Review

Current application of neurofilaments in amyotrophic lateral sclerosis and future perspectives

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Abstract

Motor neuron disease includes a heterogeneous group of relentless progressive neurological disorders defined and characterized by the degeneration of motor neurons. Amyotrophic lateral sclerosis is the most common and aggressive form of motor neuron disease with no effective treatment so far. Unfortunately, diagnostic and prognostic biomarkers are lacking in clinical practice. Neurofilaments are fundamental structural components of the axons and neurofilament light chain and phosphorylated neurofilament heavy chain can be measured in both cerebrospinal fluid and serum. Neurofilament light chain and phosphorylated neurofilament heavy chain levels are elevated in amyotrophic lateral sclerosis, reflecting the extensive damage of motor neurons and axons. Hence, neurofilaments are now increasingly recognized as the most promising candidate biomarker in amyotrophic lateral sclerosis. The potential usefulness of neurofilaments regards various aspects, including diagnosis, prognosis, patient stratification in clinical trials and evaluation of treatment response. In this review paper, we review the body of literature about neurofilaments measurement in amyotrophic lateral sclerosis. We also discuss the open issues concerning the use of neurofilaments clinical practice, as no overall guideline exists to date; finally, we address the most recent evidence and future perspectives.

Key Words: amyotrophic lateral sclerosis; biomarkers; motor neuron disease; neurofilament light chain; phosphorylated neurofilament heavy chain

Introduction

Motor neuron disease (MND) spectrum embraces a heterogeneous group of fatal neurodegenerative disorders defined by the degeneration of motor neurons. Amyotrophic lateral sclerosis (ALS) is the most common and aggressive form of MND, involving both upper (UMN) and lower motor neurons (LMN). Muscle weakness leads to respiratory failure and death, which usually occur within 3–5 years after symptom onset (Riva et al., 2016; Hardiman et al., 2017). Notwithstanding this theoretical definition, the broad clinical spectrum ranges from classical to atypical phenotypes, different site of symptoms onset and genetic background, extramotor involvement, including cognitive impairment, which result in heterogeneous diseases, progression rates and survival.

Currently, the diagnosis of ALS is mainly based on the clinical findings (Brooks et al., 2000; Riva et al., 2016). No specific diagnostic test is available yet. However, neurophysiological tests, neuroimaging investigations and genetic analysis are helpful to support the diagnosis and to rule out ALS disease mimics. The mean diagnostic delay is currently about 9–12 months from symptom onset, far from being acceptable considering the short survival time (Hardiman et al., 2017). An early diagnosis is essential to avoid unnecessary treatments/investigations and for an early institution of appropriate therapies and patient's management. Indeed, it has been demonstrated that an early diagnosis have a positive impact on quality of life and survival (Nzwalo et al., 2014). Lastly, a prolonged diagnostic delay leads to a late patient enrollment in clinical trials, affecting the evaluation of potential novel disease-modifying treatments. Therefore, an implementation of reliable wet biomarkers to speed up the diagnosis of ALS is urgently needed.

To date, only few clinical and genetic factors have been shown to influence ALS prognosis: age at disease onset, site of symptoms onset, functional and respiratory performance, cognitive dysfunction, clinical phenotypes and the detection of chromosome 9 open reading frame 72 (*C9orf72*) hexanucleotide repeat expansion have been recognized as predictors of survival and are included in a recently developed complex prognostic model (Westeneng et al., 2018). However, the variability of clinical phenotypes and disease progression makes it difficult to accurately predict individual outcome and consequently to obtain a homogeneous population in clinical trials. For these reasons, a wet biomarker able to predict ALS may be of great usefulness for patient selection and stratification.

In recent years, the search for a diagnostic and prognostic wet biomarker in ALS has taken important steps forward. Many molecules, including amyloid β 1–42 peptide, phospho-

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tau/total tau ratio, TAR DNA-binding protein-43 (TDP-43), C-reactive protein and chitinase family proteins, have been investigated as potential diagnostic and prognostic biomarkers (Lunetta et al., 2017; Andres-Benito et al., 2018; Majumder et al., 2018; Gille et al., 2019; Thompson et al., 2019; Lanznaster et al., 2020; Vu et al., 2020); however, neurofilaments (NFs) are the most promising and validated in the perspective of clinical translation. NFs are neuron-specific proteins that form the scaffold of the axons. To date, four subunits of NFs have been recognized: NF light (NfL), NF medium (NfM) and NF heavy (NfH) chain and alpha-internexin (**Figures 1** and **2**). Each of these subunits contains a tail domain implicated in the protein–protein interactions and contributes to the assembly of polymers and to form the cytoskeletal structure. Cytoskeletal impairment and disrupted axonal trafficking have already been associated with ALS pathogenesis and several ALS-related genes codify for proteins involved in these mechanisms. Spatacsin (SPG11) is reported to have a role in cytoskeletal stability and transport regulation; Profilin 1(PFN1) regulates actin polymerization; NIMA Related Kinase 1 (NEK1) is necessary for cilia formation and cytoskeletal stability. Dinactin subunit 1 (DCTN1) codifies for a motor protein in retrograde axonal transport. Tubulin alpha 4a (TUBA4A) and peripherin (PRPH) are structural components of the cytoskeleton, respectively, TUBA4A (that composes microtubules) and PRPH (an intermediate neurofilament expressed in the peripheral nervous system); variants in neurofilament heavy chain (NEFH) itself is associated with ALS (Peters et al.,2015; Gentile et al., 2019).

The accumulation of NFs into cerebrospinal fluid (CSF) and serum is a known marker of neuronal injury. Elevated levels of NFs have been detected in ALS and in other neurological

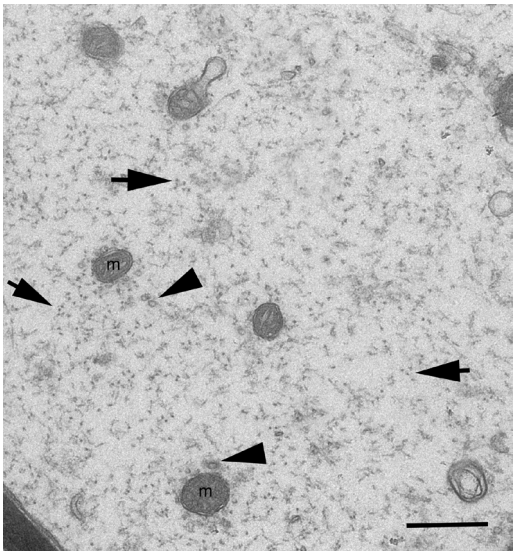

Figure 1 | **This transmission electronic microscope image shows a transverse section of a human motor axon.**
The image highlights the cytoskeletal scaffold, which is pivotal for both the structure and the function of the axon. The cytoskeleton of every eukaryotic cell is formed by three different structural components, from the larger to the smaller: microtubules, intermediate filaments and microfilaments (not visible in this image). Neurofilaments, the intermediate filaments of neurons, are visible as small dark dots, pointed out by arrows. Microtubules, the largest caliber constituents of the cytoskeleton, are visible as ring-like structures, indicated by arrowheads. Mitochondria, which generate the energy that allows the axonal transport along the cytoskeleton, are labeled with the letter "m". The myelin sheath (darker and thicker) and the axolemma (thinner, in the inner side) are visible on the left. Sourced from the authors' laboratory.

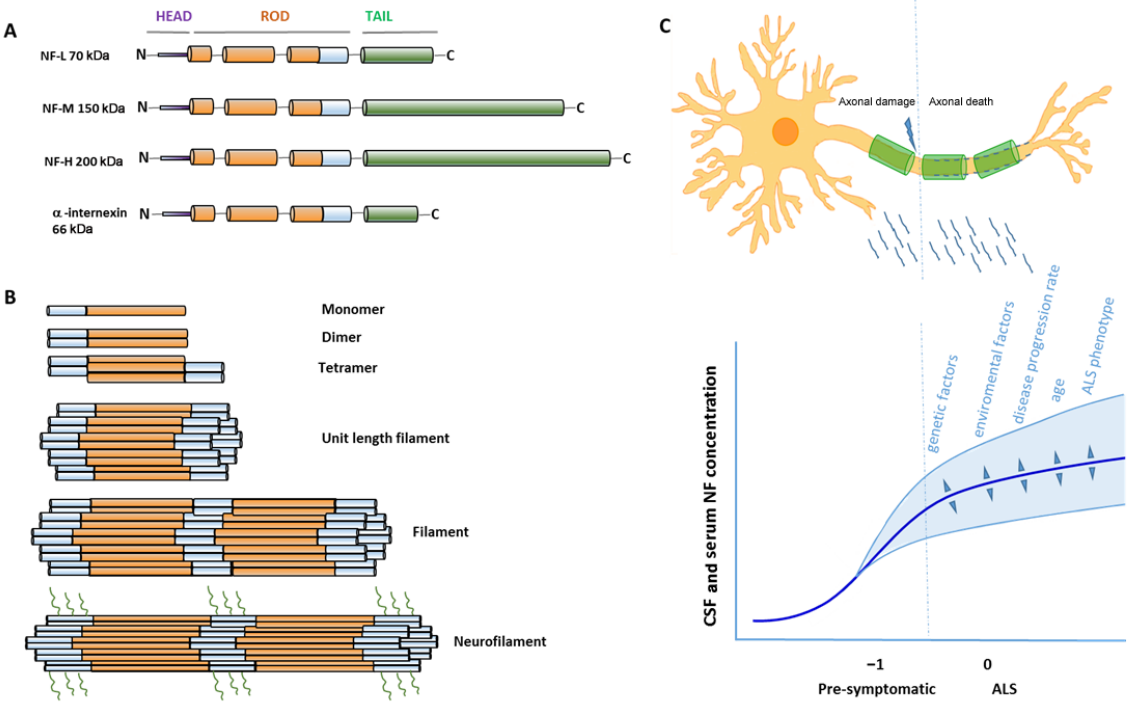

Figure 2 | **Overview of neurofilaments structure.**
(A) Neurofilament (NF) structure: Nfs are classified in neurofilament light chain (NF-L), neurofilament medium chain (NF-M), neurofilament heavy chain (NF-H) and α-internexin (α-int) according to the molecular mass of their subunits. All NF proteins have an N-terminal head domain, a central α-helical rod domain and a C-terminal tail domain. (B) Assembly of NFs: Monomer subunits form parallel dimers between subunit core domains. Two staggered, antiparallel dimers form tetramers and the lateral association of eight tetramers form cylindrical structures known as unit length filaments. The annealing of the unit length filaments forms long filaments which are further compacted to form mature neurofilaments. (C) NF kinetics in amyotrophic lateral sclerosis (ALS): During the pre-symptomatic stage, axonal damage takes place and NFs are released into the extracellular space and subsequently into cerebrospinal fluid (CSF) and blood. When ALS symptoms appear, large cell death causes extensive release of NF resulting in higher concentration of this biomarker in CSF and serum. NFs levels in the pre-symptomatic and disease phase are influenced by different factors as genetic and environmental factors, disease progression rate, age and ALS phenotype. Sourced from the authors' laboratory.

disorders such as acquired and hereditary peripheral neuropathies and other neurodegenerative diseases (Mariotto et al., 2018; Sandelius et al., 2018; Didonna and Opal, 2019; Altmann et al., 2020). Several studies have already explored NFs as potential biomarkers in ALS: the aim of this review is to dissect the most recent evidence about NFs in ALS, focusing on their role in the diagnostic process and in the prediction of prognosis.

Search Strategy and Selection Criteria

We searched PubMed database up to May 31, 2020 using the terms "amyotrophic lateral sclerosis AND neurofilament," "ALS AND biomarkers". For clinical trials, the https://clinicaltrials.gov/webpage was searched.

Analytical Methods of Neurofilaments Quantification

NFs concentration can be measured in peripheral blood (plasma, serum) and CSF. NFs concentrations in the CSF of ALS patients are remarkably elevated (usually more than 1×10^{-9} g/mL) and easily detectable. Conversely, the detection of serum levels, in the order of 10^{-12} g/mL, represents a potential pitfall. Enzyme-linked immunosorbent assay (ELISA) is the most used and widely available method for NF quantification. However, the analytical sensitivity (represented by the detection threshold) of ELISA may be not always sufficient to detect the low NF concentrations in the peripheral blood.

Electrochemiluminescence (ECL) assays have been used to study NfL on CSF and blood (Gaiottino et al., 2013; Gille et al., 2019). Their analytical sensitivity is reported to be superior to ELISA and they also have the advantage of requiring lower sample volume; ECL demonstrated a higher correlation between CSF and serum levels compared to ELISA (Kuhle et al., 2016).

Single-molecule array (Simoa) is a digital form of ELISA. Immobilizing single immunocomplexes on paramagnetic beads, it allows detection at single molecule-level, significantly improving analytical sensitivity. Simoa was employed in the study of NFs for the first time in 2015 (Gisslen et al., 2016). Commercial Simoa has a sensitivity of 6–8 pg/mL; laboratory developed Simoa yielded detection thresholds lower than 1 pg/mL. When compared with ELISA this technology demonstrated a lower detection threshold for both NfL and pNfH and also a better correlation between CSF and serum levels, boosting the study of NFs on blood (Kuhle et al., 2016; Wilke et al., 2019). Moreover, Simoa is automated, assuring a good repeatability of results. A recent study, which tested pNfH on serum of ALS patients using both Simoa and ELISA, observed a lesser inter-assay variability with Simoa (Benatar et al., 2020).

Even though ECL and ELISA tests on serum have a lower analytical sensitivity compared with Simoa, they might still be adequate for the assessment of NF levels in clinical practice. pNfH ELISA tests with good analytical sensitivity, in the order of 20 pg/mL, are available on the market. Furthermore, pNfH ELISA detection threshold can be lowered from 20 pg/mL to 6 pg/mL using biotin-streptavidin (De Schaepdryver et al., 2019). Conversely, ELISA performance has been found inadequate to accurately quantify NfL serum concentration (Gaiottino et al., 2013), whereas ECL was used with a declared analytical sensitivity of 15.6 pg/mL (Gille et al., 2019). In light of these considerations, being serum pNfH and NfL median concentration in ALS about 170 and 125 pg/mL respectively (De Schaepdryver et al., 2019; Falzone et al., 2020), pNfH assessment with ELISA and NfL assessment with ECL might yield satisfactory results in the prognostic and diagnostic evaluation of most ALS patients, without resorting more expensive technologies.

The use of NFs in longitudinal settings, for instance as a biomarker of pharmacological response in clinical trials, appears promising and relies on performing repeated sequential sampling (Benatar et al., 2020). Therefore, in this context it will be crucial that NF analytical method should be highly accurate, assuring high repeatability and reproducibility of the measurements. Further studies comparing Simoa with ELISA and ECL are needed to clarify which technology should be applied in clinical practice.

Diagnostic Value

Several studies have consistently shown that both pNfH and NfL levels are significantly elevated in the CSF of ALS patients compared to healthy and disease controls (Poesen et al., 2017; De Schaepdryver et al., 2018; Verde et al., 2019) (**Table 1**). However, accordingly to the first report, performed in a small cohort of patients, neither pNfH nor NfL seemed sufficiently accurate in distinguishing ALS from ALS-mimic disorders (Tortelli et al., 2012). In spite of that, the interest in the diagnostic role of NFs significantly increased in the last decade and subsequent studies performed in larger cohorts reconsidered NFs as potential diagnostic biomarkers (Poesen et al., 2017; Steinacker et al., 2017; Feneberg et al., 2018b; Li et al., 2018). Despite being both promising biomarkers, pNfH slightly outperformed NfL in the CSF when the two were compared (sensitivity: 78–100% pNfH *vs*. 85.4–96.2% NfL; specificity: 68.8–88.0% pNfH *vs*. 53.5–78.0% NfL) (Poesen et al., 2017; Feneberg et al., 2018b).

A blood-based biomarker would be preferable to a CSF biomarker to avoid invasive procedure and in order to perform repeated sampling over time in longitudinal studies. Serum pNfH and NfL are also increased in ALS patients compared to healthy and disease controls (De Schaepdryver et al., 2018; Verde et al., 2019). Although NFs serum concentrations are five to ten-fold lower compared to CSF, a correlation between serum and CSF levels has been demonstrated, being stronger when Simoa assay was used (Boylan et al., 2013; Gaiottino et al., 2013; De Schaepdryver et al., 2019). However, CSF pNfH performed better than serum pNfH in ALS as a diagnostic biomarker (sensitivity: 88.2% *vs*. 71.8%; specificity: 85.3% *vs*. 78.3%) (De Schaepdryver et al., 2019). Serum NfL proved accurate in the diagnosis of ALS versus healthy controls (sensitivity 100% and specificity 92.0%); however, accuracy was lower when compared with ALS mimic disorders (sensitivity 88.5% and specificity 75.8%) (Feneberg et al., 2018b; Gille et al., 2019; Verde et al., 2019). A similar performance was detected for CSF NfL measurement, although only one work compared them (Feneberg et al., 2018b). Notably, a study comparing the diagnostic performance of the two NFs on serum is still lacking.

More recent works aim to improve the diagnostic performance of NFs by the concomitant measurement of other biomarkers. The simultaneous evaluation of a set of biomarkers, an approach already employed for Alzheimer's disease (AD), might increase both sensitivity and specificity (Blennow and Zetterberg, 2018). TAR DNA-binding protein-43 (TDP-43) accumulation in motor neurons is the neuropathological hallmark of ALS (Riva et al., 2016); therefore, the pivotal role of TDP-43 in the pathogenesis of ALS suggests promise for the development of novel TDP-43-based biomarkers for ALS, even if attempts in this direction have been non-conclusive so far (Feneberg et al., 2018a; Majumder et al., 2018). Interestingly, a recent study performing a combined measurement of NfL and TDP-43 (quantified by Simoa) on CSF tested more accurate than NfL alone (Kasai et al., 2019).

In a landscape so abundant of promising evidence, some issues have prevented measurement of NF levels from entering clinical practice so far. On the analytical side, more standardization is needed, even if large multicenter studies

have already taken some steps towards this direction. Moreover, as discussed above, different assays are available and few studies conducted a direct comparison between them, even if available data suggest that Simoa may be more accurate compared with ELISA for serum pNfH measurement (Wilke et al., 2019; Benatar et al., 2020). Notably, a reference standard method for the quantification of NFs, i.e. mass spectrometry, has not been employed in ALS studies so far. From a clinical point of view, NFs performances in discriminating ALS from its mimics were not always satisfactory. This is reflected by the differing choice of the optimal cut-off values, depending on the different control group, and hence diagnostic task, considered by the previous studies (i.e. distinction from healthy controls, neurologic diseases or MND mimics). Therefore, regarding CSF NFs, the risk-benefit ratio of an invasive maneuver might still be questioned. Serum NFs, especially NfL, seem very promising, but only a few study compared them with CSF NfL and pNfH (Poesen et al., 2017; Li et al., 2018); further comparative studies are needed in order to investigate whether they are as reliable as CSF NFs.

Neurofilaments in the Pre-Symptomatic Amyotrophic Lateral Sclerosis Stage

Although ALS pathogenesis and the kinetics of MN neurodegeneration are largely unknown, the hypothesis that ALS might have a long pre-symptomatic phase of unknown duration is consistently accredited (Benatar et al., 2018). Growing evidences from animal models and patients with ALS indicate that axonal degeneration occurs in the disease early disease stage, before symptoms onset (Riva et al., 2011; Maglemose et al., 2017; Gentile et al., 2019). In this context, the development of an ALS biomarker able to detect the earliest events indicating MN degeneration, since the pre-symptomatic disease stages, would be of great value, opening the window for early therapeutic interventions. The few pioneering studies addressing this issue suggested that both CSF pNfH and NfL and serum NfL are already elevated in early symptomatic and pre-diagnostic (between onset and diagnosis) phase (Feneberg et al., 2018; De Schaepdryver et al., 2019). Two studies enrolling pre-symptomatic mutation carriers evaluated the dynamic of NF levels elevation in CSF and blood. No difference was observed between pre-symptomatic carriers and healthy controls when the sampling was performed too early before phenoconversion (Weydt et al., 2016; Benatar et al., 2018). NF levels rise some months before appearance of symptoms while no significant elevation was observed in asymptomatic mutation carriers 24 months before onset, suggesting that the change is linked with incipient onset (Weydt et al., 2016). The pre-symptomatic elevation of NF levels is earlier in C9orf72 expansion carriers, compared to fused in sarcoma (FUS) and super superoxide dismutase 1 (SOD1) mutation carriers (up to 3.5 years before the first symptoms) (Benatar et al., 2018).

Prognostic Value

There is wide agreement on the fact that the detection of high levels of pNfH/NfL predicts an aggressive disease course and shorter survival. Higher levels of NFs are observed in both CSF and serum of patients that exhibit a faster decline of the ALS Functional Rating Score–Revised (ALSFRS-R) (Gaiani et al., 2017; Steinacker et al., 2017; Gille et al., 2019; Falzone et al., 2020). The impact of NF levels on survival prediction is clearly demonstrated by Kaplan-Meier survival curves, showing a significant separation of cumulative survivals between subgroups of ALS patients when divided in accordance with different NF levels (Steinacker et al., 2017; Falzone et al., 2020; Thouvenot et al., 2020). Furthermore, multivariate Cox regression models, corrected for well characterized clinical and genetic prognostic factors, demonstrated that serum pNfH and NfL concentrations are independently associated with a reduced survival and are the main predictors of patients survival (Falzone et al., 2020; Thouvenot et al., 2020). Despite several encouraging evidence, NFs are not currently part of the prognostic assessment in ALS. To date, most of the prognostic studies on NFs have been single center, evaluating pNfH or NfL alone, performing measurement on only one biofluid and using a single assay. Moreover, a longitudinal assessment of the correlation between NF levels and ALSFRS-R decline has been explored in only one recent study (Benatar et al., 2020). Indeed, future studies must be addressed to fill these gaps. However, a recent multicenter study partly addressed these issues and compared NfL and pNfH prognostic performances in both CSF and serum, suggesting that NfL in either CSF and serum are the main predictors of the ALSFRS-R slope and might be a future biomarker for the prognostic evaluation in ALS (Benatar et al., 2020).

Neurofilaments across Amyotrophic Lateral Sclerosis Disease Spectrum

NFs concentration, in both serum and CSF, is heterogeneous across the ALS disease spectrum (**Table 2**). Several features might lead to the NFs variability. Disease progression rate, differential upper and LMN and extra-motor involvement affect NF levels, probably as a consequence of more widespread disease. Specific genetic variants influence NF concentrations, because of different underlying pathogenic mechanisms or of a more aggressive disease course. The study of the association between NF levels and ALS phenotypes is not only potentially relevant from a diagnostic and prognostic point of view, but also opens a window for a better understanding of the potentially heterogeneous pathogenetic processes underlying the different disease phenotypes.

There is broad agreement on the fact that both pNfH and NfL correlate with disease progression rate; a faster decline on the ALSFRS-R results in higher NF concentrations in both serum and CSF (Menke et al., 2015; Poesen et al., 2017; Steinacker et al., 2017; Schreiber et al., 2018; De Schaepdryver et al., 2019; Verde et al., 2019; Falzone et al., 2020). Conversely, NF levels do not mirror the progression through disease stages; indeed, they are stable over the disease course and tend to decrease in the late stages (Menke et al., 2015; Gaiani et al., 2017; Poesen et al., 2017). Many studies reported higher NFs levels in patients with greater UMN burden, both in serum and CSF (Menke et al., 2015; Gaiani et al., 2017; Schreiber et al., 2018; Gille et al., 2019; Falzone et al., 2020). It has been postulated that the axonal dying-back in the corticospinal tract (CST) might be one of the major determinants of the elevation of NFs, especially in the CSF (Gaiani et al., 2017). Consistently, NFs correlate with CST involvement as evidenced by diffusion tensor imaging (DTI) and motor evoked potentials studies (Menke et al., 2015; Falzone et al., 2020). Notably, ALS-mimicking UMN syndromes (primary lateral sclerosis and hereditary spastic paraplegia) exhibit significantly lower NFs levels despite sharing a common involvement of the motor cortex/CST (Zucchi et al., 2018) (**Table 2**). Hence, the rate of the degenerative process itself might also be a relevant determinant of the NFs concentration.

NFs are elevated in both CSF and serum of patients with predominant UMN impairment while predominant LMN phenotypes exhibit low NF concentration in both biofluids (Gaiani et al., 2017; Falzone et al., 2020). The poor CST involvement occurring in LMN and the slower disease course of these phenotypes suggest that elevated NF concentration reflects a rapid axonal disruption in the CST. Taken together, these data indicate that LMN involvement seems to make a small contribution in determining NFs concentration both in the CSF and in the peripheral blood. NFs levels in the CSF are higher than in serum, suggesting that the main source

Table 1 | **Diagnostic performance of neurofilaments in the most recent ALS studies**

Reference	Sample size	Biofluid	Assay	Neurofilament	Cutoff value	Diagnostic sensitivity (%)	Diagnostic specificity (%)
Steinacker et al. (2017)	253 ALS *vs.* 85 DM	CSF	ELISA	pNfH	pNfH: 560 pg/mL	83.00	80.00
				NfL	NfL: 2200 pg/mL	77.00	88.00
Poesen et al. (2017)	220 ALS *vs.* 50 DM	CSF	ELISA	pNfH	pNfH: 768 pg/mL	90.70	88.00
				NfL	NfL: 2453 pg/mL	85.40	78.00
Feneberg et al. (2018b)	54 eALS *vs.* 64 OND	CSF	ELISA	pNfH	pNfH: 625 pg/mL	98.00	91.00
				NfL	NfL: 2300 pg/mL	94.00	86.00
	135 lALS *vs.* 64 OND	CSF	ELISA	pNfH	pNfH: 597 pg/mL	93.00	89.00
				NfL	NfL: 2146 pg/mL	89.00	84.00
	45 eALS *vs.* 48 OND	Serum	Simoa	NfL	NfL: 128 pg/mL	88.00	92.00
	118 lALS *vs.* 64 OND	Serum	Simoa	NfL	NfL: 116 pg/mL	79.00	92.00
Li et al. (2018)	53 ALS *vs.* 25 OND	CSF	ELISA	pNfH	pNfH: 1662 pg/mL	82.90	87.50
				NfL	NfL: 1307 pg/mL	91.40	59.00
De Schaepdryver et al. (2018)	85 ALS *vs.* 31 DM	CSF	ELISA optimized using biotin/streptoavidin	pNfH	pNfH: 750 pg/mL	92.90	96.00
		Serum			pNfH: 81.9 pg/mL	71.80	85.20
Verde et al. (2019)	124 ALS *vs.* 44 DM	Serum	Simoa	NfL	NfL: 62.0 pg/mL	85.50	77.30

ALS: Amyotrophic lateral sclerosis; CSF: cerebrospinal fluid; DM: disease mimics; eALS: early symptomatic ALS with disease duration from symptoms onset lower than 6 months; ELISA: enzyme-linked immunosorbent assay; lALS: ALS with disease duration from symptoms onset longer than 6 months; NfL: neurofilament light chain; OND: other neurological diseases; pNfH: phosphorylated neurofilament heavy chain.

Table 2 | **Mean NF concentration in studies comparing ALS and disease mimics**

Study	ALS	PLS	MMN	CIDP	HSP	SBMA
De Schaepdryver et al. (2018)	CSF pNfH 2451 (314–17247); serum pNfH 173 (6–1024)	NA	CSF pNfH 385 (36–750); serum pNfH 29 (11–81)	CSF pNfH 530 (81–5042); serum pNfH 73 (6–518)	CSF pNfH 146 (20–1302); serum pNfH 30 (6–226)	Serum pNfH 37 (10–95)
Feneberg et al. (2018b)	CSF pNfH 4545 (409–12670);	CSF pNfH 833 (201–6195);	CSF pNfH 369 (188–657);	CSF pNfH 528;	CSF pNfH 147;	
	CSF NfL 6802 (1053–25650);	CSF NfL 1898; (100–8557);	CSF NfL 434 (219–920);	CSF NfL 737;	CSF NfL 707	NA
	serum NfL 252 (51–879)	serum NfL 84 (34–95)	serum NfL 40 (15–66)	serum NfL 42		
Poesen et al. (2017)	CSF pNfH 2966 (414–18089);	CSF pNfH 440 (95–3365);	NA	CSF pNfH 212 (24–6343);	NA	NA
	CSF NfL 9427 (370–93574)	CSF NfL 2189 (570–10180)		CSF NfL 2212 (345–35384)		
Verde et al. (2019)	Serum NfL 125125 (14–908)	NA	NA	Serum NfL 135135 (132–155)	Serum NfL 30 (12–41)	
Steinacker et al. (2017)	CSF pNfH 1825 (62–19160);	CSF pNfH 1340; (62–8940);	NA	CSF pNfH 1264 (205–10000);	CSF pNfH 189 (62–318); CSF NfL 495 (168–937)	NA
	CSF NfL 4990 (100–38350)	CSF NfL 3750 (100–25650)		CSF NfL 3548 (330–10000)		
Clinical features		UMN only, usually slower	LMN only, Ab anti-GM1, conduction blocks	LMN only, sensory symptoms, EMG slowing, IVIG or CS response	Usually young onset, slow, lower limb onset	X-linked, LMN only, slow, androgen deficit

NFs mean concentration is reported in pg/mL. ALS: Amyotrophic lateral sclerosis; CIDP: chronic inflammatory demyelinating polyneuropathy; CSF: cerebrospinal fluid; HSP: hereditary spastic paraplegia; MMN: multifocal motor neuropathy; NA: not applicable; NF: neurofilament; NfL: neurofilament light chain; PLS: primary lateral sclerosis; pNfH: phosphorylated neurofilament heavy chain; SBMA: spinal and bulbar muscular atrophy.

of NFs is in the central nervous system and that NFs are not completely free to diffuse through the blood-brain barrier (BBB). NF levels do not correlate with BBB permeability expressed as CSF/serum albumin ratio (Kalm et al., 2017) and the current mechanism underlying NFs diffusion across the BBB are still to be investigated.

One alternative hypothesis may be that NFs levels might reflect the extent of extra-motor involvement in ALS. However, to date, only one study explored the serum concentration of pNfH across the cognitive phenotypes. Although ALS patients with a concurrence of cognitive dysfunction and/or frontotemporal dementia (FTD) showed higher serum pNfH levels compared to patients with normal cognition and behavior, this difference did not reach a statistical significance, suggesting that extra-motor areas involvement may not be a major determinant (Falzone et al., 2020). On the other hand, a significative difference in NfL CSF levels between ALS and ALS/FTD, with higher concentrations in the former, is reported in a more recent work (Delaby et al., 2020).

Previous studies have demonstrated that patients harboring the *C9orf72* hexanucleotide repeat expansion have higher pNfH levels in both CSF (Gendron et al., 2017) and serum (Falzone et al., 2020). Moreover, both pNfH and NfL concentrations are also increased in the presymptomatic stage compared with sporadic ALS patients (Benatar et al., 2018). *C9orf72* patients display a faster mean progression rate and

show a widespread disease pathology with the involvement of extramotor areas (Agosta et al., 2017). Consequently, the higher levels of NFs in these patients may be explained by combination of one or more of these factors. Recently, an article showed a previously unknown link between the activation of the STING pathway in myeloid cells and C9orf72 protein function, suggesting that reduced C9orf72 levels cause an alteration in the immunophenotype in the macrophages and in the brain tissue of patients with C9-ALS/FTD (McCauley et al., 2020). Indeed, there is a growing interest in molecules involved in inflammation as candidate biomarkers for ALS. A test combining a biomarker of axonal damage (NFs) with a biomarker of inflammation might yield improved diagnostic and prognostic performances.

Application in Clinical Trial: Current Status and Lessons from Other Diseases

NFs represent a promising surrogate biomarker in clinical trials, reflecting neuronal death and axonal degeneration, which ultimately underpins disease progression and the achievement of clinical milestones. Clinical trials mainly employ clinical measures such as survival or the rate of decline on ALSFRS-R as primary endpoint to assess drugs therapeutic effect. As a consequence, they require large sample size and long follow up study period making it difficult to evaluate properly which experimental therapy to advance. Biomarkers capable of predicting prognosis and tracking pharmacological response are urgently needed to help overcome these challenges.

The measurement of NfL levels at baseline, in consideration of their prognostic value, might be useful in order to reduce sample size, as highlighted by a recent study; in the same work, a variation in NfL slope in response to treatment is suggested as possible outcome measure (Benatar et al., 2020). The evaluation of NfL, both on CSF and serum, has become part of the standard measurements in multiple sclerosis (MS) trials. Indeed, in MS it has been consistently demonstrated that higher NFs levels are associated with long-term disability and their association with clinical and MRI-related measures of disease activity supports their use as markers of response to treatment (Kuhle et al., 2015, 2019; de Flon et al., 2019). More recently plasma pNfH levels were observed to correlate with disease activity and treatment response in infants with spinal muscular atrophy treated with nusinersen (Darras et al., 2019). In the last years NF levels evaluation has entered also ALS clinical trials and is contemplated in some recent protocols (Mandrioli et al., 2019; Morimoto et al., 2019). Importantly, pNfH were measured in a pre-clinical trial of SOD1 antisense oligonucleotide and their increase stopped after treatment (McCampbell et al., 2018).

The development of novel therapeutics strategies, including gene therapy, highlights the need for biomarkers able to accurately predict prognosis and to track pharmacological response in the ALS scenario.

Conclusion and Future Perspectives

There is an urgent need for the implementation of reliable biomarker in ALS. NFs promise to be helpful in the diagnostic process, in delineating prognosis and for improving clinical trial design. Moreover, the dynamics of NFs levels before and during the disease offer a unique opportunity for a better comprehension of the physiopathology of both ALS and its clinical variants.

Clinical application of NFs seems forthcoming, but some issues, including standardization of the analytical techniques, which of the two NF subunits should be evaluated and delineation of precise practice-oriented cut-offs, still need to be addressed.

In a future perspective, NFs might be included in a clinical diagnostic protocol or even as supportive supplemental diagnostic criterion. While prediction models for ALS prognosis are already available (Calvo et al., 2017; Westeneng et al., 2018), future studies might evaluate if NF levels might be incorporated as an additional variable in order to further improve the productivity such models.

Finally, NFs measurement might also be useful within the context of clinical trials, for reducing the sample size and, most importantly, as a pharmacodynamic biomarker for novel potential disease-modifying treatments.

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