

Patients group	ALS	NPH	Axonal neuropathies	Dementia
n°	52	10	8	10
Sex (M/F)	30/22	5/4	5/2	10/9
Age at T0 ± SD (y)	62.2 ± 12	70 ± 8	65 ± 15	65 ± 15
n° genetic mutation	10	n/a	2	n/a
ALSFRSr at diagnosis ± SD	40.5 ± 4.4	n/a	n/a	n/a
ALSFRSr_tot T0 ± SD	37 ± 6.7	n/a	n/a	n/a
ALSFRSr_noresp T0 ± SD	26.0 ± 5.1	n/a	n/a	n/a
ALSFRSr_tot T6 ± SD	30.2 ± 8.7	n/a	n/a	n/a
ALSFRSr_noresp T6 ± SD	20.1 ± 7.2	n/a	n/a	n/a
PR at T0 ± SD	0.83 ± 0.74	n/a	n/a	n/a
PR_noresp T0 ± SD	0.77 ± 0.63	n/a	n/a	n/a
PR at T6 ± SD	1.40 ± 1.36	n/a	n/a	n/a
PR_noresp T6 ± SD	1.17 ± 1.21	n/a	n/a	n/a
MRC_tot at T0 ± SD	121 ± 13	n/a	n/a	n/a
ΔMRC at T0 ± SD	1.5 ± 1.3	n/a	n/a	n/a
MRC_tot at T6 ± SD	100 ±21	n/a	n/a	n/a
ΔMRC at T6 ± SD	3.4 ± 3.0	n/a	n/a	n/a
FVC ± SD	99 ± 21	n/a	n/a	n/a
UMNS at T0 ± SD	8.1 ± 5.0	n/a	n/a	n/a
UMNS at T6m ± SD	8.4 ± 5.8	n/a	n/a	n/a
Diagnostic delay (months)	9.9 ± 6.0	n/a	n/a	n/a
Survival (months)	34 ± 15	n/a	n/a	n/a

Legend: ALSFRSr_tot: ALS Functional Rating Scale-revised total score; ALSFRSr_noresp: ALSFRSr without the respiratory items; PR: progression rate of disease; PR_noresp: PR without considering the respiratory items; MRC_tot: total Medical Research Council scale; ΔMRC: monthly loss of point at MRC scale; UMNS: Upper Motor Neuron; T0: at the moment of sampling; T6: after 6 month from sampling; n/a: not assessed.

Our initial analysis involved comparing CSF levels of INA between ALS patients and those with other neurological disorders. We observed statistically significant differences (p=0.002; **Figure 1**). Post Bonferroni's correction, it was noted that INA levels were significantly elevated in ALS patients compared to those with NPH (p=0.012) and neuropathies (p=0.047). INA levels in dementia were

comparable to ALS (p=0.71). Within the ALS subgroup, patients harboring mutations in one of the four major known genes exhibited higher INA levels compared to those without genetic mutations (p=0.025).

No statistically significant differences in CSF INA levels were identified between ALS cases with bulbar and spinal onset (p=0.68) or between male and female patients (p=0.58).

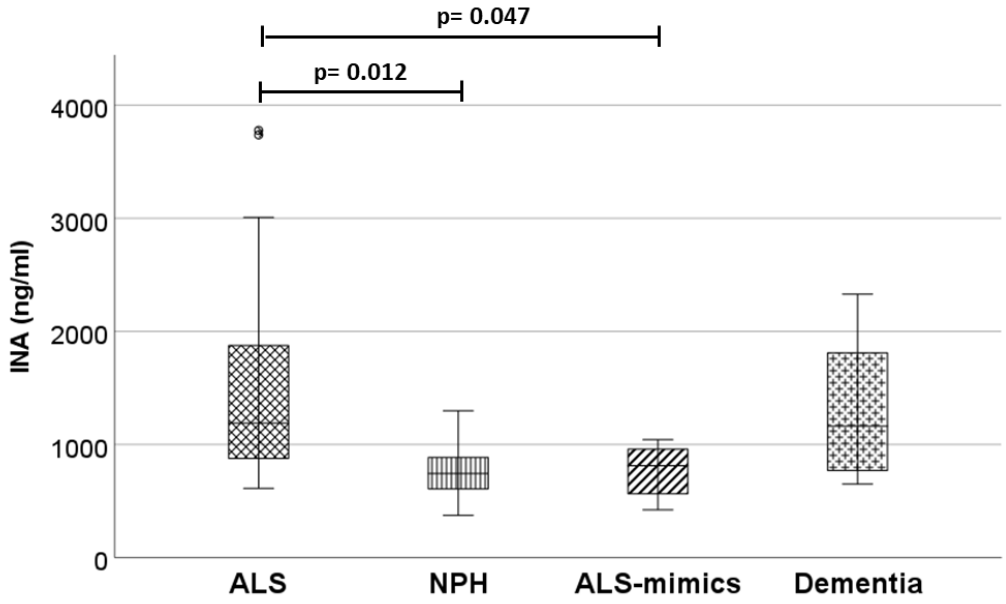


Figure 1 – Levels of INA in ALS and other neurological disorders, measured in CSF

Legend: NPH= Normal Pressure Hydrocephalus; INA= alpha-internexin.

5.3.3 Correlation of INA CSF levels with clinical and instrumental parameters in ALS patients

Despite the modest number of patients enrolled in this pilot study, a significant positive correlation between CSF INA levels and ALSFRSr scores after 6 months was evident (r_s= 0.51; p=0.004; **Figure 2B**), as well as with ALSFRSr_noresp after 6 months (r_s= 0.49; p=0.005; **Figure 2C**). Simultaneously, an inverse correlation was observed with PR after 6 months (r_s= -0.39; p=0.03; **Figure 2D**), PR_noresp after 6 months (r_s= 0.49; p=0.005; **Figure 2E**), UMNS at the moment of sampling (r_s= -0.49; p=0.003 **Figure 2I**), and UMNS after 6 months (r_s= -0.40; p=0.019; **Figure 2L**). Furthermore, a noteworthy inverse correlation of INA with ΔMRC after 6 months was observed (r_s= -0.44; p=0.027; **Figure 2H**), along with a correlation with total MRC score after 6 months (r_s= 0.38; p=0.049; **Figure 2G**), and with the disease stage after 6 months, as assessed using the MiToS scale (r_s= -0.43; p=0.02; **Figure 2F**). To mitigate potential selection bias arising from the limited number of ALS patients, we conducted a

multiple regression analysis, correcting for ALSFRSr at the time of diagnosis. Notably, all the aforementioned correlations remained significant, and the Spearman coefficient slightly increased. While no statistically significant correlation of INA with ALSFRSr scores at the moment of sampling was found, a trend of correlation was noted (r_s=0.28; p=0.08; **Figure 2A**). Following correction for ALSFRSr at the time of diagnosis using a multiple regression analysis, a significant correlation between CSF INA levels and ALSFRSr at the time of sampling was observed (r_s=0.31; p=0.019).
Finally, no correlations were observed with spirometry parameters, age, weight, BMI, or blood examination values, including the neutrophil to lymphocyte ratio, monocyte to lymphocyte ratio, and platelets to lymphocyte ratio (p>0.05).

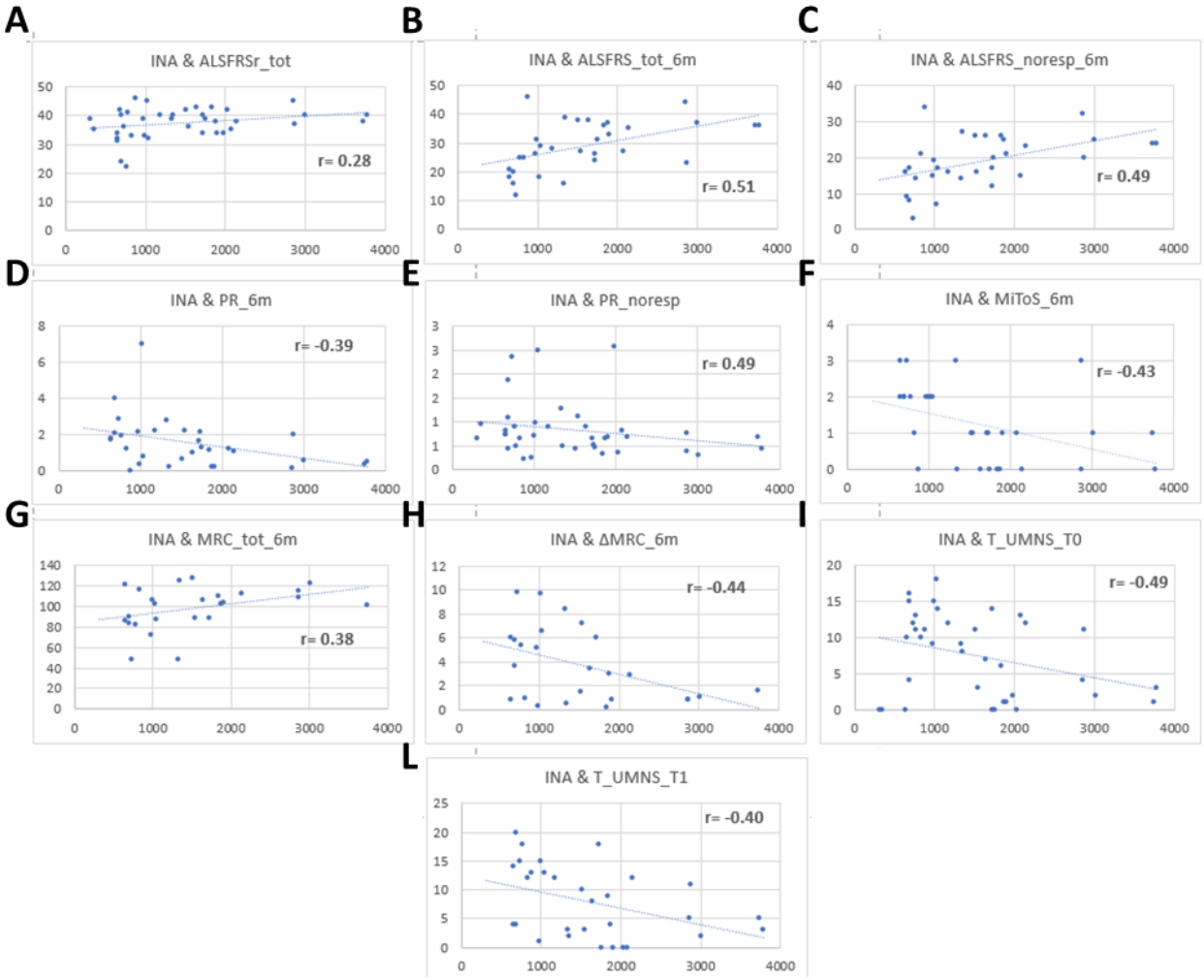


Figure 2 - Correlation between INA CSF levels, clinical, and laboratory data in ALS patients

Legend: ALSFRSr_tot: ALS Functional Rating Scale-revised total score; ALSFRSr_tot _6m: ALSFRSr after 6 month from sampling; ALSFRSr_noresp_6m: ALSFRSr after 6 month from sampling without the respiratory items; PR_6m: progression rate of disease after 6 months; PR_noresp_6m: progression rate of disease after 6 months without considering the respiratory items; MRC_tot_6m: total Medical Research Council scale after 6 month from sampling; ΔMRC_6m: monthly loss of point at MRC scale after 6 month from sampling; UMNS_T0: Upper Motor Neuron Score at the time of sampling; UMNS_T6: Upper Motor Neuron Score after 6 month from sampling.

5.4 Discussion

This preliminary cross-sectional study constitutes the first characterization of alpha-internexin in human biofluids.

In the preliminary investigation, employing commercially available ELISA kits proved insufficient for quantifying INA in the plasma of both healthy controls and ALS patients, likely due to the notably low levels of circulating protein. Conversely, INA measurement in CSF was feasible. This data might reflect the predominant location of INA in the neurite cytoskeleton of CNS neurons [145].

Building upon these findings, we conducted a retrospective longitudinal study that demonstrated the capacity to distinguish ALS patients from those with other neurological disorders, some of which are ALS-mimics (axonal neuropathies, myasthenia gravis with bulbar onset, cervical myelopathy, primary progressive multiple sclerosis, and functional disorders). Despite the limited sample size, statistically significant differences emerged between ALS and ALS-mimics, suggesting that expanding the patient cohort may amplify these distinctions.

Compared to individuals with various forms of dementia, no statistically significant differences were observed, potentially attributable to the limited sample size. It does not appear that INA levels vary significantly with the sex and age of individuals, but further analyses are warranted.

Based on these data, a potential diagnostic role for alpha-internexin in suspected cases of ALS is conceivable. However, confirmation on a broader scale, encompassing not only ALS but also other motor neuron diseases, and collecting samples in the early stages of disease, is imperative.

The most noteworthy result of this study pertains to the correlation with certain ALS clinical parameters. Specifically, we observed a direct proportionality between CSF levels of INA and the ALSFRSr at baseline. Furthermore, these levels correlated even more strongly with ALSFRSr_total and ALSFRSr_noresp scores at T6, as well as the MRC total score at T6. Simultaneously, an inverse correlation was noted between the PR and the PR_noresp at T6 and INA levels. Additionally, in line with previous data, there was an inverse correlation between INA and the MiToS staging system at 6 months. Finally, we observed an inverse correlation between INA and the UMNS calculated at T6.

These correlation data presented allow us to hypothesize a prognostic role for INA in ALS subjects. Specifically, an inverse correlation was observed between INA levels and both the severity of the disease at T0 and at T6, as well as the rate of disease progression in the subsequent months. Moreover, the lower are CSF INA levels the higher is the UMNS at T6 and, therefore, the higher is

the upper motor neuron damage. This phenomenon may be explained by postulating that INA is a neurofilament that tends to increase following axonal damage, allowing for cytoskeleton structure replacement at the injury site. Hence, INA might be a factor involved in the regeneration process or it could merely be an epiphenomenon of the underling regeneration.
Indeed, there is an elevation in INA expression within damaged neurons, facilitating the substitution of the cytoskeletal structure at the injury site [153]. The observed decrease in INA levels in more severely affected ALS patients, as noted in our study, aligns with neuropathological findings. In advanced stage of sALS, Wong et al. reported a reduction in INA, along with NfL and peripherin, within motor neurons [154].

Another noteworthy observation is the lower CSF concentration of INA in ALS patients with pathogenic genetic mutations (specifically, mutations in the *C9orf72*,). While speculative due to the limited number of patients involved, our study revealed a statistically significant difference in INA levels between genetic and non-genetic forms, suggesting a biological basis for this disparity. Differences in INA levels between genetic and non-genetic forms of ALS may be justified by distinct pathogenic mechanisms, especially considering that neuropathological mechanism may act in genetic forms from an early age, potentially reducing the efficiency of motor neuron regeneration over time. Some studies on C9orf72 patients support this hypothesis, indicating neuropathological changes several years before clinical symptom onset, compromising regeneration mechanisms [155,156]. These speculative hypotheses require confirmation through larger studies, including presymptomatic patients with genetic forms.
Therefore, based on these pieces of evidence, we can only speculate on the potential role of INA not merely as a marker of damage but rather as an indicator of motor neuron repair.

Despite these intriguing findings, the study has many limitations. It represents only the initial attempt to measure a novel protein in human biofluids, employs a pilot study design with a limited number of patients and controls, lacks completely healthy controls and other type of MND, follows a cross-sectional design, does not compare INA with other known biofluid markers, and do not include either patients in the very early phases of the disease or presymptomatic patients.

In conclusion, based on the presented data, we can only speculate about the potential diagnostic and prognostic roles of INA in ALS. A longitudinal multicentre study, encompassing a huger number

and type of MND, MND-mimics and healthy controls, including patients in the early phases of the disease and presymptomatic carriers of genetic mutations, is warranted. Furthermore, the development of a kit using more sensitive platforms, such as Meso Scale and Simoa, would be beneficial in order to try to measure INA in plasma or serum, which are more affordable and obtainable with less invasive procedures.

Chapter 6. Peripherin: a novel early diagnostic and prognostic plasmatic biomarker in Amyotrophic Lateral Sclerosis

In this chapter I'm presenting the unpublished results of the measurement in cohort of MND patients and other MND-mimics visited in our MND Center of a type of neurofilament mainly expressed in peripheral nervous system (PNS), called peripherin. This is the third study known, in which I measured in plasma this recently studied protein, using standard ELISA Kit. I confirmed in our huger cohort of patients the possible role of peripherin as a diagnostic biomarker in MND, replicating data previously described in literature. Moreover, I observed that peripherin could be considered a prognostic biomarker in MND.

6.1 Introduction

As above mentioned in Chapter 4, there is a strong need of prognostic and diagnostic biomarkers able to early differentiate MND from MND-mimics, to stratify patients and to predict disease outcome, in order to improve patients' inclusion in clinical trials.

Beyond the widely studied neurofilament triplet proteins (NFTPs) the renowned NfL, NfM, and pNfH, and the alpha-internexin (studied in Chapter 4), another type III intermediate filament belong to the group of neurofilaments, the peripherin (PRPH) [145]. It forms heteromers and constitute the fourth subunit of the Nfs in peripheral nervous system. As well as all the other NFTPs, PRPH present a stoichiometry, fundamental for an adequate axonal transport and for an appropriate cell structure [146]. Alterations to this stoichiometric balance of intermediate filament is associated with the formation of cytoplasmatic inclusions in motor neurons of ALS-models [147]. This phenomenon seems to be the result of the loss of proper regulation of intermediate filament mRNA metabolism [146,148]. Moreover, as occurs with alpha-internexin, PRPH is fundamental not only in axonal transport and constitution of neuron cytoskeleton, but it fulfil a role in axonal development and in neuronal cells differentiation [157].

PRPH has been recently studied in human biofluids by two different teams. Sabbatini et al. [158] evaluated its values in serum and CSF of MNS patients, demonstrating its possible diagnostic role in differentiating ALS from dementia, spinal bulbar muscle atrophy and polyneuropathies. Another

important paper by Keddie et al. showed that PRPH could also be useful in the early evaluation of patients affected by Guillame-Barrè syndrome [159].

Looking at absolute levels of PRPH, there are three orders of magnitude of differences between the two papers. Due to this high variability and to different platform used (standard ELISA Kit and a new developed Simoa assay), future studies might compare the two methods, using Bland-Altman plots.

Starting from these evidences and due to the important role of PRPH in the health of motor neurons and in their capability to react to dangerous stimuli, I decided to try to replicate the above presented data in plasma of our cohort of MND patients, since I set up during the last four years a huge biobank of plasma samples in our Centre.

6.2 Materials and Methods

6.2.1 Design of the study and clinical assessment

Firstly, we set up a preliminary test in order to evaluate the capability of the Human Peripherin (PRPH) ELISA Kit ELISA commercial Kit (Abbexa, Cambridge, UK) to measure PRPH in human biofluids and we found the best dilution factor for ALS patients.

Secondly, we realised a retrospective, longitudinal study on a cohort of 193 patients and 38 healthy controls (HCs) referring to the Department of Neurosciences of the University of Turin (Italy), from June 2019 to March 2023, who underwent blood collection for diagnostic purposes. Blood samples from MND patients were taken at the moment of the diagnosis or some month earlier.

A cohort of 46 ALS patients underwent blood sampling after 6 months, too.

Demographic and clinical characteristics of all patients were obtained from medical records and are listed in **Table 3**.

ALS patients or individuals in the comparative neurological control groups that exhibited active cancer, infectious diseases, or autoimmune disorders were excluded from the study.

ALS patients received their diagnosis following the Gold Coast criteria[109]. Disease severity was assessed using Amyotrophic Lateral Sclerosis revised Functional Rating Scale (ALSFRSr)[138]. ALSFRSr without respiratory items (ALSFRSr_noresp), ALSFRSr bulbar score obtained adding item 1 and 3 (ALSFRSr_B), and ALSFRSr limbs score obtained adding items 4, 5, 6, 7, 8, and 9 (ALSFRSr_4limb) were calculated.

Moreover, all ALS patients underwent: a clinical evaluation (neurological examination, including the Medical Research Council scale [MRC][139] measurement, and ALSFRSr scale) at the moment of

diagnosis, at the moment of blood sampling (T0), and after 6 months from sampling (T6), an instrumental evaluation (electromyography, cerebral MRI with tractography and functional-MRI, spirometry), a neuropsychological evaluation[140], and a genetic test for the most common mutation (*C9orf72, SOD1, TARDBP* and *FUS* genes). Patients' phenotypes have been classified as follows: classic ALS, bulbar ALS, respiratory ALS, flail arm, flail leg, prevalent upper motor neuron ALS[23].

Progression rate of disease (PR) and progression rate without respiratory items (PR_noresp) at T0 were calculated as follow:

$$PR\_T0 = \frac{48 - \text{ALSFRSr}_{\text{T0}}}{\text{Date T0} \ - \text{Date symptom onset}}$$

$$PR\_noresp\_T0 = \frac{36 - \text{ALSFRSr\_noresp}_{\text{T0}}}{\text{Date T0} \ - \text{Date symptom onset}}$$

Progression rate of disease (PR) and progression rate without respiratory items (PR_noresp) at T1 were calculated as follow:

$$PR\_T1 = \frac{\text{ALSFRSr}_{\text{T0}} - \text{ALSFRSr}_{\text{T1}}}{\text{Date T1} \ - \text{Date T0}}$$

$$PR\_noresp\_T1 = \frac{\text{ALSFRSr\_noresp}_{\text{T0}} - \text{ALSFRSr\_noresp}_{\text{T1}}}{\text{Date T1} \ - \text{Date T0}}$$

Moreover, we calculated in ALS patients an index of upper motor neuron (UMN) impairment, called UMN burden score (UMNBS), ranging from 0 to 24, collecting retrospectively data on burden and atrophy distribution from medical records of our Centre (**Table 1**). This is a readaptation of the Penn Upper Motor Neuron Score (PUMNS [150], based on the availability of our data. We rated limb spasticity using the Modified Ashworth Scale (MAS)[151].

We also calculated a lower motor neuron index (LMNI; **Table 2**), implementing the scoring system proposed by Devine et al.[160], whose score range between 0 to 12 points, and adding the bulbar lower motor neuron score proposed by Zoccolella et al.[161], whose score range between 0 to 3 points. The resulting LMNI consists in a complete scale able to evaluate the lower motor neuron involvement both in bulbar and in limbs regions, scoring between 0 to 15 points.

Stage of disease was assessed using the two most known system: the KINGS[35] and the MiToS[36] staging systems.

Table 1 – Upper Motor Neuron Burden Score (UMNBS)

Limb reflexes (*bicipital, brachioradialis, patellar, ankle*)	0 if absent, reduced or normal in a normotrophic area 1 if brisk or retained in an atrophic area
Pathological reflexes • *Palmomental reflex (bilateral), Hoffmann's sign (bilateral), Babinski's sign (bilateral)* • Jaw jerk	0 if absent 1 if present 0 if absent 1 if present 2 if brisk
Spasticity at four limbs (as PUMNS)	0 if normal muscle tone 1 if MAS 2-3 2 if MAS 4-5

Table 2 - Lower motor neuron index (LMNI)

		Lower Motor Neuron Signs
Limb Score (for each limb) [0-12]	0	No clinically significant involvement
	1	Definite, but trace involvement Weakness ≥ 4/5, involving one or more segments (with no segments < 4/5), and mild wasting
	2	Moderate involvement Weakness ≥ 3/5, involving one or more segments (with no segment < 3/5), and moderate wasting
	3	Significant and severe involvement Little or no movement (LMN weakness ≤ 2/5) involving one or more segments, and severe wasting
Bulbar Score [0-3]	0	No clinically significant involvement
	1	Tongue atrophy and/or fasciculations
	2	Score (1) + tongue hypomobility
	3	Score (2) + jaw and/or face weakness

6.2.2 Laboratory evaluation

Plasma samples used for preliminary evaluation of the kit were collected in EDTA tube, centrifuged at 18 °C at 1500× *g* for 15 min within 1 h from collection, and then stored at −80 °C.

To uphold participant privacy, an immediate anonymization process is implemented through the assignment of a unique code. Only authorized members of the research group possess the capability to correlate these codes with participants' identities.

The samples were kept at 4 °C overnight before the ELISA test to allow for slow thawing; then they were left at room temperature for 30 minutes and used to run the test, following the same protocol for gathering, storage and thawing previously described[124]. Shortly before using, samples were centrifuged at 3000x g for 15 minutes, in order to remove platelets and any cellular debris.

6.2.2.1 Assay

We used the Human Peripherin (PRPH) ELISA Kit ELISA commercial Kit (Abbexa, Cambridge, UK), following the manufacturer's instructions.

Each plate contained calibrators (0.312 - 20 ng/ml) in duplicate and two samples with known concentration of INA, provided in each kit. Manufacturer declared a Sample recovery range after spiking of 91–105% and a linearity range of 90–116% in dilutions up to 1:8 both in serum and in plasma.

After preliminary analysis, useful for validation of this kit in our samples and led following protocols adapted from Andreasson et al.[152], all samples were distributed on the plate using a dilution of 1:2 in 0.01 mol/L of phosphate-buffered saline (as recommended in the manual kit), and measured in duplicate. The inter-assay and intra-assay coefficients of variance were all below 10%. According to manufacturer's instructions, analytical sensitivity was set <0.156 ng/mL.

The plates were read using a CLARIOstar Plus plate reader (BMG LABTECH, Ortenberg, Germany). The standard curves were fitted with 4-parameter logistic regression, using MARS data analysis software (BMG LABTECH). The median intra-assay and inter-assay coefficients of variation were below 15% for all the assays.

General blood tests were performed by our hospital laboratory.

6.2.3 Statistical analysis

A Shapiro–Wilk test showed that the data were not normally distributed.

Kruskal–Wallis and Mann-Whitney tests were used to compare the groups. A Bonferroni' comparisons test was performed following the Kruskal–Wallis test, in case of any significant differences. The correlations between PRPH levels and clinical, laboratory and instrumental parameters were calculated by the Spearman rank correlation (r_s). Multiple regression analyses were led. The level of significance for all the statistical tests was set at 0.05.

Kaplan Meier curves were generated in patients who underwent death or tracheostomy.

Wilcoxon signed-rank test was assessed for longitudinal analysis.

We used the program SPSS Statistic V29 (Chicago, IL, USA) to perform the statistical calculations.

All the patients signed a written informed consent form. The study design was approved by the Ethical Committees of the Turin ALS Center (Comitato Etico Azienda Ospedaliero-Universitaria Città della Salute e della Scienza, Torino) (n° 0011613, 03/02/2020).

6.3 Results

6.3.1 Preliminary evaluations in biofluids

We led different dilution (from no dilution to 1:8 dilution) of CSF and of plasma of 3 HCs and of 3 MND patients in different stages of disease, conforming to the MiToS system[36]. Using a standard human PRPH ELISA Kit we observed that this Nfs is not measurable in CSF, since its levels are under the lower detectable threshold of the kit. Contrarily, in line with PRPH physiological distribution in peripheral nervous system[145], we were able to measure it in plasma of MND patients and of HCs. We observed that, using the Abbexa Human Peripherin (PRPH) ELISA Kit, the best dilution for plasma samples seems to be 1:2 or undiluted.

6.3.2 Evaluation of plasma PRPH in MNS patients, MND-mimics and healthy controls

We conducted a retrospective, longitudinal investigation involving 120 MND patients (103 ALS, 13 PLS, 4 PMA), 73 cases of MND-mimics (including myelopathies, radiculopathies, axonal neuropathies, inclusion body myositis, post-poliomyelitis syndrome, myasthenia gravis with bulbar onset, frontotemporal diseases, primary progressive aphasia, Parsonage-Turner disease, Hirayama disease, syringomyelia, progressive supranuclear palsy, benign fasciculation syndrome, functional disorder, and hereditary spastic paraplegia [HSP]), and 38 HCs.

Among MND 71 were male (59%) and 33% had a bulbar onset of disease. Among ALS patients 22 (22%) had a genetic mutation in one of the four major genes: 8 had mutation in *C9orf72*, 3 in TARDBP, 4 in *FUS* and 7 in *SOD1*. No differences in sex and age between MND, MND mimics, and HCs were observed ($p<0.05$).

Demographic and clinical characteristics of the participants are summarized in **Table 3**.

Table 3. Participant demographic and clinical information

	MND (120)	**ALS (103)**	**MND mimics (73)**	**HCs (38)**	**p-value**
Sex (M/F)	71/49	60/43	44/29	20/18	0.93$^{\xi}$
Age at T0 ± SD (y)	65.3 ± 11.4	64.5 ± 11.9	62.4 ± 13.9	64.2 ± 9.3	0.27$^{\xi}$
Presence of genetic mutation	22	22	13	n/a	-
ALSFRSr_tot at diagnosis ± SD	39.1 ± 4.9	38.8 ± 4.8	n/a	n/a	0.90*
FVC ± SD	88.7 ± 22.3	90.2 ± 20.1	n/a	n/a	0.98*
Diagnostic delay (months)	12.9 ± 7.8	11.2 ± 7.4	n/a	n/a	0.58*

*Legend: ALSFRSr_tot: ALS Functional Rating Scale-revised total score; FVC: Forced Vital Capacity; SD: Standard Deviation; n/a: not assessed; *Mann-Whitney test performed between MND and ALS; $^{\xi}\chi^2$ test.*

Our initial analysis involved comparing plasma levels of PRPH in MND patients (1.49 ± 0.63 ng/ml), MND mimics (0.81 ± 0.31 ng/ml) and HCs (0.96 ± 0.46 ng/ml). We observed statistically significant differences (Kruskal-Wallis: chi-squared= 68,80, df= 2, p= 1.11×10^{-15}; **Figure 1**); post Bonferroni's correction, it was noted that PRPH levels were significantly elevated in MND patients compared to MND mimics ($p=1.20 \times 10^{-14}$) and to HCs ($p=2.72 \times 10^{-6}$). These differences are still more pronounced if we exclude PLS and PMA and we consider only ALS (PRPH levels 1.55 ± 0.64 ng/ml; ALS vs MND mimics: chi-squared= 69,04, df=2, $p=9.99 \times 10^{-16}$). Looking at different types of MND (PLS, PMA), we did not observe statistical differences, although in PLS and PMA patients (1.17 ± 0.39 ng/ml) PRPH levels resulted slightly lower compared to ALS (1.55 ± 0.64 ng/ml). No differences between ALS/FTD and the other ALS patients (Mann-Whitney, p=0.132).

Based on these findings, we tried to better clarify the capability of plasma PRPH to differentiate ALS patients and MND mimics realizing the ROC curves: AUC 0.851 (IC 0.795-0.906) and best cut-off level of PRPH 1.05 ng/ml with a Youden index of 0.56.

Moreover, we looked at the 13 PLS and at the 12 HSP included in our study, two overlap diseases with a similar onset and similar clinical characteristics during their course not easy to discriminate one each other without the genetic test. We can observe that, although the limited number of patients considered, PRPH levels are statistically significant higher (Mann Whitney, p=0.006) in PLS (1.15 ± 0.38 ng/ml) than in HSP (0.66 ± 0.24 ng/ml).

Within the ALS subgroup, patients harboring mutations in one of the four major known genes did not exhibit different levels of PRPH compared to those without genetic mutations (p>0.05).

No statistically significant differences in PRPH plasma levels were identified between ALS cases with bulbar and spinal onset (Mann-Whitney, p=0.91) nor among ALS with different phenotype (based on Chiò classification[23]) nor between male and female patients (Mann-Whitney, p=0.98).

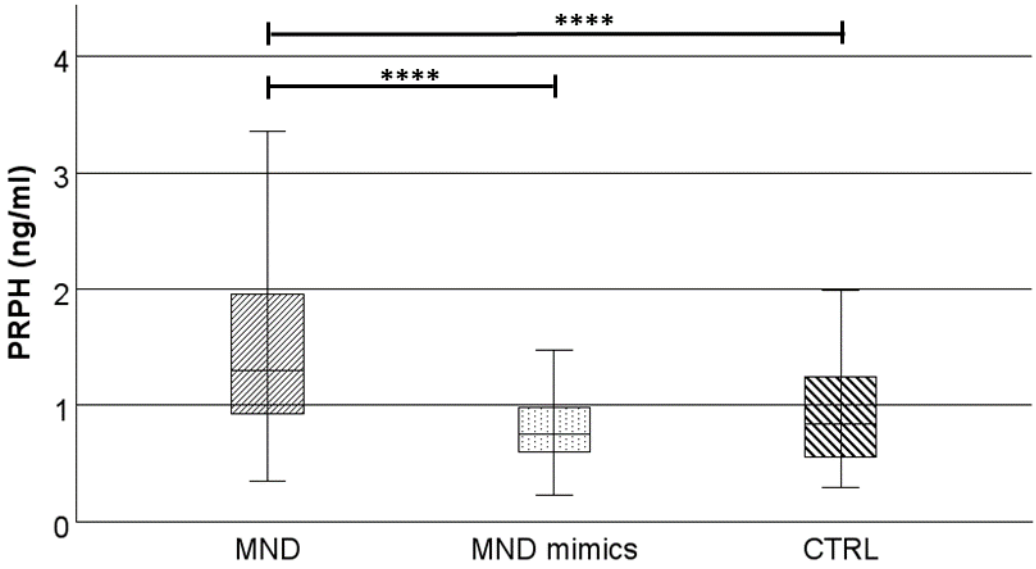


Figure 1 – Levels of plasma PRPH in MND, MND-mimics, and HCs

*Legend: PRPH= Peripherin; MND=motor neuron disease; HCs= healthy controls; ****= p≤0.0001.*

Box plots show median and interquartile range.

6.3.3 Correlation of plasma PRPH levels with clinical, laboratory, and instrumental parameters in ALS patients

In examining the relationship between PRPH levels and clinical parameters, our focus centred on ALS to minimize the clinical heterogeneity that would arise if ALS were not delineated from other motor neuron diseases.

A modest positive correlation was noted between plasma PRPH levels and ALSFRSr scores at T0 (r_s=0.387; p=0.0001; see **Figure 2A**), as well as ALSFRSr_noresp scores at T0 (r_s=0.343; p=0.0008; see **Figure 2B**), ALSFRSr_4limbs at T0 (r_s=0.276; p=0.008; see **Figure 2C**), ALSFRSr scores at T6 (r_s=0.283; p=0.017; see **Figure 2D**), and ALSFRSr_noresp scores at T6 (r_s=0.244; p=0.04; see **Figure 2E**), while a negative correlation was observed between PRPH levels and PR at T0 (r_s=-0.270; p=0.01; see **Figure 2F**). A weak negative correlation was also reported with LMNI at T0 (r_s=-0.272; p=0.015; see **Figure 2G**). These results are confirmed after multiple regression analysis covarying for sex and age.

Moreover, when comparing ALS patients in an early disease phase (MiToS 0) with those in a more advanced stage (MiToS >0), higher PRPH levels were observed in patients in the early phase (Mann Whitney test, MiToS p=0.01; see **Figure 2I**).

In contrast, no correlation was observed at either baseline or at six-month follow-up with PRPH levels and MRC score, change in MRC score (ΔMRC), UMNBS, duration of disease until blood sampling, or duration of disease between blood sampling and death or tracheostomy. We also explored potential correlations between PRPH plasma levels and other laboratory parameters, but no significant associations were found, including neutrophil-to-lymphocyte ratio, monocyte-to-lymphocyte ratio, platelets-to-lymphocyte ratio, and creatine phosphokinase levels (p>0.05).

Furthermore, we found no correlation between plasma PRPH levels and age at blood sampling.

However, a positive correlation was observed between forced vital capacity (FVC) and PRPH (r_s=0.382; p=0.0008; see **Figure 2H**). This result is confirmed after multiple regression analysis covarying for ALSFRSr at T0, sex, and age.

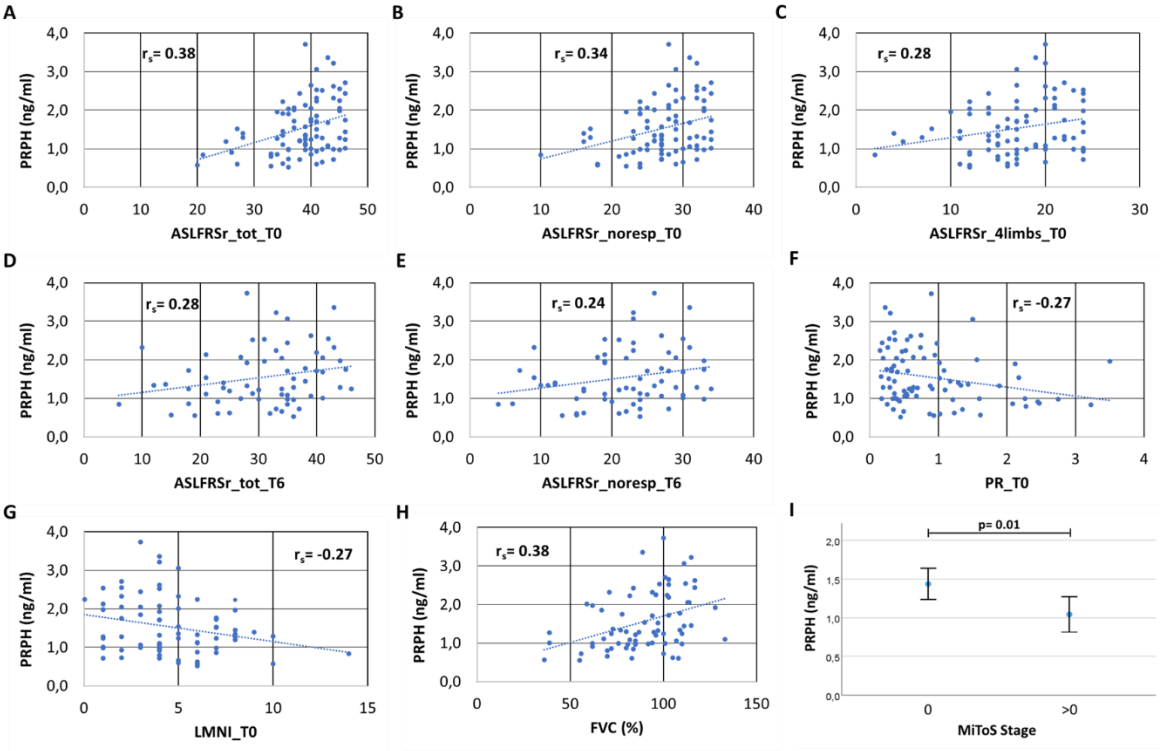


Figure 2 – Correlation between clinical parameters and PRPH in ALS patients

Legend: PRPH= Peripherin; ALSFRSr_tot_T0: ALS Functional Rating Scale-revised total score at T0; ALSFRSr_noresp_T0: ALSFRSr without the respiratory item at T0; ALFRSr_4limb_T0: sum of item 4,5,6,7,8,9 of the ALSFRSr at T0; ALSFRSr_tot_T1: ALS Functional Rating Scale-revised total score at T1; ALSFRSr_noresp_T1: ALSFRSr without the respiratory item at T1; FVC: Forced Vital Capacity; LMNI: Lower Motor Neuron Index; MiToS: Milano-Torino staging system.

6.3.4 Survival analysis

Among the 105 subjects with ALS, 55 have either deceased or undergone tracheostomy by February 2024. Consequently, interim survival analyses were conducted in this subgroup of patients, considering the limited sample size. Due to the higher level of plasma PRPH in patients with a lower functional impairment, we decided to assess Kaplan-Meier curves dividing patients above and below median value (1.40 ng/ml). We generated both curves considering the time from disease onset to death/tracheostomy and the time from blood sampling to death/tracheostomy (see **Figure 3A,B**). A statistically significant increase in overall survival (Log Rank test, χ^2=4.385, p=0.036; Breslow test, χ^2=5.039, p=0.025; Tarone-Ware test, χ^2=5.123, p=0.024; **Figure 3A**) and in survival after sampling (Log Rank test, χ^2=5.831, p=0.016; Breslow test, χ^2=8.272, p=0.004; Tarone-Ware test, χ^2=7.470, p=0.006; **Figure 3B**) was noted among patients with higher plasma PRPH levels. Although the small sample size, the statistical significance is maintained also stratifying for sex (**Figure 3C,D**).

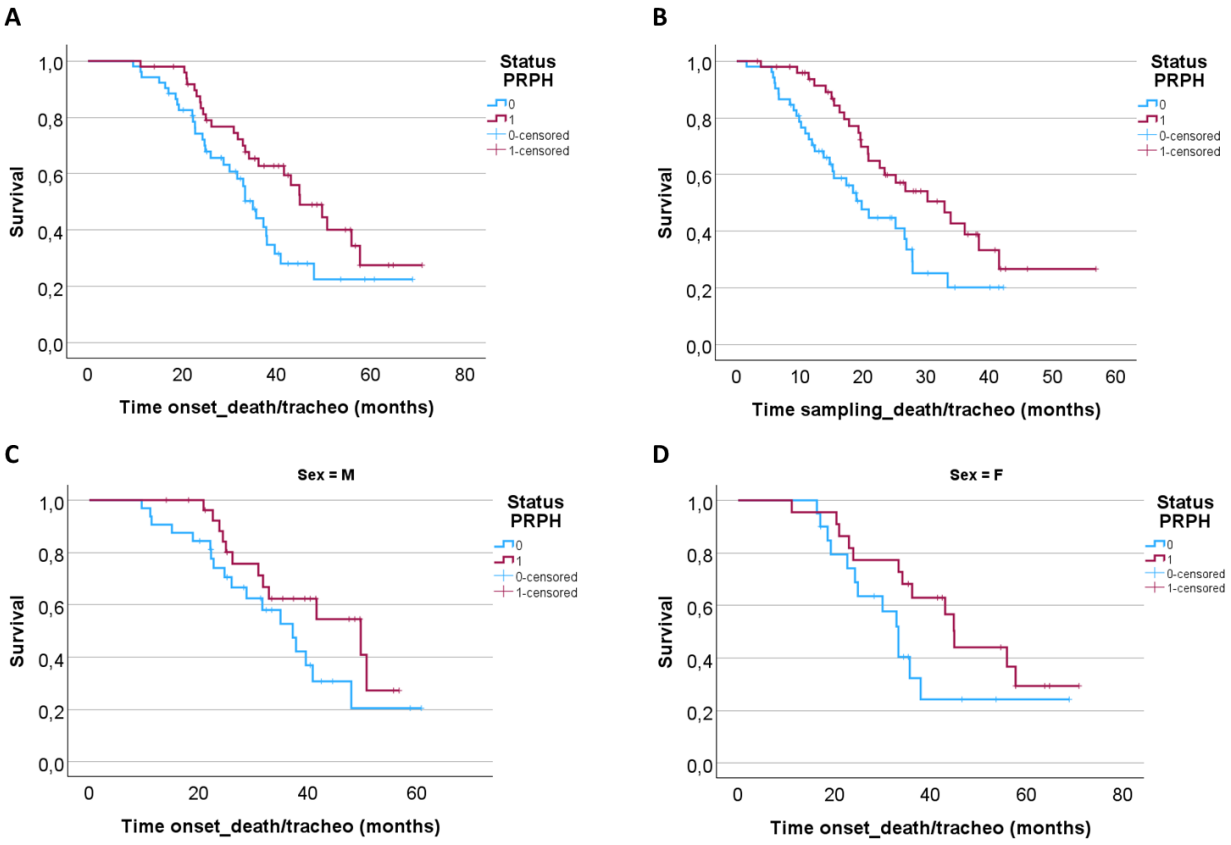


Figure 3 – Kaplan-Meier analysis

Light-blue line (status PRPH 0) correspond to levels of PRPH below median value (0.52-1.40 ng/ml), while red line (status PRPH 1) to levels of PRPH above median value (1.40-3.73 ng/ml). Patients with higher levels of PRPH (above median) show a longer survival.

6.3.5 Longitudinal evaluation

No differences (Mann-Whitney p>0.1) are observed at baseline in terms of gender (M:F ratio 0.58 vs 0.54), age (64.5 ± 11.9 vs 64.1 ± 9.5), ALSFRSr (38.8 ± 4.8 vs 38.3 ± 5.7), PR (0.90 ± 0.93 vs 0.96 ± 0.98), MRC (126 ± 11 vs 127 ± 11), and FVC (89 ± 20 vs 90 ± 20) between the general group of 105 ALS and the subgroup of 46 subjects with ALS, for whom we measured PRPH levels approximately 6 months after the initial collection. Analysing the longitudinal trend of plasma PRPH levels, no significant variations are observed between T0 and T6 (p= 0.09), although the trend is decreasing (T0 1.55 ± 0.70 ng/ml; T1 1.34 ± 0.55 ng/ml; **Figure 4A**).

In order to better evaluate the possible role of plasmatic PRPH as a dynamic index of the attempt of the lower motor neuron's regeneration, we calculated the percentage change in PRPH levels between T0 and T6 (*ΔPRPH%*) and the percentage change in PR values between T0 and T6 (*ΔPR%*).

A moderate inverse correlation was observed between ΔPRPH% and ΔPR% (r_s= -0.423, p= 0.01; **Figure 4B**). Since we previously reported that PRPH plasmatic levels are influenced by the stage of the disease in ALS, we performed multiple linear regression analysis, covarying based on the MiToS score at T0 ($r_{adjusted}$= -0.465; F= 4.137, p= 0.026). Despite the small sample size, the significance of this correlation was maintained.

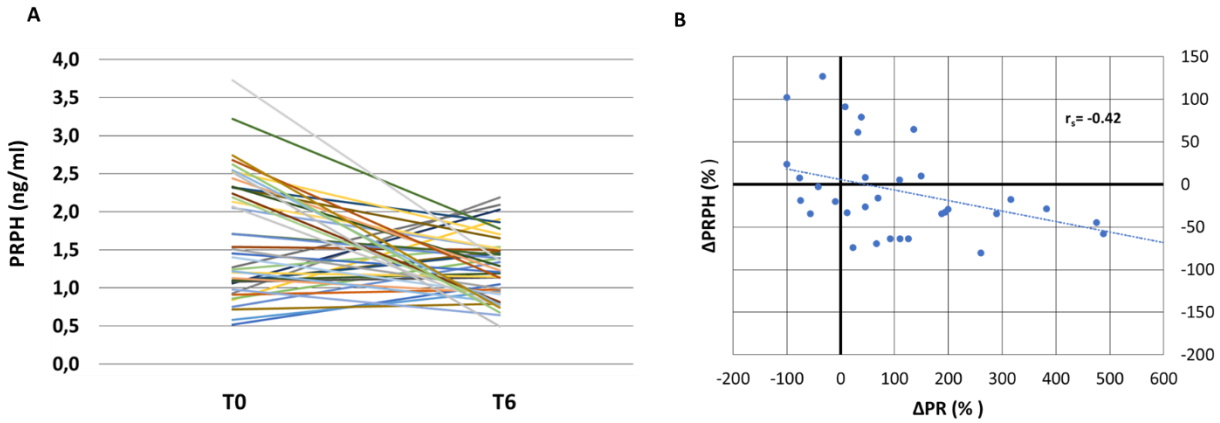


Figure 4 – Longitudinal changing of plasma PRPH and correlation with progression of disease

Legend: PRPH= Peripherin; ΔPRPH%= percentage of change in PRPH levels between T0 and T6; ΔPR%= percentage of change in PR values between T0 and T6; T0: at the moment of sampling; T6: after 6 months from sampling.

6.4 Discussion

In our investigation, we have examined the capacity of plasma PRPH in distinguishing MND from MND-mimics and HCs. The most important finding is the satisfactory ability of PRPH to distinguish individuals affected by MND, and most of all by ALS, from those with MND-mimicking conditions, identifying, in our data, the optimal cut-off value as 1.05 ng/ml. These findings corroborate the prior data presented by Sabbatini et al.[158], albeit their measurements were conducted in serum rather than plasma. As noted by Sabbatini and consistent with histological and cellular findings[122,158], PRPH was not detectable in CSF.

Drawing from these observations, the potential diagnostic utility of PRPH in suspected cases of ALS is conceivable. Nevertheless, broader confirmation is imperative, paralleled by adherence to standard laboratory techniques, that still need to be defined, as reported by Keddie at al.[159]

An additional finding gleaned from our study pertains to the potential future utility of PRPH in distinguishing between PLS and HSP prior to genetic testing, a process typically necessitating several months and incurring higher costs. While these findings require validation through larger-scale

multicentre studies, they hold promise for bolstering early diagnostic efforts in patients affected by a pure upper motor neuron disease.

Analysing the absolute levels of PRPH, we observed a substantial variability among the three available studies, including the present one, akin to the report by Keddie et al[159]. These discrepancies may stem from variations in laboratory techniques, different kit employed, variances in sample processing methodologies, different atmospheric conditions, different dilutions, and distinctions in the type of material employed (plasma in our study versus serum in the other two). Speculation may also extend to a potential blood matrix-effect resulting in the sequestration of PRPH in aggregates or its degradation by proteases or immunological binding, consequently reducing the free fraction, akin to what happen in plasma for NfL and pNfH[91].

Focusing on ALS, we found no associations between plasma PRPH levels and variables such as gender, age, ALS phenotype, disease onset pattern, cognitive impairment, PR of disease, UMNS, LMNI, and common blood examinations. In contrast to the findings reported by Sabbatini et al., our study revealed a modest correlation between PRPH levels and specific clinical parameters. Specifically, an inverse association between plasma PRPH levels and disease severity at the time of sampling (measured in our study through the two well-known staging system), and a positive correlation between PRPH levels and the size of functional impairment (measured by ALSFRSr and its sub-scores) were observed. Furthermore, a higher plasma PRPH level was associated with a lower progression rate of the disease and with a reduced lower motor neuron damage at the moment of sampling.

Another noteworthy finding was the direct correlation between PRPH and FVC, indicating that ALS patients with less compromised respiratory function exhibit higher levels of this protein.

Upon observing the survival analyses conducted, it is notable that ALS patients with higher levels of PRPH at T0 exhibit greater survival compared to the other patients, as evidenced by the graphs concerning both the time elapsed between onset and death/tracheostomy and that between sampling and death/tracheostomy. This data will require updating in this cohort in a few years (nowadays, fortunately, 69 patients with ALS out of 105 are alive).

All the above mentioned data suggest that, unlike neurofilaments, which directly correlate with disease progression rate and with the number of regions affected by damage[162–164], plasma PRPH levels may not indicate the extent of motor neuron damage but rather associate with the repair

attempts initiated by the motor neurons at the beginning of the disease. If this hypothesis holds true, PRPH levels could potentially increase as part of an upregulation process in response to neuronal injury, a phenomenon already observed in cellular studies[165–167].

Furthermore, the higher levels of PRPH in SBMA patients compared to ALS, observed by Sabbatini and colleagues[158], lend support to the notion of PRPH not as a marker of damage but as a marker of regeneration. In fact, given the less aggressive nature and the lower PR of SBMA compared to ALS, this observation suggests that PRPH may indeed signify attempts at motor neuron regeneration.

Moreover, the longitudinal data concerning plasmatic PRPH levels, as described above, support the aforementioned findings. Specifically, in ALS patients, the variation in plasma PRPH levels from T0 to T6 is inversely proportional to the change in progression rate before T0 and after. This finding persists even after adjusting for disease stage at baseline and for the time elapsed between onset and T0. This strengthens the hypothesis that PRPH may reflect the activation of compensatory mechanisms/attempts at motor neuron damage repair. Given the limited sample size of the longitudinally studied cohort, this finding requires validation in a larger external cohort.

Unfortunately, based on this study, we cannot determine the directionality of the causal effect: whether peripherin increases due to its involvement in the supposed repair process or its increase is merely an epiphenomenon.

However, such interpretations remain speculative, and comprehensive and multicentre studies with extended follow-up periods, along with histological and neuropathological data and with confirmation in cellular and animal models, are necessary to validate these hypotheses.

The limitations of our study encompass the absence of additional fluid biomarkers concurrently measured (such as NfL and pNfH), the lack of validation evidence in induced pluripotent stem cell-derived motor neuron models and in animal models of ALS, the monocentric design of the study, and challenges in comparing these findings with the other two studies due to the utilization of plasma instead of serum and to the absence of a validated and standard protocol.

In conclusion, plasma levels of PRPH may serve as a useful biomarker in the differential diagnosis of ALS, in order to do an early diagnosis, and in prognosis definition. Further investigations, enrolling a larger patient cohort and employing more advanced PRPH quantification techniques such as single-molecule array technology (SiMoA), are undoubtedly necessary to validate our findings.

Thesis Conclusions

The studies presented in this thesis, some of which are already published, others pending publication, aim to identify new instrumental and fluid biomarkers in motor neuron diseases. The first study, focusing on longitudinal audiometric evaluation in ALS patients without bulbar signs/symptoms, demonstrates that the alteration of stapedial reflex decay test is an important early marker of subsequent bulbar impairment. The second study, regarding longitudinal polysomnographic assessment in ALS patients, reveals early micro and macrostructural sleep alterations in these individuals. Moreover, this study highlights that nighttime apnea and desaturation indices are early markers of respiratory impairment, preceding symptoms or alterations in spirometry and ABG.

Studies on fluid biomarkers, conducted on different populations, have expanded knowledge in this field. Particularly, the study on CHI3L1, a known biomarker in MND, shows that its plasma measurement may play a useful role in the early diagnosis of ALS. The study on peripherin, the second known study in MND, demonstrates the utility of plasma peripherin measurement both diagnostically in early disease stages and prognostically. Lastly, the study on alpha-internexin, the first known study in humans, highlights its potential role in early diagnosis and prognosis determination. Unlike peripherin, alpha-internexin can only be measured via classical ELISA methods in cerebrospinal fluid. Both peripherin and alpha-internexin, along with NfL, NfM, and pNfH, constitute the fourth subunit of neurofilament heteromers and are expressed, respectively, in the peripheral and central nervous systems. From the studies conducted, it appears that both these proteins highlight processes of neuronal damage regeneration occurring in MNDs. Whether they are directly implicated in the regenerative process or if their levels are merely an epiphenomenon of this process cannot be deduced from these data. However, the interesting finding is that they seem to be involved in cellular damage response phenomena in MNDs in some way.

Further investigations, encompassing a larger patient cohort, involving multiple centres, and measuring all these biomarkers together are undoubtedly necessary to validate our findings.

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