UNIVERSITA' VITA-SALUTE SAN RAFFAELE

CORSO DI DOTTORATO DI RICERCA INTERNAZIONALE IN MEDICINA MOLECOLARE

Curriculum in Neuroscienze e Neurologia Sperimentale

Peripheral Nervous System Involvement in Amyotrophic Lateral Sclerosis: from diagnosis to disease understanding and development of novel biomarkers

DoS: Dr. Nilo Riva

Second Supervisor: Professor Philippe Van Damme

Tesi di DOTTORATO DI RICERCA di Yuri Matteo Falzone

Matr. 013850

Ciclo di dottorato XXXIV

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This thesis has been fully written by myself and it has never been used for previous applications for a degree.

Preliminary results that were obtained during my first PhS year, which overlapped with my last year of residency in Neurology, were included in my residency neurology thesis.

Figures 1.1, 1.2 and 1.3 were taken from previously published articles and they were included in my thesis accordingly with the fair use principle (decreto legislativo 9 aprile 2003, n.68, attuazione della direttiva 2001/29/CE sull'armonizzazione di taluni aspetti del diritto d'autore e dei diritti connessi nella società dell'informazione).

Throughout the thesis, I have used the terms 'I' and 'We' interchangeably.

All the data and results shown here were obtained by myself.

All sources of information are acknowledged by means of references

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ABSTRACT

The mainstay of Amyotrophic lateral sclerosis (ALS) diagnosis is the clinical evaluation, and it can be challenging when its first manifestations overlap with those of ALS mimic disorders (ALSmd). The lack of specific diagnostic test prevents an early diagnosis. The definition of prognosis in ALS is hampered by the heterogeneity of its clinical features, with variability in survival being the most salient feature. Therefore, wet biomarkers are needed to aid clinical decision, track disease progression, and better define disease trajectories. Recent advances highlighted neurofilaments as the most promising biomarkers for ALS. In the first part of our results, we assessed serum phosphorylated neurofilament heavy chain (pNfH) in a large ALS cohort (n=219). pNfH was an independent predictor of survival for ALS and its concentration was heterogenous across the ALS phenotypes, patients with fast disease progression and a predominant upper motor neuron burden showed the highest serum concentration. Subsequently, we performed gene expression and pathways analyses, on 8 motor nerve biopsies of patients with ALS and compared with 7 motor neuropathies as controls, identifying Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1) as a potential candidate biomarker for ALS. Therefore, we tested UCHL1 in an independent large ALS and control cohorts to assess its diagnostic and prognostic performances. At the same time, we also tested serum Neurofilament light chain (NfL) and Glial fibrillary acidic protein (GFAP). In our analysis, UCHL1 resulted not promising as diagnostic biomarkers, conversely, it was an independent prognostic factor, proving itself helpful in the stratification of survival for patients with lower NfL levels. NfL consistently reached the best diagnostic performance for ALS showing almost optimal performance in discriminating ALS from healthy controls and other neurodegenerative diseases. Conversely, the diagnostic yield in distinguishing ALS from ALSmd was lower with specificity decreasing to 78.0%. As similarly observed for the pNfH, NfL concentrations were heterogenous across the ALS phenotypes and higher concentrations were detected in patients with a fast disease progression and a predominant upper motor neuron burden. Serum GFAP, a known marker of astrogliosis, differs among cognitive phenotypes, namely ALS with concomitant cognitive impairment or FTD had higher levels compared with ALS with normal cognition. Therefore, GFAP might be instrumental in tracking the occurrence of cognitive decline in ALS.

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ACRONYMS AND ABBREVIATIONS

ALS = Amyotrophic Lateral Sclerosis

MND = Motor Neuron Disease

UMN = Upper Motor Neuron

LMN = Lower Motor Neuron

sALS = sporadic Amyotrophic Lateral Sclerosis

fALS = familial Amyotrophic Lateral Sclerosis

PLMN = Pure Lower Motor Neuron

PUMN = Pure Upper Motor Neuron

FA = Flail Arm

FL = Flail Leg

PMA = Progressive Muscle Atrophy

PLS = Primary Lateral Sclerosis

TDP-43 = TAR DNA-binding protein 43

FTD = Frontotemporal dementia

C9orf72 = Chromosome 9 open reading frame 72

ECAS = Edinburgh Cognitive and Behavioral ALS Screen

ALSci = ALS with cognitive impairment

ALSbi = ALS with behavioural impairment

ALS-cbi = ALS with combined cognitive and behavioural impairment

ALS-FTD = ALS with frontotemporal dementia

SOD1 = Superoxide Dismutase 1

FUS = FUS RNA Binding Protein

CNS = Central Nervous System

DPRs = Dipeptide Repeat Proteins

CSF = Cerebrospinal Fluid

NfL = Neurofilament Light Chain

pNfH = Phosphorylated Neurofilament Heavy Chain

rEEC = El Escorial criteria revised

EMG = Electromyographic

GC = Gold Coast

ALSFRS = ALS Functional Rating Scale revised

FVC = Forced Vital Capacity

NFs = Neurofilaments

MN = Motor neuropathies

PNS = Peripheral Nervous System

ALSmd = ALS mimic disorders

DEGs = Differently Expressed Genes

MRC = Medical Research Council

UMNs = Upper Motor Neuron score

cMAP = Compound Muscle Action Potential

MEP = Motor-Evoked Potential

CL = Classic

PY = Pyramidal

B = Bulbar

R = Respiratory

HR = Hazard Ratio

M = Male

F = Female

IVIG = Intravenous Immune Globulin

RTX = Rituximab

AZA = Azatioprine

GO = Gene Ontology

UCHL1= Ubiquitin Carboxyl-Terminal Hydrolase Isozyme L1

GFAP = Glial Fibrillary Acidic Protein

tTAU = total TAU protein

DEG = Neurodegenerative Disorders

HC = Healthy Controls

AD = Alzheimer's disease

PD = Parkinson's disease

NIV = Non-Invasive Ventilation

HSPG = Hereditary Spastic Paraparesis

PCR = Polymerase Chain Reaction

ELISA = Enzyme-Linked Immunosorbent Assay

UPS = Ubiquitin-Proteasome System

LIST OF FIGURES AND TABLES

1. INTRODUCTION

1.1 Amyotrophic Lateral Sclerosis, a tangled world

Amyotrophic lateral sclerosis (ALS) was firstly described by the French neurologist Charcot in the nineteenth century as a disease selectively involving the motor system. However, this perspective completely changed over time, and it is now considered a complex neurodegenerative disorder, marked by wide variability in clinical manifestations, extra-motor involvement, genetic background and neuropathological features (Masrori & Van Damme, 2020; Brown & Al-Chalabi, 2017; Hardiman *et al*, 2017).

ALS is caused by a relentless motor neuron degeneration which leads to progressive paralysis at the four limbs, dysphagia, respiratory failure and eventually death occurring three to five years from symptoms onset (Hardiman *et al*, 2017; Gentile *et al*, 2019). Despite this description, ALS manifestations embrace heterogenous phenotypes ranging from different upper motor neuron (UMN) and lower motor neuron (LMN) burden, site of symptoms onset, variable disease progression and prognosis. In consideration of this wide variability, a more generical term, such as motor neuron disease (MND) seems to be more appropriate for this condition (Gentile *et al*, 2019). Therefore, ALS might be only a particular phenotype being part of a wider and tangled MND spectrum. Defining MND phenotypes is important because they might reflect different pathogenetic mechanisms and consequently variable responses to pharmacological treatment.

Nowadays, the mainstay of ALS/MND diagnosis is clinical evaluation. Specific diagnostic tests are currently lacking, electrodiagnostic and neuroimaging investigations are helpful to rule out some conditions which may mimic ALS. The diagnosis of ALS can be problematic mainly when its clinical presentation overlap to those of ALS mimic disorders and the lack of specific diagnostic tests prevents an early diagnosis (Traynor *et al*, 2000). The definition of prognosis in ALS/MND is hampered by the heterogeneity of its clinical features (Westeneng *et al*, 2018a; Beghi *et al*, 2011). Several clinical and genetic factors are known to influence patients prognosis and were recently grouped in a prognostic multivariate model (Westeneng *et al*, 2018a). However, most of these factors are not available at the early disease stage and they require a clinical follow-up or further investigations to be collected. An accurate prediction of the individual outcome is crucial

to establish early interventions as well as in clinical trial design. Against this background, wet biomarkers are urgently needed to aid clinical decisions, achieve early diagnosis, track disease progression, and define disease trajectories (Falzone *et al*, 2021a).

1.2 ALS clinical presentation

Although ALS might affects patients across all ages (Logroscino *et al*, 2010), the median age at the onset for sporadic ALS cases (sALS) is about 65 years, and approximately ten years earlier for the familial ALS cases (fALS) (Logroscino *et al*, 2010; Chiò *et al*, 2013; O'Toole *et al*, 2008; Vazquez *et al*, 2008). Age at symptoms onset has been proved to be a prognostic factor in ALS, so that a decreased survival correlates with older age at the disease onset (Haverkamp *et al*, 1995; Preux *et al*, 1996). Most of ALS patients are considered sporadic, while fALS account for a minority of the whole cases (five to ten percent). sALS and fALS have indistinguishable clinical courses and survival.

ALS clinical presentation relies on a continuum ranging from pure lower motor neuron (PLMN) to selective pure upper motor neuron (PUMN) syndromes (Chio *et al*, 2011). In agreement with the literature, eight different clinical phenotypes have been proposed (Chio *et al*, 2011): Classic (Charcot type) accounts for 30-35% of the phenotypes and is defined by a spinal onset with a clear coexistence of UMN and LMN involvement. Bulbar represents the second most frequent form, about 20-25% of the ALS phenotypes. These patients are mainly characterized by progressive dysarthria, swallowing dysfunction, tongue hypotrophy and fasciculation with no involvement of the spinal regions for at least six months from disease onset. Flail Arm (FA) and Flail Leg (FL) account for approximately 20% of the phenotypes; both are mostly lower motor neuron syndromes with symptoms restricted to the proximal region of the upper limbs and the distal region of the lower limbs for at least twelve months from the symptoms onset, respectively (Wijesekera *et al*, 2009). Pyramidal accounts for approximately 10% of the ALS phenotypes, these patients show a clearcut prevalence of the UMN signs such as spinal and bulbar spasticity. PLMN or progressive muscular atrophy (PMA), a rare phenotype accounting for 5-10% of the ALS patients, patients exhibit selective LMN signs with no clinical or electrophysiological UMN involvement (Chio *et al*, 2011; Visser *et al*, 2007). Despite the selective involvement of the LMN, neuropathological investigations have consistently demonstrated the degeneration of the corticospinal tract even in the absence

of UMN symptoms or signs. Therefore, UMN signs might be masked by extensive LMN degeneration (Brownell *et al*, 1970; Ince *et al*, 2003; Saberi *et al*, 2015). In consideration of the clinical and genetic similarities between PLMN and ALS, the hypothesis that they are two distinct entities is unlikely (Kim *et al*, 2009; van Blitterswijk *et al*, 2012c). PUMN or primary lateral sclerosis (PLS) is marked by selective UMN degeneration without any clinical and electrophysiological findings suggestive of LMN involvement. This is a rare ALS form accounting for approximately 5% of the ALS phenotypes (Pringle *et al*, 1992; Tartaglia *et al*, 2007). These patients have distinctive features compared to the other ALS phenotypes such as a longer disease duration and survival, sparing of respiratory function, the absence of familiarity. Nevertheless a proportion of PUMN develops LMN signs, usually within three to four years from symptoms onset, making the distinction from ALS spectrum difficult (Gordon *et al*, 2006). Finally, the respiratory phenotype is an extremely rare form accounting for less than 1% of the ALS patients. These patients have a prevalent respiratory dysfunction at the onset with minimal spinal or bulbar impairment at least for six months after onset (Shoesmith *et al*, 2007).

It has been demonstrated that patients survival is strictly correlated to the phenotype, therefore ALS stratification accordingly to the phenotype is a pivotal step of the clinical assessment (Chio *et al*, 2011). Bulbar and respiratory are the most aggressive forms showing the shortest survival while PUMN has a benign disease course with a median survival longer than ten years (Chiò *et al*, 2009; Sabatelli *et al*, 2008a). FA phenotype has an intermediate outcome, with a mean survival time of four years (Wijesekera *et al*, 2009; Katz *et al*, 1999). Classic and FL phenotypes have a similar outcome with a mean survival of 36 months (Chio *et al*, 2011; Chiò *et al*, 2009; Millul *et al*, 2005). The mean cumulative survival of the different ALS phenotypes is shown in Figure 1.1.

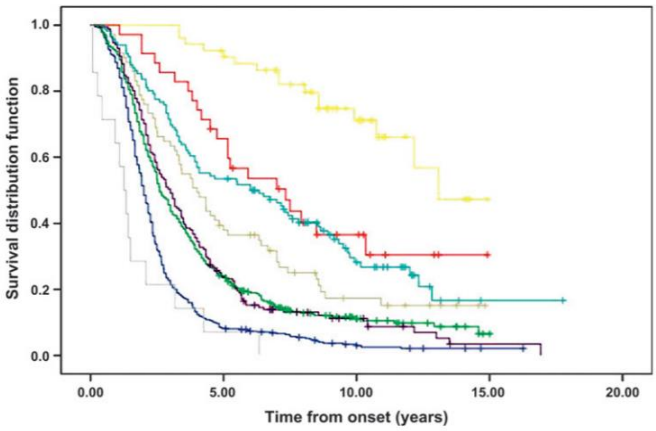

Figure 1.1 ***Cumulative survival stratification based on the ALS phenotype.*** *Yellow, pure upper motor neuron (PUMN); red, pure lower motor neuron (PLMN); light blue, pyramidal; grey, flail arm (FA); violet, classic; green, flail leg (FL); blue, bulbar; cyan, respiratory. Censored patients are represented with crosses. Taken from Chiò, A et al; 2011(Chio et al, 2011).*

1.3 ALS and the extra motor involvement

Although ALS has been considered a paradigm of selective motor neuron disease for a long time, it is currently considered a multisystem disease with considerable extra-motor involvement. Cognitive and behavioural dysfunction have been described since early reports, however, these symptoms were initially considered as atypical manifestations of ALS (David & Gillham, 1986; Gallassi *et al*, 2009). When positive ubiquitinated cytoplasmic inclusion comprised primarily of TAR DNA-binding protein 43 (TDP-43), were documented in most of ALS patients and in approximately half of the patients affected by frontotemporal dementia (FTD) these neurodegenerative disorders were settled on the so-called "ALS-FTD continuum," evidencing the noticeable clinical, pathophysiological, genetical and neuroimaging overlap between the two disorders (Saberi *et al*, 2015; Arai *et al*, 2006; Neumann *et al*, 2006), Figure 1.2. It is now clearly evident that the neurodegenerative process occurring in ALS can spread from the motor cortex to the frontal and anterior temporal lobes or vice versa, causing a variable severity of executive and language dysfunctions or behavioural changes (Jo *et al*, 2020). Further proof of the existence of the ALS-FTD spectrum was the identification of a mutual hexanucleotide repeat expansion in the chromosome 9 open reading frame 72 (*C9orf72*) gene.

Up to fifty percent of ALS patients have significant cognitive/behavioural dysfunction while about ten percent fulfil the diagnostic criteria for FTD (Trojsi *et al*, 2019; Montuschi *et al*, 2015; Goldstein & Abrahams, 2013; Phukan *et al*, 2012). ALS patients typically manifest executive dysfunction, social cognition impairment, lack ok fluency, language and semantic memory deficits and behavioural changes, conversely visuo-perceptive and visuo-constructive functions are usually spared (Abrahams *et al*, 2014, 1995a, 1995b; Consonni *et al*, 2019; Taylor *et al*, 2013; Girardi *et al*, 2011; Beeldman *et al*, 2016). Several ALS dedicated screening tests like the Edinburgh Cognitive and Behavioural ALS Screen (ECAS) and the ALS Cognitive and Behavioural Screen (ALS-CBS) are validated and are instrumental for the neurologist to detect macroscopic cognitive and behavioural dysfunction (Abrahams *et al*, 2014; for the ALS-CBS Italian Study Group *et al*, 2020; Strong *et al*, 2017). ECAS has shown a sensitivity and specificity of 85% in detecting cognitive or behavioural dysfunction in patients with ALS (Lulé *et al*, 2015). A thorough neuropsychological assessment by an expert neuropsychologist is needed to detect less evident cognitive and behavioural deficits and for accurate patient classification (Christidi *et al*, 2018). In 2017, an international consensus revised the previously published Strong criteria for the diagnosis of frontotemporal impairment in ALS (Strong *et al*, 2017, 2009). Accordingly with these criteria, ALS patients can be categorized in different cognitive phenotypes: ALS with cognitive impairment (ALSci), ALS with behavioural impairment (ALSbi), ALS with combined cognitive and behavioural impairment (ALS-cbi), ALS with frontotemporal dementia (ALS-FTD) and ALS with comorbid dementia (ALS-dementia) (Strong *et al*, 2017).

Patients with ALS must be carefully investigated and followed up for cognitive involvement to target patient care. The occurrence of cognitive dysfunction in ALS is a well-known unfavourable prognostic factor and lead to increase caregiver burden and quality of life decline (Montuschi *et al*, 2015; Elamin *et al*, 2013, 2011). Only few longitudinal studies on cognitive performance in ALS are available. Patients with normal cognition at diagnosis remain typically unaffected, while social cognitive and executive function decline over the disease course (Elamin *et al*, 2013; Beeldman *et al*, 2020).

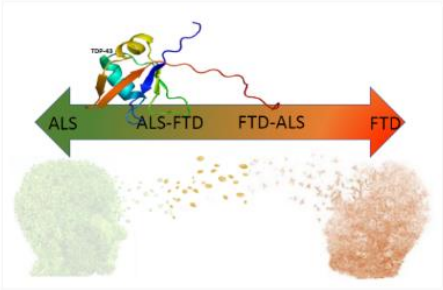

Figure 1.2. ALS-FTD spectrum disorder. *Taken from Dharmadasa, T et al; 2017(Dharmadasa et al, 2017).*

1.4 ALS Genetic Background

ALS is currently recognized as a complex genetic disorder with a Mendelian pattern of inheritance occurring only in a minority of the patients. A variable proportion of patients with ALS, ranging from 5 to 20%, show a positive family history of ALS or FTD and are considered as possible, probable, or definite fALS depending on the definition of familial ALS (Byrne *et al*, 2013; Boylan, 2015). Approximately 60-80% of patients with fALS are carriers of a mutation in the most common ALS-related genes, of which *C9orf72* 40%, superoxide Dismutase 1 *(SOD1)* accounts for 20%, FUS RNA Binding Protein *(FUS)* for the 1–5%, and *TARBDP* 1–5% (Renton *et al*, 2014). Only in a minority of patients with sALS, about10%, a mutation in the ALS-related genes can be detected (Renton *et al*, 2014).

In patients with fALS genetic plays a fundamental role in the pathogenetic mechanism, conversely in sALS the role of genetic has to be further elucidated. Evidence coming from studies performed on twins shows that the genetic contribution to sALS is 61% (Graham *et al*, 1997; Al-Chalabi *et al*, 2010). The genetic architecture of ALS has been explored in a genome-wide association study that divided the explained heritability by allele frequencies; this study suggested that the presence of rare gene variants might explain the remaining percentage of the genetic variability (PARALS Registry *et al*, 2016). Therefore, ALS might be a complex oligogenic disorder as it has been proposed for neuropsychiatric disorders such as schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium *et al*, 2015). The hypothesis of an oligogenic model as causative mechanism for ALS is supported by the incomplete penetrance observed in

several ALS families, the lower risk of ALS in different populations, and the co-segregation of several ALS-associated genes with the disease in some families (van Blitterswijk *et al*, 2012a, 2012b). Additionally, mathematical models have identified that patients with ALS are likely to harbour a variable number of gene variants that interplay with other environmental factors through a series of a minimum of six steps leading to ALS occurrence (Chiò *et al*, 2018). Interestingly, first-degree relatives of patients affected by sALS have an eight-time higher risk of developing ALS in their lives (Hanby *et al*, 2011). In light of these findings, dichotomizing ALS into sporadic and familial forms might represent an over-simplification because all the findings highlight similarities in the genetic background of both sALS and fALS.

The first gene identified to be causative of ALS was *SOD1* discovered in 1993 (Rosen *et al*, 1993). In most of the *SOD1* families, ALS is transmitted in an autosomal dominant manner and the penetrance is higher than 90% by the age of 70 (Cudkowicz *et al*, 1997). Mutation in the *SOD1* gene can be found in approximately 20% of fALS and 1-2% of sALS cases (Ghasemi & Brown, 2018). The substitution of valine for alanine in codon 4(A4V) is the most frequent *SOD1* mutation in North America, this mutation has been associated with an aggressive ALS form marked by short survival (Ghasemi & Brown, 2018). Although uncommon, *SOD1* can be causative of ALS also when transmitted as a recessive trait. In Scandinavia, about 1% of the population carries the D90A mutation. In that setting, ALS is triggered when both alleles are mutated (Andersen *et al*, 1995). In opposition to the A4V *SOD1* mutation, the D90A/D90A is associated with a slowly progressive ALS phenotype with a survival longer than ten years (Ghasemi & Brown, 2018).

In 2006, the protein TDP-43, encoded by the *TARDP* gene, was identified as the major component of the pathological cytosolic ubiquitinated inclusions in both sALS and sporadic FTD (Neumann *et al*, 2006). After this discovery, several *TARDBP* mutations were found in patients with ALS and FTD (Sreedharan *et al*, 2008). *TARDBP* mutations account for approximately 10% of the fALS and 1% of the sALS cases (Renton *et al*, 2014). Nowadays, more than 60 mutations in *TARDBP* have been reported and they are predominantly inherited in an autosomal dominant manner (Van Deerlin *et al*, 2008). TDP-43 is multifunctional protein involved in multiple cellular pathways such as gene transcription and translation, microRNA biogenesis, gene silencing, RNA binding and

transport, and suppression of aberrantly spliced cryptic RNA transcripts (Ling *et al*, 2015). In both sALS and fALS, except for cases harbouring mutations in *SOD1* or *FUS* genes, TDP-43 is cleaved and hyperphosphorylated in the cytoplasm of motor neurons where it aggregates and deposits leading to the deposition of insoluble cytoplasmic aggregates (Neumann *et al*, 2006).

In 2011, two independent studies identified the most frequent genetic cause of ALS and ALS-FTD: an expansion of a hexanucleotide intronic repeat (GGGGCC) within the first intron of the *C9ORF72* gene (Renton *et al*, 2011; DeJesus-Hernandez *et al*, 2011). *C9orf72* expansion accounts for 30-40% of the fALS and 8-10% of sALS cases (Renton *et al*, 2014). The biologic function of *C9orf72* and the pathogenic mechanisms inducing motor neuron degeneration are not fully understood yet. A reduction of the *C9orf72* mRNA transcript levels was demonstrated in the central nervous system (CNS) of patients carrying the *C9orf72* expansion suggesting that haploinsufficiency might be a pathogenic mechanism (Ghasemi & Brown, 2018). However, mouse model ablated for the *C9orf72* gene did not show any significant motor neuron loss (Koppers *et al*, 2015). A second intriguing hypothesis, rising from pathological studies in C9orf72-ALS, is an RNA gain-of-function mechanism due to intranuclear deposition of abnormally expanded RNA and the production of dipeptide repeat proteins (DPRs) that sequester RNA-binding proteins (Freibaum & Taylor, 2017; Fumagalli *et al*, 2021). The existence of the DPRs was firstly described in postmortem tissue from patients with spinocerebellar atrophy type 8 (Koob *et al*, 1999). Further evidence in different animal models demonstrated the toxicity of DRPs at different system levels (Tran *et al*, 2015; Mizielinska *et al*, 2014). Patients with ALS harbouring *C9orf72* expansion show peculiar clinical phenotypes prone to significant cognitive and behavioural dysfunction, associated with bulbar phenotype and with aggressive disease forms leading to a shorter survival compared to sporadic and other genetic determined ALS (Millecamps *et al*, 2012; Byrne *et al*, 2012; Chio *et al*, 2012). Indeed, *C9orf72* was demonstrated as a negative independent factor for ALS survival (Umoh *et al*, 2016).

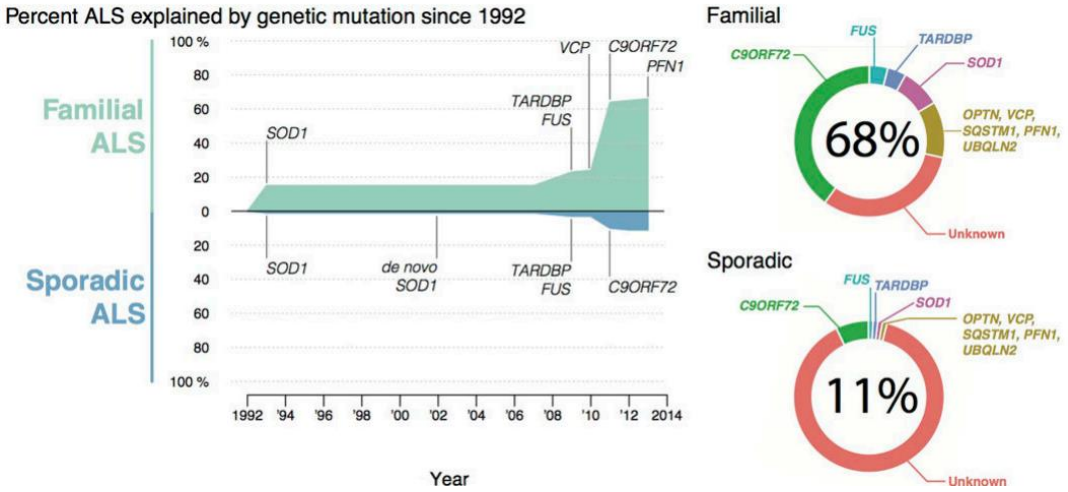

Figure 1.3*. *Timeline of gene discoveries in fALS and sALS. *It represents the percentage of the fALS and sALS cases in which genetic mutations are detectable in populations of European ancestry. Taken from Renton, A et al, 2014(Renton et al, 2014).*

1.5 The diagnostic work-up in ALS

Nowadays, the mainstay of the ALS diagnosis is the clinical evaluation; progressive upper and lower motor neuron dysfunctions without an alternative better explanation for the presenting symptoms and signs are required to establish the diagnosis of ALS (Brooks *et al*, 2000). Blood examination, neurophysiological and neuroimaging investigations are helpful to rule out some neurological disorders that may resemble signs and symptoms suggestive of ALS. The diagnosis can be straightforward when both UMN and LMN are clear in multiple regions. However, particularly at the initial disease stage, UMN or LMN can be clinically undetectable; the disease spreading can be very slow, the symptoms can be restricted only to one region and UMN signs can be completely masked by an extensive LMN degeneration. Nonetheless, the heterogeneity of ALS clinical features, the presence of several neurological conditions mimicking ALS and the lack of specific diagnostic tests are major limitations in establishing an early diagnosis (Traynor *et al*, 2000). As a matter of fact, the mean diagnostic delay still remains relevant, about ten to twelve months from symptoms onset, which is unacceptable considering the short survival of the patients with ALS (Westeneng *et al*, 2018b; Knibb *et al*, 2016).

In the last decade, several studies attempted to identify serum or cerebrospinal fluid (CSF) candidate biomarkers for ALS. Consistent evidence highlighted neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH) as the most

promising ones (Falzone *et al*, 2021a; Poesen & Van Damme, 2019). Patients with ALS showed significantly higher NfL and pNfH concentrations in both serum and CSF and a satisfying diagnostic yield compared with most of the other neurological conditions and ALS mimic disorders (Sferruzza *et al*, 2021; Forgrave *et al*, 2019). CSF NfL and pNfH and serum NfL provided similar diagnostic accuracy in discriminating patients with ALS from its mimic disorders, whereas serum pNfH showed to be slightly less precise (Halbgebauer *et al*, 2021). Despite this evidence, both NfL and pNfH are not currently available in clinical practice due to issues yet to be solved. Standardization of the analytical techniques is lacking, which of the two neurofilaments subunits should be used and in which biofluids and delineation of precise practice-oriented cut-offs (Falzone *et al*, 2021a). Wet biomarkers are urgently needed in clinical practice to support clinicians in the diagnostic work-up and to speed up the diagnosis of ALS.

The international diagnostic criteria have been proposed many years ago to speed up ALS diagnosis and to enhance clinical trials (Brooks *et al*, 2000; Brooks, 1994; de Carvalho *et al*, 2008). In May 1990, a three-day workshop "Clinical limits of ALS" took place in the Spanish city of El-Escorial aimed at defining the first diagnostic criteria. After this meeting the El-Escorial criteria were developed and later published in 1994 (Brooks, 1994). These diagnostic criteria were based on the identification of the UMN and LMN signs and their extension in different spinal and bulbar regions, thereby defining four levels of diagnostic category namely suspected, possible, probable, and definite ALS (Brooks *et al*, 2000). To improve the diagnostic sensitivity, the El Escorial criteria were revised (rEEC) in December 2000. rEEC excluded the category suspected ALS and included a new category named probable laboratory supported which allowed an integrated evaluation of the electromyographic (EMG) findings with the neurological examination (Brooks *et al*, 2000). These criteria showed high specificity; however, concerns were immediately raised about the low sensitivity, mainly in the initial disease stages of ALS, ending up in delays in diagnosis and recruitment in clinical trials (Chiò, 1999; Aggarwal & Cudkowicz, 2008). In 2008, the neurophysiological Awaji diagnostic criteria were proposed to address these perceived issues, aimed at improving diagnostic performance, particularly during the initial stages of the disease (de Carvalho *et al*, 2008). Awaji criteria considered both clinical and EMG findings; in particular, neurophysiological evidence of LMN involvement was deemed analogous to clinical

LMN signs. Several studies explored the diagnostic sensitivity of the Awaji criteria showing controversial results concerning spinal onset ALS (Higashihara *et al*, 2012; Jang & Bae, 2015; Krarup, 2011; Chen *et al*, 2010; Geevasinga *et al*, 2016), conversely an improvement was clearly observed in patients with predominant bulbar onset (Geevasinga *et al*, 2016; Johnsen *et al*, 2019). Awaji and El-Escorial criteria shared few limitations; first of all, they are intricate criteria with low interrater-variability (Johnsen *et al*, 2019); secondly, it has been shown that about 22% of patients with possible-ALS reach the end disease stages without fulfilling diagnostic categories for probable or definite-ALS (Traynor *et al*, 2004). Lastly, patients with only UMN signs in two regions are classified as possible-ALS despite the lack of clinical LMN dysfunction. These patients might be eventually diagnosed with PLS or PUMN which has a completely different disease course and survival compared with ALS (Turner *et al*, 2020). The above-mentioned limitations may have a significant negative impact on clinical trial enrolment and findings. To overcome these limitations, the new Gold Coast (GC) diagnostic criteria for ALS were published in 2020 (Shefner *et al*, 2020). Accordingly with GC criteria, ALS diagnosis can be established in presence of progressive UMN and LMN signs in at least one body region (UMN and LMN dysfunction have to be simultaneous in the same region) or LMN signs in at least two body regions and mimic disorders have to be ruled out through appropriate examinations (Shefner *et al*, 2020). The LMN signs can be detected either by clinical or EMG evaluations. The GC criteria have several advantages, better diagnostic sensitivity compared with the Awaji and rEEC criteria (Hannaford *et al*, 2021), exclusion of PLS/PUMN from the ALS diagnostic criteria, while accordingly with prior criteria PLS/PUMN could have been classified as possible-ALS, lastly they seem to be more robust in selecting classic ALS for recruitment into clinical trials. The above-mentioned diagnostic criteria for ALS are reported in Table 1.1

ALS diagnostic criteria	**rEEC, 2000**	**[a]Awaji, 2008**	**[b]Gold Coast, 2019**
	Definite-ALS UMN and LMN signs in three spinal regions, or Bulbar region and two spinal regions		**Presence of ALS** 1) progressive motor impairment 2)UMN and LMN signs in at least one region, or LMN signs in at least two regions 3) exclusion of other diseases
	Probable-ALS UMN and LMN signs in at least two regions with some UMN signs necessarily rostral to the LMN signs		
	Probable laboratory supported ALS UMN and LMN signs in one region, OR UMN signs alone present in one region, and LMN signs defined by EMG criteria present in at least two regions	Not applicable	
	Possible-ALS UMN and LMN signs in one region, or UMN signs in two or more regions; or LMN signs are found rostral to UMN signs and the diagnosis of clinically probable ALS laboratory-supported cannot be proved	**Possible-ALS** UMN and LMN signs in one region, or UMN signs in two or more regions; or LMN signs are found rostral to UMN signs and other diagnoses must have been excluded	

Table 1.1 ALS diagnostic criteria. *Abbreviations: rEEC, revised El-Escorial criteria. Regions indicate bulbar, cervical, thoracic, and lumbar districts. [a] LMN impairment was defined at the clinical, electrophysiological, or neuropathological assessment. Fasciculation potentials were considered as equivalent to ongoing neurophysiological changes such as fibrillation potentials and positive sharp waves in the presence of chronic neurogenic changes. [b]LMN impairment was defined clinically or by electrophysiological assessment. ALS diagnostic criteria were taken from previous published articles(de Carvalho et al, 2008; Shefner et al, 2020).*

1.6 Prognostic determinants and the state of play of wet biomarkers

As previously mentioned, ALS is characterized by an intrinsic heterogeneity in phenotypes, variable cognitive impairment, and genetic background. In consideration of this variability, patients affected by ALS manifest a great variability in disease progression and survival which can range from months to more than ten years (Chio *et al*, 2011; Westeneng *et al*, 2018b). Nowadays, the most widely used outcome measure in clinical trial is the disease progression rate assessed through the ALS Functional Rating Scale revised (ΔALSFRS-R). In this background, an accurate prediction of the individual outcome is crucial to establish prognosis, implementation of specific interventions and to obtain homogenous populations in clinical trials (Falzone *et al*, 2021a).

A recent large multicentre European study assessed in an ALS cohort sixteen clinical variables as potential predictors of survival to provide estimates of prognosis for individual patients affected by ALS. Survival was defined as the period between symptoms onset and the need for non-invasive ventilation more than twenty-three hours per day, tracheostomy placement, or death (Westeneng *et al*, 2018b). A multivariate comprehensive model was finally developed and validated including eight of sixteen predictors which resulted statistically significant. According to the model, the patient can be categorized into five prognostic categories: very long, long, intermediate, short, and very short times to the composite outcome. Six of the eight variables included in the model were clinical: higher age at symptoms onset, lower diagnostic delay (time between symptoms onset and diagnosis), faster progression rate evaluated with ALS Functional Rating Scale revised (ΔALSFRS-R), presence of bulbar phenotype, presence of dementia, and definite-ALS at the El-Escorial criteria (Westeneng *et al*, 2018b; de Carvalho *et al*, 2008). The two remaining predictors were the presence of *C9orf72* hexanucleotide repeat expansion and reduction of the respiratory function measured with forced vital capacity (FVC). Unfortunately, some of these parameters are not available at an early disease stage, consequently resulting unhelpful for an early patient's stratification; secondly, bulbar phenotype and definite-ALS cannot be established at the first clinical evaluation requiring an observational time for the classification; thirdly, FVC decreases only in the later stage of the disease; finally, genetic analysis is not immediately available. Furthermore, the prediction performance of the model is not as accurate as expected,

providing a broad range of the estimated survival outcome. In addition, information concerning Riluzole was not included even if the authors underlined its limited effect on prognosis. Lastly, prognosis was heterogeneous across countries and it could have been influenced by several factors (Mitsumoto, 2018).

In this setting, a wet biomarker would be extremely helpful to simplify prognosis assessment at the initial disease stage. Neurofilaments (NFs) are pivotal in maintaining the cytoskeletal structure of neurons and are considered reliable markers of acute and chronic axonal injury. NFs structure is shown and described in detail in Figure 1.4. Despite axonal degeneration being a non-specific pathogenetic mechanism of ALS, shared by several neurological diseases, elevated NFs levels in biofluids have been consistently associated with ALS, supporting their introduction as a promising biomarker for this neurodegenerative disorder (Falzone *et al*, 2021a). Many studies have investigated the prognostic role of NfL and pNfH pointing out the reliability of both biomarkers in predicting ALS survival when measured in both serum or CSF (Falzone *et al*, 2020; Gaiani *et al*, 2017; Gaiottino *et al*, 2013; Gagliardi *et al*, 2021; Lu *et al*, 2015; Rossi *et al*, 2018; Steinacker *et al*, 2017). As widely observed, an elevation of NFs in both biofluids correlated with a faster decline of the ALSFRS-R (Falzone *et al*, 2020; Gaiani *et al*, 2017; Gille *et al*, 2019; Steinacker *et al*, 2017). The ability of NFs to predict survival in patients with ALS is consistently demonstrated by the clear separation of the Kaplan-Meier survival curves when ALS patients are sub-grouped according to different NFs concentrations (Falzone *et al*, 2020; Steinacker *et al*, 2017; Thouvenot *et al*, 2020). The prognostic performance of NFs was also confirmed in multivariate models; they independently correlated with reduced survival and were the main predictors of patients' survivals along with the disease progression rate (Falzone *et al*, 2020; Thouvenot *et al*, 2020). However, most of the prognostic studies on NFs have been monocentric, did not assess pNfH and NfL simultaneously, explored NFs concentration only in one biofluid and lacked correlation between NFs and disease progression rate longitudinally. A recent study partially overcame these limitations demonstrating that NfL levels in either CSF or serum are the main predictor of the ALSFRS-R slope (Benatar *et al*, 2020). In light of these findings, NfL might be considered in clinical practice to predict patients' trajectories and for patient stratification in clinical trials. The primary outcome measure in ALS trial a is survival or the decline on ALSFRS-R. In consideration of the amount of time required

for these outcome measures to become informative clinical trials result to be lengthy and expensive. Easy detectable and reliable biochemical biomarkers could reduce the duration of trials and make them more efficient (Benatar *et al*, 2020). The upcoming introduction of NFs in clinical practice needs the definition of standardized cut-off values, to provide consistency and allow comparisons between measurements from different laboratories.

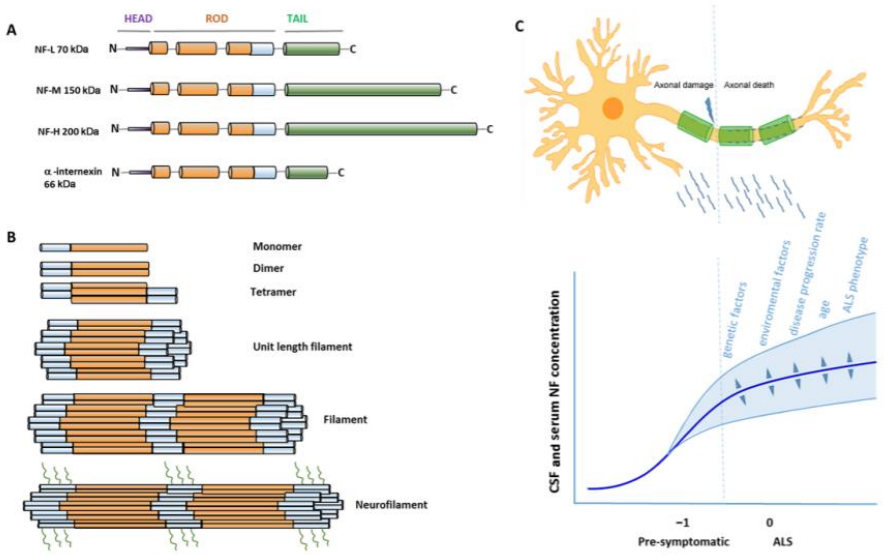

Figure 1.4. Assembly and structure of neurofilaments. *(A) Neurofilament (NFs) are formed by four subunits namely phosphorylated neurofilament heavy chain neurofilament, neurofilament medium chain, neurofilament light chain (NfL), and α-internexin. Each of the subunits is composed by an N-terminal head domain, a central α-helical rod domain and a C-terminal tail domain. (B) Neurofilament monomers form parallel side-to-side heterodimers. These dimers line up with antiparallel orientation forming tetrameric structures. Eight tetramers associate laterally giving rise to the unit length filament aggregation of the unit length filaments results in filament elongation and compaction forming mature neurofilament of a 10 nm diameter. (C) In the ALS asymptomatic stage, NFs are mainly released from the degenerating motor neurons and their axons and flow into the extracellular space and consequently into cerebrospinal fluid and blood. In the ALS symptomatic stage, extensive motor neuron and corticospinal tract degeneration lead to massive release of NFs and consequent a further elevation of NFs in both biofluids. Several modifying factors may interplay influencing NFs concentration in biofluids. Taken from Falzone* et al, 2021*(Falzone et al, 2021a)*.

1.7 Motor nerve biopsy

The diagnosis of ALS can be trivial in patients presenting with progressive UMN and LMN dysfunction in more than one region, whereas it could be challenging when patients manifest selectively LMN signs. PLMN or PMA is an adult-onset LMN phenotype characterized by asymmetric presentation, distal and/or proximal onset, and a fast disease

progression compared to other LMN syndromes (Swinnen & Robberecht, 2014). This phenotype is thought to represent about 5% of all patients with MND (Rowland, 2010). Whether PLMN and ALS have a distinct pathogenetic mechanism and thereby are different disorders, or whether PLMN represents the end of a spectrum of LMN versus UMN involvement has been debated for a long time. There is extensive evidence supporting the latter hypothesis: a consistent proportion of PLMN/PMA patients have a disease progression and a pattern of disease spreading ''ALS-like''; patients carrying *SOD1* mutations typically lack or present minimal UMN involvement; neuroimaging studies demonstrated widespread frontotemporal alterations in PLMN that are similar to those in ALS; neuropathological studies of patients with PLMN frequently present the degeneration of the lateral spinal cord tracts (Ince *et al*, 2003; Rowland, 2010; Riva *et al*, 2011; Cudkowicz *et al*, 1998; Spinelli *et al*, 2016; van der Graaff *et al*, 2011).

Motor neuropathies (MN) are miscellaneous group of conditions mainly characterized by the involvement of the motor nerves. MN and PLMN, may have similar clinical manifestations marked by progressive weakness, wasting, and fasciculations, and reduction of the deep tendon reflexes. In most MN cases the neurological examination, laboratory and neurophysiological investigations are sufficient to discriminate them from those with PLMN. However, in selected cases, only patient follow-up can lead to the diagnosis; establishing an early diagnosis in MN is crucial because there are several effective therapeutic approaches (Riva *et al*, 2011). The neuropathological evaluation of the motor branch of the obturator nerve has been demonstrated to be a useful tool in discriminating patients with an establish diagnosis of MN from PLMN (Corbo *et al*, 1997). A later study proved that the neuropathological examination was also promising in the diagnostic phase in differentiating PLMN from MN (Riva *et al*, 2011). The neuropathological features of the motor nerve biopsy of patients with PLMN are active axonal degeneration, focal fibers loss and low/absent axonal regeneration. On the contrary MN is marked by signs of demyelination/remyelination and a high number of regenerating clusters suggestive of axonal regeneration (Riva *et al*, 2011). Therefore, in selected cases, despite this being a surgical procedure, this investigation may be helpful for neurologist to establish an early diagnosis. In the last decades, it has been proved that neighbour non-motor neuron cells might be involved in ALS pathogenesis, thereby suggesting a concomitant presence of a non-cell autonomous mechanism in this disease

(Clement *et al*, 2003; Ilieva *et al*, 2009). As known, neurons are specialized and polarized cells, their soma dimension represents approximately 1% of the entire motor neuron volume, while the remaining part is constituted by the length of the axons (Riva *et al*, 2016). Indeed, the peripheral nervous system (PNS) with its cells and environment constitute an intriguing and alternative approach to explore the non-cell autonomous mechanisms in ALS. Most of the tissues, taken from patients affected by ALS, are collected post-mortem. These specimens have a reduced chance to be informative and prevent studies aimed at identifying the earliest pathogenetic mechanisms underlying this disease. As above-mentioned, in the last years motor nerve biopsy has been proved to be a reliable technique for an early diagnosis in ALS and it takes the chance to collect in-vivo human tissue to investigate the molecular pathways underlying ALS pathogenesis within the peripheral nervous system, using a transcriptomic and system biology approach. Unravelling the early events in the pathogenesis of ALS may be highly valuable in understanding the disease and developing novel candidate biomarkers.

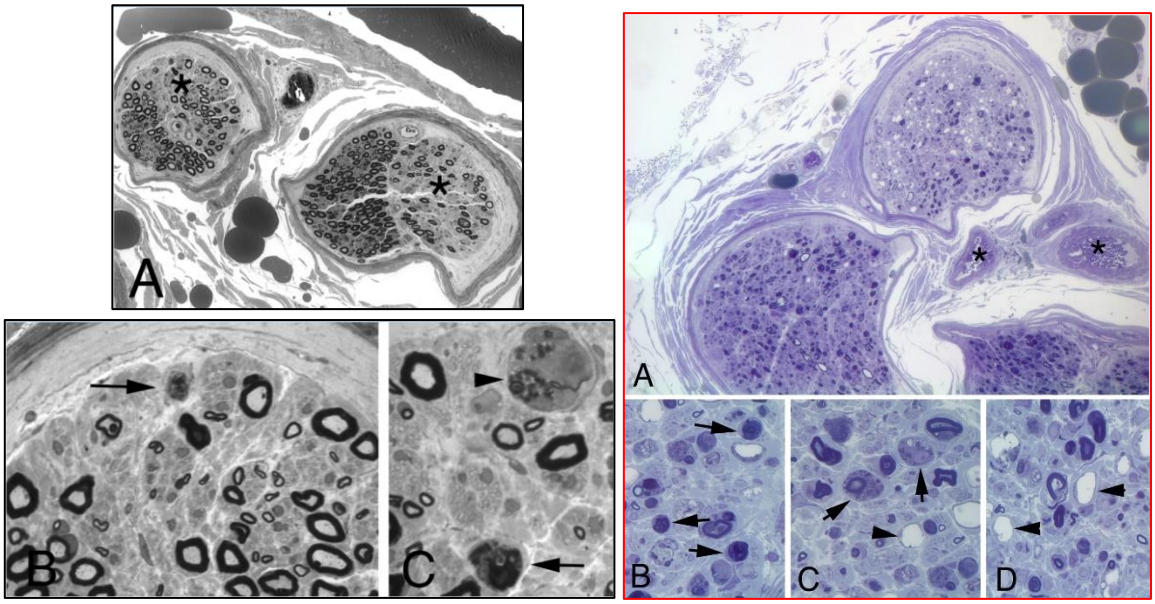

Figure 1.5. Similar neuropathological changes observed in motor and sensitive degenerating nerves. *Black boxes indicate degenerating features occurring in a patient with ALS while red box indicates degenerating features occurring in a patient with axonal sensory neuropathy. A black box) focal decreased density of myelinated nerve; B black box) arrows indicate axonal degeneration signs, namely ovoids, evident at the higher magnification; C black box) arrows indicate macrophages clearing axonal and myelin components evident with higher magnification. A red box) widespread decreased density of myelinated nerve; B red box) arrows indicate axonal degeneration signs, namely ovoids; C red box) arrows indicate macrophage; D red box) arrows indicate ghost fibers sign of axonal loss. Courtesy of A. Quattrini,*

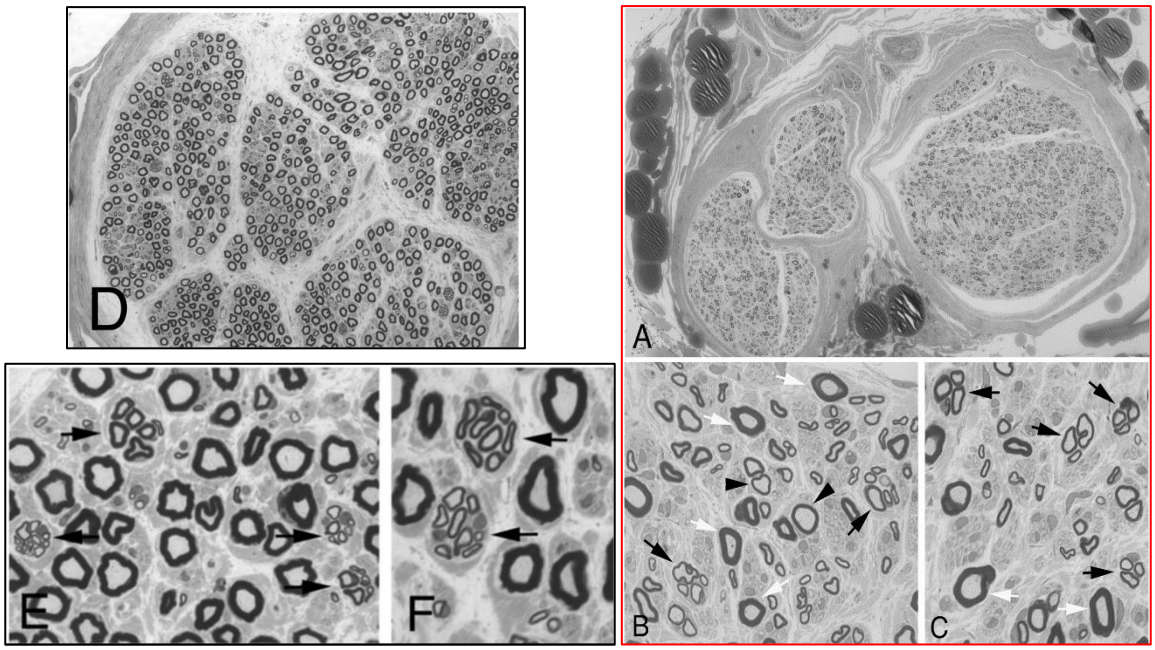

Figure 1.6. Neuropathological changes in regenerating motor and sensitive nerves. *Black boxes show a motor axonal regenerating nerve while red box show a sensory regenerating nerve. D) mild reduction of large myelin nerve fibers; E and F) arrows indicate abundant clusters of axonal regeneration evident with higher magnification; A) mild reduction of large myelin nerve fibers; B and C) arrows indicate abundant clusters of axonal regeneration evident with higher magnification. Courtesy of A. Quattrini.*

2.0 AIMS OF THE STUDY

As previously mentioned, the mainstay of the ALS diagnosis is the clinical evaluation, progressive upper and lower motor neuron dysfunctions have to be documented in absence of an alternative explanation for the presenting symptoms and signs (Brooks *et al*, 2000; de Carvalho *et al*, 2008). The diagnosis of ALS can be challenging when its first manifestations overlap with those of ALS mimic disorders and the lack of specific diagnostic testing prevents an early diagnosis (Falzone *et al*, 2020). The definition of prognosis in ALS is hampered by the heterogeneity of its clinical features, with variability in survival being the most salient feature (Verde *et al*, 2019). An accurate prediction of the individual outcome is crucial to establish early interventions as well as in clinical trials design (Falzone *et al*, 2021a; Gaiottino *et al*, 2013). Therefore, wet biomarkers are needed to aid clinical decision and achieve early diagnosis, track disease progression and better define disease trajectories. Consistent evidence supported NfL and pNfH, markers of axonal injury, as the most promising biomarkers for prognostic assessment in ALS (Falzone *et al*, 2020; Gaiottino *et al*, 2013; Lu *et al*, 2015; Wilke *et al*, 2016). Studies aimed at investigating the diagnostic applicability of NFs showed adequate diagnostic performance in distinguishing ALS from other neurodegenerative diseases, while not always satisfactory accuracy in discriminating ALS from its mimic disorders (ALSmd) has been obtained (Rossi *et al*, 2018; Gille *et al*, 2019; Verde *et al*, 2019; Feneberg *et al*, 2018). These diverging results may be due to the heterogeneity of ALSmd and the lack of specificity of NFs. Against this background, we perform this study with the following aims:

- To further explore the diagnostic and prognostic performance of serum pNfH and NfL currently considered the benchmark biomarkers
- To explore the serum NFs concentration across the ALS phenotypes, genotypes, and different cognitive status
- To correlate serum NFs concentration with several ALS clinical parameters
- To identify the differentially expressed genes (DEGs) on motor nerve biopsies from patients with ALS and MN by a gene expression analysis
- To perform pathway analysis on the DEGs and identify the up and downregulated pathways

- To select candidate biomarkers from the performed pathways analysis
- To test the diagnostic and prognostic performance of novel candidate biomarkers against NFs

3. RESULTS

3.1. Evaluation of serum pNfH concentrations in a large cohort of patients with ALS

3.1.1 ALS cohort, descriptive statistics

We explored serum pNfH in a large cohort consisted of two hundred and nineteen consecutive patients with ALS that were considered for the current study. Of them, eighty-seven were female and 132 were males. The demographics and the descriptive data of our cohort are reported in Table 3.1. The median serum pNfH concentration in our cohort was 174.3 pg/ml (IQR 40.1-363.3 pg/ml).

	ALS n = 219
Age at the serum sampling (years)	64.0 (57.0-71.0)
Diagnostic delay (months)	9.0 (6.0-15.0)
ALSFRS-R (points)	37.0 (32.0-42.0)
ΔALSFRS-R (points/month)	0.7 (0.4-1.2)
MRC score (points)	100.0 (84.0-111.0)
ΔMRC (points/month)	1.3 (0.6-2.5)
UMNs (points)	8.0 (3-11)
ECAS ALS SPECIFIC (points)	75.0 (56.0-85.0)
Total ECAS score (points)	99.0 (79.0-112.0)
Mean MEP/cMAP	0.2 (0.1-0.3)
Mean cMAP four limbs	6.0 (3.2-8.2)
C9orf72 expansion (no/yes)	199/20 (91.1%/8.9%)
Disease duration at the sampling (months)	14.0 (9.0-24.0)
Serum pNfH (pg/ml)	174.2 (40.1-363.6)

Table 3.1. Demographics and clinical characteristics of the ALS cohort. *Values are represented as numbers and percentages or median and interquartile ranges. M, male; F, female; ΔALSFRS-R, ALS functional rating scale progression rate; ΔMRC, medical Research council scale progression rate; ECAS, Edinburgh cognitive and behavioral ALS Screen; UMNs, upper motor neuron score; MEP/cMAP, motor evoked potential/compound muscle action potential; C9orf72, chromosome 9 open reading frame 72; pNfH, phosphorylated neurofilament heavy chain.*

pNfH concentration was not significantly influenced by the age at sampling as shown in figure 3.1 (p=0.452). Similarly, gender did not have any effect on the pNfH concentration as shown in Figure 3.1 (p=0.383).

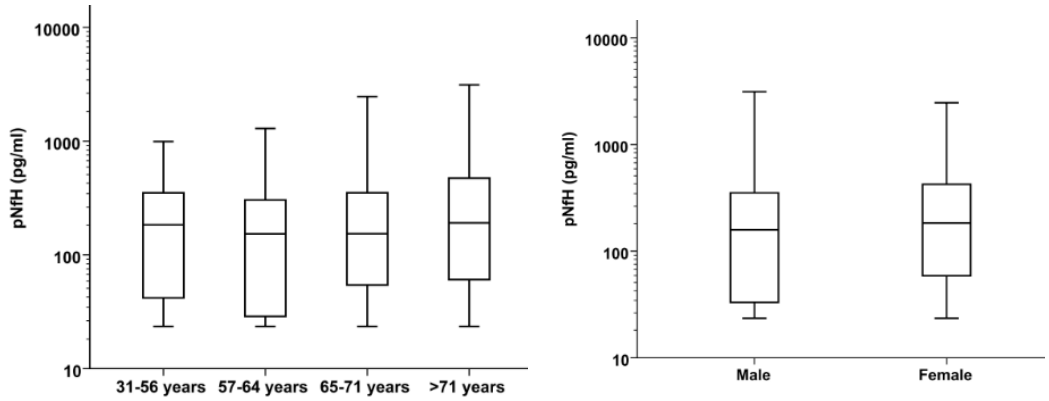

Figure 3.1. Serum pNfH concentrations among ALS patients grouped for age at sampling and gender. *Boxes indicate median pNfH concentration and interquartile range. Whiskers are the lowest and highest values. Biomarker levels are shown on a 10-logarithmic scale.*

3.1.2 Serum pNfH concentrations are different across ALS phenotypes

We explored the serum pNfH concentration across the ALS phenotypes evidencing a significant difference among the investigated groups, overall comparison p<0.001. Groups differences are shown in figure 3.2 while median concentrations and respective interquartile ranges are reported in Table 3.2.

Patients that were categorized as pyramidal, bulbar, and classic showed the highest serum concentration of pNfH. On the contrary, patients categorized with restricted and atypical phenotypes such as FA, PLMN, and PUMN showed the lowest concentration of serum pNfH. Focusing on single group differences, patients with pyramidal phenotype showed significant higher pNfH concentrations in comparison with PUMN (p<0.001) and PLMN (p<0.001); Patients with bulbar phenotype manifested significant higher pNfH concentration when compared with PUMN (p=0.023), PLMN (p<0.001) and FA (p=0.032); Classic had significantly higher levels in comparison with PUMN (p=0.01) and PLMN (p=0.04).

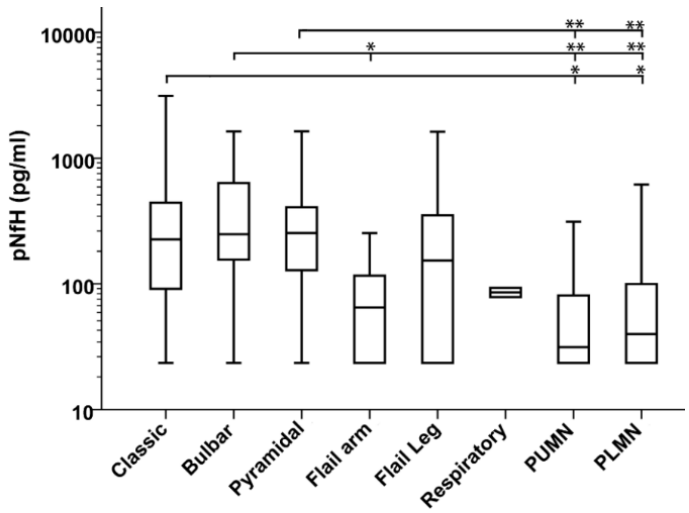

Figure 3.2 Serum pNfH concentrations comparison among ALS phenotypes. *Boxes indicate median pNfH concentration and interquartile ranges. Whiskers are the lowest and highest values. pNfH concentrations are shown on a 10-logarithmic scale. * p value<0.05; ** p value<0.01. Patients were categorized accordingly to previously published criteria(Chio et al, 2011).*

ALS phenotypes	**n**	**Serum pNfH (pg/ml)**
Classic	82 (37.4%)	226.2 (89.6-449.5)
Bulbar	31(14.2%)	248.2 (153.0-651.6)
Pyramidal	31 (14.2%)	254.3 (108.4-407.6)
FA	10 (4.6%)	70.6 (23.5-120.4)
FL	30 (13.7%)	153.4 (23.5-351.1)
Respiratory	2 (0.9%)	85.8 (-)
PLMN	23 (10.5%)	40.0 (23.5-112.2)
PUMN	10 (4.6%)	32.7 (23.5-127.6)

Table 3.2 Serum pNfH concentrations across ALS phenotypes. *Serum pNfH concentrations are reported as median values, the interquartile range is in brackets. Abbreviations: pNfH, phosphorylated neurofilament heavy chain; FA, flail arm; FL, flail leg; PLMN, pure lower motor neuron; PUMN, pure upper motor neuron.*

3.1.3. Patients with the C9orf72 hexanucleotide expansion have higher serum pNfH concentration

In our ALS cohort, twenty patients carried the hexanucleotide repeat expansion in the *C9orf72* gene (9.1%), four harbored pathogenic mutations in *TARDBP* (1.8%), and two in *SOD1* (1%). *C9orf72*-ALS showed significantly higher pNfH concentrations compared with patients harboring no genetic mutation (p=0.011) as shown in Figure 3.3. *TARDBP* and *SOD1* carriers were not compared with the apparent sporadic ALS in consideration of their small sample sizes.

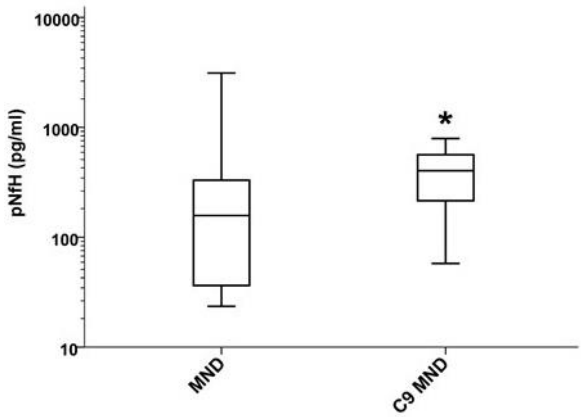

Figure 3.3. Serum pNfH differences between C9orf72 carriers and non-carriers. *Boxes indicate median pNfH concentration and interquartile range. Whiskers are the lowest and highest values. pNfH concentrations are shown on a 10-logarithmic scale. * p value<0.05*

3.1.4. Serum pNfH concentrations did not differ across ALS cognitive phenotypes

One hundred and eighteen (53.9%) patients with ALS were screened and consequently assessed with a thorough neuropsychological evaluation to detect the presence of a concomitant cognitive impairment. Patients with ALS manifesting the co-occurrence of a cognitive/behavioral or FTD had higher serum pNfH concentration, however, the difference did not reach the statistical significance as shown in Figure 3.4. The median concentrations of each cognitive category with respective interquartile ranges are reported in Table 3.3.

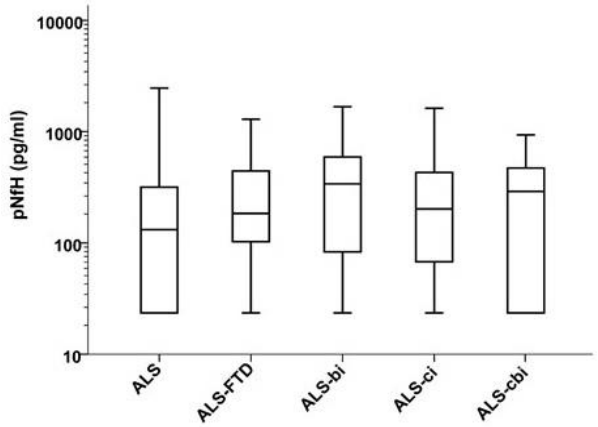

Figure 3.4. Serum pNfH concentrations comparison among ALS cognitive phenotypes. *Boxes indicate median pNfH concentrations and interquartile ranges. Whiskers are the lowest and highest values. pNfH concentrations are shown on a 10-logarithmic scale.*

ALS Cognitive	**n**	**Serum pNfH (pg/ml)**
ALS motor	52 (44.1%)	131.6 (23.5-318.7)
ALS-FTD	21 (17.8%)	184.1 (90.4-523.7)
ALS-bi	11 (9.3%)	339.2 (74.2-688.0)
ALS-ci	24 (20.3%)	202.3 (66.9-435.3)
ALS-cbi	10 (8.5%)	292.3 (23.5-586.8)

Table 3.3. pNfH values across ALS cognitive phenotypes*. pNfH concentration is given in median values and interquartile range between brackets. pNfH, phosphorylated heavy chain; Frontotemporal dementia (FTD) (ALS-FTD); behavioral impairment (ALS-bi); cognitive impairment (ALS-ci); combined cognitive and behavioral impairment (ALS-cbi).*

3.1.5. Serum pNfH concentration correlates with the disease progression rate, UMN burden and disease duration

We performed a univariate correlation analysis between serum pNfH concentrations and several clinical parameters. pNfH concentrations moderately correlated with the disease progression rate measured with ΔALSFRS-R (r=0.317, <0.001). pNfH correlated with the clinical and neurophysiological UMN burden measures, a moderate inverse correlation was observed with MEP/cMAP 4 limbs (r=-0.342, <0.001) and a weak

correlation with the UMNs (r=0.201, *p*=0.003). A further weak inverse correlation was observed between pNfH and the disease duration at serum sampling (r=-0.204, *p*=0.002) and with the diagnostic delay (r=0.173, *p*=0.01). All the correlations between pNfH concentration and the ALS clinical parameters that were performed are reported in Table 3.4.

	Pearsoncorrelation, r	**p value**	**Case number**
Age at sampling	0.092	0.173	219
Disease duration at sampling	- 0.204	**0.002**	219
Diagnostic delay	-0.173	**0.01**	219
MRC	- 0.019	0.786	219
UMNs	0.201	**0.003**	219
ALSFRS	- 0.178	**0.009**	219
ΔALSFRS-R, progression rate	0.317	**<0.001**	219
ECAS specific score	0.031	0.759	118
ECAS total score	- 0.027	0.778	118
BMI	- 0.132	0.102	155
MEP/cMAP 4 limbs	- 0.342	**<0.001**	130
cMAP 4 limbs	- 0.126	0.128	148

Table 3.4. Correlation between serum pNfH and ALS clinical parameters. *MRC, medical research council UMNs, upper motor neuron score; ALSFRS, amyotrophic lateral sclerosis function rating scale; ECAS, Edinburgh Cognitive and Behavioural ALS Screen; BMI, body mass index; MEP/cMAP, motor evoked potential/compound muscle action potential. In bold are reported the significant correlations.*

3.1.6. Disease progression rate and the UMN burden are the main determinants of serum pNfH variability

A hierarchical multiple regression analysis was performed including the clinical parameters that significantly correlated with pNfH at the univariate analysis. Therefore,

disease progression rate, UMNs, MEP/cMAP 4 limbs, disease duration at sampling and the diagnostic delay were included in the multivariate model. We excluded one of the two measures of UMN burden due to their high collinearity, therefore only the MEP/cMAP 4 limbs was considered in the model due to its higher correlation with pNfH compared to UMNs. The model including the above-mentioned variables, with the exclusion of UMNs, was significant R^2 =0.188; p <0.001. According to the multivariate model, disease progression rate and MEP/cMAP 4 limbs were the most significant variables in explaining approximately 18.8% of serum pNfH concentration variability. All the variables included in the model with their respective p value and beta value are reported in Table 3.5.

	β	***p* value**	**model *p* value**	**R**	**R^2**
MEP/cMAP 4 limbs	- 0.272	**0.002**	**<0.001**	0.433	0.188
ΔALSFRS-R, disease progression rate	0.246	**0.017**			
Diagnostic delay	0.059	0.625			
Disease duration at sampling	- 0.060	0.623			

Table 3.5. Multivariate hierarchical regression model to detect the serum pNfH determinants. Abbreviations: MEP/cMAP, motor evoked potential/compound muscle action potential; ALSFRS, amyotrophic lateral sclerosis function rating scale. Bold indicates statistical significance.

3.1.7. Serum pNfH significantly stratify ALS survival

We assessed the performance of serum pNfH concentration in stratifying patients' survival. Survival was defined as the time occurring between serum sampling and death or tracheostomy. ALS patients were divided in four groups accordingly with serum pNfH concentrations. Patients with the lowest serum pNfH were grouped in the first quartiles. The Kaplan-Meier survival analysis showed a significant stratification of the cumulative survival, Log-rank test (Mantel-Cox), X^2=53.0, p<0.0001 (Figure 3.5). The median survival of the first quartile was 33.0 months (95% CI 16.1-49.9 months) while the fourth quartile had a median survival of 9.0 months (95% CI 7.0-11.0 months). Median survival of each quartile is reported in Tables 3.6.

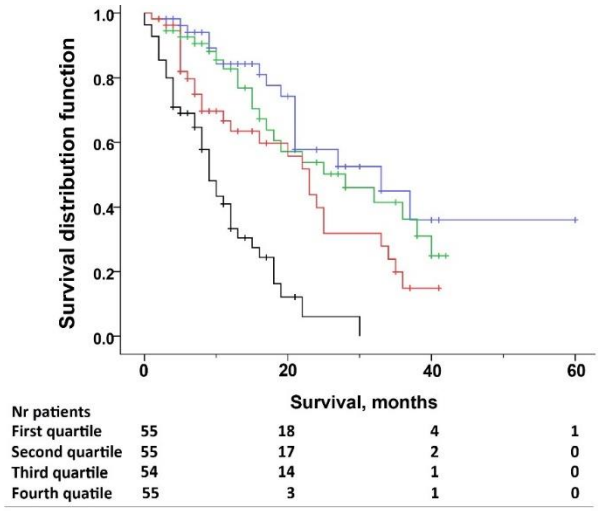

Figure 3.5. Kaplan-Meier cumulative survival in patients with ALS grouped accordingly with serum pNfH concentrations*. Blue line indicates first quartile, green line indicates second quartile, red line indicates third quartile and black line indicates the fourth quartile.*

Serum pNfH concentration	**Median survival (m)**	**95% C.I.**
First quartile, 23.5 – 40.1 pg/ml	33.0	16.05-49.95
Second quartile, 40.08 – 174.3 pg/ml	28.0	13.42-42.58
Third quartile, 174.4 – 363.6 pg/ml	23.0	18.58-27.42
Fourth quartile, > 363.6 pg/ml	9.0	7.02-11.10
ALS cohort	20.0	16.88-23.12

Table 3.6. Median survival in patients with ALS grouped accordingly to serum pNfH concentration. *Abbreviations: pNfH, phosphorylated neurofilament heavy chain; ALS, amyotrophic lateral sclerosis, C.I, confidence interval.*

To further assess the effect of the pNfH on ALS patients' survival, we performed an additional Kaplan-Meier analysis considering the time to King's stage four as survival event. Time to King's stage four was defined as the time occurring between serum collection and feeding or respiratory failure. Kaplan-Meier curves showed a significant stratification of patient's cumulative survival, Log-rank (Mantel-Cox) $X^2=68.1$, $p<0.0001$ (Figure 3.6).

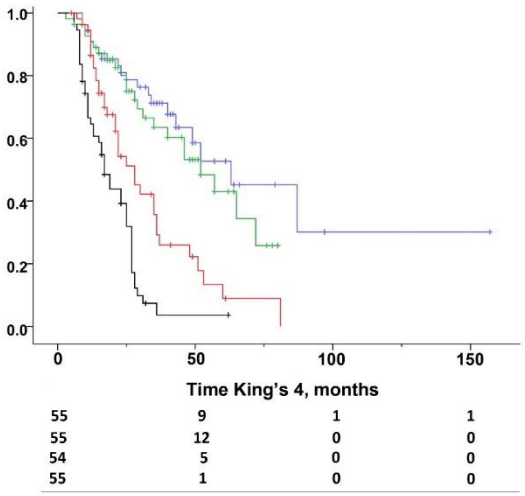

Figure 3.6. Kaplan-Meier time to King's stage 4 in patients with ALS grouped accordingly with serum pNfH concentrations*. The blue line indicates first quartile, green line indicates second quartile, red line indicates third quartile and black line indicates the fourth quartile.*

3.1.8. Serum pNfH is an independent determinant for survival in ALS

We performed a multivariate survival analysis carrying out a Cox regression model to further explore the role of pNfH in determining ALS prognosis. Along with serum pNfH, we included several ALS clinical parameters, known to impact negatively on ALS survival, in the model: serum pNfH concentrations (divided in quartiles); disease duration at sample collection in months (dichotomized accordingly with median value); age at the serum sample in years (dichotomized accordingly with median value); diagnostic delay in months (dichotomized accordingly with median value); disease progression rate measured with the ΔALSFRS-R (dichotomized accordingly with median value); concomitant presence of dementia (yes or no); detection of *C9orf72* expansion (yes or no); ALS phenotype (grouped based on median survival of each phenotype).

Serum pNfH concentration was one of the significant independent prognostic factors of survival at the Cox regression analysis, overall significance $p<0.001$. ALS patients

showing pNfH levels higher than 363.6 pg/ml (fourth quartile) had a proportional hazard ratio (HR) of 3.67 (95% CI 1.96-6.90) compared with the first quartile group to reach the survival event. Faster disease progression rate and older age at serum sample were the other two independent negative prognostic factor in our cohort. As expected, we also confirmed that serum pNfH is a significant independent negative prognostic factor for ALS survival (defined as the time from serum sample and King's stage 4) at the Cox regression analysis. All the variables included in the models with respective HR and *p* value are reported in Table 3.7.

Prognostic factor	Survival		Time to King's stage 4	
	HR (95% CI)	***p* value**	**HR (95% CI)**	***p* value**
pNfH (pg/ml)		**<0.001**		**<0.001**
First quartile	1		1	
Second quartile	1.27 (0.66-2.43)	0.480	1.41 (0.75-2.63)	0.288
Third quartile	1.55 (0.83-2.89)	0.167	2.45 (1.37-4.39)	**0.003**
Fourth quartile	3.67 (1.96-6.90)	**<0.001**	3.55 (1.97-6.37)	**<0.001**
Disease duration at the serum sample (months)				
>14	1		1	
≤14	1.45 (0.88-2.42)	0.148	1.74 (1.09-2.77)	**0.021**
Diagnostic delay (months)				
≤ 9	1.04 (0.66-1.64)	0.874	1.23 (0.82-1.84)	0.321
> 9	1		1	
Disease progression rate (points/month)				
≤ 0.74	1		1	
> 0.74	2.80 (1.74-4.50)	**<0.001**	4.32 (2.74-6.82)	**<0.001**
Dementia				
No	1		1	
Yes	1.61 (0.92-2.82)	0.095	1.18 (0.70-1.99)	0.542
C9orf72 expansion				
No	1		1	
Yes	1.30 (0.62-2.69)	0.488	1.16 (0.59-2.27)	0.662
Age at the venipuncture (years)				
31-64	1		1	
> 64	1.70 (1.13-2.57)	**0.011**	1.62 (1.11-2.36)	**0.013**
ALS phenotype		0.058		**0.006**
PUMN/PLMN/FA	1		1	
CL/PY/FL	2.43 (1.16-5.09)	**0.019**	2.15 (1.10-4.20)	**0.025**
B/R	2.51 (1.07-5.91)	**0.035**	3.61 (1.63-8.00)	**0.002**

Table 3.7. Multivariate Cox regression analysis on survival. *Abbreviations: pNfH, phosphorylated neurofilament heavy chain; ALS, amyotrophic lateral sclerosis; PUMN, pure upper motor neuron; PLMN, pure lower motor neuron; FA, flail arm; CL, classic; PY, pyramidal; FL, flail leg; B, bulbar; R, respiratory; HR, hazard ratio. Bold indicates statistical sigficance.*

3.2. Gene expression and pathway analyses to identify candidate biomarkers for ALS.

3.2.1 Gene expression analysis in motor and sensitive nerve biopsies

We performed a gene expression analysis on eight obturator motor nerve biopsies taken from patients with ALS and seven taken from patients affected by MN. The demographics and clinical data of the enrolled patients are reported in Table 3.8.

Age	Gender	Neuropathological diagnosis	Disease duration (m)	Symptoms onset	Treatment
69.0	M	Definite-MN	11	LL, as	IVIG
68.0	M	Probable-MN	30	LL, s	Steroids
76.0	M	Probable-MN	12	LL, as	Steroids
73.0	F	Probable-MN	12	LL, s	None
39.0	F	Definite-MN	108	LL, as	IVIG and RTX
47.0	F	Definite-MN	56	LL, s	AZA
32.0	F	Definite-MN	7	LL, s	IVIG and RTX
44.0	M	Probable-ALS	49	LL, as	Riluzole
44.0	M	Probable-ALS	12	LL, as	Riluzole
50.0	M	Probable-ALS	12	LL, as	Riluzole
66.0	M	Probable-ALS	25	LL, s	Riluzole
57.0	M	Probable-ALS	36	LL, as	Riluzole
69.0	F	Probable-ALS	10	LL, as	Riluzole
67.0	M	Probable-ALS	10	UL, as	Riluzole
63.0	M	Probable-ALS	12	LL, s	Riluzole

Table 3.8. Clinical and histopathological characteristic of the ALS and MN patients enrolled for the gene expression analysis. *The neuropathological diagnosis was established according to previous published criteria (Riva et al, 2011). Disease duration was defined as time between symptoms onset and the nerve biopsy. Abbreviations: M, male; F,female; MN, motor neuropathy; ALS, amyotrophic lateral sclerosis; LL, lower limbs; UL, upper limbs; as, asymmetric; s, symmetric; IVIG, intravenous immunoglobulin; RTX, rituximab; AZA, azathioprine.*

Gene expression analysis through Limma methods identified 3669 DEGs; of them 2177 were upregulated in patients with ALS and the remaining 1492 DEGs were downregulated. We performed a further gene expression analysis on five sural nerve

biopsies showing neuropathological acute axonal degeneration signs in absence/or minimal presence of regenerating features (resembling the neuropathological conditions of ALS) and four with clear regenerating signs with absent or low degenerating features (resembling the neuropathological conditions of MN). We obtained 3522 DEGs from the second analysis, 2022 DEGs were upregulated in degenerating sural nerves while 1500 DEGs were downregulated. Subsequently, we merged the DEGs that were obtained by the two independent analysis and the genes that resulted shared by both analyses were filtered out. Therefore, 1083 DEGs were eliminated because they might be representative of the biological axonal degeneration/regeneration process instead of being specific of the pathogenic mechanisms of ALS (Figure 3.7). Of the remaining 2586 DEGs, 1587 were upregulated and 999 downregulated in patients with ALS.

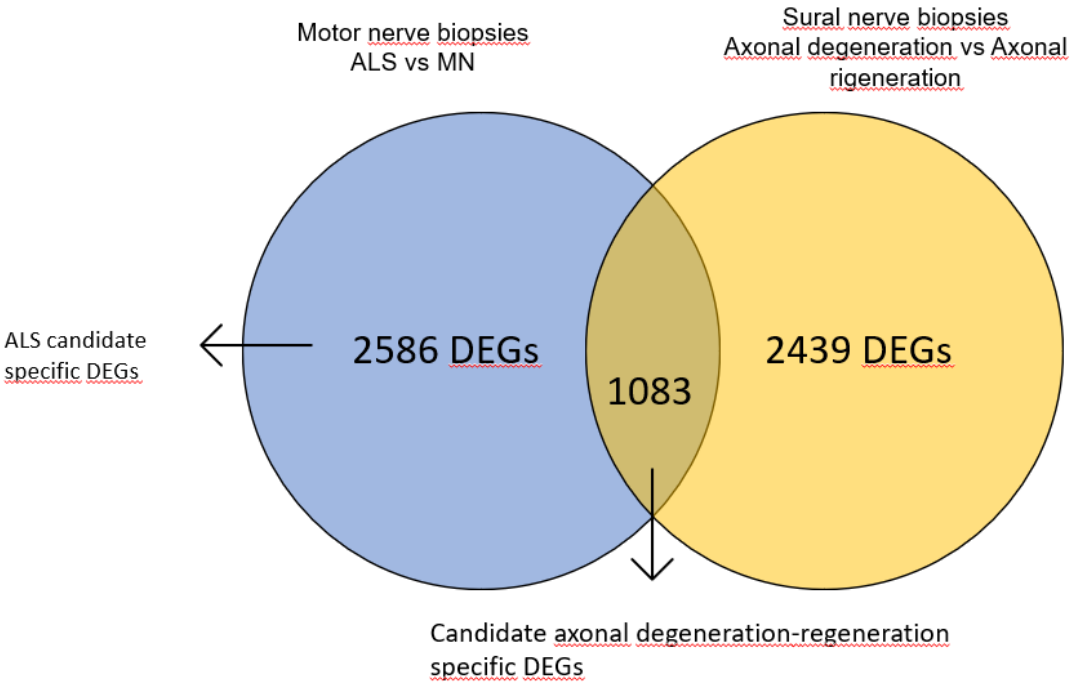

Figure 3.7. Total of differentially expressed genes (DEGs) and shared DEGs that were obtained in the two independent analysis. *Abbreviations: ALS, amyotrophic lateral sclerosis; MN, motor neuropathies.*

3.2.2. Pathway analysis

The identified differentially expressed genes were categorized into functional pathways according to Reactome and Gene Ontology (GO) databases. The analysis was perfomed using WebGestalt database(Wang *et al*, 2013). We assessed the upregulated and downregulated DEGs in two independent analyses. Functional enrichment analysis performed with GO detected four significantly enriched upregulated pathways, and two significant pathways were identified consulting the Reactome database. Conversely, no significant downregulated pathways were identified with both databases. The top upregulated pathways are reported in Table 3.8 and 3.9.

Pathway	**ID**	**Expected**	**Ratio**	**Adj.p**	**FDR**
ncRNA processing	GO:0034470	24.5	2.1	<0.001	<0.001
tRNA metabolic process	GO:0006399	12.4	2.3	<0.001	0.004
Cellular response to topologically incorrect protein	GO:0035967	10.2	2.5	<0.001	0.02
Response to endoplasmic reticulum stress	GO:0030968	18.1	2.0	<0.001	0.04

Table 3.8. Top upregulated pathways in ALS according to GO biological process database. *The statistical analysis was performed using WebGestalt. GO, gene ontology; ratio is obtained dividing the observed genes and the expected genes. Abbreviations: ID, identification code; Adj.p, adjusted p value; FDR, false discovery rate.*

Pathway	**ID**	**Expected**	**Ratio**	**Adj.p**	**FDR**
Cytosolic tRNA aminoacylation	R-HSA-379716	1.8	3.1	<0.001	0.04
Unfolded protein response (UPR)	R-HSA-381119	3.1	3.5	<0.001	0.05
Post translational protein modification	R-HSA-597592	3.9	3.9	<0.001	0.08

Table 3.9. Top upregulated pathways in ALS according to Reactome database. *The statistical analysis was performed using WebGestalt. GO, gene ontology; Ratio is obtained dividing the observed genes and the expected genes. Abbreviations: ID, identification code; Adj.p, adjusted p value; FDR, false discovery rate.*

3.2.3. Selection of the candidate genes

We focused our attention on the genes that resulted upregulated in the gene expression analysis and annotated in the significant upregulated pathways. Consequently, we studied the encoded proteins of the selected genes. Of these proteins, we evaluated several parameters aimed at identifying a candidate biomarker for ALS. We evaluated for each

protein the expression levels in the central and peripheral nervous systems and other tissues; if the protein was specifically expressed in the CNS/PNS; if the protein was expressed in neurons or other cells; the protein subcellular localization: We evaluated also the presence of previous studies demonstrating the role of the protein in any neurodegenerative diseases.

Our investigation aimed at identifying a protein with a promising profile for a biomarker, thereby one that is selectively and highly expressed in axons or neurons, mainly localized in the cytosol or plasma membrane of neurons and previously documented as involved in some neurodegenerative diseases or ALS. Considering the above-mentioned parameters, we focused our attention on Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1). UCHL1 is a neuron-specific protein mainly expressed in its cytoplasm and involved in the ubiquitin pathway as deubiquitinating enzyme (Genç *et al*, 2015; Liu *et al*, 2002). However, several other functions have been related to UCHL1: it is essential for axonal repair, it interplays with neuronal cytoskeleton proteins, it is involved in axonal transport and in maintaining axonal integrity (Bheda *et al*, 2010; Liu *et al*, 2015; Pukaß & Richter-Landsberg, 2015). Furthermore, UCHL1 is thought to be involved in the pathogenesis of several neurodegenerative diseases and it has been found raised in CSF of patients with ALS, in two different proteomics studies (Setsuie & Wada, 2007; Oeckl *et al*, 2020). Considering these findings UCHL1 has been proposed as a potential novel candidate biomarker for ALS. Therefore, we decided to assess the diagnostic and prognostic performance of UCHL1 in the serum of patients with ALS benchmarking its performances against NfL. To assess UCHL1 serum levels we used an ultrasensitive SIMOA four-plex platform which allowed us to measure NfL, Glial fibrillary acidic protein (GFAP) and Total tau protein (tTAU) at the same time.

3.3. Evaluation of UCHL1, NfL, GFAP and tTAU in ALS and control cohorts

3.3.1 UCHL1 concentrations are not elevated in ALS compared to the disease control cohorts.

In this analysis we assessed a panel of serum neurochemical biomarkers, namely NfL, ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1), Glial fibrillary acidic protein (GFAP) and total tau protein (t-TAU) in a large ALS and control cohorts. We

measured the above-mentioned serum biomarkers concentration in one hundred and forty-three patients with ALS and a control cohort consisting of forty-five healthy controls (HC), seventy patients affected by other neurodegenerative disease (DEG) and seventy patients affected by ALS mimic disorders (ALS-md). ALS and the control groups were age (p=0.27) and sex matched (p=0.89). The demographics and the clinical characteristics of the ALS and control cohorts are reported in Table 3.10 and 3.11. Serum biomarkers groups comparison are reported in Table 3.11.

	ALS n = 143
Gender, M/F	87/56
Age at the serum sampling (years)	64.0 (55.0 - 72.0)
Disease duration at the sampling (months)	9.0 (7.0-12.0)
Diagnostic delay (months)	7.0 (5.0 - 10.0)
ALSFRS-R (points)	41.0 (36.0 - 44.0)
ΔALSFRS-R (points/month)	0.9 (0.5 - 1.5)
MRC total score (points)	106.0 (94.8 - 114.0)
UMNs (points)	8.0 (4.0 – 11.0)
ECAS ALS SPECIFIC (points)	71.0 (56.0 - 84.0)
Total ECAS score (points)	93.0 (76.0 - 109.0)
Mean MEP/cMAP	0.2 (0.1-0.3)
Mean cMAP four limbs	6.0 (3.2-8.2)
C9orf72 expansion (no/yes)	123/20 (84.0%/16.0%)

Table 3.10. ALS group demographics and clinical characteristics. *Given numbers are median values and interquartile range and total cases with percentage. Abbreviations: ALS, amyotrophic lateral sclerosis; M, male; F, female; ΔALSFRS-R, ALS Functional Rating Scale progression rate; ΔMRC, Medical Research Council Scale progression rate; ECAS, Edinburgh Cognitive and Behavioural ALS Screen; UMNs, upper motor neuron score; MEP/cMAP, motor evoked potential/compound muscle action potential, C9orf72, chromosome 9open reading frame 72.*

Study group	ALS (n=143)	HC (n=45)	DEG (n=70)	ALSmd (n=70)
Gender, M/F	87/56	25/20	42/28	44/26
Age	64.5 (54.8–72.2)	62.0 (58.0 – 70.0)	66.0 (61.8 – 72.3)	68.0 (54.8 – 77.0)
NfL	112.1 (70.8–184.9)	14.1 (10.9–19.2)**	24.5 (15.8–37.4)**#	27.6 (16.9–53.0)**##
UCHL1	41.9 (28.0-62.8)	25.8 (17.2-36.2)**	49.7 (26.3–62.9)##	44.5 (24.5 -67.4)##
GFAP	131.4 (92.6-173.4)	125.9 (84.2-156.5)	206.2 (118.5–277.2)**##	149.8 (96.0–212.3)
tTAU	0.8 (0.5-1.3)	0.9 (0.7-2.0)	1.2 (0.4–1.8)**	0.6 (0.3–1.1)§

Table 3.11. Demographic characteristics and biomarkers serum concentrations in the study groups. *Median and interquartile range in brackets are given. NfL, neurofilament light chain; UCHL1, ubiquitin C-terminal hydrolase L1; GFAP, glial fibrillary acidic protein; tTau, total tau protein; ALS, amyotrophic lateral sclerosis; HC, healthy controls; DG, neurodegenerative disorders; ALSmd, ALS mimic disorders. *p < 0.05 vs ALS; **p < 0.01 vs ALS; # p < 0.05 vs HC; ## p < 0.01 vs HC; § p < 0.05 vs DEG; §§ p < 0.01 vs DEG.*

Serum NfL levels were significantly elevated in the ALS group compared with HC, DEG and ALSmd cohorts (p<0.01) (Table 3.11). UCHL1 was significantly higher in ALS compared with HC (p<0.01), however the concentrations were not different between ALS and the remaining control groups. GFAP was significantly raised in the DEG groups compared to the ALS and HC groups (p<0.01). tTAU as significantly elevated in the serum of DEG compared with ALS (p<0.01) and ALSmd groups (p<0.05). Serum biomarkers median levels and interquartile range (IQR) of ALS and control cohorts are summarized in Table 3.11 and Figure 3.8. Univariate Pearson's pairwise analysis identified a weak correlation between NfL and tTAU (r=0.19; p=0.02;), and a moderate correlation between NfL and GFAP (r=0.39; p<0.001). UCHL1 showed a weak correlation with GFAP (r=0.18; p=0.03) and tTAU (r=0.18; p=0.03).

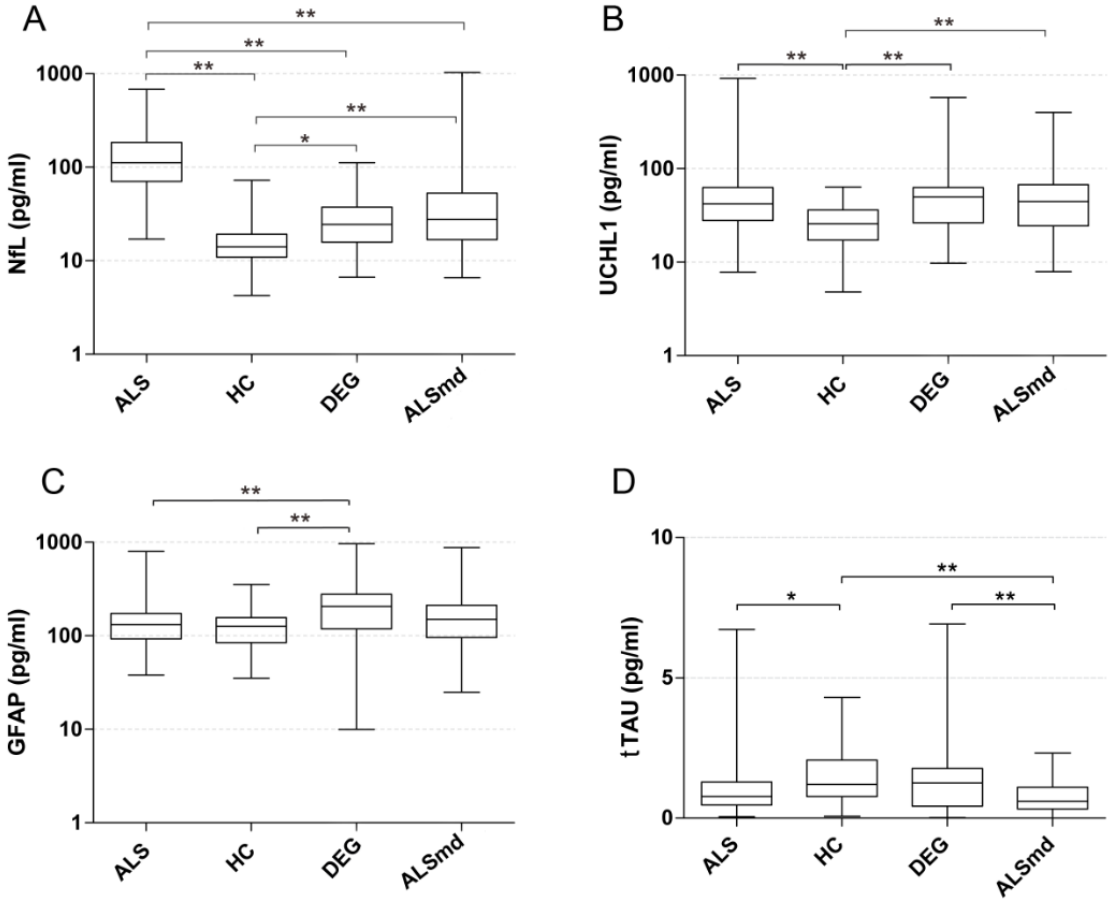

Figure 3.8. Panel of serum biomarkers concentration among different study groups. *Abbreviations: ALS, amyotrophic lateral sclerosis; HC, healthy control; DEG, other neurodegenerative disorder; ALSmd, ALS mimic disease; NfL, neurofilament light chain; UCHL1, ubiquitin C-terminal hydrolase L1; GFAP, glial fibrillary acidic protein; tTau, total tau protein. ** indicates significance <0.01; * indicates significance <0.05.*

3.3.2 Correlation between ALS clinical parameters and serum biomarkers concentrations

We performed a correlation among the serum biomarkers levels and several ALS clinical characteristics and functional parameters of disease progression. In addition, to further assess the relation among the clinical parameters and the biomarkers, we performed a group differences analysis sub-grouping the clinical variables accordingly with tertile values or normal/impaired as appropriate. NfL correlated with both clinical (r=0.31; *p*=0.02) and neurophysiological (r=-0.33; *p*=0.01) measure of UMN burden, ALS patients with an impaired MEP/cMAP ratio had significant higher concentration of NfL compared with those that had normal MEP/cMAP ratio. Coherently, the same result was obtained with the UMNs, patients who obtained a score higher than six points had an elevation of the NfL compared to those that had less than six points. Lastly, we observed a moderate correlation of NfL with disease progression rate (r=0.47; p<0.001), patients with higher disease progression rate showed an elevation of serum NfL concentrations

UCHL1 weakly correlated with the age at sampling (r=0.21; p=0.03), ALS patients that were sampled at older age than 69 years had significant higher levels of UCHL1 compared with younger patients. UCHL1 weakly correlated with the clinical measure of UMN burden (r=0.22; p=0.02) but not with the neurophysiological measure. ALS patients with six or higher points at the UMNs evaluation showed an elevation of UCHL1 compared to those with less than six points.

GFAP correlated with the age at sampling (r=0.42; p<0.001), ALS patients that were sampled at older age than 69 years had significant higher levels of GFAP compared with younger patients. GFAP inversely correlated with ECAS total score (r=-0.40; p<0.001). ALS patients with an impaired score at the cognitive screening test had significant higher GFAP concentrations compared with those who obtained a normal score. Lastly. GFAP correlated with the disease progression rate (r=0.27; p=0.01), ALS patients with ΔALSFRS higher than 1.22 showed a significant elevation of GFAP compared with the first tertile group.

tTAU did not correlated with any ALS clinical variables. All the groups differences that were perfomed are reported in Table 3.12.

		NfL	UCHL1	GFAP	TAU
Age at VP	35 - 39	126.9 (76.0–188.8)	38.0 (20.5-56)	99.0 (68.5-117.3)	0.6 (0.3-1.2)
	60 - 69	95.2 (64.2-137.7)	39.3 (27.4-61.0)	131.4 (98.5-160.9)	0.7 (0.5-1.3)
	> 69	135.4 (80.5-285.2)	51.2 (35.2 74.7)*	176.8 (132-285)**#	0.9 (0.6-1.5)
Disease Duration at VP	< 8	135.4 (83.2-213.5)	40.3 (26.1-54.8)	132.8 (98.9-180.1)	1 (0.6-1.4)
	8 -11	95.8 (63.2-184.7)	40.1 (29.3-64.5)	117.6 (89.9-166.3)	0.6 (0.4-1)
	> 12	106.5 (73.4-114.0)	49.7 (34.0-72.6)	133.4 (85.7-171.0)	0.8 (0.5-1.5)
UMNs	< 5	86.7 (57.1-118.6)	35.2 (22.7-58.5)	113.1 (75.8-151.8)	0.6 (0.4-1.1)
	6 -10	121.5 (77.5-251.7)**	46.3 (33.0-67.4)**	141.3 (93.9-176.8)	0.8 (0.5-1.5)
	> 11	156.2 (109.8-232.6)**	45.2 (27.3-69.1)**	143.5 (100-227)*	0.9 (0.5-1.3)
ΔALSFRS	< 0.63	79.0 (52.1-112.6)	35.5 (24.6-66.5)	116.7 (81.2-145.5)	0.6 (0.4-1.3)
	0.64 – 1.22	112.1 (81.0-162.6)**	42.2 (28.2-59.4)	116.6 (79.5-174.4)	0.7 (0.5-1.1)
	> 1.22	174.0 (104.3-322)** #	46.7 (33.3-64.9)	159.4 (110-226)*#	0.8 (0.6-1.4)
ECAS	impaired	152.6 (92.5-236.7)	41.9 (25.2-65.6)	165.8 (104.7-214.1)	0.9 (0.7-1.3)
	normal	114.8 (71.6-297.9)	49.4 (29.0-77.7)	111.6 (80.8-161.0)*	0.6 (0.5-1.5)
MEP/ cMAP 4 limbs	normal	87.9 (63.2-156.5)	42.9 (28.5-64.5)	133.1 (87.4-176.7)	0.7 (0.4-0.9)
	Impaired	166.0 (94.8-285.2)*	39.0 (27-74.7)	132.8 (91.9-173.4)	0.9 (0.5-1.3)

Table 3.12. Biomarkers level in different ALS subgroups*. ALS clinical variables were divided accordingly with tertile values or in normal and impaired as appropriate. Given are median values and interquartile range. Abbreviations: VP, venipuncture; UMN, upper motor neuron score; ΔALSFRS-R, ALS functional rating scale progression rate; ECAS, Edinburgh Cognitive and Behavioural ALS Screen; mean MEP/cMAP, motor evoked potential/compound muscle action potential. * indicates statistical significance compared with the first category of each variable (p<0.05); ** indicates statistical significance compared with the first category of each variable (p<0.01); # indicates statistical significance compared with the second category of each variable (p<0.05); ## indicates statistical significance compared with the second category of each variable (p<0.05)*

3.3.3 NfL concentrations differ across the ALS phenotypes

We explored biomarkers serum concentration across the ALS phenotypes. Higher NfL levels were detected in pyramidal, classic, and bulbar patients compared with atypical or restricted phenotypes such as FA, FL, pure PLMN and PUMN. FL and FA were grouped

together as well as PLMN and PUMN due to the small sample size of the single categories and their clinical similarities. We observed that classic and pyramidal phenotypes had significant higher levels of NfL compared with FA/FL and PUMN/PLMN. All the groups comparison with statistical significance are shown in Figure 3.9 and Table 3.13. Conversely, UCHL1, GFAP and tTAU showed homogenous levels regardless of the ALS phenotypes (Table 3.13).

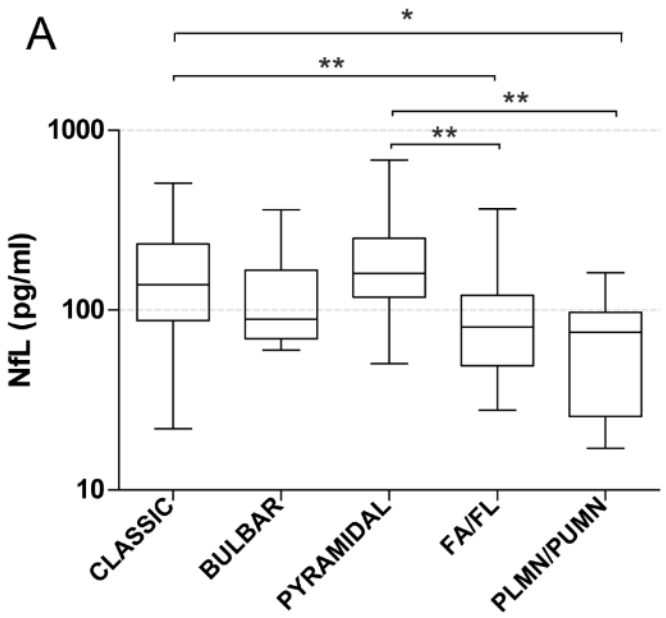

Figure 3.9. NfL serum concentrations across the ALS phenotypes. *Boxes are median and interquartile range. Whiskers are highest and lowest values. Biomarker levels are shwon on a 10-logarithmic scale. * p value <0.05, ** p value <0.01. Abbreviations: NfL, neurofilament light chain; FA, flail arm; FL, flail leg; PLMN, pure lower motor neuron; PUMN, pure upper motor neuron.*

		NfL	**UCHL1**	**GFAP**	**tTAU**
Phenotype	Classic (n=62)	140.6 (87.0-246.0)	41.8 (29.4-68.2)	143.5 (102.6-185.8)	0.9 (0.6-1.3)
	Bulbar (n=27)	88.9 (69.4-167.0)	34.7 (26.4-53.9)	129.5 (101.5-176.8)	0.9 (0.5-1.9)
	Pyramidal (n=19)	160.1 (118.0-251.0)	43.4 (21.3-67.4)	116.2 (92.6-179.3)	0.9 (0.5-1.7)
	FA/FL (n=23)	80.5 (49.0-121.0)**##	48.0 (32.1-74.9)	131.6 (74.8-167.5)	0.5 (0.4-0.9)
	PLMN/PUMN (n=10)	75.4 (25.6-97.0)* ##	42.1 (25.6-65.2)	98.0 (54.8-136.2)	0.4 (0.2-0.7)

Table 3.13. Biomarkers concentrations across the ALS phenotypes. *** indicates difference <0.01 with pyramidal; * indicates difference <0.05 with pyramidal; ## indicates difference <0.01 with classic.*

3.3.4 GFAP elevation reflects the presence of cognitive involvement

In our cohort seventy-four (52.7%) patients underwent neuropsychological assessment and were consequently classified as defined by previous published criteria (Strong *et al*, 2017). We investigated biomarkers serum concentration across ALS cognitive phenotypes demonstrating that GFAP was significantly higher in ALS patients presenting a concomitant cognitive/behavioural impairment (ALS-bi; ALSci and ALS-cbi) or FTD, compared with patients with ALS showing normal cognitive function at the neuropsychological evaluation (Figure 3.10). This result was confirmed also after correction for the age at sampling. Serum NfL, UCHL1 and tTAU levels did not differ among cognitive phenotypes (Table 3.14).

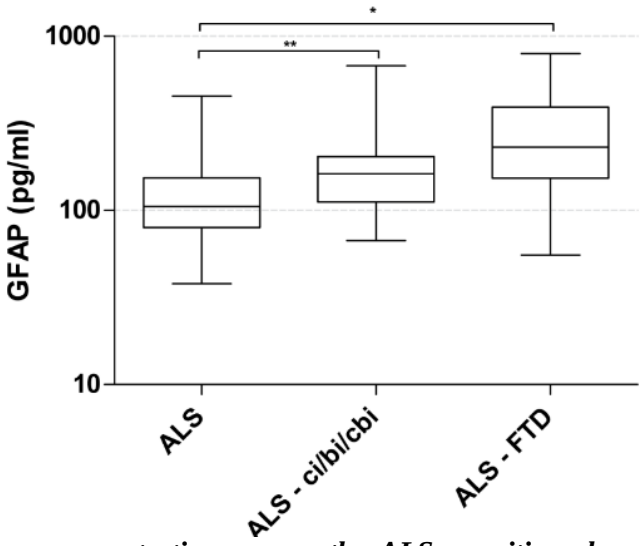

Figure 3.10. Biomarkers concentrations across the ALS cognitive phenotypes. *Boxes are median and interquartile range. Whiskers are highest and lowest values. Biomarker levels are plotted on a 10-logarithmic scale. * p value <0.05, ** p value <0.01. Abbreviations: ALS-bi, ALS behavioral impairment; ALS-ci, ALS cognitive impairment; ALS-cbi, ALS combined cognitive and behavioral impairment; ALS-FTD, ALS Frontotemporal dementia (FTD)*

		NfL	UCHL1	GFAP	tTAU
Cognitive phenotype	0 (ALS motor, n=27)	114.2 (72.9-198.8)	44.1 (26.4-62.2)	105.3 (80.0-154.0)	0.6 (0.5-0.9)
	1 (ALS-bi, n=16)	139.0 (60.9-243.3)	35.0 (27.9-49.7)	169.8 (140-207)*	1.0 (0.7-2.0)
	2 (ALS-ci, n=5)	100.7 (63.3-309.3)	39.1 (30.4-70.6)	152.5 (119-186) *	0.7 (0.4-1.6)
	3 (ALS-cbi, n=19)	138.4 (94.6-231.9)	47.5 (34.6-73.5)	141.3 (96.1-208.4)	1.1 (0.5-1.4)
	4 (ALS-FTD, n=7)	184.9 (109.0-362.1)	57.5 (41.9-65.0)	231.4 (153-391)*	0.9 (0.6-1.1)

Table 3.14 Biomarkers concentrations across the ALS cognitive phenotypes. ** indicates difference <0.05 with ALS motor.*

3.3.5 Biomarkers are not influenced by the ALS genotype

In our ALS cohort, twenty patients were detected to carry the *C9orf72* hexanucleotide expansion (C9-ALS). C9-ALS and C9-negative-ALS groups had indistinguishable serum biomarkers profile as shown in Table 3.15.

VARIABLE		NfL	UCHL1	GFAP	tTAU
C9ORF72	Non-C9 (123)	110.2 (70.2-192.9)	42.3 (28.2-64.3)	131.5 (886.8-177.5)	0.8 (0.5 -1.3)
	C9-ALS (20)	118.2 (72.5-163.3)	39.7 (25.6-59.6)	127.8 (106.2-167.6)	0.7 (0.3-0.9)

Table 3.15. Biomarkers concentrations in C9-ALS and non-C9 carriers. *Abbreviation: C9ORF72, chromosome 9 open reading frame 72*

3.3.6 Biomarkers longitudinal evaluation

Thirty-four patients underwent longitudinal serum biomarkers evaluation. The median time occurring between baseline (T0) and the second sampling (T1) was 6 months (range 5-7 months). A Wilcoxon matched-pairs signed-rank test to evaluate significant changes of repeated measures of biomarkers concentration showed no difference for NfL (p=0.197), UCHL1 (p=0.939) and GFAP (p=0.109). Conversely, tTau levels were significantly increased at the second time point (p<0.005) (Figure 3.11 and Table 3.16).

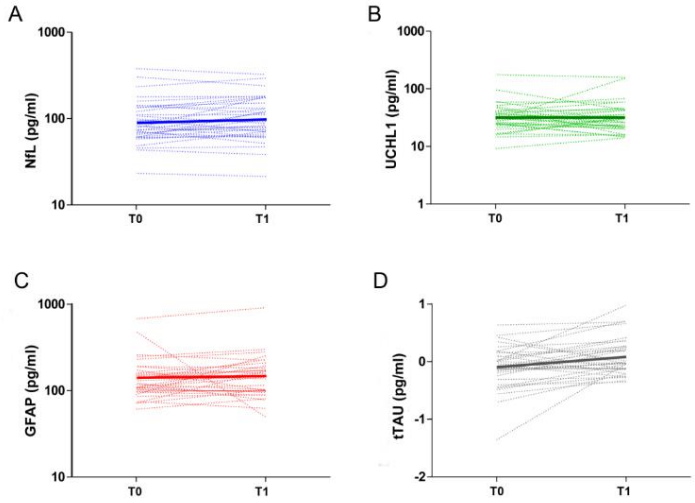

Figure 3.11. Longitudinal biomarkers trajectories. *A) NfL, B) UCHL1, C) GFAP and D) tTAU levels in the follow-up period (6 months) for each subject with ALS (dashed lines) and predicted average trajectories (solid lines). Biomarkers level are plotted on a 10-logarithmic scale. T0, first blood sample; T1, second blood sample*

Biomarker	**T0**	**T1**
NfL	79.1 (62.3-129.3)	89.9 (67.2-146.1)
UCHL1	30.7 (24.2-41.1)	30.0 (21.0-43.3)
GFAP	126.2 (101.5-169.6)	145.0 (99.6-190.3)
tTAU	0.8 (0.6-1.3)	1.1 (0.7-1.8)

Table 3.16. Biomarkers longitudinal comparison in the ALS cohort. *Median and interquartile range in brackets are given. NfL, neurofilament light chain; UCHL1, ubiquitin C-terminal hydrolase L1; GFAP, glial fibrillary acidic protein; tTau, total tau protein*

3.3.7 Serum biomarkers diagnostic performances

We explored the diagnostic performances of each serum biomarker in distinguishing ALS from HC, ALS from DEG and ALS from ALSmd. Serum NfL displayed the best diagnostic performance among biomarkers when discriminating ALS from HC (AUC=0.990; 95% CI: 0.978-1.00). NfL showed an excellent diagnostic yield with a sensitivity of 96.0% and a specificity of 98% when an optimal cut-off was set at 31.7 pg/ml (Table 3.17). UCHL1 showed a good diagnostic performance (AUC=0.761; 95% CI: 0.765-0.837), however this biomarker showed a low sensitivity (57.0%) but a good specificity (86.0) when the optimal cut-off was set at 39.3 pg/ml (Table 3.17). Conversely GFAP and tTAU had lower performances in the differentiating ALS from HC (Table 3.17 and Figure 3.12-3.14). NfL also had the highest diagnostic yield in distinguishing ALS from DEG (AUC=0.946; 95% CI: 0.916-0.976) with a sensitivity 87.0% and specificity of 94.0% when the optimal cut-off was set at 56.9 pg/ml (Table 3.17). Conversely, UCHL1, GFAP and tTAU were not helpful in discriminating ALS from DEG (Figure 3.12-3.14). When distinguishing ALS from ALSmd the highest AUC value was observed for NfL (AUC=0.850; 95% CI: 0.785-0.914) with a sensitivity of 87.0% and a specificity of 78.0% when an optimal cut-off was set at 56.6 pg/ml. UCHL1, GFAP, and tTAU showed lower AUC values (Figure 3.12-3.14). The optimal cut-off, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and AUC of each biomarker are given in Table 3.17.

ALS vs HC				
	NfL	**UCHL1**	**GFAP**	**tTAU**
Cut-off	31.7 pg/ml	39.3 pg/ml	164.1 pg/ml	1.2 pg/ml
Sensitivity	96.0% (91.0%-98.0%)	57.0% (48.0%-65.0%)	31.0 % (23.0%-39.0%)	72.0 % (48.0%-65.0%)
Specificity	98.0% (88.0%-100%)	86.0% (73.0%-95.0%)	84.0% (70.0%-93.0%)	61.0% (73.0%-95.0%)
PPV	99.0% (96.0%-100.0%)	93.0% (86.0%-97.0%)	86.0% (70.0%-93.0%)	86.0% (78.0%-91.0%)
NPV	98.0% (75.0%-95.0%)	38.0% (28.0%-48.0%)	27.0% (20.0%-35.0%)	40.0% (28.0%-53.0%)
AUC	0.990 (0.978-1.00)	0.761 (0.675-0.837)	0.561 (0.466-0.656)	0.679 (0.595-0.775)
ALS vs DEG				
Cut-off	56.9 pg/ml	44.1 pg/ml	200.3 pg/ml	1.1 pg/ml
Sensitivity	87.0% (80.0%-92.0%)	55.0% (47.0%-64.0%)	84.0% (77.0%-90.0%)	70.0% (62.0%-78.0%)
Specificity	94.0% (86.0%-98.0%)	60.0% (47.0%-72.0%)	53.0% (40.0%-65.0%)	57.0% (44.0%-69.0%)
PPV	97.0% (92.0%-99.0%)	75.0% (65.0%-82.0%)	79.0% (72.0%-85.0%)	78.0% (69.0%-84.0%)
NPV	77.0% (67.0%-86.0%)	38.0% (29.0%-49.0%)	61.0% (47.0%-73.0%)	47.0% (36.0%-59.0%)
AUC	0.946 (0.916-0.976)	0.539 (0.464-0.626)	0.687 (0.606-0.767)	0.585 (0.495-0.675)
ALS vs ALSmd				
Cut-off	56.6 pg/ml	52.7 pg/ml	145.6 pg/ml	0.6 pg/ml
Sensitivity	87.0% (80.0%-92.0%)	66.0% (58.0%-74.0%)	61.0% (52.0%-69.0%)	65.0% (57.0%-73.0%)
Specificity	78.0% (67.0%-87.0%)	45.0% (33.0%-57.0%)	55.0% (43.0%-67.0%)	54.0% (41.0%-66.0%)
PPV	89.0% (83.0%-94.0%)	71.0% (63.0%-79.0%)	74.0% (65.0%-81.0%)	75.0% (66.0%-82.0%)
NPV	74.0% (62.0%-84.0%)	39.0% (28.0%-51.0%)	40.0% (30.0%-51.0%)	42.0% (32.0%-54.0%)
AUC	0.850 (0.785-0.914)	0.508 (0.422-0.594)	0.557 (0.470-0.643)	0.583 (0.499-0.668)

Table 3.17. Diagnostic performances of each serum biomarker in discriminating ALS from HC, DEG and ALSmd. *Given are cut-off, sensitivity, specificity, PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve and between brackets 95% confidence interval. Abbreviations: ALS, amyotrophic lateral sclerosis; HC, healthy controls; DEG, other neurodegenerative disorders; ALSmd, amyotrophic lateral sclerosis mimic disorders*

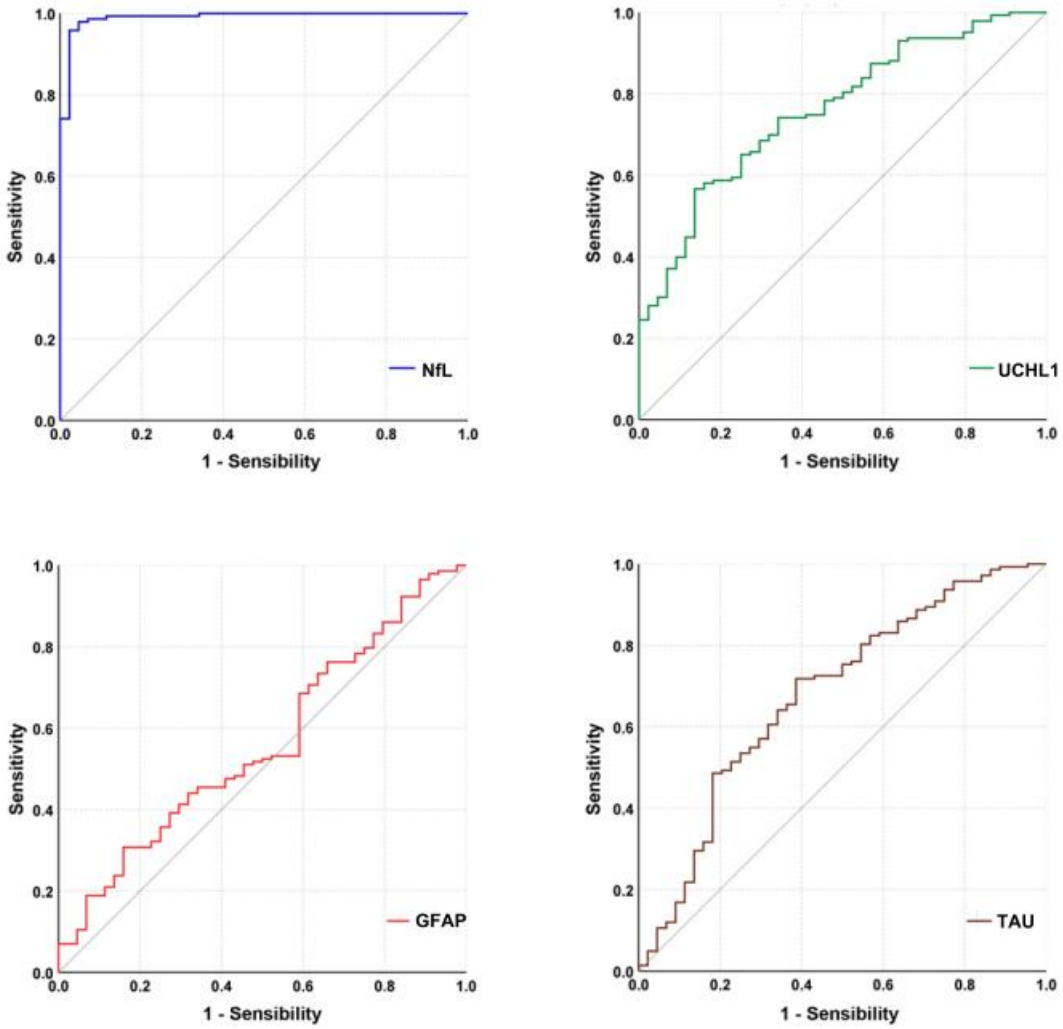

Figure 3.12. Receiver operating characteristic (ROC) curves to discriminate patients with ALS from HC. *based on NfL upper left, UCHL1 upper right, GFAP lower left, tTAU lower right serum levels.*

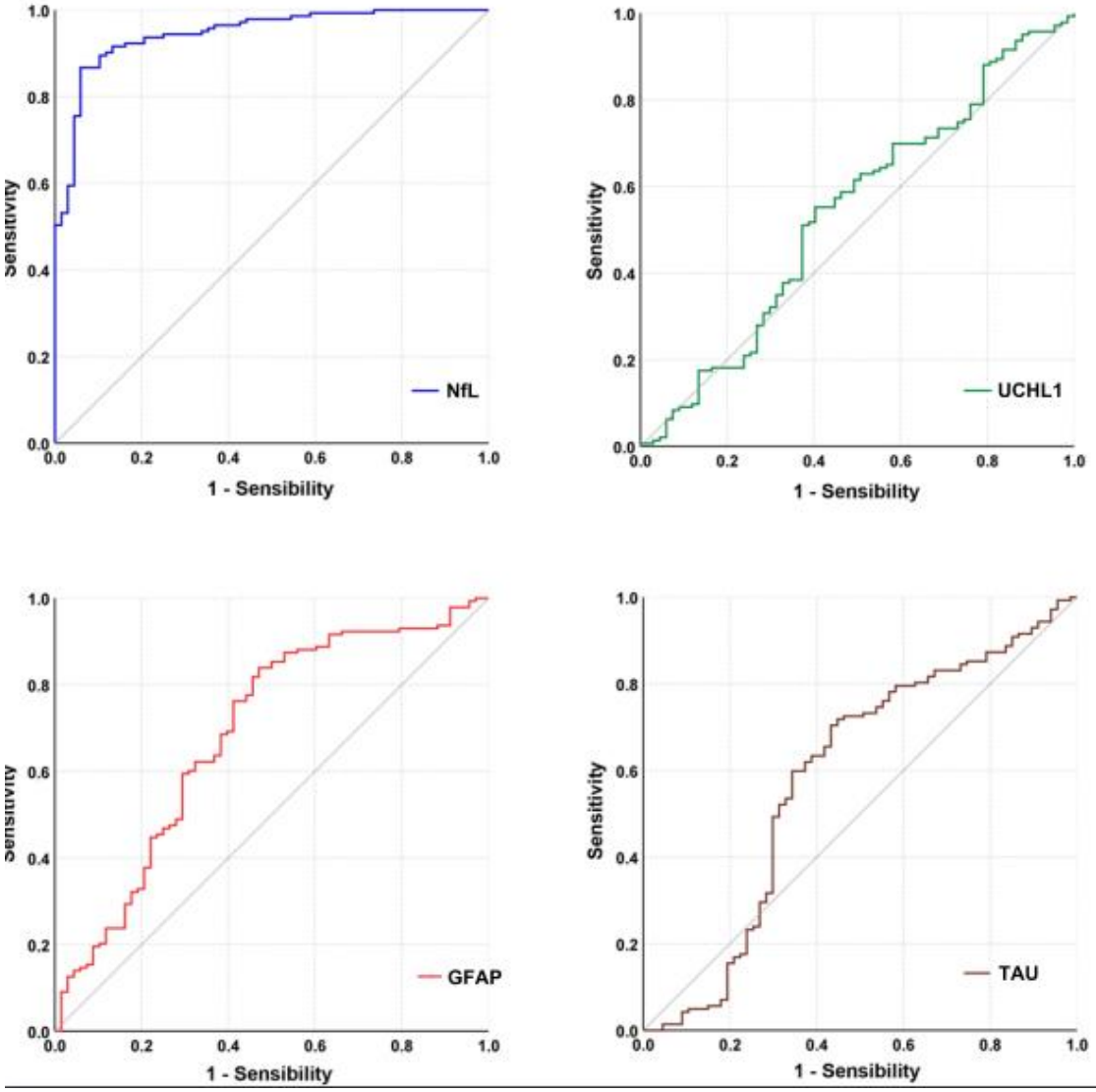

Figure 3.13. Receiver operating characteristic (ROC) curves to discriminate patients with ALS from DEG. *based on NfL upper left, UCHL1 upper right, GFAP lower left, tTAU lower right serum levels.*

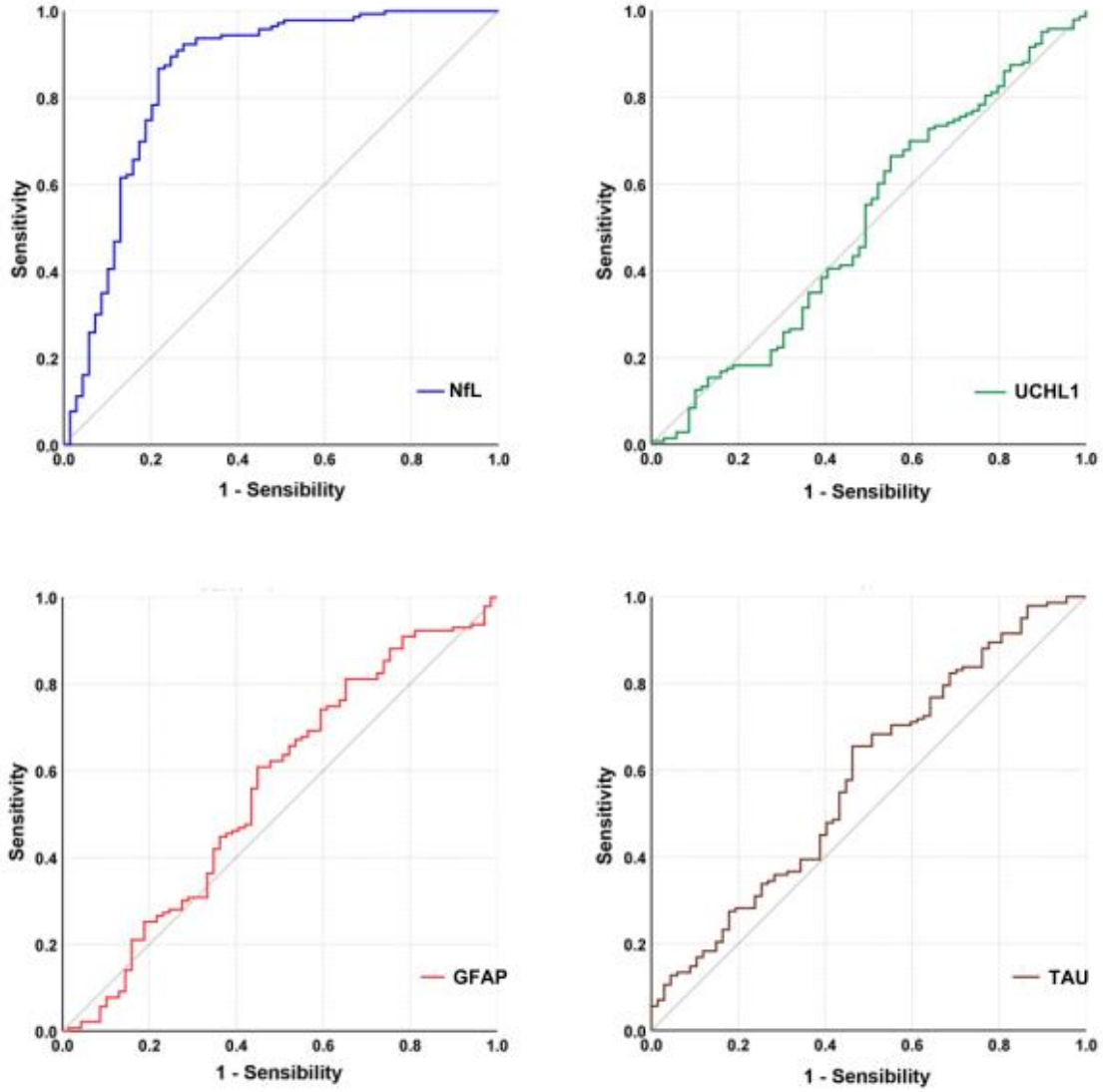

Figure 3.14. Receiver operating characteristic (ROC) curves to discriminate patients with ALS from ALSmd. *based on NfL upper left, UCHL1 upper right, GFAP lower left, tTAU lower right serum levels.*

3.3.8. Serum NfL and UCHL1 are both prognostic predictors for ALS

In a univariate analysis, higher NfL, UCHL1 and GFAP negatively affected prognosis. First, Kaplan-Meier survival curves were obtained with ALS patients stratified according to serum NfL concentrations tertile (Mantel–Cox; $\chi 2=42.3$, $p<0.001$). Survival estimations for NfL concentration tertile were as follows: first tertile (lower values) 40.3 months (95% CI: 25.1-57.0); second tertile 21.0 months (95% CI: 14.2-25.8); third tertile 9.0 months (95% CI: 5.9-12.1 months) (Figure 3.15). Survival estimations for serum UCHL1 concentration tertile (Mantel–Cox; $\chi 2=11.3$, $p=0.004$) were as follows: first tertile (lower values) 30.0 months (95% CI: 22.7-37.3); second tertile 18.0 months (95%

CI: 13.9-22.1); third tertile 15.0 months (95% CI: 10.7-19.2 months) (Figure 3.15). For GFAP concentrations (Mantel–Cox; $\chi 2=7.6$, $p=0.02$): first tertile (lower values). 36.0 months (95% CI: 16.1-55.8); second tertile 18.0 months (95% CI: 12.3-23.6); third tertile 13.0 months (95% CI: 4.0-22.0 months) (Figure 3.16). These results were confirmed when Kaplan-Meier curves were adjusted by age at onset and disease duration at serum sample (data not shown). Conversely, tTau concentration did not show any effect on survival. To further explore the prognostic role of UCHL1, we performed a Kaplan-Meier analysis combining NfL and UCHL1 (Figure 3.17). We clustered patients according to serum median NfL and UCHL1 levels in four different groups. The survival analysis resulted significant (Mantel–Cox; $\chi 2=35.4$, $p<0.001$). Patients with above median concentrations of NfL had similar prognosis regardless of UCHL1 concentrations (NfL-high/UCHL1-low and NfL-high/UCHL1-high median survival was 12.0 and 11.0 months, respectively); in contrast, with below median NfL levels, UCHL1 concentrations allowed an effective stratification of patients. In this NfL range, the median survival for ALS patients with below median UCHL1 concentrations (NfL-low/UCHL1-low) was 40.0 months (95% CI 21.7-58.3), while it was 22.0 months (95% CI 18.8-25.1) for ALS patients with UCHL1 concentration above the median (NfL-low/UCHL1).

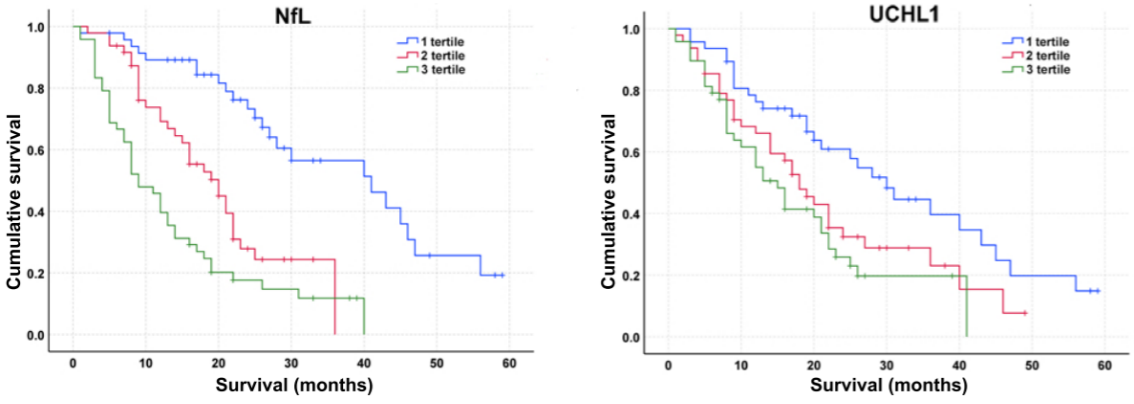

Figure 3.15. Kaplan-Meier (KM) curves estimates ALS cumulative survival according to NfL and UCHL1 concentrations. *Survival was defined as the time occurring between serum sampled and death/tracheostomy.*

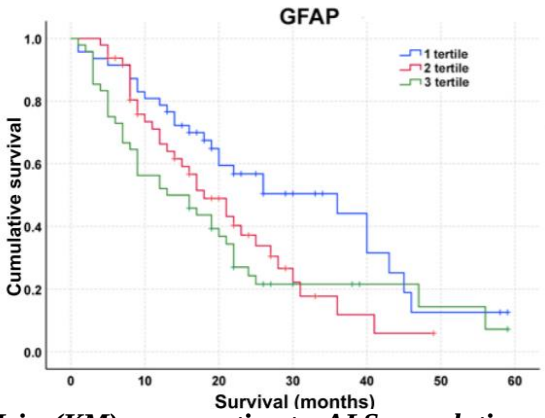

Figure 3.16. Kaplan-Meier (KM) curves estimates ALS cumulative survival according to GFAP concentrations. *Survival was defined as the time occurring between serum sampled and death/tracheostomy.*

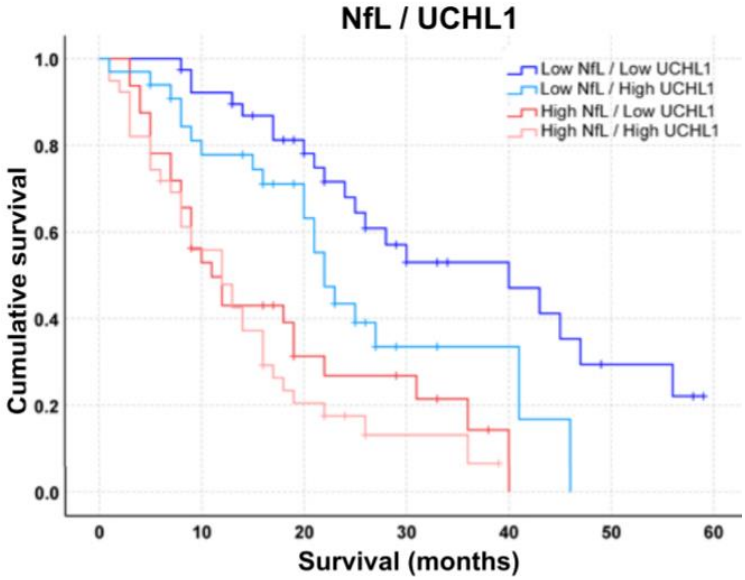

Figure 3.17. Kaplan-Meier. survival curves in ALS patients combining NfL and UCHL1 levels. *Below median values for both biomarkers (Low NfL/Low UCHL1); with NfL below median values and UCHL1 above median values (Low NfL/High UCHL1); with NfL above median values and UCHL1 below median values (High NfL/Low UCHL1); above median values for both biomarkers (High NfL/High UCHL1). Survival was defined as the time from blood sample to death or tracheostomy.*

We performed a multivariate survival analysis applying the Cox regression model to further explore the role of NfL, UCHL1 and GFAP in determining ALS prognosis. Along with serum biomarkers, we included in the model several ALS clinical parameters known to influence survival of patients with ALS: NfL chain divided in tertiles; UCHL1 divided in tertiles; GFAP divided in tertiles; age at venipuncture, diagnostic delay, disease progression rate, *C9ORF72* hexanucleotide expansion dichotomized in yes or no; ALS

phenotype subdivided in two different groups spinal and bulbar. Multivariate Cox regression models showed that both NfL and UCHL1 are independent prognostic factor for ALS along with the disease progression rate and the diagnostic delay. Conversely, GFAP did not reach statistical significance. All the variables included in the analysis with respective p-value, hazard ratio, and 95% CI are reported in Table 3.18.

Covariates	**Survival (from serum sample to death or tracheostomy**	
	HR (95% CI)	***p* value**
NfL levels (pg/ml)		**<0.001**
1 tertile	1	
2 tertile	3.24 (1.67 – 6.3)	**<0.001**
3 tertile	5.62 (2.93 – 10.77)	**<0.001**
GFAP levels (pg/ml)		0.649
1 tertile	1	
2 tertile	1.15 (0.66 – 2.0)	0.621
3 tertile	0.89 (0.48 – 1.65)	0.716
UCHL1 levels (pg/ml)		**0.038**
1 tertile	1	
2 tertile	1.04 (0.59 – 1.85)	0.80
3 tertile	1.88 (1.06 – 3.33)	**0.032**
Age at venipuncture	1.017	0.18
Diagnostic delay	1.09	**0.03**
Disease progression rate	1.86	**<0.001**
C9orf72 expansion		
YES	1	
NO	0.57	**0.09**
Phenotype		
Spinal	1	
Bulbar	1.49	0.15

Table 3.18. Cox proportional hazards regression multivariate analysis on ALS survival. *Variables included in the model: NfL, neurofilament light chain subdivided in tertile; UCHL1, ubiquitin C-terminal hydrolase L1 subdivided in tertile; GFAP, glial fibrillary acidic protein subdivided in tertile; age at venipuncture; diagnostic delay; progression rate; C9orf72, chromosome 9 open reading frame 72 hexanucleotide expansion dichotomized in no or yes; ALS phenotype, subdivided in two different groups spinal and bulbar. HR, hazard ratio; CI, confidence interval. Bold indicates statistical significance.*

4.DISCUSSION

Wet biomarkers are needed in clinical practice to easily diagnose ALS and better define patient's prognosis. In the last decade the research on this topic has taken relevant steps forward identifying NFs as the most promising biomarkers for ALS in the perspective of clinical translation. NFs are specific neuronal cytoskeletal proteins, exclusively expressed in the peripheral and central nervous systems, essential for structural support and in maintaining axonal structure integrity (Poesen & Van Damme, 2019). NFs elevation in biofluids mirrors an acute or chronic axonal degeneration regardless of the ongoing specific pathogenetic mechanism (Falzone *et al*, 2021b). As known, most of the neurological conditions lead to neuronal or axonal damage consequently causing an elevation of NFs in the biofluids (Bridel *et al*, 2019). Despite NFs being non-specific disease biomarkers, patients with ALS show one of the highest concentrations in both CSF and serum among all the neurological conditions (Verde *et al*, 2019; Bridel *et al*, 2019). Therefore, setting a high diagnostic cut-off value was proposed as a promising strategy to have high sensitivity and specificity diagnostic yield. Previous studies showed that NFs yield a high diagnostic performance in discriminating ALS from HC in both biofluids (Gaiottino *et al*, 2013; Gagliardi *et al*, 2021; Verde *et al*, 2019; Wilke *et al*, 2016). Conversely, studies aimed at investigating the applicability of CSF and serum NfL in distinguishing ALS from its mimic disorders achieved controversial results, mainly evidencing a wide heterogeneity in diagnostic specificity and sensitivity (Halbgebauer *et al*, 2021; Rossi *et al*, 2018; Gille *et al*, 2019, 201; Feneberg *et al*, 2018; Poesen *et al*, 2017; Brodovitch *et al*, 2021). To overcome this limitation, an evaluation of a set of biomarkers, as already employed for the diagnosis of Alzheimer's disease (AD), might be a promising approach to improve the diagnostic performance in differentiating ALS from its mimics disorder (Blennow & Zetterberg, 2018).

In the first part of our results, we demonstrated in a large ALS cohort (n=219) that serum pNfH is an independent prognostic factor for ALS and its concentration are heterogenous across the ALS phenotypes. Interestingly, patients showing a concomitant fast disease progression and a predominant upper motor neuron burden had the highest pNfH concentration.

In the second part we attempted to identify further promising biomarkers for ALS. Through our gene expression and pathways analyses, performed on motor nerve biopsies of patients with ALS and compared with MN as controls, we identified UCHL1 as a potential protein involved in the ALS pathogenesis. This gene was found to be significantly upregulated in motor nerve biopsies of ALS patients and annotated in the upregulated pathways named post-translational protein modification. Conversely, UCHL1 was not upregulated in the degenerating sensitive nerves fibers suggesting that this protein might be specifically involved in the ALS pathogenic mechanism. There is also interesting evidence in literature that pointed out UCHL1 as a promising biomarkers for ALS supporting our results. Firstly, UCHL1 is a protein involved in the ubiquitin-proteasome system (UPS) which is fundamental in regulating the elimination of the intracellular misfolded proteins (Bendotti *et al*, 2012). ALS along with other neurodegenerative disorders is marked by the neuropathological accumulation of ubiquitin-positive misfolded proteins thought to be the leading cause of the neuronal damage. Although proteolysis is a complex mechanism, the massive accumulation of ubiquitinated proteins strongly indicates a relevant role of the UPS in the ALS pathogenic mechanism (Kato, 2007). Secondly, UCHL1 has been related to several other functions such as axonal repair, interplay with neuronal cytoskeleton proteins, axonal transport and in maintaining axonal integrity (Bheda *et al*, 2010; Liu *et al*, 2015; Pukaß & Richter-Landsberg, 2015). Lastly, UCHL1 has been found raised in the CSF of patients with ALS in two different proteomics studies (Setsuie & Wada, 2007; Oeckl *et al*, 2020).

In the last part of our study, we tested UCHL1 in an independent large ALS (n=143) and control cohorts (n=185) to assess its diagnostic and prognostic performances. At the same time, we also assessed the NfL, GFAP and tTAU serum concentrations in both cohorts. UCHL1 resulted to be elevated in the ALS compared with HC achieving good diagnostic sensitivity and specificity performance. Unfortunately, its serum concentrations did not differ among ALS and ALSmd resulting to be not helpful in discriminating these two groups. Conversely, UCHL1 was an independent prognostic factor for survival, proving itself helpful in the stratification of survival for patients with lower NfL levels.

Among the investigated biomarkers, NfL consistently reached the best diagnostic performance for ALS. NfL showed almost optimal diagnostic yield in distinguishing ALS

from HC (AUC=0.990) and DEG (AUC=0.946) with excellent sensitivity and specificity values. Conversely, the diagnostic yield in discriminating ALS from ALSmd was lower (AUC=0.850) with specificity decreasing to 78.0%. Furthermore, NfL was an independent predictor of survival, and its levels were heterogeneously distributed across ALS motor phenotypes as similarly observed for the pNfH.

Interestingly, we observed that serum GFAP concentrations, a known marker of astrogliosis, were different among cognitive phenotypes, namely ALS with concomitant cognitive impairment (ALS-bi, ALS-ci and ALS-cbi) or FTD had higher levels compared with ALS with normal cognition. At the contrary, both NfL and pNfH concentrations did not reflect the cognitive involvement in ALS. Therefore, GFAP might be instrumental in tracking the occurrence of cognitive decline in ALS.

4.1. Serum Neurofilaments concentrations are heterogenous across ALS phenotypes

We observed that both serum neurofilaments subunits concentration was not homogenous across the ALS motor phenotypes. Higher concentrations were detected in patients showing pyramidal, bulbar, or classic phenotypes compared with atypical or restricted phenotypes such as FA, PLMN and PUMN.

The clinical parameters that showed the highest correlation with NFs were the disease progression rate and the clinical and neurophysiological measures of upper motor neuron burden. Specifically, NFs showed an inverse correlation with the MEP/cMAP and a positive correlation with ΔALSFRS-R. Furthermore, these variables resulted to be the major determinants of serum pNfH concentration in a multivariate model. Even though a throughout explanation is probably more intricate, our results suggest that a rapid axonal degeneration of the corticospinal tract might be the major determinant of the serum NFs concentrations. This hypothesis might explain the heterogeneous distribution of NFs across ALS motor phenotypes, with pyramidal and classic phenotypes, characterized by a consistent and relentless UMN involvement, showing higher NFs levels compared with restricted or predominantly lower phenotypes such as PLMN, FA and FL. Consistently with our hypothesis, previous MRI investigations demonstrated a correlation among NFs and the degree of the corticospinal tract involvement (Gille *et al*, 2019).

Our data consistently conclude that serum NFs are influenced by the ALS phenotypes. Therefore, the diagnostic performance of NFs might be significantly different across ALS phenotypes. Atypical phenotypes or patients with selective involvement of the LMN or UMN, which inherently represent primary challenge for the clinician at the time of diagnosis, may somewhat lower the diagnostic yield of NF, therefore reducing their transability in clinical practice.

4.2. Serum GFAP but not NFs reflects the presence of a concomitant cognitive impairment in ALS

Based on our data, ALS patients presenting a concomitant cognitive impairment have similar serum NFs concentration of patients with normal cognition. Therefore, both NFs do not reflect the presence of a concomitant cognitive involvement. Conversely, GFAP seemed to be a promising biomarker to track the co-occurrence of a cognitive impairment.

GFAP is a specific brain protein and established marker of astrogliosis (Heller *et al*, 2020). The abnormal proliferation of astrocytes, consequence of the neuronal damage, has been documented to be increased in frontal cortical tissue of patients with FTD (Umoh *et al*, 2018). GFAP has been found elevated in both CSF and serum of patients with symptomatic FTD (Ishiki *et al*, 2016; Oeckl *et al*, 2019; Consortium for Frontotemporal Lobar Degeneration German *et al*, 2019). As expected, serum GFAP concentration was raised in our DEG group compared to ALS and HC groups and its serum concentration correlated with the age at the symptom's onset. Interestingly, we observed that GFAP concentrations were different among cognitive phenotypes, namely ALS with concomitant cognitive impairment (ALS-bi, ALS-ci and ALS-cbi) or FTD had higher levels compared with ALS with normal cognition. Considering these results, higher levels of GFAP might be suggestive of a wider neurodegenerative process with reactive astrogliosis, extended to the frontotemporal regions, which typically occurs in patients with ALS and concomitant cognitive impairment. Therefore, monitoring GFAP along with the neuropsychological status might be instrumental in tracking the occurrence and the evolution of cognitive decline in ALS. Further longitudinal studies are needed to better address this hypothesis.

4.3. Biomarkers diagnostic performance

In our study, among the investigated biomarkers, NfL considerably reached the best diagnostic performance for ALS. NfL showed almost an optimal diagnostic yield in distinguishing ALS from HC (AUC=0.990, CI 0.978-1.00) with a sensitivity and specificity of 96.0% (CI 91.0-98.0%) and 98.0% (CI 88.0-100%) when a cut-off value was set at 31.7 pg/ml. Similarly, NfL showed a good diagnostic performance in distinguishing ALS from other neurodegenerative disorders. The AUC was 0.946 (0.916-0.986), sensitivity and specificity were 87.0 (CI 80.0-92.0%) and 94.0 (CI 86.0-98.0%) respectively when the cut-off was set at 56.9 pg/ml. Conversely, the diagnostic yield of NfL in discriminating ALS from ALS-md was significantly lower compared to the previous comparison with HC and DEG. The AUC was 0.850 (CI 0.715-914), the sensitivity was 87.0 (CI 80.0-92.0%) and the specificity dropped at 78.0% (CI 67.0-87.0%).

A previous study assessed UCHL1 as diagnostic biomarker showing promising performances in discriminating ALS from its mimic disorders in both CSF and serum (Li *et al*, 2020). On the contrary, in our study, serum UCHL1 showed good diagnostic yield only in discriminating ALS from HC (AUC=0.761; CI 0.675-0.831), unsatisfactory performance in distinguishing ALS from DEG (AUC=0.539; CI 0.464-0.626) and ALS-md (AUC=0.557; CI 0.470-0.643) with low sensitivity and specificity values were detected. Therefore, accordingly to our data serum UCHL1 is not a promising diagnostic biomarker for ALS. These diverging results might be explained by the higher sample size of our ALS and control cohorts.

Serum GFAP and tTau were not helpful in differentiating ALS from any of the control cohorts. All the AUC, sensitivity, specificity and PPV and NPV of each biomarker is reported in Table 3.17. We also attempted to combined NfL to the other remaining biomarkers without improving the diagnostic performances of NfL when measured alone (data not shown).

NfL seems to be a promising diagnostic biomarker. The upcoming introduction of NfL in clinical practice needs the definition of standardized cut-off values, to provide consistency and allow comparisons between measurements from different laboratories. Furthermore, future investigations should be addressed on finding novel biomarkers to

improve the diagnostic specificity when combined with NfL in differentiating ALS from its mimic disorders.

4.4. Biomarker prognostic performance, UCHL1 allows better survival stratification of patients with low NfL

In univariate analysis, serum NfL, UCHL1 and GFAP correlated with patents survival and significantly stratified the prognosis of patients with ALS, higher levels of biomarkers reflected a shorter disease duration (Figure 3.15-3.16). In addition, we evaluated the prognostic prediction of the biomarkers in a multivariate model confirming both serum NfL and UCHL1, but not GFAP, as independent prognostic factors for ALS. In the multivariate model were included several ALS parameters known to negatively influence patients 'survival (Table 3.18). Serum NfL was one of the strongest predictors of survival together with the disease progression rate. Patients with ALS grouped in the third tertile (higher NfL concentration) and second tertile have an increased proportional HR to reach the survival event of 5.62 (2.93 – 10.77) and 3.24 (1.67 – 6.3) when compared to the first tertile group (lower NfL concnetration). As above mentioned, UCHL1 was as well a predictor of survival showing lower HR ratio compared to NfL. Patients with ALS grouped in the third tertile (higher UCHL1 concentration) and second tertile have an increased proportional HR to reach the survival event of 1.88 (1.06 – 3.33) and 1.04 (1.04 (0.59 – 1.85) when compared to the first tertile group (lower UCHL1 concentration)

Lastly, we performed a survival analysis combining both NfL and UCHL1 to better stratify the survival of patients with ALS. Our data shown that UCHL1 better defined ALS prognosis in patients showing low NfL concentration (below median value). Conversely, patients with high NfL concentration (above median value) have similar survival regardless of UCHL1 levels (Figure 3.17). Therefore, UCHL1 might be an independent prognostic factor for ALS, and its evaluation combined with NfL might be reach a better definition of patient's prognosis.

4.5. Limitations

Our study is not exempt from limitations. First, we were not able to evaluate both the neurofilaments subunits in the same ALS cohort, thereby the combined diagnostic and prognostic performances of both NFs were not explored. We decided to evaluate separately the NFs subunits since they were assessed with diverse assays, namely NfL was measured with SIMOA and pNfH with ELISA, with different analytical sensitivity. A previous study evaluated both NFs subunit with SIMOA assays demonstrating the higher performance of NfL in predicting disease progression and survival in ALS(Benatar *et al*, 2020). In light of this evidence, we decided to benchmark the other biomarkers to NfL instead of using pNfH.

Although we performed our analysis in a large ALS cohort, we were not able to validate our results in an independent cohort. Therefore, further independent studies are needed to confirm UCHL1 as prognostic determinant of the ALS survival. Also, our results on GFAP as mirrors of the co-occurrence of a cognitive impairment has to be validated in an independent cohort, also because were obtained in a part of our ALS cohort (52.7%) which underwent neuropsychological evaluation.

Our data indicate that UCHL1 is not a promising diagnostic biomarker when assessed in serum of patients with ALS, however, we were not able to assess its diagnostic and prognostic performance in the CSF due to the lack of serum and CSF matched samples in our cohort. Therefore, future studies addressing this question are needed. Despite that, we fully believed that a blood-based biomarker would be preferable than a CSF one, because it would be easily collected, monitored over the time, and would avoid invasive procedure such as the lumbar puncture.

We performed a longitudinal evaluation of the biomarkers demonstrating that NfL, UCHL1 and GFAP are stable at two different time points. Conversely, we observed a slight elevation of tTau at the second time point. Unfortunately, our longitudinal study has a short period of observations and was performed in subset of our ALS patients (20%).

Lastly, we acknowledge that our gene expression analysis was performed on a small sample size of patients with ALS and MN that were considered as controls. However, motor nerve biopsy is performed only in selected case in which the clinical evaluation and the neurophysiological and neuroimaging studies failed to establish a diagnosis.

Furthermore, in our analysis the lack of normal control motor nerves, by comparing two groups of patients with both diseases involving the PNS could also have limited the possibility of highlighting conserved, unspecific response-to-injury process.

4.6. Future perspectives

In our study, serum NfL showed the better diagnostic performance for ALS among the investigated biomarkers. It had an almost optimal diagnostic sensitivity and specificity in discriminating ALS from HC and other neurodegenerative disease, conversely its diagnostic performances significantly dropped when it was evaluated in distinguishing ALS from its mimic disorders. Unfortunately, when the remaining biomarkers were combined with NfL they did not improve the diagnostic performance of NfL when measured alone. The distinction of ALS from its mimic disorders represents a daily challenge for clinicians, therefore further steps have to be taken in this direction.

To overcome this issue, our efforts should be focused on identifying novel specific disease biomarkers or a non-specific ALS biomarker to be combined with NfL resembling the approach that was employed for the diagnosis of Alzheimer's disease. Through our gene expression analysis and filtering approach, we obtained a group of genes that were specifically up or downregulated in the peripheral nervous system of patients with ALS. These genes have to be further investigated because they might be potentially involved in the disease pathogenesis or being specific candidate biomarkers. A second interesting approach would be to identify specific biomarker of the axonal regeneration process to be combined with NfL, an established marker of axonal regeneration. With the second approach we would be able to detect the presence of axonal regeneration that completely lack in ALS while it is the neuropathological hallmark of the motor neuropathies which represents the main diagnostic challenge for clinicians.

4.7. Conclusions

In conclusion, we confirmed NfL as a potential diagnostic and prognostic biomarker for ALS. The upcoming introduction of NfL in clinical practice needs the definition of standardized cut-off values, to provide consistency and allow comparisons between measurements from different laboratories. Although we confirm NfL as the strongest predictor of survival, UCHL1 is an independent prognostic factor for ALS and may be

helpful in stratifying survival of patients with low NfL. Finally, GFAP might be useful to detect extra-motor, namely cognitive impairment or FTD, in ALS. Future investigations should be addressed on finding novels, more specific biomarkers, to improve the diagnostic specificity when combined with NfL in differentiating ALS from its mimic disorders

5. MATERIAL AND METHODS

5.1 Study population and clinical evaluation

5.1.1 ALS and control cohorts

A total of two hundred and nineteen consecutive patients with ALS, diagnosed according to the revised El Escorial and Awaji criteria, were recruited at the Department of Neurology and Neurorehabilitation of San Raffaele Scientific Institute, Milan, Italy, from January 2014 to March 2021(Brooks *et al*, 2000; de Carvalho *et al*, 2008). ALS diagnosis was established after a neurological and neurophysiological assessment performed by neurologists with expertise in MND. A control cohort consisting of seventy patients with other neurodegenerative disorders (DEG), seventy with ALSmd and forty-five healthy controls (HC) were included in the study (total patients enrolled in the control cohort one hundred and eighty-five). ALSmd were patients presenting signs and symptoms resembling those of ALS, a diagnosis which was excluded after a throughout diagnostic work-up and follow-up. DEG cohort consists of patients with other neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and FTD, these diagnoses were established after a thorough neurological and neuropsychological evaluation by neurologists with expertise in neurodegenerative disorders. HC were recruited among patients' caregivers or spouses genetically unrelated to patients with ALS. All the patients included in the study underwent blood sample collection for biomarkers quantification and genetic analysis at the first visit performed at our center. Serum samples were processed within 1 h of blood collection and kept at − 80 °C until analysis.

Participants were excluded in case of patients' refusal to undergo the examination or blood sample or withdrawal of the informed consent. The study was performed according to the Helsinki Declaration and approved by the ethic committee of our San Raffaele Scientific Institute. All patients gave informed written consent to participate in the study.

5.1.2. Demographics and clinical history

Demographics and clinical history information of the ALS and control cohorts were registered at the first evaluation performed at the San Raffaele Scientific Institute outpatient or inpatient clinic. The following information was collected by expert neurologists: date of birth, sex, age at the symptoms onset, familial history for ALS or other neurodegenerative diseases, site of symptoms onset, disease duration, neurological ALS history, prior medical history, and medications. Disease duration was defined as the period between symptoms onset and date of sampling/first neurological evaluation. Survival was defined as the time from sampling to death/tracheostomy. Patients were followed up with periodical neurological evaluation and phone calls, and survival status was updated in April 2021.

5.1.3 Evaluation of the muscle strength

Muscle strength was evaluated with the Medical Research Council (MRC) scale (Kleyweg *et al*, 1991). MRC is a validated scale for muscle strength assessment in patients affected by neuromuscular conditions. MRC scale grades muscle power on a scale of 0 to 5 in relation to the maximum expected for that muscle. Grade 5 indicates normal muscle contraction while Grade 0 indicates total paralysis with no visible muscle contraction. This score was defined as the sum of MRC scores from twelve muscles in the upper and lower limbs on both sides so that the score ranged from 120 (normal) to 0 (quadriplegic) (Riva *et al*, 2019). Furthermore, MRC progression (ΔMRC) was calculated as [(120−MRC score)/disease duration].

5.1.4 Measures of patient's functionality and disease progression

ALSFRS-R, a validated questionnaire-based scale, was used to assess disability progression in patients with ALS (Cedarbaum *et al*, 1999). Nowadays, change from baseline in the total ALSFRS-R is the most commonly used primary endpoint measure in clinical trials and is considered the gold standard measure of disability progression (van Eijk *et al*, 2020). This scale encompasses bulbar, motor, and respiratory functionality rating twelve daily activities from 0 to 4, where 0 means no function at all, and 4 means normal function. A reduction of the ALSFRS-R total score (range 0-48 points) indicates a worsening of the clinical condition (Cedarbaum *et al*, 1999; van Eijk *et al*, 2020, 2018).

The ALSFRS-R progression slope (ΔALSFRS-R), which normalizes the ALSFRSR by the duration of symptoms, has been demonstrated to be one of the strongest predictors of survival in patients with ALS (Westeneng *et al*, 2018b; Kimura *et al*, 2006). Therefore, changes in the ALSFRS-R over time (ΔALSFRS) is widely accepted as disease progression proxy. Disease progression rate (ΔALSFRS-R) was calculated as (48−ALSFRS-R)/disease duration).

ALS patients were classified accordingly to the King's clinical staging system which evaluates the spread of the disease through anatomical regions, based on the number of affected regions, and encompasses advanced stages defined by nutritional or respiratory failure. Stages one to three are defined by the number of regions involved according to El-Escorial criteria; Stage four equals nutritional failure, defined by the need for gastrostomy, or respiratory failure, defined by the need for non-invasive ventilation (NIV), with stage five equals to death (Roche *et al*, 2012).

5.1.5 Upper motor neuron burden assessment

Upper motor neuron score (UMNs) was calculated totaling the number of pathological UMN signs at the examination. At the neurological evaluation the presence of the following was counted as pathological: brisk deep tendon reflexes of muscles biceps, supinator, triceps, finger, knee and ankle reflexes, and extensor plantar responses assessed bilaterally and brisk facial and jaw jerks (score range from 0 to 16). A higher score indicates extensive UMN involvement (Turner *et al*, 2004).

5.1.6 Classification of ALS motor phenotypes

The phenotypic classification was applied after the clinical examination and revised during follow-up in accordance with the temporal criteria. The following phenotypic criteria were used:

Classic: progressive and clear UMN and LMN involvement with bulbar or spinal onset. In this category we also included patients with bulbar onset but with a spinal involvement in the first six months of disease after symptom onset (Chio *et al*, 2011).

Bulbar: patients complaining dysarthria and/or dysphagia, tongue wasting and/or fasciculations without spinal involvement for the first six months after symptom onset (Chio *et al*, 2011).

Pyramidal: patients showing a clear and evident prevalence of the UMN signs. Spastic paresis must coexist with one or more of the following signs: Babinski or Hoffmann sign, exaggerated deep tendon reflexes, clonic jaw jerk, spastic dysarthria and pseudobulbar syndrome. Spastic paresis might be present at the initial or in the late stages of the disease. In the meantime, these patients are marked by evident signs of LMN dysfunction, such as muscle weakness and hypotrophy and by chronic and active denervation at the EMG examination in at least two distinct regions (Gordon *et al*, 2006; Sabatelli *et al*, 2008b).

FA: predominant proximal upper limbs onset with weakness and atrophy. Neurological signs are confined to upper limbs for twelve months. UMN signs in upper limbs can appear at some point during the disease course except for clonus or hypertonia (Wijesekera *et al*, 2009; Hübers *et al*, 2016).

Flail leg (FL): predominant distal lower limb onset with weakness and atrophy. Signs of the disease are confined to lower limbs for twelve months. UMN signs in lower limbs can appear at some point during the disease course except for clonus or hypertonia (Chio *et al*, 2011; Wijesekera *et al*, 2009).

PLMN also known as PMA: selective clinical and electrophysiological LMN involvement. The following features must be ruled out: motor conduction blocks, UMN signs, a positive family history of spinal muscular atrophy or detection of SMN1 gene deletion or CAG repeat expansion (>40) in the androgen receptor gene (Chio *et al*, 2011).

PUMN: only clinical signs of UMN involvement are evident, such as spastic paresis, Babinski or Hoffmann sign, exaggerated deep tendon reflexes, clonic jaw jerk, spastic dysarthria, and pseudobulbar syndrome. Patients with clinical or EMG signs of LMN dysfunction are excluded from this category and so are those with a history of disease that mimics MND, family history of hereditary spastic paraparesis (HSPG) and mutation of genes related to HSPG (Pringle *et al*, 1992; Gordon *et al*, 2006; Turner *et al*, 2020).

Respiratory: patients showing a predominant respiratory involvement at the disease onset, establish as orthopnoea or shortness of breath at rest or exertional, with minimal or mild spinal or bulbar involvement in the first six months after onset (Shoesmith *et al*, 2007).

5.1.7 Classification of ALS cognitive phenotypes

The cognitive status of patients with ALS was screened with the validated Edinburgh Cognitive and Behavioural ALS Screen (ECAS) (Poletti *et al*, 2016). This scale encompasses a spectrum of functions usually impaired in ALS (ALS specific domains), namely Fluency, Executive Functions, Language Functions and Social Cognition. ECAS also evaluates functions normally spared in ALS (ALS non-specific domains) namely Memory and Visuospatial Functions. Lastly, it assesses the presence of behavioural impairment through a structured interview that must be performed by the caregiver independently from the patient and is developed on the five cardinal behavioural domains for diagnosing FTD (Rascovsky *et al*, 2011). Pathological cut-off scores were set at 105/136 for the total score and 77/100 for specific domains accordingly to the literature (Poletti *et al*, 2016).

A thorough neuropsychological assessment was performed if patients with ALS obtained pathological score at the ECAS. Based on these evaluation patients were categorized into five different cognitive phenotypes, consistently with the neuropsychological results and published criteria (Strong *et al*, 2017):

ALS-ci was established on the presence of either executive impairment (considering also social cognition) or language impairment or the co-occurrence of both dysfunctions.

ALS-bi was established on the presence of either apathy (independently from the coexistence of other behavioural abnormalities) or two or more of the following behavioural manifestations: 1) disinhibition attitude, 2) lack of sympathy or empathy, 3) perseverative or compulsive/ritualistic manner, 4) hyperorality or nutritional modifications, 5) loss of insight, 6) psychiatric manifestations.

ALS-cbi: patients fulfilling criteria for both cognitive and behavioural impairment.

ALS-FTD: patients showing FTD manifestations defined as: A) detection of progressive worsening of behavioural and/or cognition by neurological evaluation or history and B) identification of at least three of the behavioural/cognitive manifestations above-mentioned. or C) presence of at least two of the above-mentioned behavioural/cognitive manifestations, along with lack of insight and/or psychiatric manifestations or D) presence of language dysfunction fulfilling previously published

criteria for semantic dementia/semantic variant primary progressive aphasia (PPA) or non-fluent variant PPA (Neary *et al*, 1998; Gorno-Tempini *et al*, 2011).

ALS-pure motor: patients showing no signs or symptoms of cognitive and behavioural dysfunction at the screening test.

Only patients who underwent neuropsychological testing in a period between ± 2 months from serum sample collection were included in the study.

5.1.8. Neurophysiological evaluation

Patients with ALS underwent routine motor nerve conduction studies to assess bilateral median and common peroneal compound muscle action potentials (cMAPs) amplitude (peak to peak). Distal cMAPs were registered from the abductor pollicis brevis and extensor digitorum brevis muscle through surface electrodes after a supramaximal peripheral stimulation of the median and peroneal nerves. Mean cMAP at the four limbs was calculated as follows: left median cMAP + right median cMAP + left common peroneal cMAP + right common peroneal cMAP/4.

Routine transcranial magnetic stimulation was used to assess motor-evoked potential (MEP) amplitude at the four limbs. MEP/cMAP amplitude ratio was calculated for each limb, measuring the proportion of the cMAP elicited after peripheral nerve stimulation in the same target muscle (MEP/cMAP ratio) (Riva *et al*, 2012; de Carvalho *et al*, 2005). Subsequently, mean MEP/cMAP at the four limbs was calculated as follows: left upper limb MEP/cMAP + right upper limb MEP/cMAP + left lower limb MEP/cMAP + right lower limb MEP/cMAP /4.

Only patients who underwent neurophysiological testing in a period between ± 2 months from serum sample collection were included in the study.

5.2 Genetic analysis

Blood samples and DNA extraction were obtained from all patients with ALS using NucleoSpin Blood L (MACHEREY –NAGEL). ALS patients were screened for the intronic hexanucleotide expansion in the *C9orf72* gene using a two-step protocol. Firstly, a repeat-primed polymerase chain reaction (PCR) was performed using primers in Table 5.1 comprising one fluorescently labeled primer (Renton *et al*, 2011). An amplicon-length

PCR (primers on Table 5.1) was carried out and performed on an automated ABI3730DNA genetic analyzer (Applied Biosystems, Foster City, CA, USA) to determine the length of the alleles (Akimoto *et al*, 2014). Additionally, when the expansion was identified, DNA was reextracted and an independent repeat-primed PCR assay was carried out (primers on Table 5.1) (DeJesus-Hernandez *et al*, 2011). Raw data were analyzed using GeneMapper software (version 5, Thermofisher Scientific). The expansion was considered pathological when larger than 30 repeats and in the presence of a sawtooth amplification shape with a periodicity of 6bp on the electropherogram. All coding exons and adjacent intronic regions of *SOD1* and *TARDBP* genes, were analyzed by sequencing PCR products on both strands by analysis using the Big Dye Terminator v1.1 Cycle Sequencing Kit. PCR and sequencing reactions were purified with Ampure (Agencourt, Beckman-Coulter) and Big Dye XTerminator (Applied Biosystems), respectively, on a liquid handling system (Biomeck FX, Beckman-Coulter) Dye terminator reaction sequences were loaded on a 3730 AB Genetic Analyzer (Applied Biosystems). Variants were examined on ALSod database (https://alsod.ac.uk/) and dbSNP (https://www.ncbi.nlm.nih.gov/SNP/)

	FORWARD 5'>>>3'	
C9ORF72	6-FAM-AGTCGCTAGAGGCGAAAGC	(Renton *et al*, 2011)
C9ORF72	TACGCATCCCAGTTTGAGACGGGGGCCGGGGCCGGGGCCGGGG	
C9ORF72	TACGCATCCCAGTTTGAGACG	(DeJesus-Hernandez *et al*, 2011)
C9ORF72	FAM-TGTAAAACGACGGCCAGTCAAGGAGGGAAACAACCGCAGCC	
C9ORF72	CAGGAAACAGCTATGACC	
C9ORF72	CAGGAAACAGCTATGACCGGGCCCGCCCCGACCACGCCCCGGCCCCGGCCCCGG	
C9ORF72	FAM-CAAGGAGGGAAACAACCGCAGCC	(Akimoto *et al*, 2014)
C9ORF72	GCAGGCACCGCAACCGCAG	

Table 5.1 PCR primers used for the *C9orf72* gene analysis

5.3. Serum pNfH quantification

Serum pNfH concentrations (pg/ml) were determined using an enzyme-linked immunosorbent assay (ELISA). Commercial kits applied human phosphorylated neurofilament H antibody (Biovendor, RD191138300R). The sensitivity of the Elisa kit was 23.5 pg/ml. All tested samples were run in duplicate and the mean intra-assay variation was lower than 15%. Samples were run with appropriate standards and controls included in the commercial kit and the analysis was performed blinded to clinical data. Samples showing pNfH serum levels the analytical sensitivity were considered as having a value of 23.5 pg/ml. The mean inter-plate coefficient of variation was 6.8%.

5.4. Serum NfL, GFAP, UCHL1 and t-TAU quantification

Single-protein array technology (Quanterix Corporation, Lexington, MA, USA) was used to quantify serum GFAP, UCHL-1, NF-L, and tTAU levels (pg/mL). The analysis was performed with the fully automated instrument HD-1 Analyzer (Quanterix). Samples were run with appropriate standards and controls and the technician performing the assays was blinded to clinical data. For the longitudinal assay, samples were assessed on the same plate for each ALS patient to reduce batch effects. The inter-assay coefficient of variation (CV) was lower than 15%.

5.5 Gene Expression analysis

5.5.1 Motor and sural nerve biopsies sample selection

Eight obturator motor nerve biopsies from patients with ALS and seven from patients with MN were selected and included in the study. Patients fulfilling the neuropathological diagnostic criteria for probable ALS and probable or definite MN were considered eligible for the study. The histopathological criteria were previously published(Riva *et al*, 2011). All the patients underwent clinical follow-up also to confirm a diagnosis of ALS or MN coherently with appropriate clinical criteria (Brooks *et al*, 2000; de Carvalho *et al*, 2008).

We also selected nine sural nerve biopsies. Of them, five were showing clearcut active axonal degeneration with no regenerating signs and four showed clear axonal regenerating signs at the neuropathological examination. All the biopsies were collected, after informed consent, during the diagnostic work-up for diagnostic purposes and were

retrieved from our tissue bank were previously stored. All the patients who underwent motor nerve biopsy had strict clinical indication for the diagnostic procedure. The experimental protocol was approved by our Ethical Committee, and the procedures were performed in line with the approved guidelines.

Obturator nerve biopsy was carried after regional anaesthesia and with minimal sedation. In brief, the anterior branch of the nerve was surgically collected, and then split into three portions. The first part was fixed in ten percent formalin for histological evaluation using paraffin-embedded tissue. A second part was fixed in two percent buffered glutaraldehyde and in one percent osmium tetroxide. These segments were eventually embedded in EPON after alcohol dehydration protocol. Transverse sections (size from 0.5 to 1 micrometre) were stained with toluidine blue and examined by light microscopy. Ultrathin sections were stained with uranyl-acetate and lead citrate and analysed with the electron microscope. The last segment was frozen and stored in liquid nitrogen and used for gene expression studies. A similar protocol was applied for the sural nerve biopsies

5.5.2 RNA isolation protocol

RNA was extracted from the selected obturator and sural nerves samples in the study using the RNAeasy kit (Qiagen, Venlo, Netherlands). Nanodrop-2000 spectrophotometer (Celbio, Milan, Italy) was used for RNA quantification and purity. To establish the RNA integrity Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) was used. Gene expression analysis was carried out using the Illuminal HumanHT-12 v4 Bead-Chips (Illumina Inc., San Diego, California, USA), each of the arrays specifically targeted more than forty-seven thousand transcripts that were previously selected from the NCBI RefSeq database. The mean RIN of samples RNA was 5, indicating that a part of RNA was somehow degraded. For this reason we chose to use the Illumina Whole-Genome Gene Expression DASL΄ HT Assay being an optimal kit for the evaluation of the degraded RNA(April *et al*, 2009). Two hundred and fifty nanograms of total RNA were retrotranscribed to cDNA using biotinylated oligo (dT) and random nonamer primers.,We further annealed the biotinylated cDNA with the DASL Assay Pool (DAP) probe groups, with specific oligonucleotides of fifty bases specifically designed to interrogate each target sequence in the transcript. Then, universal PCR amplification and Cy3 staining

steps were applied. Lastly, labelled PCR products were obtained and hybridized to the BeadChips, which were imaged using the Illumina® BeadArray Reader. The software Illumina® GenomeStudio version 2011.1 was used to generate fluorescent hybridization signals.

5.5.3 Gene expression analysis

R statistical environment and Bioconductor package Lumi were applied for quality controls and the pre-processing steps. Outliers were identified through means of principal component analysis (PCA) and signal intensities distribution analysis with R package Array Quality Metrics functions. Signals normalization was done with the quantile procedure as available in Lumi package. Then, probes showing a similar fluorescent signal to the estimated background were eliminated. We considered all the probes that were called 'present' by the Genome Studio algorithm (detection call p-value<0.05) on at least thirty percent of samples in at least one of the two groups. We performed PCA on the normalized filtered set of 27.679 probes. The dataset was filtered, and a differential expression analysis was carried out applying the Limma method, which relies on a linear model and pooled estimate of gene variance to reveal the differently expressed genes (DEGs). Correction for multiple testing was performed by controlling the False Discovery Rate (FDR) with Benjamini-Hochberg analysis. DEGs were defined when FDR was lower than 0.05 and fold-change (FC) higher than 1.5 or lower than 0.66**.**

As mentioned, we performed a gene expression analysis on eight motor nerves obtained from patients with ALS and seven MN patients. Comparing the gene expression profiles of these two groups we obtained a pool of DEGs. Consequently, we decided to further filter our DEGs dataset to identify which of these genes were specifically up or downregulated in patients with ALS and thereby involved in the degenerative process occurring in the peripheral motor nerves of patients with ALS. To do so, we approached the matter starting from some preliminary neuropathological considerations. The histopathological findings of ALS consist of clear signs of axonal degeneration in absence of regenerating features, conversely patients with MN show abundant clusters of regeneration and remyelinating fibers. However, these changes are not specific to ALS or MN but are also shared by other neuropathies/neuronopathies. Therefore, we performed an independent gene expression analysis on five sural nerves showing only

active axonal degeneration (resembling the neuropathological findings of ALS) and four with regenerating features (resembling the neuropathological findings of MN). The DEGs, shared by both independent analyses, may be specific to the axonal degeneration/regeneration process and not of ALS. Therefore, we filtered these DEGs out and we focused our attention on the remaining genes.

5.5.4 Pathway analysis

Once the DEGs were filtered, the remaining upregulated and downregulated genes were assessed with a functional enrichment analysis performed on Gene Ontology and Reactome databases, using WebGestalt. The gene over-representation was assessed with hypergeometric test with Benjamini-Hochberg multiple testing adjustment in both databases, the filtered set of genes were considered as the customized background set. For Gene Ontology, we considered for the analysis only terms with at least three annotated genes.

5.6. Statistical methods

The distribution of the data was assessed with Kolgomorov-Smirnov and Shapiro–Wilk tests. We represent with median and interquartile range (IQR) or with mean and standard deviation the continuous variables as appropriate. Categorical variables were described as numbers and relative frequencies. Mann–Whitney U test and Kruskal–Wallis test with Bonferroni post hoc comparison were performed to assess differences among two or more than two groups as appropriate. Pearson correlation *r* was used to perform correlation between parameters. Pairs of basal and follow-up serum biomarker levels were assessed with the Wilcoxon matched-pairs signed-rank test. Clinical variables were categorized accordingly with their tertile values to assess differences among groups.

Receiver operating characteristic (ROC) curve analysis was carried out to assess diagnostic sensitivity, specificity, positive and negative predictive values (PPV and NPV) and to calculate the area under the curve (AUC) of serum biomarkers, with corresponding 5-95% CI. The highest Youden index was used to calculate the optimal cut-off of each biomarker on a ROC analysis.

Kaplan–Meier (KM) univariate analysis was carried out to estimate the biomarkers effect on survival. Patients were clustered according to biomarkers tertile values. Log

rank test (Mantel–Cox) was used to test for significant differences among groups. Patients who were alive at last follow-up were censored. Multivariable analysis with Cox proportional hazards model (enter method) was performed to estimate the proportional hazard ratios of biomarkers on survival. Cox regression was adjusted for factors that negatively influenced ALS survival.(Westeneng *et al*, 2018a; Falzone *et al*, 2020)

All statistical tests were carried out using SPSS 26.0 software (Technologies, Inc., Chicago, IL, USA). Statistical significance was set at $p<0.05$.

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