DOTTORATO TOSCANO DI NEUROSCIENZE

Ciclo XXXVI

Plasma Neurofilament Light Chain as a noninvasive biomarker in neurodegenerative diseases

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Index

1. Introduction.....pg 3

1.1 Neurodegenerative diseases.....pg 3

1.1.1 Amyotrophic Lateral Sclerosis.....pg 5

1.1.2 Alzheimer's Disease.....pg 7

1.1.3 Subjective Cognitive Decline: the preclinical stage of Alzheimer's Disease.....pg 11

1.1.4 Mild Cognitive Impairment: the intermediate stage of Alzheimer's Disease.....pg 12

1.2 Biomarkers for neurodegenerative diseases.....pg 14

1.3 Neurofilament Light Chain.....pg 15

1.3.1 Neurofilament Light Chain as a biomarker in Neurodegenerative diseases.....pg 17

2. Hypothesis and specific aims.....pg 21

3. Materials and methods.....pg 22

3.1 Patients.....pg 22

3.2 Plasma sample collection and NfL analysis.....pg 24

3.3 Statistical analysis.....pg 24

4. Results.....pg 26

4.1 ALS patients, clinical and plasma analysis.....pg 26

4.2 Cognitive Decline patients, clinical and biomarker analysis.....pg 28

4.3 Comparison in plasma NfL levels between ALS and cognitive decline groups.....pg 37

5. Discussion.....pg 38

6. Conclusions.....pg 41

7. Bibliography.....pg 42

Introduction

Neurodegenerative diseases

Neurodegenerative diseases (NDD) are a varied set of disorders of the nervous system, characterized by a progressive and unstoppable neuronal cell dysfunction and death. NDD are the most common and growing cause of morbidity and mortality worldwide, mainly among elderly people (**Agnello, 2022**). In normal physiological conditions, neuronal cell death is limited in the adult brain, even during the aging process (**West, 1994**). In NDD, the fundamental pathological feature is the increasing death of specific vulnerable populations of neurons and loss of neurons is usually associated with misfolded protein accumulation in the central (CNS) and in the peripheral nervous system (PNS) (**Morrison, 1997; Caccamo, 2017**). Misfolded proteins are altered in their physicochemical properties, resulting in dysfunction in their activity or in their interactions with other proteins (**Carrell, 1997**). Moreover, misfolded proteins may elude the ubiquitin-proteasome system or the autophagy-lysosome pathway, designate to protein elimination, resulting in misfolded proteins deposition at intra/extracellular level, with a consequent toxicity. Genetic mutations in genes that encode for these proteins are responsible for the familiar form of NDD (**Soto, 2003**). For these reasons, NDD are also defined as proteinopathies. Furthermore, NDD can involve a wide range of functional systems of the nervous system and different disorders can be characterized by dysfunction of different functional systems. Based on the area of the nervous system involved, clinical manifestations vary in NDD and neuronal deterioration can lead to cognitive deficits, dementia, motor impairments, behavioral and psychological disorders.

So, NDD can be classified according to the clinical manifestations or according to the specific protein depositions. According to clinical manifestation, two main groups can be distinguished:

1. Cognitive impairment and dementia, which involved anatomic regions such as hippocampus, limbic system, entorhinal cortex and neocortical area;
2. Movement disorders, including both hyper- and hypokinetic disorder, associated with upper and lower motor neuron dysfunction or cerebellar dysfunction. Anatomic regions involved are brainstem nuclei, thalamus, basal ganglia, cerebellar cortex and nuclei, lower motor neurons and motor cortical areas.

However, a combination of both these symptoms usually appears during the progression of disorders.

Regarding protein deposits, the accumulation can be intracellular, extracellular or synaptic. Intracellular deposition can take place in nuclear, cytoplasmic or in neurotic (dendrites or axonal) compartments, or in cellular processes (in astrocytes).

The principal proteins associated with the majority of sporadic and genetic adult-onset NDD are:

- the microtubule-associated protein tau encoded by a single gene (*MAPT*) on chromosome 17q21;
- Ab, which is cleaved from a large transmembrane precursor protein (amyloid precursor protein or *APP*). The *APP* gene has been mapped to the centromeric region of chromosome 21q21.3;
- a-synuclein, which is encoded by a single gene (*SNCA*) on chromosome 4;
- prion protein (PrP), which is a 253-amino acid protein encoded by the gene of PrP (*PRNP*) located to chromosome 20;
- transactive response (TAR) DNA-binding protein 43 (TDP-43), a highly conserved nuclear protein, encoded by the *TARDBP* gene on chromosome 1;
- FET proteins, which include the fused-in sarcoma (FUS), Ewing sarcoma RNA-binding protein 1 (EWSR1), and TATA-binding protein-associated factor 15 (TAF15). The most examined is FUS, which is a 526-amino acid-long protein encoded by a gene on chromosome 16;
- there are further proteins associated mostly with hereditary disorders. These include, for example, proteins encoded by genes linked to neurologic trinucleotide repeat disorders, neuroserpin, ferritin-related NDD, and familial cerebral amyloidosis.

Based on the brain area involved in protein deposition, clinicopathologic features and clinical manifestations of the disease can be different.

NDD can be named according to the major protein showing deposits in the nervous system. Accordingly, we distinguish tauopathies, a-synucleinopathies, TDP-43 proteinopathies, FUS/FET proteinopathies, prion diseases, trinucleotide repeat diseases, neuroserpinopathy, ferritinopathy, and cerebral amyloidosis (**Kovacs, 2017**). Among the best known NDD, there are Alzheimer's disease (AD) and other dementias, Parkinson's disease (PD) and PD-related disorders, Prion disease, Motor neuron diseases (MND), Huntington's disease (HD), Spinocerebellar ataxia (SCA), Spinal muscular atrophy (SMA). AD

with other dementia and Amyotrophic Lateral Sclerosis (ALS), the most frequent MND, are the main contributors to neurologic disability and finally to death (**Brett, 2018**). As characterized by accumulation of pathologically altered protein, their defined diagnosis is based on the neuropathological evaluation. Numerous in vivo biomarkers, including cerebrospinal fluid (CSF) and blood, are currently in development. Biomarkers detected in biofluids, in combination with both neuroimaging and genetics, are under study in order to be routinely implemented in clinical practice (**Wiltfang, 2016**). Moreover, AD and ALS degeneration is progressive and diagnosis arrives only when symptoms appear, that means disease is in an advanced stage. There is an enormous need to find biomarkers useful for early diagnosis, before the first symptoms appear, and to develop new therapeutic targets, which would guarantee improving patients' quality of life. Researchers from all around the world are looking for biomarkers that can be identified in different biological fluids such as plasma, serum, and CSF, specific for dementia and ALS, as well as for NDD in general. In the management of NDD, there is a compelling need for reliable biomarkers that can improve the accuracy of differential diagnosis and of prognostic assessment as well as predict the response to treatments.

Amyotrophic Lateral Sclerosis

ALS is a NDD characterized by a progressive deterioration and loss of upper and lower motor neurons in the brain and spinal cord, leading to paralysis. The first symptom of ALS is progressive, unilateral weakness in the distal legs and arms without remission or relapse. The disease onset can be spinal/limb or bulbar (**Goutman, 2022**). About one-third of ALS patients showed a bulbar process, manifesting difficulty in speaking, chewing and swallowing. The spinal ALS is characterized by cytoplasmic inclusions in corticospinal neurons. ALS patients with limb onset exhibit difficulty with simple actions, such as holding a cup or buttoning a shirt, stumbling more easily, and experiencing changes in their running or walking gaits. Unfortunately, patients with ALS show a gradual decline in their ability in function (**Feldman, 2022**). To evaluate if the physical abilities of ALS patients are functioning the Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS or ALSFRS-R) score is investigated. The ALSFRS-R is a 12-item scale with each item scored from 0 (unable) to 4 (normal ability) with a possible

total score range of 0 to 48. Higher the score, the better the patient is physically functioning. After the diagnosis of ASL, usually the survival is 2 to 5 years. An older age at disease onset, bulbar-onset, lower ALSFRS or ALSFRS-R score, and early dysfunction of the respiratory muscles are associated with a shorter survival in ALS. Instead, patients with spinal onset, younger age at symptom onset and difficulty in diagnosis are associated with a longer survival (**Gosselt, 2020**). ALS prevalence is greater in Caucasian population, in male sex, and after 60 years old. Risk factors that can increase ALS incidence are unhealthy habits (smoking), traumatic events and heavy manual labor, agricultural work, military service (particularly in the Gulf War), football, pesticides, chemicals, heavy metals, geography, and electric shock. ALS can be divided into familial and sporadic (idiopathic) forms. The familiar form is about 5 to 10% of ALS cases, caused by an autosomal dominant mutation and has an earlier onset. All the other ALS cases are sporadic. The sporadic ALS has no known cause, the onset occurs in the late age, and 67% of patients are males (**Ryan, 2019**). The diagnosis is determined toward clinical examinations, laboratory tests, and nerve conduction (NCSs)/electromyography (EMG) studies. A normal NCSs, without block, and normal sensory nerve action potentials (SNAPs) determines ALS diagnosis. Acute and chronic denervation, evaluated with needle EMG can give ALS diagnosis before the first symptoms appear. The spinal-onset ALS cannot be diagnosed until a minimum of 2 body regions are affected. Bulbar-onset ALS can be diagnosed when both upper and lower motor neuron dysfunction is determined. To exclude other conditions, laboratory tests are performed. Research tests are MRI, DTI and transcranial magnetic stimulation (**Williams, 2013**). CSF biomarker analysis is requested only in case of malignancy, HIV and chronic inflammatory demyelinating polyneuropathy. The El Escorial criteria provides a standard diagnostic process for ALS. These criteria supply a staging system of ALS in possible, probable, and definite condition (**Brooks, 2000**). Genetic analysis is performed if the patient has a family history of ALS, with one first- or second-degree relative is affected by ALS and/or frontotemporal dementia (FTD). Roughly 25 genes have been associated with sporadic ALS, familial ALS, or both. Ubiquitinated inclusions resulting from gene mutations are found in the spinal cord of ALS patients. Chromosome 9 open reading frame 72 *(C9orfT2), FUS, SOD 1,* and *TARDBP* are the most common genes associated with ALS (**Chia, 2017**).

Alzheimer's Disease

AD is the main cause of dementia worldwide, accounting for 60%-80% of cases, and is a condition of the elderly life, doubling in prevalence every 5 years after age 65. Since the population ages, AD is estimated to increase, tripling, by 2050 (**2023 Alzheimer's disease facts and figures**). AD is a progressive and unstoppable NDD and nowadays there is no cure. The hallmark of the disease is the accumulation of plaques of protein amyloid beta (Aβ) in the extracellular compartment of the neurons and neurofibrillary tangles of protein tau (NFTs) in the cytoplasm of neuronal cells (**Villemagne, 2013**). These cellular deposits are accompanied by damage and, finally, death of neuronal populations. Based on the area involved, brain tissues affected interfere with memories, thoughts, sensations, emotions, movements and skills. The brain damage begins years before symptoms onset. In a slow and progressive way, Aβ plaques, damaging the outside of neurons, destroy the synapses and may interfere with neuron to neuron communication. Protein tau accumulation in the inside of neurons blocks the transport of nutrients and other molecules essential for normal function and neurons' survival (**Braak , 2011**). Studies suggest that the first event is the formation of Aβ plaques and the increasing of these deposits is followed by the formation of NFTs (**Hanseeuw, 2019**). AD is also characterized by a lower brain volume, linked to brain changes caused by inflammation and atrophy. Abnormal protein tau and Aβ deposits, toxic for the neuronal cells, activate the reaction of brain immune system cells, known as microglia. Microglia reaction against toxic proteins and death cells causes AD chronic inflammation. Instead, the atrophy and the decrease in brain volume is due to the progressive cell loss. Another consequence is an affected and inefficient glucose metabolism (**Kapasi, 2017**). Early AD manifestations are memory impairment, in particular difficult to remember names, events, recent conversation, and changes in personality, characterized by depression and apathy. In advanced stages, impaired communication, disorientation, confusion, poor judgment, behavioral changes and, ultimately, difficulty speaking, swallowing and walking appear (**Bateman, 2012**).

In most cases, AD develops from 65 years old and older, known as late-onset AD (LOAD). LOAD is a result of multifactorial causes and the greatest risk factors are the advanced age, genetics and having a family history of AD (**Hebert, 2010; Saunders, 1993; Farrer, 1997**). AD incidence increases dramatically

with age. The percentage of AD cases grows with the advancing age in a direct proportional manner: 5% in the range of age 65-75, 13.1% in the range 75-84, 33.2% are older than 85. In any case, the older age alone is not sufficient to cause AD (**Hebert, 2013; Nelson, 2011**). The strongest genetic risk factor is the ε4 isoform of apolipoprotein E (ApoE) (**Loy, 2014**). Inheriting one allele of *APOE*-ε4 may increase the risk to develop AD of three times, while for those who have both alleles with *APOE*-ε4 form, the risk is eight- to 12-fold higher (**Michaelson, 2014**). ApoE is a multifactorial protein involved in lipid transport and metabolism, in maintenance of cell membrane and synapses, in facilitating neuronal communication and repair after injury (**Belloy, 2019**). The *APOE* gene contains three most common single nucleotide polymorphisms (SNPs) that lead to three different protein isoforms: ε2, ε3 and ε4. The three ApoE forms differ by only one or two amino acids at residues 112 or 158, these differences alter ApoE structure and function (**Mahley, 2006**). The ApoE-ε4 is associated with a higher accumulation of Aβ proteins and an onset at younger age (**Jansen, 2015; Spinney, 2014**). Studies reported that ApoE-ε4 colocalizes with amyloid plaques, hypothesizing that ε4 form could play as a Aβ-binding protein and, so, it could induce a pathological β-sheet conformational change in Aβ protein (**Wisniewski, 1992; Sawmiller, 2023**). Nowadays, in the USA, the ε4 allele frequency among AD patients is 56% who have one copy and 11% who have two copies (**Ward, 2012**). Moreover, genome wide association studies (GWAS) identified more than 20 genetic risk factors, implicating in a wide range of different pathways (synaptic function, APP and tau processing, inflammatory, immune response, cholesterol metabolism and endosomal vesicle recycling). The identified risk loci are: for neural-immune system, *CLU, CR1, ABCA7*, *EPHA1, CD33, INPP5D, TREM2,* HLA complex; for synaptic function, *PICALM, BIN1, EPHA1, CD2AP, MEF2C, PTK2B, AKAP9*; for endocytosis, *PICALM, BIN1, EPHA1, CD33, CD2AP, SORL1, RIN3*; and for lipid metabolism, *APOE, CLU, ABCA7, PLD3* (**Kunkle, 2019; Hinz, 2017**). These common risk loci each confer a very small increased risk, but when combined they can almost double AD incidence (**Escott-Price, 2015**).

Having a family history of AD is a risk factor, but is not necessarily to develop the disease during lifespan. However, individuals with first-degree relatives with AD have a higher probability to develop the disease than those who do not have AD family history. Genetic predisposition with worse lifestyle habits,

such as sedentary and non-healthy diet, may have an impact (**Wolters, 2017**). Furthermore, women have an increased risk of developing AD than men (**Gauthier, 2021**). Age, genetics, sex and family history are risk factors that cannot be modified, but others can reduce AD incidence. Moreover, The Lancet Commission on Dementia Prevention estimated 12 modifiable risk factors: cardiovascular risk factors (obesity, hypertension, diabetes), unhealthy habits (smoking, physical activity, diet), mentally activity, socializing and education are some of those. The prevention by intervening on modifiable risk factors is of great relevance, it can delay or prevent up to 40% of AD cases (**Livingston, 2020**).

Less than 1% of AD patients (all ages) and 11 % of early onset AD (EOAD), diagnosed before the age of 65, have rare genetic mutations on any of three causative genes, responsible for autosomal dominant AD. These genes are *APP* (Amyloid precursor protein) located on chromosome 21, *PSEN1* (Presenilin 1) located on chromosome 14 and *PSEN2* (Presenilin 2) located on chromosome 1 (**Bekris, 2010; Uddin, 2021**). Loss of function mutations or mutations that may change the production of proteins coded by *APP, PSEN1* and *PSEN2* may result in affected processing or production of Aβ protein (**Tanzi, 2005**). The *APP* gene codes for a type 1 transmembrane protein that can be cleaved toward two different pathways:

-in the non-amyloidogenic pathway, APP protein is cleaved by a-secretase, resulting in two peptides, the secreted extracellular sAPPa and the membrane-bound C83 (a 83 amino acid peptide). C83 is subsequently cleaved by y-secretase in soluble fragments.

-in the amyloidogenic pathway, APP is first cleaved by b-secretase in sAPPb, secreted, and in C99, bound to the cell membrane. In the second step, C99 is cleaved by y-secretase in fragments with a variable length, from 37 to 42 amino acids. The longer fragments, 40 and especially 42, self-aggregate in toxic insoluble plaques and form fibrils. Their downstream effects are the hyperphosphorylation of the protein tau, inflammation, excitotoxicity and oxidative stress (**Harris, 1995; McLean, 1999**).

PSEN1 and *PSEN2* genes code for presenilin proteins, catalytic subunits of y-secretase. Mutations altering presenilin proteins are the most common and cause the most aggressive form of inherited AD (**Selkoe, 2016**). Pathogenesis of AD was suggested to begin with the increasing deposits of the pathological form of Aβ, generated by sequential cleavage of the APP by the b- and y-secretase enzymes in the brain, and due to an imbalance between Aβ production and Aβ clearance. The formation of NFTs

and subsequent neuronal dysfunction and neurodegeneration, perhaps mediated via inflammation, are thought to be downstream processes (**Hardy, 2002**).

The diagnosis of AD is based on clinical assessment, in particular the clinical interview with the patient, and a cognitive and physical examination. To exclude that cognitive impairment is caused by other conditions, blood tests are performed that include full blood count, renal function, thyroid function, vitamin B12 and folate (**Albert, 2011; Hort, 2010**). Structural imaging such as computed tomography and Magnetic Resonance Imaging (MRI) is investigated to evaluate cognitive impairment. The presence of focal symmetrical medial temporal atrophy is typical of AD (**Frisoni, 2010**). 18F-fluorodeoxyglucose (FDG)-positron emission tomography (PET) hypometabolism in the parieto-temporal association areas, posterior cingulate and precuneus is supportive of an AD diagnosis (**Kato, 2016**). Amyloid PET imaging is not routinely used in many countries. Three agents (florbetapir, flutemetamol and florbetaben) bind fibrillary Aβ and correlate with amyloid burden (**Sabri, 2015; Villemagne, 2017**). CSF biomarker examination is used for a molecular diagnosis of AD. The typical CSF pattern in AD is low Aβ42 and elevated levels of phosphorylated-tau (p-tau) and total tau (t-tau). In addition to these, there are no other blood based biomarkers, specific for AD, in routine clinical use (**Olsson, 2016**). The clinicians require genetic analysis when there is the suspicion of an autosomal dominant cause of AD (**Albert, 2011**).

The pathophysiology process and structural brain alterations of AD have an evolution during the years and the beginning of the disease is many years before the development of the clinical manifestations and first symptoms (**Dubois, 2016**). In 2011, the National Institute on Aging-Alzheimer's Association (NIA-AA) group proposed that AD can be subdivided in three clinical stages based on clinical characteristics and psychometric concomitants of patients: preclinical AD or Subjective Cognitive Decline (SCD), Mild Cognitive Impairment (MCI) and AD-dementia (**Jack, 2018**). From stage 1 to stage 3, symptoms increase gradually in severity (**Khan, 2020**). Moreover, NIA-AA established in the research framework that the AD diagnosis is not defined by symptoms/signs but by biological construct along a temporal continuum. Individuals can be categorized in 3 groups by biomarkers (imaging and biofluids) using a system namely A/T/N. The "A" refers to Aβ biomarkers, amyloid PET or CSF Aβ42; "T" to tau

biomarkers, CSF p-tau, or tau PET); and "N," to biomarkers of neurodegeneration or neuronal injury (FDG-PET, structural MRI, or CSF t- tau). Each biomarker category is rated as positive or negative. Eight different combinations are possible: A+/T+/N+, A+/T+/N-, or A+/T-/N-, etc (**Jack, 2018**). Studies explored the prognostic possibilities for clinical progression and cognitive decline using ATN along AD continuum (**Yu, 2019**).

Subjective Cognitive Decline: the preclinical stage of Alzheimer's Disease

SCD is defined as the self-perception of cognitive decline in memory and/or other cognitive abilities compared with a previously normal status and unrelated to an acute event, in the absence of objective neuropsychological deficits (**Jessen, 2014**). SCD is a heterogeneous condition and at present there is no validated test. The 8-10% of affected subjects may convert to AD after several years (**Earl Robertson, 2023**). SCD occurs in the late stage of preclinical AD, not detectable on standardized neuropsychological assessment, as individuals have sustained only mild neuronal damage and are able to functionally compensate. At the same time, SCD is the earliest clinical manifestation of AD, when the underlying pathology begins to impinge upon perceived cognitive function (**Jessen, 2014**). Personality traits of individuals affected by SCD are anxiety, depression and other emotional factors. SCD has a complex and heterogeneous presentation and the researchers are working to establish a common framework and criteria for classifying SCD. Targeted diagnostic self-rated questionnaires and tests have been developed. Gifford and colleagues proposed the Subjective Cognitive Decline Questionnaire (SCD-Q) to distinguish a reliable objective cognitive impairment from SCD (**Gifford, 2015**). In the same way, the Memory Complaint Questionnaire (MAC-Q) and Perception Questionnaire—Revised (IPQ-R) were proposed to identify SCD cases. These questionnaires established that the first manifestation of SCD is episodic memory, followed by executive function (**Hurt, 2010; Reid, 2012; Vogel, 2016**). Other clinical tools for testing individuals with SCD are the SCD-Questionnaire 9 (SCD-Q9), the Auditory Verbal Learning Test-Long Delay Free Recall (AVLT-LR) and the Everyday Cognition scales (ECog). A paired associative memory test was produced by Sanabria and colleagues, called the Face-Name Associative Memory Exam (FNAME), able to detect memory deficits. A lower score at FNAME test was associated

with increased Aβ burden (**Farias, 2008; Sanabria, 2018; van Harten, 2018**), The standardization and validation of these tests are particularly important considering that fluid biomarkers and neuroimaging biomarkers, used for the biological diagnosis of MCI and AD, were not sufficiently sensible to detect SCD. Many studies reported that there are no significant differences in CSF biomarkers between individuals with SCD and healthy older adults, nor for the most accurate tau protein levels (**Antonell, 2011; Scheef, 2012; Visser, 2009**). Instead, Schoonenboom et al. affirmed that SCD individuals have significantly higher levels of Aβ42 relative to those with AD (**Schoonenboom, 2012**). As for AD, the *APOE*-ε4 is a genetic risk factor for SCD (**Ali, 2018; Mandecka, 2016**). In fact, carriers of the *APOE*-ε4 allele have worse episodic memory and smaller right hippocampal volumes than healthy controls without this allele (**Risacher, 2015**). Regarding neuroimaging biomarkers, studies on brain changes with structural MRI showed cortical thickness reduction in the bilateral entorhinal cortex and the parahippocampal cortex of SCD individuals (**Meiberth, 2015**). Moreover, an atrophy in the cornu ammonis 1(CA1) region was found in subjects with AD and SCDS (**Dorè, 2013**). Cherbuin and colleagues reported that SCD was correlated with longitudinal hippocampal atrophy that tends to worsen over time upon follow-up (**Cherbuin, 2015**). Using the Diffusion tensor imaging (DTI), studies revealed extensive changes in SCD patients compared with controls, detecting topological alterations of the brain structural connectome in SCD (**Selnes, 2013; Shu, 2018**). With Functional MRI (fMRI), SCD showed decreased activity in the resting-state network (**Rodda, 2011**). Furthermore, SCD patients exhibit hypometabolism in the right precuneus lobe and left parietal lobe, assessed with the FDG-PET method (**Scheef, 2012**). Thanks to the Amyloid PET analyses, the Aβ deposition in the brain of SCD was found to be increased compared to that of the controls (**Snitz, 2015**).

Mild Cognitive Impairment: the intermediate stage of Alzheimer's Disease

MCI is the intermediate stage between preclinical AD and dementia. The main cognitive domains are six: learning and memory, social functioning, language, visuospatial function, complex attention, or executive functioning. Generally, in MCI, the affected domain is the one who deals with learning and memory, classified as "amnestic". Nonamnestic MCI is characterized by impairment in one or more of

the other cognitive domains, while memory remains relatively intact (**Petersen, 2005; Sachdev, 2014**). Nonamnestic MCI is less common and more difficult to diagnose with respect to amnestic MCI. The prevalence in individual's older than 65 is approximately 3 to 22%. The strongest risk factor is age (**Ganguli, 2004; Hänninen, 2004; Petersen , 2010**). Other greater risk factors are male sex, carrying *APOE*-ε4 allele, having a family history of cognitive impairment, and the presence of vascular risk factors (hypertension, hyperlipidemia, coronary artery disease, and stroke) (**Caselli, 2009; Ng, 2015; Roberts, 2012; Vassilaki, 2015**). In addition, other conditions that can increase the risk of MCI are unhealthy habits (cognitively and physically sedentary lifestyle), diabetes mellitus, depression and chronic obstructive pulmonary disease (**Geda, 2010; Geda, 2014; Roberts, 2014; Singh, 2014; Verghese, 2006**). Clinicians establish the diagnosis after a thorough interview regarding the patient's history and evaluating change in cognition, abnormal cognitive function in one or more domains, normal daily activity, and absence of dementia. Standard cognitive screening tests are not available, but a used screening tool is the Montreal Cognitive Assessment (MoCA) that is a 30-point questionnaire that takes approximately 10 minutes to administer, with a cutoff point of 24/25. The sensitivity and specificity of the test have been found to be 80.48% and 81.19%, respectively. The Mini-Mental Status Examination (MMSE) and the Dementia Rating Scale (DRS), specific for abnormal cognitive function, are not recommended to detect MCI. A direct comparison of the MoCA and the MMSE also reported that MoCA was more sensitive for precisely differentiating persons with MCI from those with normal cognitive function (**Espino, 2001; Langa, 2014; Nelson, 2008**). The diagnostic criterion for AD can be applied also for MCI, characterized by 3 categories of biomarkers: amyloid ligands, functional imaging, and structural MRI. MRI studies suggested that volumetric measures of the hippocampus that show atrophy are suggestive of MCI (**Jack, 2009**). FDG-PET can detect regions of hypometabolism in the brain which may be characteristic of MCI due to AD (**Small, 2008**). Studies have suggested that CSF biomarker levels (protein tau and Aβ42) may help identify patients with MCI who are more likely to progress to AD (**Hansson, 2009**).

Biomarkers for neurodegenerative diseases

A biomarker is a biochemical tool that reflects altered pathophysiological processes of a disorder. Biomarkers include proteins, metabolites, genetic targets and imaging data. Biomarkers can be useful for the diagnosis, the prognosis and also for the therapeutic management and monitoring of the progression of a particular disorder (**Baldacci, 2020**). Ideally, a biomarker is disease specific, sensitive in discriminating different disorders, accurately measurable with little or no variability, simple to manage and inexpensive (**Tohkin, 2015**). The NDD pathogenesis, as in AD with other dementia and in ALS, is characterized by pathological protein aggregation, synaptic and neuronal network dysfunction, aberrant proteostasis, cytoskeletal abnormalities, altered energy metabolism, DNA and RNA defects, inflammation, and neuronal cell death (**Wilson, 2023**). So, there is a wide range of clinically applicable biomarkers for NDD. Despite that, biomarkers in ALS diagnosis and prognosis are lacking. Currently, the only one available diagnosis for ALS is based on neurologist's observations. Genetic targets are utilized to define ALS pathogenesis. These genetic biomarkers include genes, such as *TARDBP*, *FUS*, and *C9orf72*, encoding proteins that have a role in RNA misprocessing in ALS pathogenesis (**van Blitterswijk, 2012**). A series of biomarkers are available for diagnosis of AD and cognitive decline-related diseases. The collection, toward a lumbar puncture, of CSF to analyze amyloid and tau protein levels are used to assess AD pathophysiology (**McKhann, 2011**). Additional AD pathophysiological biomarkers are neuroimaging tools (amyloid-PET, brain MRI and FDG-PET, DTI). Imaging techniques are useful to evaluate structural and functional changes in the brains of AD patients, but they are expensive and not easily repeatable (**Elahi, 2017**). Genetic analysis includes the screening of main associated genes (*APP*, *PSEN1* and *PSEN2*), but it is required only in familiar forms. The *APOE*-ε4 is investigated as a strongest genetic risk factor for sporadic AD, when it is too late and the first symptoms have already appeared (**Greenberg, 1995**). However, NDD available biomarkers are, in general, expensive, invasive, lacking specificity, sensitivity, and validity, and are not able to fully explain the pathogenesis underlying the disorder (**Sharma, 2023**). A minimally invasive and globally accessible screening tool is needed. Noninvasive and inexpensive examinations such as peripheral blood biomarker analyses may be ideal to diagnose and differentiate NDD. Peripheral blood neurofilament light chain (NfL), neuronal proteins

reflecting axonal damage, are evolving to be robust candidate biomarkers to detect NDD. During the last decades, NfL were studied for their use in diagnosis, even in preclinical stages, in prognosis and monitoring treatment response (**Gaetani, 2019**).

Neurofilament Light Chain

NfL are neuronal cytoplasmic proteins highly expressed in large caliber myelinated axons (**Barro, 2020; Khalil, 2018**). NfL are pivotal for cytoskeleton formation and for maintenance of neuronal structure. After a neuronal damage, NfL are released in biofluids (CSF and blood) proportionally to the degree of axonal damage (**Gafson, 2020**). For these reasons, NfL are a promising biomarker of neuronal injury and death in multiple NDD.

NfL are subunits of Neurofilaments (Nfs), cylindrical scaffold proteins classified as Intermediate filaments (IF), characterized by 10nm of diameter, between actin (6 nm) and myosin (15 nm). Nfs are expressed in dendrites, neuronal soma and, primarily, in axons. Nfs confer structural stability to neurons, enable axon's radial growth and allow nerve conduction and, expanding their caliber, they permit a higher conduction velocity. Moreover they maintain the cellular shape during mechanical stress, so confer cell resistance, and regulate intracellular traffic between dendrites and axons **(Barry, 2012; Liu, 2004; Rao, 2003; Yuan, 2012b; Zetterberg, 2016;)**. Nfs are composed of 5 subunits: NfL, neurofilament middle chain (NfM), neurofilament heavy chain (NfH), α-internexin (α-int) and peripherin. All the subunits are constituted by a central conserved rod domain (310 amino acid residues with a α-helical motif and with hydrophobic heptad repeats, responsible for the formation of coiled-coil structures) flanking by a variable short amino-terminal head (98 amino acids, rich in sites for O-linked glycosylation or phosphorylation) and by a variable tail at carboxy-terminal domain (68-618 amino acids, rich in glutamic-acid and lysine stretches). Based on tail length, the molecular weight differs between each subunit (**Geisler, 1983; Steinert, 1988**). The NfH tail is composed of 3 segments: a glutamic-acid-rich segment (E segment), multiple lysine-serine-proline (KSP) repeats and a lysine-glutamic acid-proline (KEP) segment. The tail of NfM is shorter and has two E segments (E1 and E2),

two KSP repeat segments and a serine-proline (SP) and lysine-glutamic acid (KE) segment. NfL tail has only a E segment. The α-int tail is made of E segment and a KE. Peripherin has only a single phosphotyrosine residue (Figure 1) (**Angelastro, 1998; Gaetani, 2019; Gafson, 2020**).

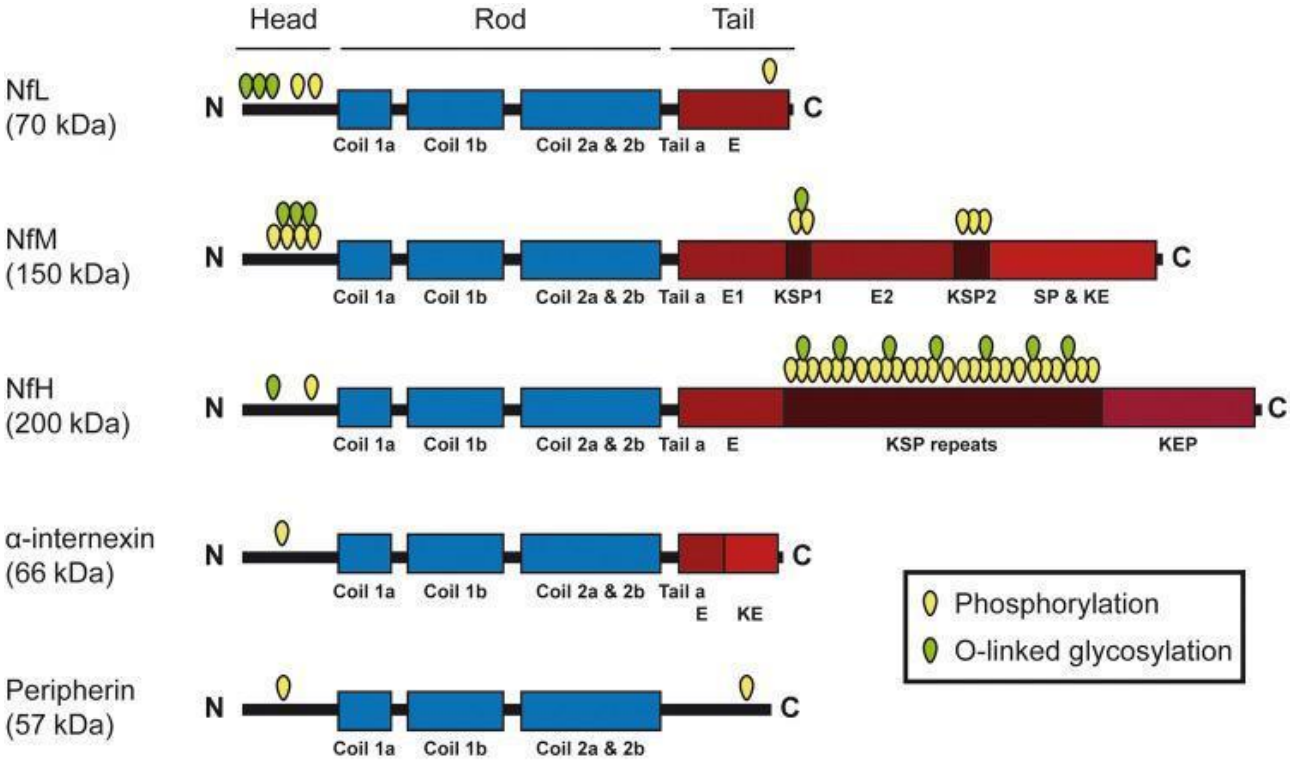


Figure 1. Neurofilaments (Nfs) structure. Gafson AR, Barthélemy NR, Bomont P, Carare RO, Durham HD, Julien JP, Kuhle J, Leppert D, Nixon RA, Weller RO, Zetterberg H, Matthews PM. Neurofilaments: neurobiological foundations for biomarker applications. Brain. 2020 Jul 1;143(7):1975-1998. doi: 10.1093/brain/awaa098. PMID: 32408345; PMCID: PMC7363489.

NfL are the backbone of Nfs. NfL interact with α-int or peripherin to constitute the core of the filament, forming a homopolymer of 10nm, and co-assemble with NfH and NfM, that are the peripheral side arms projecting radially outwards from the filament core (**Yuan 2006; Yuan 2012a**). Side-arm projections (NfH and NfM phosphorylated) interconnect Nfs, maintain inter-space between Nfs and preserve the organization in parallel structure of the axons (**Chen, 2000**; **Leermakers, 2010; Gafson, 2020**; **Yuan , 2017**). Despite the key role of NfH and NfM in axonal organization, among Nfs subunits, NfL are the fundamental. NfL knockout mice showed a severe atrophy and a retarded maturation of axonal sprouts (**Zhu, 1997**). The targeted disruption of NfL gene in NfL -/- transgenic mouse model demonstrated a smaller axonal diameter with a consequent significant decrease in velocity conduction (**Kriz, 2000**).

Instead, in NfH -/- transgenic mice, the absence of NfH does have no impact on the number of Nfs or axonal elongation or targeting. NfH loss showed only a little reduction in space between Nfs, resulting nearer (**Rao, 1998**). Abnormal Nfs structure and function in brain disease highlighted their pivotal role for neuronal cells. NfL are encoded by the *NEFL* gene. Studies reported that mutation in *NEFL* gene may contribute to neuronal loss in Charcot-Marie-Tooth (CMT) disease types 2E (**Mersiyanova, 2000**) and type 1F (**Jordanova, 2003**) and G (**Zuchner, 2004**). Instead, SNPs on *NEFH* gene, which encode for NfH, were associated with the development of ALS (**Figlewicz, 1994**),

Neurofilament Light Chain in Neurodegenerative diseases

After an axonal injury or degeneration, NfL concentration increases in CSF and blood. NfL levels rise proportionally to the entity of axonal damage, reflecting the NDD or inflammatory, traumatic and cerebrovascular diseases. For this reason, NfL were widely studied, especially in NDD, to evaluate their value as a diagnostic and prognostic biomarker and to monitor response to a treatment (**Gaetani, 2019**). Under normal physiological conditions, due to neuronal cell turnover, NfL release may occur by means of exosomes or after neuronal membrane integrity loss (**Faure, 2006; Lachenal, 2011**). Studies suggest that NfL arrive in peripheral circulation, constantly released, via perivascular drainage along basement membranes of arteries to drain into cervical or lumbar lymph nodes and then into the blood (**Carare, 2008; Szentistvanyi, 1984**). Their levels in biofluids are higher in older age under normal conditions, with a statistically significant and positive correlation. NfL levels increase twofold in 50 years old and sixfold in 80 years old compared with 20 years old subjects (**Salvioli, 2023**). In NDD, NfL concentration increases suddenly and clearly. Since NfL are the backbone of Nfs, they are the most abundant subunits and are also the most soluble one, which makes NfL the most reliably measurable Nfs subunits in biofluids. For many years, NfL were studied and analyzed only in CSF, where their concentration is about 20–50-fold greater than in peripheral blood, due to the crossing of the blood-brain barrier (**Bergman, 2016**). The standard Enzyme-Linked Immunosorbent Assay (ELISA) sandwich method is not sensitive enough to detect NfL concentration in blood and plasma samples.

Electrochemiluminescence (ECL) assay technology is more sensitive than ELISA, but it is not sufficient for detecting the lowest concentration of NfL in blood (**Gafson, 2020**). An ultrasensitive Single Molecule Assay (Simoa) platform, provided by Quanterix corp., was developed. Simoa can detect proteins at femtomolar concentration in biofluids, providing an analytical sensitivity of 0.6 pg/mL compared with 78.0 pg/mL for the corresponding ELISA. Simoa technology is a digital ELISA, where a sandwich antibody complex, specific for the target protein, is captured on microscopic beads (2.7 μm diameter) and labeled with an enzyme (one labeled immunocomplex per bead) generating a fluorescent product. The fluorescent reaction is produced and isolated in a 50 femtoliter-size chamber designed to hold only a single bead and detected with fluorescence imaging. The extremely small volume (~50 fl) of reaction chambers permits a high local concentration of fluorescent product, corresponding to an increase in sensitivity (**Rissin, 2010**). Thanks to Simoa assay, NfL can be detected also in plasma samples, accelerating the discovery and their possible use as a diagnostic biomarker. Many studies analyzed NfL concentration, previously in CSF and then in plasma, in patients affected by different NDD, such as AD, FTD, Multiple Sclerosis (MS), ALS, HD, atypical parkinsonian disorders (APD) and traumatic brain injury (TBI). Data showed that NfL measurement cannot distinguish between NDD with similar degree of myelinated axonal damage or same progression rate or disease intensity. Regarding their potential as a diagnostic biomarker, NfL can discriminate between NDD patients and Healthy Controls (HCs), with a greater concentration in CSF and plasma (**Gaetani, 2019**). In MS, NfL increase in CSF and plasma, and a significant difference between MS patients and HCs were reported. Moreover, a strong association between CSF NfL and plasma NfL were found in MS (**Disanto, 2017; Kuhle, 2013**). NfL showed the ability to discriminate MS from MS mimics, both in CSF and in plasma (**S M, 2017**). A positive association between NfL levels and cognitive impairment was found in MS patients: a higher NfL concentration in CSF and plasma was correlated with a higher cognitive deterioration and an inferior performance in memory, speaking, executive function and information processing speed (**Gaetani, 2019b**). Studies conducted on genetic MS form, reported that NfL may predict disease onset 6 years before clinical manifestation (**Gaetani, 2021**). In AD, NfL levels are higher in patients compared with HCs. Studies reported increased NfL levels in MCI and in the other dementia stages of AD. The higher NfL

concentration was not correlated to Aβ plaques pathology (**Mattsson, 2016**; **Olsson, 2016**). Moreover, AD patients demonstrated greater NfL levels with respect to the preclinical stage of MCI (**Bos, 2019**). NfL reflect cognitive deterioration, higher NfL concentration was correlated with a higher cognitive impairment in AD patients. A significant association was found between NfL and MMSE test performance (**Ramani, 2021**). Data reported poor cognition relative to specific domains, such as domains of attention, memory, visuospatial, language, information processing, and executive functions, in AD patients with altered NfL levels (**Mattsson, 2017; Rolstad, 2015**). Longitudinal studies showed that NfL increase sixteen years before first symptoms appear in blood of AD pathogenic mutation carriers in *PSEN1*, *PSEN2* and *APP* genes, with the highest concentration near the disease onset (**Gaetani, 2019; Weston, 2019**). NfL may predict MCI 3.8 years before onset with a greater accuracy with respect to CSF biomarkers t-tau and p-tau (**Kern, 2019**). In monitoring response to treatment, NfL demonstrated a reduction in plasma level in AD mouse models treated with a β-secretase (BACE) inhibitor (**Bacioglu, 2016**). NfL are also higher in CSF and plasma in FTD patients compared to HCs. NfL may discriminate between cognitive decline caused by neurodegeneration and cognitive disturbances, such as depression, not associated with NDD. NfL can help in distinguishing between behavioral variant of FTD (bvFTD) and psychiatric disorders, not characterized by neuronal loss, with a high sensitivity (**Olsson, 2019**). NfL concentration is different also in patients affected by FTD with respect to AD. AD patients showed a higher increase of CSF NfL than in FTD (**de Jong, 2007**). NfL better discriminate between AD and FTD than CSF biomarkers (**Paterson, 2018).** NfL can distinguish also between different Primary Progressive Aphasias (PPAs): non-fluent and semantic variant PPAs (nfvPPA and svPPA) showed higher NfL levels than logopenic variant PPA (lvPPA) (**Steinacker, 2017**). Moreover, NfL increase near the symptom onset in FTD mutation carriers (**Meeter, 2016**). The GENFI (Genetic Frontotemporal dementia Initiative) study reported an increase in NfL levels in *C9ORF72* mutation carriers 30 years prior FTD clinical onset, instead 15 years before for Granulin (*GRN*) pathogenic mutation carriers. *MAPT* mutations are associated with an altered value of NfL only near the FTD onset (**Staffaroni, 2022; Van Der Ende, 2019; Wilke, 2022**). NfL concentration negatively correlates with FTD patient survival (**Pijnenburg, 2015**). In ALS patients, NfL are increased compared to HCs, both in

CSF and in plasma. Moreover, NfL levels in ALS are elevated with respect to other MND and ALS mimics, such as multifocal motor neuropathy, cervical myeloradiculopathy and chronic inflammatory demyelinating polyneuropathy. NfL may distinguish between patients with first symptoms of ALS, with an onset within 6 months, and patients with ALS mimics (**Feneberg, 2018**). The sensitivity and specificity of NfL as a diagnostic tool was higher than the 80% in ALS (**Feneberg, 2018; Weydt, 2016**). Elevated NfL was detected also in the preclinical stage of ALS familiar form. In particular, asymptomatic ALS mutation carriers had no difference in NfL concentration compared with HCs, instead symptomatic ALS mutation carriers showed higher NfL value immediately before disease onset with respect to HCs. Asymptomatic ALS mutation carriers who developed the disease within 1 year showed elevated NfL (**Benatar, 2018**). High plasma NfL levels were detected 2 years before ALS onset in *FUS* mutation carriers, 3.5 years in *C9ORF72* mutation carriers and 1 year for *SOD1* (**Benatar, 2019**). These data suggest that, using NfL as a biomarker, ALS neurodegeneration may be identified 1 year before and patients can be treated with neuroprotective therapies. No differences were found in PD patients compared with HCs in NfL levels, neither in CSF nor in plasma. CSF NfL were elevated in movement disorders such as progressive supranuclear palsy (PSP), MSA and corticobasal syndrome (CBS), as compared with HCs and to PD patients (**Paterson, 2015**). MSA, PSP and CBS patients showed higher NfL also than patients affected by dementia with Lewy body (DLB) and Parkinson's disease dementia (PDD) (**Hall, 2012**). Moreover, in PD patients and in PD- related disorders no association was found between NfL levels and cognition, tested with MMSE as a global screening of cognition and Fluency Tests/A Quick Test of Cognitive Speed to evaluate specific domains (**Hall, 2015; Hall, 2016**). Increased CSF NfL concentration was found in HD patients, but due to the slow progression of the neurodegenerative process of the disease, the detection of plasma NfL is a challenge (**Constantinescu, 2008**). A negative association was reported between NfL and measures of attention and processing speed (SDMT and Stroop) in HD patients (**Byrne, 2017**).

Hypothesis and specific aims

The major aim of the project is to study plasma NfL as a biomarker in NDD, focusing on ALS and cognitive decline affected patients, in a diagnostic and prognostic way, with the advantage of using the innovative and ultrasensitive Simoa technology.

Study specific aims are:

Aim1: to demonstrate that plasma NfL could be a simple, non-invasive, inexpensive and useful biomarker for early diagnosis, before the first symptoms appear, in subjects with a family history of NDD.

Aim2: to improve the accuracy of differential diagnosis in NDD, establishing a plasma NfL reference value.

Aim3: to use plasma NfL at the individual level to longitudinally monitor within-subject change to follow the progress of the disease in different NDD.

Materials and Methods

Patients

The study involved a total of 161 patients, 21 affected by ALS and 140 patients affected by different stages of cognitive decline, subdivided in 45 with SCD, 73 with MCI, and 22 with AD. All study participants were consecutively enrolled from July 2018 to November 2022 at the Neurological Clinic I of Careggi Hospital in Florence. ALS patients received a defined diagnosis according to El Escorial diagnostic criteria for ALS (**Brooks, 1994**). SCD (**Jessen, 2014**), MCI (**Albert, 2011**) and AD (**McKhann, 2011**) patients met the criteria for a clinical diagnosis according to the NIA-AA classification. Exclusion criteria from the ALS group was the presence of all other disease processes. Patients excluded from the cognitive decline-group had a history of head injury, all other neurological diseases, major depression or substance use disorder. All study patients underwent comprehensive family and clinical assessment, neurological and functional examination, and extensive neuropsychological investigation. A positive family history was defined as to have one or more first-degree relatives with a diagnosed NDD. Blood samples were collected for genetic testing and for plasma isolation. A follow-up sample was collected for 48 patients (19 SCD, 29 MCI) of cognitive decline-group two years after the first visit. Cognitive decline-group underwent CSF collection for Aβ42, Aβ42/Aβ40, t-tau and p-tau measurement. CSF was collected by lumbar puncture, centrifuged within 1 h from collection and immediately stored at -80 °C until performing the analysis. CSF biomarkers were measured using a chemiluminescent enzyme immunoassay (CLEIA) analyzer LUMIPULSE G600 (Fujirebio). Normal values of CSF biomarkers are set as follow: Aβ42> 670 pg/mL, Aβ42/Aβ40ratio > 0.062, t-tau < 400 pg/mL and p-tau < 60 pg/mL (**Alcolea, 2019**). Among the cognitive decline-group, 28 patients (16 SCD, 9 MCI, 3 AD) also underwent amyloid-PET and 93 patients (23 SCD, 51 MCI, 19 AD) also underwent FDG-PET brain scan. After biomarker analysis, patients were classified according to the A/T/N (amyloid/tau/neurodegeneration) system, as follow:

- A+ = Aβ pathology presence
- A- = Aβ pathology absence

- T+ = p-tau concentration was higher than the cut-off value
- T- = p-tau concentration was lower than the cut-off value
- N+ = t-tau higher than the cut-off value or positive FDG-PET, as neurodegeneration biomarkers
- N- = negative neurodegeneration biomarkers, which means low t-tau value and negative FDG-PET.

Finally, four groups were defined:

- AD, when A+/T+/N+
- AD pathological change, when A+, including patients with A+/T-/N- and A+/T-/N+;
- non-AD pathological change, when A-/T+/N+
- normal biomarkers, when A-/T-/N-

Progression to MCI and to AD was diagnosed according to the NIA-AA criteria (**Jessen, 2014; McKhann, 2011**).

About genetic analysis, genomic DNA, isolated from blood samples using the automated QIAcube method, was PCR amplified and analyzed by Sanger sequencing on SeqStudio Genetic Analyzer (ThermoFisher, Waltham, MA, USA) and analyzed with the GeneMapper version 4.0 software (Applied Biosystems, Foster City, CA, USA. Main disease-associated genes (AD: *APP, PSEN1,PSEN2*; ALS: *C9ORF72, SOD1 and TDP43*) were investigated. Variants were checked on population databases (1000Genomes, gnomAD) and nonsynonymous variants were evaluated using in-silico prediction tools (SIFT, Polyphen-2, CADD). *APOE* genotyping was investigated in the cognitive decline-group by High Resolution Melting Analysis (HRMA) (**Sorbi, 1994**). The regions encompassing rs7412 [NC_000019.9:g.45412079C > T] and rs429358 (NC_000019.9:g.45411941T > C) of *APOE* were amplified with two sets of designed primers and examined with samples with known *APOE* genotypes as standard references.

The study procedure was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki.

Plasma sample collection and NfL analysis

Peripheral blood sample, collected in polypropylene EDTA-treated tubes (Sarstedt, Nümbrecht, Germany), was centrifuged within 2 h of collection, at 1300 rcf at 4°C for 10 min, to isolate plasma. Plasma samples were immediately frozen and stored at -80° C until testing. Plasma NfL analysis was performed using Simoa NF-Light SR-X kit (cat. No. 103400; Quanterix Corp., Lexington, USA) for human samples on the automatized Simoa SR-X platform (GBIO, Hangzhou, China), following the manufacturer's instructions. Quality criteria of NF-Light kit performance was: kit lower limit of quantification (LLOQ) was 0.316 (pooled CV 19%; mean recovery 102%) and the lower limit of detection (LOD) was 0.0552 pg/mL (range 0.0152-0.108 pg/mL); Plasma Dynamic range was 0– ~2000 pg/mL; expected Coefficient of Variation (CV) of duplicates (intra-assay precision) was <20%. To assess inter-assay precision and kit accuracy, Quanterix corp. provided triplicate measurements of 3 plasma based panels and 2 controls, 3 runs each across 2 instruments for 6 runs in total and 18 measurements, and certificated reproducibility (intra-assay variability) and repeatability (inter-assay variability) of the assay. Two provided quality controls, one with low NfL concentration and the other with high NfL concentration, were run with our plasma samples, and they had a mean concentration of 5.08 pg/ml and 169 pg/ml, respectively. Serially diluted calibrators were included and measured to calculate a calibration curve. All plasma samples were analyzed on a single run basis. Plasma samples and controls were diluted at a 1:4 ratio and measured in duplicate with calibrators. The NfL assay results exhibited a coefficient of variation below 20%, consistent with the expected values.

Statistical analysis

Statistical analysis was performed using IBM SPSS software version 27 (IBM SPSS Statistics). For all analyses, p values were set at $p < 0.05$ as significant and were two-tailed. Shapiro–Wilk's test was run to test the data normal distribution. As NfL was not normally distributed, the log10 transformation was performed. Descriptive statistics were conducted using means and standard deviation for continuous variables and frequencies or percentages and 95% confidence intervals (CIs) for categorical variables.

Continuous variables were correlated using Pearson's correlation analysis. Categorical variables were compared using chi-squared tests. Independent-samples *t*-test and Mann–Whitney *U*-test were used for comparison between two groups, one-way analysis of variance (ANOVA) with Bonferroni post hoc test was used for comparisons among three or more groups. Welch *t*-test was run when the assumption of homogeneity of variances was violated. Receiver-operating characteristic (ROC) curves were used to evaluate the performance of plasma NfL in predicting disease status and progression. Kaplan–Meier survival analyses with pairwise log-rank were run to compare proportions of progression of cognitive decline among groups. Youden method was performed to determine the optimal cut-off value for NfL and calculated accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Cox regression analysis was investigated to ascertain that the effect of NfL on progression from SCD to MCI was independent from other covariates.

Results

ALS patients, clinical and plasma analysis

Clinical ALS data were reported in table 1. Total ALS plasma samples were 21 (8 males and 13 females), the patients mean age at onset was 67.20 ± 14.617 years old. Fourteen patients had a spinal ALS onset (SO), 7 showed a bulbar onset (BO). Genetic testing did not find any certain significant variant. The Log function of NfL concentration (LogNfL) was used for analysis to gain normally distributed data. The mean concentration of plasma ALS LogNfL was 1.98 ± 0.32 pg/ml.

Table1. ALS group clinical characteristics.

		Total ALS patients $n = 21$
Sex *n (%)*		m 8 (38,1)
		f 13 (61,9)
Age at onset *M (SD)*		67.2 ± 14.62
Site of disease onset *n (%)*	Spinal (SO)	14 (66,7)
	Bulbar (BO)	7 (33,3)
LogNfL pg/mL *M (SD)*		1.98 ± 0.32

Abbreviations: ALS, amyotrophic lateral sclerosis; BO, bulbar onset; f, female; LogNfL, Log function of NfL concentration; m, male; M, mean; n, number; SD, standard deviation; SO, spinal onset.

A Pearson's correlation analysis determined that a statistically significant positive correlation there was between LogNfL and age at onset ($\beta = 0.039$; $p < 0.05$) in ALS. In fact, a linear regression model established a significant linear relationship between LogNfL and age at onset [$F(1,18) = 4.96$, $p < 0.05$, and adjusted $R2 = 0.17$]. A linear relationship was found also between LogNfL and site of onset [$F(1,19) = 6.47$, $p < 0.05$, and adjusted $R2 = 0.21$]. No significant relationship was found with gender [$F(1,19) = 0.4$, $p = 0.535$, and adjusted $R2 = -0.031$]. Moreover, a statistically significant linear relationship emerged between age at onset and site of onset [$F(1,18) = 5.92$, $p < 0.05$, and adjusted $R2 = 0.21$].

Independent-samples *t*-test established that LogNfL was higher in BO (2.21 ± 0.32 pg/mL) than SO (1.87 ± 0.27 pg/mL) with a statistically significant difference of −0.34 (95% CI, −0.61 to −0.06), $t(19) = -2.54$, $p = 0.020$, and there was homogeneity of variances, as assessed by Levene's test for equality of variances ($p = 0.906$) (Figure 2). A ROC analysis was performed, a cutoff LogNfL level of 2.1028 pg/ml discriminated between BO and SO with 78.6% sensitivity and 71.4% specificity (95% CI 54.5–98.6%). Furthermore, a statistically significant difference in age at onset was found between BO and SO [−14.86 (95% CI, −24.78 to −4.93), $t(15.377) = -3.18$, $p = 0.006$]. The mean age at onset of SO was 62 ± 4.33 years, and BO had a mean age at onset of 76.86 ± 1.74 years, (Figure 3).

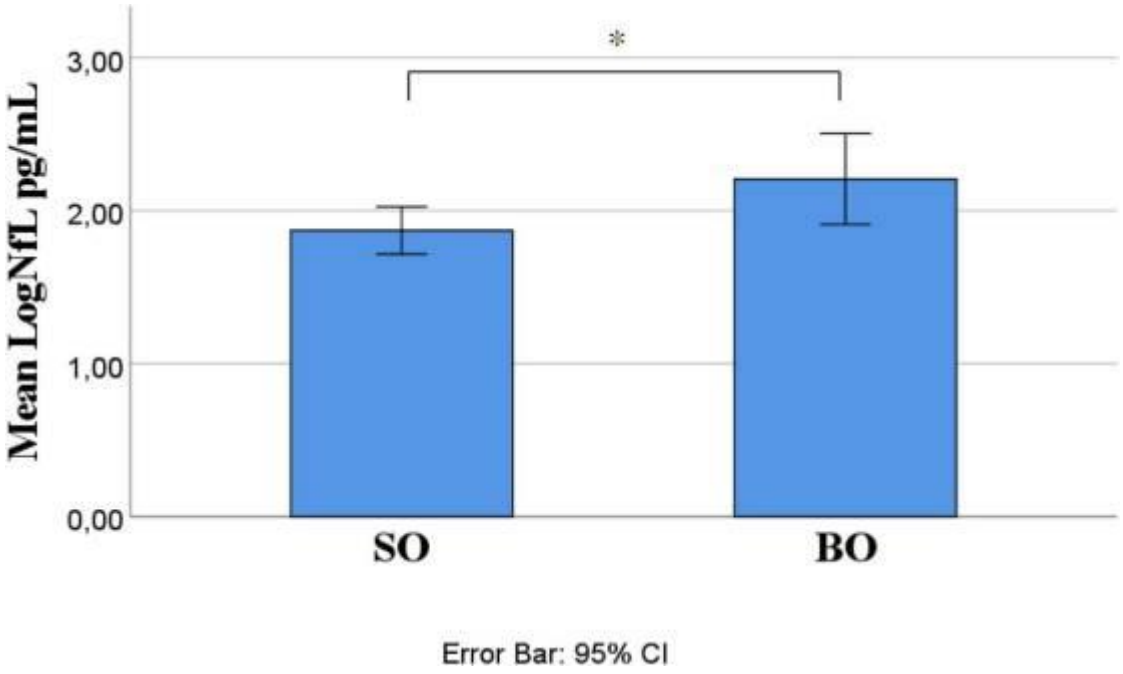


Figure 2. ALS site of onset compared for mean LogNfL levels. Mean LogNfL was higher in BO than SO (2.21 + 0.32 versus 1.87 + 0.27 pg/mL; p = 0.020). Abbreviations: LogNfL, log of NfL concentration; SO, spinal onset; BO, bulbar onset.

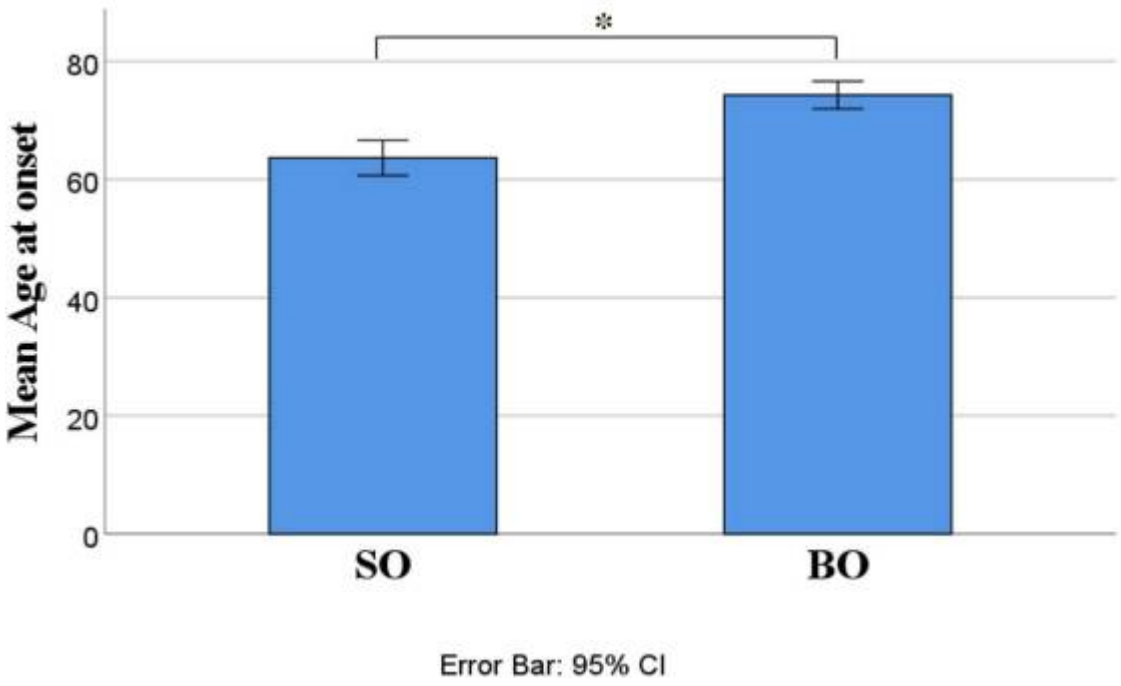


Figure 3. ALS site of onset compared for mean age at onset. Mean age at onset was higher in BO than SO (62 + 4.33 versus 76.86 + 1.74. p = 0.006). Abbreviations: LogNfL, log of NfL concentration; SO, spinal onset; BO, bulbar onset.

Cognitive Decline patients, clinical and biomarker analysis

Demographic and clinical data of cognitive decline-groups were summarized in Table 2. Total plasma samples of cognitive decline-groups were 140: 45 SCD, 73 MCI and 22 AD. A statistically significant difference emerged in age at disease onset (SCD vs MCI, $p = 0.004$; SCD vs AD, $p = 0.023$), in MMSE test score (SCD vs MCI, $p = 0.029$; SCD vs AD, $p < 0.001$) and in *APOE* ε4+ frequency (SCD vs AD, $\chi 2 = 8.77$, $p = 0.003$) between each group. Genetic analysis did not highlight any certain significant variant. A statistical significance difference in LogNfL levels was found between the SCD, MCI and AD groups ($F[2136] = 14.99$, $p < 0.001$, $\eta 2 = 0.181$) (Figure 4a): SCD vs MCI ($p = 0.002$), SCD vs AD ($p < 0.001$), MCI vs AD ($p = 0.010$).

Table 2. Clinical characteristics of cognitive decline groups.

	SCD	MCI	AD
n	45	73	22
Age at onset *M (SD)*	66.43 (9.12)	71.26 (7.96)	72.64 (7.12)
Disease duration *M (SD)*	4.61 (4.82)	3.63 (2.63)	3.85 (3.50)
Years of education *M (SD)*	12.37 (3.59)	11.42 (4.33)	9.71 (5.53)
MMSE score *M (SD)*	27.51 (2.37)	26.39 (2.11)	19.23 (4.74)
Sex *n (%)* m	16 (35.6)	25 (34.2)	11 (50)
f	29 (64.4)	48 (65.8)	11 (50)
Family history of AD *%*	73.81	63.64	52.94
APOE ε4⁺ *%*	23.81	39.73	61.90
LogNfL (pg/mL) *M (SD)*	1.11 (0.22)	1.21 (0.19)	1.4 (0.12)

Abbreviations: AD, Alzheimer's disease; APOE, apolipoprotein E; f, female; LogNfL, Log function of NfL concentration; MCI, mild cognitive impairment; m, male; M, mean; MMSE, Mini-Mental State Examination; n, number; SCD, subjective cognitive decline; SD, standard deviation.

LogNfL correlated with age at disease onset ($\beta = 0.549$, $p < 0.001$) and MMSE score ($\beta = -0.291$, $p = 0.001$). There were no differences in LogNfL between male and female ($p = 0.222$) or between *APOE*ε4+ and *APOE*ε4− subgroups ($p = 0.579$). Covariate adjusted model showed a significant effect of diagnosis group (SCD, MCI and AD-D) on LogNfL, confirmed after controlling for age, education, MMSE score and *APOE* genotype [$F(2119) = 30.51$, $p = 0.033$, partial $\eta 2 = 0.056$].

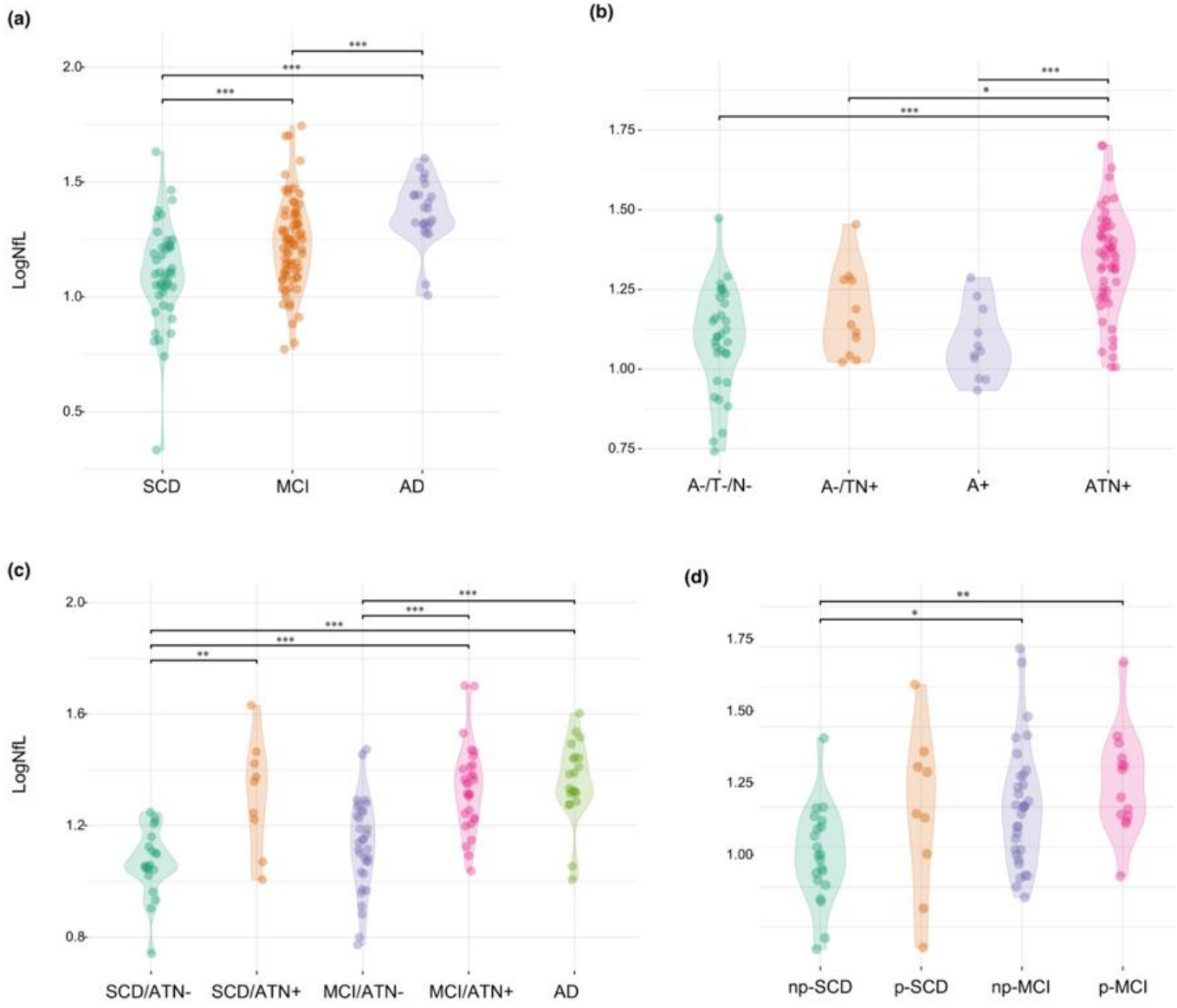


Figure 4. LogNfL levels in cognitive decline-groups. (a) Comparisons between groups based on the diagnosis. LogNfL were highest in AD patients (AD vs MCI, p = 0.010; AD vs SCD, p < 0.001), MCI had higher LogNfL than SCD (p = 0.002). (b) Comparisons between groups based on A/T/N classification. The ATN+ group showed the highest LogNfL concentration (ATN+ vs A−/T−/N−, p < 0.001; ATN+ vs A−/TN+, p = 0.015; ATN+ vs A+, p = 0.001). (c) Comparisons diagnosis/ATN groups. The SCD/ATN− patients had the lowest LogNfL levels (SCD/ATN− vs SCD/ATN+, p = 0.003; SCD/ATN− vs MCI/ATN+, p < 0.001; SCD/ATN− vs AD, p < 0.001). MCI/ATN− group had lower LogNfL than MCI/ATN+ (p < 0.001) and AD (p < 0.001). (d) Comparisons between progression groups: a difference emerged in np-SCD versus np-MCI (p = 0.020) and in np-SCD versus p-MCI groups (p = 0.003). Abbreviations: A/T/N, amyloid/tau/neurodegeneration; LogNfL, log of NfL concentration; MCI, Mild Cognitive Impairment; np-MCI, non-progressive MCI; np-SCD, non-progressive SCD; p-SCD, progressive SCD.

CSF biomarker measurement and frequencies of positive amyloid-PET and FDG-PET were shown in Table 3. A statistically significant difference was found in Aβ42 (SCD vs AD, p < 0.001; MCI vs AD, p < 0.001), in Aβ42/Aβ40 (SCD vs AD, p < 0.001; MCI vs AD, p < 0.001), in p-tau (SCD vs AD, p < 0.001; MCI

vs AD, p < 0.001) and in t-tau levels (SCD vs AD, p < 0.001; MCI vs AD, p < 0.001). Regarding the neuroimaging biomarkers, a significant difference was found in FDG-PET positive frequency on total number (SCD vs AD, p < 0.001; MCI vs AD, p < 0.001). About ATN evaluation, significant differences emerged in A+ (SCD vs AD, p < 0.001; MCI vs AD, p < 0.001), in T+ (SCD vs AD, p < 0.001; MCI vs AD, p = 0.006) and in N+ presence (SCD vs AD, p < 0.001; MCI vs AD, p = 0.002).

The criteria used to assess Aβ pathology and the frequency in each group were reported in Table 4.

Table 3. Biomarker analysis in cognitive decline-groups.

	SCD	**MCI**	**AD**
CSF			
n	30	60	20
Aβ42 (pg/mL) *M (SD)*	1066.06 (400.61)	929.21 (450.78)	518.33 (146.6)
Aβ42/Aβ40, *M (SD)*	0.08 (0.02)	0.07 (0.02)	0.04 (0.01)
p-tau (pg/mL) *M (SD)*	55.00 (31.54)	68.48 (50.89)	129.53 (60.8)
t-tau (pg/mL) *M (SD)*	417.35 (221.31)	456 (296.9)	784.38 (349.15)
Neuroimaging			
Amyloid-PET + *n (%)*	9/16 (56.25)	6/9 (66.67)	2/3 (66.67)
FDG-PET + *n (%)*	7/23 (30.43)	18/51 (35.29)	17/19 (89.47)
ATN			
A+ *n (%)*	13/30 (43.33)	33/60 (55.00)	20/20 (100)
T+ *n (%)*	9/30 (30.00)	30/60 (50.00)	19/20 (95.00)
N+ *n (%)*	11/30 (36.67)	34/60 (56.67)	19/20 (95.00)

Abbreviations: AD, Alzheimer's disease; Aβ, Amyloid-beta; ATN, amyloid/tau/neurodegeneration; CSF, cerebrospinal fluid; FDG-PET, fluorodeoxyglucose-positron emission tomography; MCI, mild cognitive impairment; M, mean; n, number; PET, positron emission tomography; p-tau, phosphorylated-tau; SCD, subjective cognitive decline; SD, standard deviation; t-tau, total-tau.

Table 4. Definition of Aβ pathology and frequency in cognitive decline-groups.

	A+ (12)	ATN+ (54)
CSF A+ / Amyloid PET N.P.	7 (6 MCI, 1 AD)	38 (1 SCD, 21 MCI, 16 AD)
CSF A+ / Amyloid PET -	1 (SCD)	2 (1 SCD, 1 AD)
CSF A- / Amyloid PET +	2 (SCD)	2 (1 SCD, 1 MCI)
CSF A+ / Amyloid PET +	2 (1 SCD, 1 MCI)	12 (6 SCD, 4 MCI, 2 AD)

Abbreviations: Alzheimer's disease; Aβ, Amyloid-beta; ATN, amyloid/tau/neurodegeneration; CSF, cerebrospinal fluid; MCI, mild cognitive impairment; N.P., not performed; PET, positron emission tomography; SCD, subjective cognitive decline.

Based on A/T/N classification, cognitive decline patients were divided in 4 different groups: 33 patients with normal biomarkers in A-/T-/N- group, 11 with non-AD pathological changes in A-/TN+, 12 with

AD pathological change in A+ (including A+/T-/N- and A+/T-/N+), and 53 with AD in ATN+ (A+/T+/N+). Demographic and clinical characteristics were reported in Table 5.

Table 5. A/T/N groups clinical analysis

	A−/T−/N−	A−/TN+	A+	ATN+
n	33	11	12	53
Age at disease onset *M (SD)*	63.71 (8.47)	69.61 (7.35)	66.73 (8.04)	72.08 (5.98)
Disease duration *M (SD)*	4.71 (4.41)	3.68 (2.87)	5.06 (4.69)	3.43 (3.08)
Years of education *M (SD)*	12.33 (3.89)	11.82 (4.58)	11.25 (4.75)	12.00 (4.56)
MMSE score *M (SD)*	27.10 (2.26)	26.54 (1.48)	25.14 (2.98)	23.95 (4.14)
Sex: *n* female/ *n* male	23/10	6/5	8/4	29/24
Family history of AD *%*	69.70	72.73	50.00	50.94
APOE ε4+ *%*	33.33	9.09	33.33	61.54
LogNfL (pg/mL) *M (SD)*	1.09 (0.16)	1.18 (0.14)	1.11 (0.15)	1.34 (0.16)

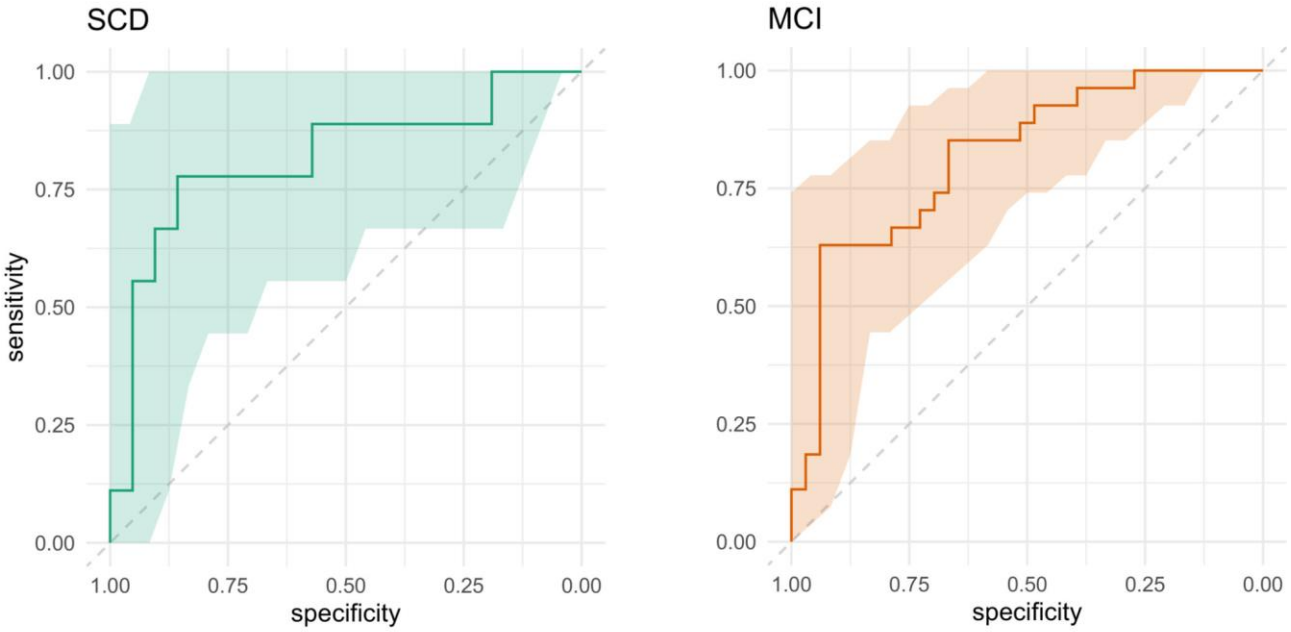
Abbreviations: AD, Alzheimer's disease; APOE, apolipoprotein E; ATN, amyloid/tau/neurodegeneration; f, female; LogNfL, Log function of NfL concentration; MCI, mild cognitive impairment; m, male; M, mean; MMSE, Mini-Mental State Examination; n, number; SCD, subjective cognitive decline; SD, standard deviation.

A statistically significant differences were found in age at disease onset and in MMSE score between A−/T−/N− and ATN+ ($p < 0.01$), in *APOE* ε4+ frequency between A−/T−/N− and ATN+ ($p = 0.011$) and between A−/TN+ and ATN+ ($p = 0.002$). LogNfL levels differed among the ATN groups ($F[3, 96] = 7.21$, $p < 0.001$, $\eta 2 = 0.184$). ATN+ had higher LogNfL than A−/T−/N− ($p < 0.001$), A−/TN+ ($p = 0.015$) and A+ ($p = 0.001$). There was no difference between A−/T−/N− and A−/TN+ ($p = 0.765$), between A−/T−/N− and A+ ($p = 1.00$) or between A−/TN+ and A+ ($p = 1.00$) (Figure 4b).

Associating diagnosis and ATN, four groups were defined. The groups consisted of 21 SCD/ATN−, nine SCD/ATN+, 33 MCI/ATN−, 27 MCI/ATN+. AD patients were 20. The SCD/ATN+ patients showed an age at disease onset higher than the SCD/ATN− patients (71.06 ±7.63 vs 61.46 ± 6.92, $p = 0.002$). MCI/ANT+ patients had a higher age at onset (73.38 ± 5.56 vs 67.95 ± 8.49, $p = 0.004$) and a higher frequency than MCI/ATN− patients of *APOE*ε4+ (66.67% vs 37.50%, $p = 0.002$). LogNfL levels were significantly different between each diagnosis/ATN groups [$F(5103) = 13.50$, $p < 0.001$, $\eta 2 = 0.396$]. . The SCD/ATN− patients had lower LogNfL than SCD/ATN+ ($p = 0.003$), MCI/ATN+ ($p < 0.001$) and AD patients

($p < 0.001$). MCI/ATN− had lower LogNfL than MCI/ATN+ ($p < 0.001$) and AD patients ($p < 0.001$). No differences were found between SCD/ATN− and MCI/ATN− ($p = 1.00$) or between SCD/ATN+, MCI/ATN+, and AD patients ($p = 1.00$) (Figure 4c).

NfL levels, detected in plasma samples at baseline, showed good accuracy in distinguishing between ATN+ and ATN− patients in the SCD and MCI groups [area under the curve (AUC) 0.815 and 0.818, respectively]. In the SCD group, NfL with a cut-off of 19.45 pg/mL discriminated ATN− and ATN+ patients with excellent specificity of 95.24%, good PPV and NPV of 83.33%, but not in sensitivity with a value of 55.56%. In the MCI patients, NfL might distinguish between ATN− and ATN+ with a cut-off of 20.49 pg/mL, with excellent specificity of 93.94%, with very good PPV, fair NPV and poor sensitivity (62.96%) (Figure 5).



N	30
AUC	0.815
cut-off (pg/mL)	19.45
Accuracy	83.33 (69.99 : 96.67)
Sensitivity	55.56 (37.77 : 73.34)
Specificity	95.24 (87.62 : 100)
PPV	83.33 (70.00 : 96.67)
NPV	83.33 (70.00 : 96.67)

N	60
AUC	0.818
cut-off (pg/mL)	20.49
Accuracy	80.00 (69.88 : 90.12)
Sensitivity	62.96 (50.74 : 75.18)
Specificity	93.94 (87.90 : 99.98)
PPV	89.47 (81.71 : 97.24)
NPV	75.61 (64.74 : 86.48)

Figure 5. LogNfL accuracy in discriminating A/T/N status. ROC curves for accuracy of LogNfL in distinguishing ATN– and ATN+ groups in SCD and MCI. Colored shapes indicate 95% CI. Cut-off values estimated by Youden's method. Accuracy, sensitivity, specificity, PPV and NPV are expressed as percentages (95% CI). Abbreviations: A/T/N, amyloid/tau/neurodegeneration; AUC, area under the curve; CI, confidence interval; LogNfL, log of NfL concentration; MCI, Mild Cognitive Impairment; NPV, negative predictive value; PPV, positive predictive value; ROC, Receiver-operating characteristic; SCD, Subjective Cognitive Decline.

Forty-eight patients (19 SCD, 29 MCI) underwent follow-up visit with a new blood sample collection for NfL measurement 2 years (T2) after baseline collection (T1). Follow-up NfL levels increased highly consistent over time ($p < 0.001$). The mean NfL change (ΔNfL) was 1.13 ± 5.47 pg/mL in 2 years (0.71 ± 2.98 pg/mL per year), with no differences between SCD and MCI. ΔNfL was correlated with age at onset ($p = 0.017$), while there was no effect of the other variables on NfL change.

Follow-up visits showed that nine (30%) SCD patients progressed to MCI (p-SCD), and fourteen (29.79%) MCI patients developed dementia (p-MCI). None of the SCD patients developed dementia. The

np-SCD (21, 70.00%) and np-MCI (33, 70.21%) were patients who did not progress. Clinical data were reported in Table 6. The p-MCI group had a higher frequency of *APOE*ε4+ ($p = 0.037$) and lower MMSE scores ($p = 0.002$). There were no differences between the np-SCD and p-SCD groups for all the variables analyzed. LogNfL levels were significantly different between patients who progressed and patients who did not progress from SCD to MCI or from MCI to dementia [$F(5103) = 5.06$, $p = 0.003$, $\eta 2 = 0.172$]. The np-SCD group had lower LogNfL than the np-MCI ($p = 0.020$) and p-MCI groups ($p = 0.003$) (Figure4d).

Table 6. Clinical data collected during follow-up.

	np-SCD	**p-SCD**	**np-MCI**	**p-MCI**
n	21	9	33	14
Age at disease onset *M (SD)*	66.44 (6.60)	68.20 (10.52)	71.04 (7.62)	73.57 (6.73)
Disease duration, years *M (SD)*	4.76 (5.97)	4.24 (4.95)	3.56 (2.47)	2.84 (1.96)
Years of education *M (SD)*	12.29 (4.23)	12.33 (3.32)	10.97 (4.11)	12.00 (4.09)
MMSE score *M (SD)*	27.61 (4.42)	27.01 (2.42)	26.61 (1.86)	23.81 (3.05)
Sex: *n* female/ *n* male	12/9	7/2	21/12	10/4
Family history of AD *%*	61.90	77.78	57.58	35.71
APOE ε4⁺ *%*	20.00	50.00	27.27	64.29
LogNfL (pg/mL) *M (SD)*	1.10 (0.15)	1.23 (0.26)	1.25 (0.19)	1.32 (0.16)

Abbreviations: AD, Alzheimer's disease; APOE, apolipoprotein E; f, female; LogNfL, Log function of NfL concentration; MCI, mild cognitive impairment; m, male; M, mean; MMSE, Mini-Mental State Examination; n, number; SCD, subjective cognitive decline; SD, standard deviation.

According to established cut-off values at baseline in distinguishing ATN+ from ATN-, during follow up, patients were classified as follows: NfL−, when levels < cut-off value; NfL+, when levels > cut-off value. Seven (15.56%) SCD patients showed NfL+ (levels > 19.45 pg/mL cut-off) and 23 (31.51%) MCI patients were NfL+ (levels > 20.49 pg/mL cut-off). A Kaplan–Meier survival analysis showed a higher proportion of progression from SCD to MCI in the SCD/NfL+ group (80.00%) as compared to SCD/NfL− (22.73%)($p = 0.002$). There was no difference in the proportion of progression from MCI (with NfL+ or without NfL-) to AD ($p = 0.25$) (Figure 6). To evaluate the effect of NfL on the rate of conversion from SCD to MCI, adjusting for possible confounding factors, we performed Cox proportional hazards regression analysis, considering progression time as time and including age at baseline, years of

education and *APOE* genotype as covariates. The regression model was significant ($p = 0.002$) and NfL group was the only significant variable ($p = 0.007$).

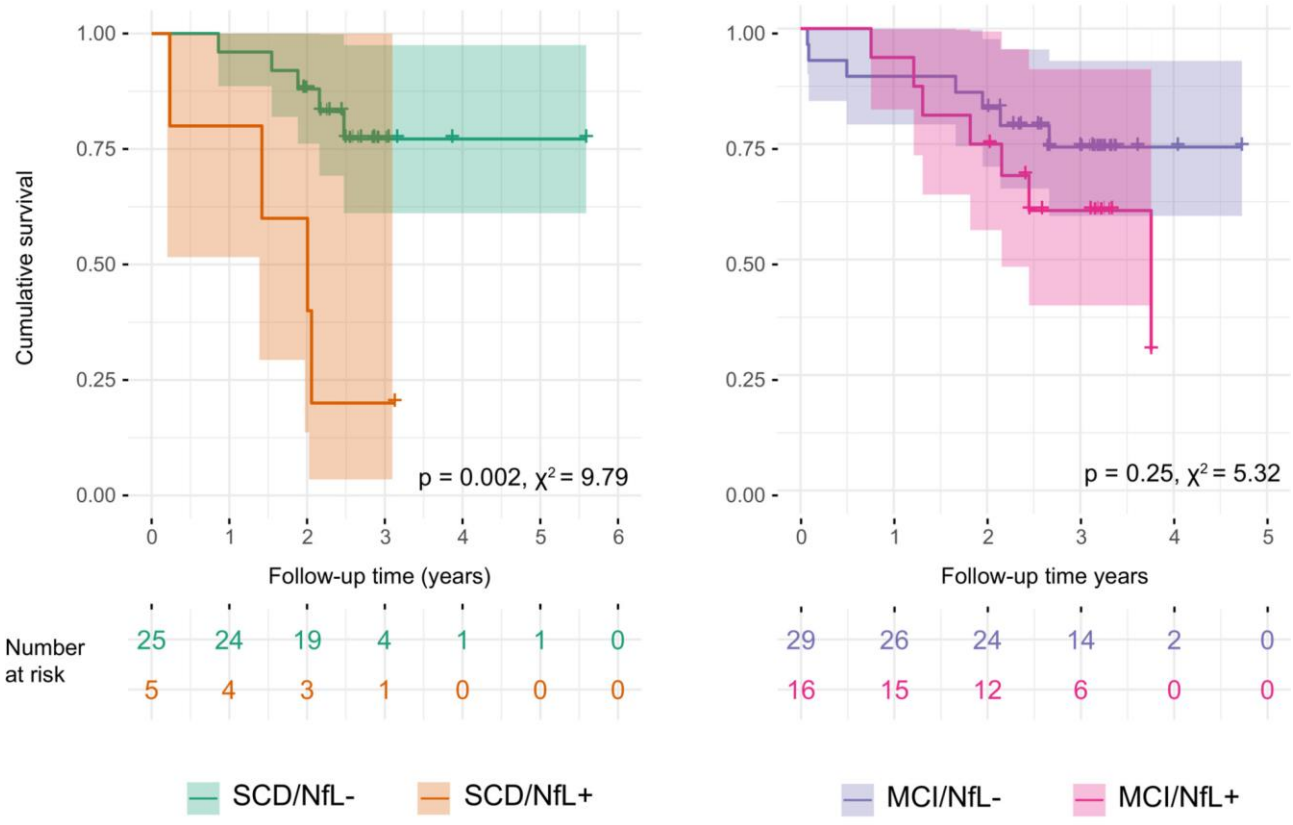


Figure 6. Kaplan–Meier survival analysis. Comparison of proportion of progression from SCD to MCI and from MCI to AD between NfL– and NfL+ groups. For patients who progressed, follow-up time indicates the time of progression. Colored shapes indicate 95% confidence interval. Abbreviations: AD, Alzheimer's Disease; MCI, Mild Cognitive Impairment; NfL, neurofilament light chain; SCD, Subjective Cognitive Decline.

A significant interaction was detected between change in NfL and progression of cognitive decline [$F(3, 44) = 5.2$, $p = 0.032$, $\eta 2 = 0.014$], confirmed also after age-adjustment [$F(3, 41) = 4.28$, $p = 0.010$, $\eta 2 = 0.239$]. A significant effect was reported between np-SCD and p-MCI ($p < 0.001$) and between np-MCI and p-MCI ($p = 0.033$) (Figure 7a). In particular, NfL concentration showed a change of 1.63 ± 2.50 pg/mL (0.81 ± 1.25 pg/mL per year) in np-SCD, a change of −1.39 ± 3.88 pg/mL (−0.13 ± 3.24 pg/mL per year) in np-MCI, and an increase of 7.05 ± 8.12 pg/mL (3.52 ± 4.06 pg/mL per year) in p-MCI. Considering the ATN groups (A−/T−/N−, A−/TN+, A+, and ATN+), a different effect was found on NfL change between A+ and ATN+ ($p = 0.024$) (Figure 7b). In particular, NfL showed a decrease

of −1.94 ± 3.32 pg/mL (−0.97 ± 1.66 pg/mL per year) in the A+ group and an increase of 3.07 ± 7.21 pg/mL (1.53 ± 3.60 pg/mL per year) in the ATN+ group.

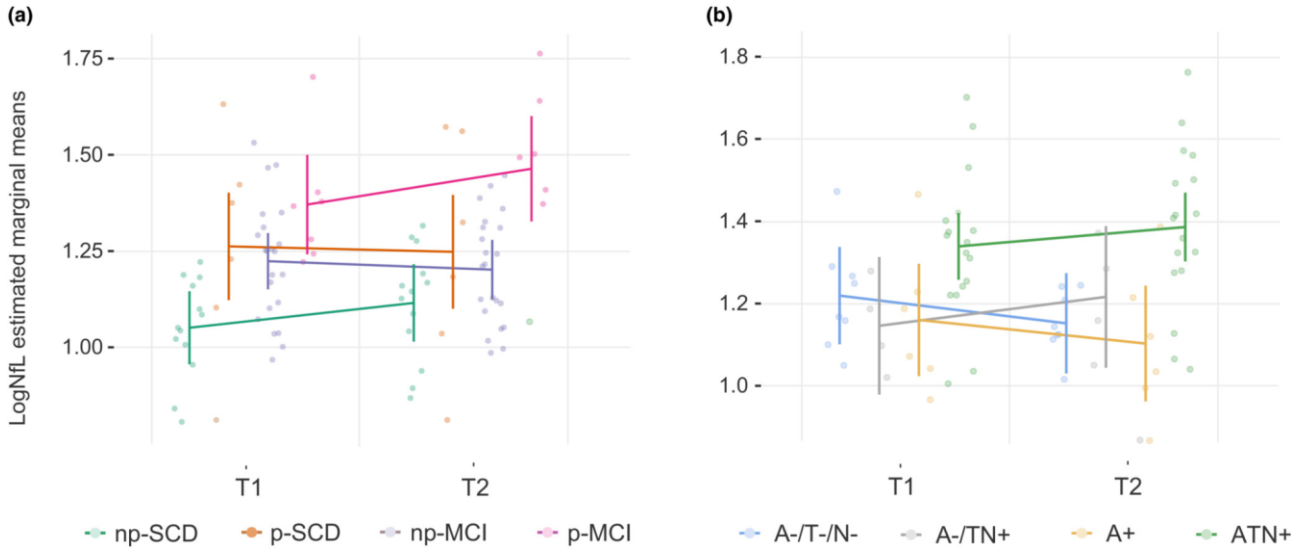


Figure 7. LogNfL change in progression and ATN groups. T1 and T2 indicate the blood collection at first and second visit for plasma NfL measurement. (a) Effect of cognitive decline progression on logNfL change: a significant effect was found in np-SCD versus p-MCI ($p < 0.001$) and in np-MCI versus p-MCI ($p = 0.033$). (b) Effect of A/T/N on NfL change: a significant effect emerged in A−/T−/N− versus ATN+ ($p = 0.077$) and in A+ versus ATN+ ($p = 0.024$). Abbreviations: A/T/N, amyloid/tau/neurodegeneration; LogNfL, log of NfL concentration; MCI, Mild Cognitive Impairment; np-MCI, non-progressive MCI; np-SCD, non-progressive SCD; p-SCD, progressive SCD.

Comparison in plasma NfL levels between ALS and cognitive decline groups.

A statistically significant difference in mean plasma LogNfL emerged between ALS and cognitive decline groups (ALS vs SCD, $p < 0.001$; ALS vs MCI, $p < 0.001$; ALS vs AD, $p < 0.001$). A difference in LogNfL was also observed between ALS and A/T/N status (ALS vs A+, $p < 0.001$; ALS vs ATN+, $p < 0.001$; ALS vs A-/TN+, $p < 0.001$; ALS vs A-/T-/N-, $p < 0.001$), and finally between ALS and progression in cognitive decline groups (ALS vs np-SCD, $p < 0.001$; ALS vs and np-MCI, $p < 0.001$; ALS vs p-SCD, $p < 0.001$; ALS vs p-MCI, $p < 0.001$).

Discussion

The main aim of this study was to investigate the diagnostic and prognostic power of plasma NfL in NDD using the ultrasensitive Simoa SR-X technology. In particular, project objectives were to determine that plasma NfL can be an useful tool in differential diagnosis of NDD, as well as in early diagnosis of NDD and in longitudinally monitoring the disease progression. NfL levels were detected in patients affected by ALS and cognitive decline at different stages, which were SCD, MCI and AD (**Ingannato, 2021; Mazzeo, 2023**). Analysis of plasma NfL levels in 21 ALS patients showed a statistically significant correlation with age at disease onset, higher NfL levels corresponded to a higher age at ALS onset. A significant association emerged with ALS site of onset. Plasma NfL levels were increased in BO, suggesting a higher neurodegeneration degree than the spinal form. In ALS patients, a linear association resulted between the ALS site of onset and age at onset. BO patients showed a later age at onset of disease (**Ingannato, 2021**). BO and higher age at symptom onset have been identified as negative prognostic factors for the disease (**Arora, 2023; Ferraro, 2021**), so we could hypothesize that elevated NfL concentration is a negative factor for the ALS progression. Moreover, the mean of plasma NfL levels in our ALS group was comparable with ALS data worldwide (**Li, 2018; Gille, 2019; Verde, 2019**).

About the cognitive decline groups, SCD, MCI and AD patients characterized by positive diagnostic biomarkers had higher plasma NfL levels than patients with normal values of CSF, neuroimaging o ATN (isolated Aβ pathology or neurodegeneration without AD pathology) biomarkers. Cut-off values were identified to distinguish ATN− and ATN+ with particularly good accuracy: 19.45 pg/mL in SCD and 20.49 pg/mL in MCI (**Mazzeo, 2023**). These plasma NfL reference values were in accordance with the published value of 20 pg/mL, identified in a large study on 60-70 aged and neurologically healthy subjects (**Simrén, 2022**). Plasma NfL was an excellent tool for the preclinical diagnosis of AD, identifying patients with SCD and MCI not associated with AD. A cuff-off plasma NfL value for the AD diagnosis was not yet determined. Moreover, plasma NfL levels were comparable in SCD and MCI patients characterized by ATN+ with AD patients (**Mazzeo, 2023**). These results suggest that NfL reflect the underlying pathology rather than cognitive levels, confirming our previous data (**Giacomucci, 2022**). Plasma NfL capability to determine an earlier diagnosis is important for SCD patients to start therapeutic

treatment and try to delay dementia onset. Furthermore, findings of this study highlight the power of using NfL in longitudinal studies, demonstrating that NfL levels increase in plasma years before the clinical onset of dementia (**Mazzeo, 2023**). In fact, literature data reported that NfL grow in biofluids a decade before first symptoms appear in AD gene mutation carriers (**Preische, 2019**). Plasma NfL also showed a prognostic utility, detecting disease progression from SCD to MCI and from MCI to AD (**Mazzeo, 2023**). In particular, NfL levels increased by 0.71 pg/mL per year, confirming data published by Khalil and colleagues (**Khalil, 2020**). In our study, NfL increasing rate was higher in patients who progressed, especially from MCI to AD, compared to patients who did not progress (**Mazzeo, 2023**). In fact, the release in plasma in MCI patients who progressed to AD was approximately 3.5 times higher than in MCI patients who did not progress, supporting previous data (**de Wolf, 2020**). Moreover, our results showed that rate of NfL increase that is lower than 1.64 pg/mL per year can exclude progression from MCI to AD with high accuracy (**Mazzeo, 2023**). Our data can confirm other studies that reported the ability of plasma NfL to distinguish between carriers of a mutation in *APP*, *PSEN1* or *PSEN2* genes and non-carriers, based on rate of levels increase (**Preische, 2019**). Moreover, plasma NfL demonstrated that the rate of increase was higher in patients with ATN+ status than in those with isolated A+. In addition, patients with only Aβ pathology showed equal NfL change during years as patients with normal values of CSF biomarkers (**Mazzeo, 2023**). These results suggest that NfL release increases when there are concomitant pathological changes, amyloid deposits and neurodegenerative processes linked to other protein deposits. Benedet and colleagues hypothesized that NfL concentration increases when Aβ pathology and tauopathy are associated (**Benedet, 2020**). These findings suggest that the biological definition of AD cannot be limited to the isolated Aβ pathology (**Jack, 2018**) and that is not sufficient for risk and progression of AD dementia (**Ebenau, 2020**).

Finally, plasma NfL can discriminate between different NDD, showing a significantly higher concentration in ALS than in cognitive decline groups. Previous studies highlighted that ALS patients had increased NfL compared with HCs or other forms of MND (**Steinacker, 2016; Xu, 2016; Rossi, 2018; Gagliardi, 2019**). So, our data extends the results of previous studies on plasma NfL levels in NDD and, also, prove plasma NfL diagnostic and prognostic value as a non-invasive biomarker.

In interpreting our study results, limitations of small sample size, lack of control group and monocentric investigations should be considered. Further analyzes are needed to extend and to confirm our results. On the other hand, all samples were collected prospectively, processed, and stored using the same standardized method, and plasma NfL analyzes were performed in a single batch, ensuring good reproducibility.

Conclusions

In conclusion, our findings prove that plasma NfL is a power tool for NDD evaluation and management. In ALS patients, plasma NfL can help to distinguish different site of disease onset with high accuracy. It can discriminate between motor disorders and cognitive disorders. In the management of preclinical disease, SCD patients with low NfL levels had a decreased risk to progress to MCI or dementia. Patients with plasma NfL value above the cut-off value had an increased risk to progress to AD. These results are important to identify patients suitable for treatment. In addition, plasma biomarkers are preferable with respect to gold standard diagnostic tools, such as CSF and neuroimaging analysis. In fact, plasma NfL is an ideal biomarker, being minimally invasive, simple to manage, inexpensive, and readily available. Plasma NfL is a promising biomarker in the diagnosis, even in the early stage of NDD~~,~~ in prognosis and in monitoring of therapeutic treatments.

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