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## Different Clinical Contexts of Use of Blood Neurofilament Light Chain Protein in the Spectrum of Neurodegenerative Diseases

Giovanni Palermo, Sonia Mazzucchi, Alessandra Della Vecchia, Gabriele Siciliano, Ubaldo Bonuccelli, Carole Azuar, Roberto Ceravolo, Simone Lista, Harald Hampel, Filippo Baldacci

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100	Abstract	<p>One of the most pressing challenges in the clinical research of neurodegenerative diseases (NDDs) is the validation and standardization of pathophysiological biomarkers for different contexts of use (CoUs), such as early detection, diagnosis, prognosis, and prediction of treatment response. Neurofilament light chain (NFL) concentration is a particularly promising candidate, an indicator of axonal degeneration, which can be analyzed in peripheral blood with advanced ultrasensitive methods. Serum/plasma NFL</p>	

concentration is closely correlated with cerebrospinal fluid NFL and directly reflects neurodegeneration within the central nervous system. Here, we provide an update on the feasible CoU of blood NFL in NDDs and translate recent findings to potentially valuable clinical practice applications. As NFL is not a disease-specific biomarker, however, blood NFL is an easily accessible biomarker with promising different clinical applications for several NDDs: (1) early detection and diagnosis (i.e., amyotrophic lateral sclerosis, Creutzfeldt–Jakob disease, atypical parkinsonisms, sporadic late-onset ataxias), (2) prognosis (Huntington’s disease and Parkinson’s disease), and (3) prediction of time to symptom onset (presymptomatic mutation carriers in genetic Alzheimer’s disease and spinocerebellar ataxia type 3).

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# Different Clinical Contexts of Use of Blood Neurofilament Light Chain Protein in the Spectrum of Neurodegenerative Diseases

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## Abstract

One of the most pressing challenges in the clinical research of neurodegenerative diseases (NDDs) is the validation and standardization of pathophysiological biomarkers for different contexts of use (CoUs), such as early detection, diagnosis, prognosis, and prediction of treatment response. Neurofilament light chain (NFL) concentration is a particularly promising candidate, an indicator of axonal degeneration, which can be analyzed in peripheral blood with advanced ultrasensitive methods. Serum/plasma NFL concentration is closely correlated with cerebrospinal fluid NFL and directly reflects neurodegeneration within the central nervous system. Here, we provide an update on the feasible CoU of blood NFL in NDDs and translate recent findings to potentially valuable clinical practice applications. As NFL is not a disease-specific biomarker, however, blood NFL is an easily accessible biomarker with promising different clinical applications for several NDDs: (1) early detection and diagnosis (i.e., amyotrophic lateral sclerosis, Creutzfeldt–Jakob disease, atypical parkinsonisms, sporadic late-onset ataxias), (2) prognosis (Huntington's disease and Parkinson's disease), and (3) prediction of time to symptom onset (presymptomatic mutation carriers in genetic Alzheimer's disease and spinocerebellar ataxia type 3).

**Keywords** Alzheimer's disease · Amyotrophic lateral sclerosis · Biomarkers · Creutzfeldt–Jakob disease · NFL · Parkinsonian syndromes

## Introduction

Neurodegenerative diseases (NDDs) are currently considered as a continuum of disorders with common pathophysiological

mechanisms, including misfolded protein deposition, neuronal synaptic disruption, axonal degeneration, neuroinflammation, and oxidative stress [1–3]. Therefore, the greatest current challenge in the field of NDDs is to provide biomarkers for the pathological mechanisms underlying each clinical picture [4], in order to improve the diagnostic and prognostic stratification of the patients and to allow early diagnosis and disease monitoring as well as to test treatment efficacy.

Within this multifaceted *scenario*, neurofilament light chain (NFL) is, at the present, the most promising candidate biomarker for an early identification of a general neurodegenerative process able to support disease diagnosis, prognosis, and progression, as well as monitoring an eventual disease-modifying treatment [5–7]. It is a component of neurofilaments (NFs) that, together with glial filaments, are the main types of intermediate filaments (IFs) of the nervous system [9–11]. Its physiological function is to confer mechanical stress resistance by preserving the characteristic cellular shape, intracellular traffic regulation between axons and dendrites, and, indirectly, nerve conduction speed modulation maintaining axon diameter [11]. Recent research suggests that they are also important for normal synaptic function [12]. Axonal dysfunction and degeneration are

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52 important steps in NDD pathogenesis, occurring long before  
53 neuronal cell death and often preceding detectable deposition  
54 of misfolded proteins [8]. During these processes, NFL is re-  
55 leased into the extracellular space and, consequently, into body  
56 fluids, such as the cerebrospinal fluid (CSF) and blood.

57 Broadly speaking, mounting data reported increased CSF  
58 NFL levels in NDDs [5, 6, 13]. In the mid-1990s, through the  
59 first enzyme-linked immunosorbent assay (ELISA) developed  
60 for NFL, Rosengren and colleagues [14] demonstrated that  
61 CSF NFL concentration was increased in amyotrophic lateral  
62 sclerosis (ALS), Alzheimer's disease (AD), and vascular cog-  
63 nitive impairment (VCI). Although with different magnitudes,  
64 further studies revealed that CSF NFL increased also in other  
65 several NDDs, such as frontotemporal dementia (FTD) [15,  
66 16], Parkinson's disease (PD) and atypical parkinsonisms  
67 (APs) [17, 18], Huntington's disease (HD) [19], mild cog-  
68 nitive impairment (MCI) [20, 21], and Creutzfeldt–Jakob dis-  
69 ease (CJD) [22, 23], as well as in non-primary neurodegener-  
70 ative disorders, such as multiple sclerosis (MS) [24],  
71 neuroinfectious conditions [25], traumatic brain injury [26],  
72 acute stroke [27], and cerebrovascular diseases [28].  
73 Moreover, in NDDs, CSF NFL levels showed to correlate  
74 with poorer cognition, short survival times, brain atrophy,  
75 and disease severity and progression [29–31], supporting the  
76 notion that it could be useful not only as a diagnostic biomark-  
77 er but also as a prognostic and progression biomarker [32]. As  
78 a result, it has been proposed as a dynamic biomarker for  
79 axonal degeneration [5, 6, 13] with the potential capacity to  
80 monitor treatment effectiveness [10, 33].

81 In the last few years, the interest in NFL research shifted  
82 toward blood. An ideal biomarker should be easily measur-  
83 able, accurate, quantitative, reproducible, and employable to  
84 exactly categorize the population in line with a certain disease  
85 [34, 35]. To this end, blood-based biomarkers would have  
86 significant advantages in time efficiency and cost efficiency  
87 compared to CSF and neuroimaging [36, 37]. Moreover, they  
88 would offer potential applications at the population level as  
89 screening tools in primary care, as well as for longitudinal  
90 evaluations with repeated sampling during follow-up. It is  
91 not surprising that brain pathophysiological processes are  
92 reflected into the periphery. However, CSF proteins partially  
93 enters the blood flow, are subsequently diluted in a greater  
94 volume compared with CSF, and go through biochemical in-  
95 teractions with a large amount of plasma proteins. They are  
96 also cleared by blood cells and metabolized by other tissues.  
97 Finally, these processes overall hamper their measurement in  
98 plasma or serum using traditional techniques. Nevertheless, in  
99 the past few years, the development of analytical tools for  
100 ultrasensitive quantification—the immunomagnetic reduction  
101 (IMR) and the single molecule array (Simoa) techniques—by  
102 allowing an efficient measurement of NFL in blood [38],  
103 charted a tight correlation between CSF and blood NFL in  
104 different NDDs [39]. Therefore, blood NFL was suggested

as a proxy of any neurodegenerative process, paving the 105  
way to its use in clinical practice as a reliable risk biomarker 106  
for neurodegeneration [40, 41]. Nonetheless, its potential ap- 107  
plication in real life remains unclear [42]. 108

Biomarker is defined as “a characteristic that is objectively 109  
measured and evaluated as an indicator of normal biologic and 110  
pathogenic processes, or pharmacologic responses to a thera- 111  
peutic intervention” [43]. From a clinical perspective, a bio- 112  
marker can be also classified in further categories with some 113  
practical and conceptual overlaps: (1) antecedent biomarkers 114  
identifying a risk of disease development (*risk biomarkers*), 115  
(2) early biomarkers screening a subclinical condition (*screen- 116  
ing biomarkers*), (3) biomarkers specifically recognizing a 117  
full-blown clinical picture (*diagnostic biomarkers*), (4) bio- 118  
markers categorizing disease severity (*staging biomarkers*), 119  
(5) biomarkers predicting future disease course (*prognostic 120  
biomarkers*), and (6) biomarkers predicting treatment re- 121  
sponse (*predicting or monitoring biomarkers*) [44]. 122  
Accordingly, it is crucial to define the context of use of a 123  
certain biomarker (primary care screening, diagnostic, risk of 124  
progression, disease monitoring, stratification for clinical tri- 125  
als, and pharmacodynamic or treatment response monitoring). 126

This review will attempt to summarize the current literature 127  
on blood (plasma or serum) NFL in NDDs, trying to translate 128  
research data in practical considerations, focusing on the con- 129  
text of use of blood NFL as a biomarker in the framework of 130  
the NDDs (Table 1). 131

## 132 Literature Research Methods

We conducted a systematic review of the literature until 133  
February 2020, using the key terms “NFL,” “neurofilament 134  
light chain,” and “neurofilament” to interrogate the PubMed 135  
database for articles published in English evaluating blood 136  
NFL concentrations (serum and plasma) in NDDs. Overall, 137  
we identified 38 studies. The use of internationally accepted 138  
clinical diagnostic criteria for each NDD, in particular AD 139  
[45–49], ALS [50–52], dementia with Lewy bodies (DLB) 140  
[53–55], FTD [56–58], PD [59–61], AP [62–65], and sporadic 141  
Creutzfeldt–Jakob disease (sCJD) [66, 67], has been checked 142  
out for any single study. The diagnostic performance of blood 143  
NFL concentrations to correctly allocate the participants to the 144  
different diagnostic groups was considered as follows: “excel- 145  
lent” (area under the ROC curve (AUROC) 0.90–1.00), 146  
“good” (AUROC 0.80–0.89), “fair” (AUROC 0.70–0.79), 147  
“poor” (AUROC 0.60–0.69), or “fail” (i.e., no discriminatory 148  
capacity) (AUROC 0.50–0.59) [68]. 149

## 150 AD

AD is the most common form of dementia in the elderly, 151  
accounting for 50–70% of prevalent neurodegenerative 152



t1.1 **Table 1** Overview on the  
t1.2 possible context of use of blood  
NFL as a biomarker in NDDs  
t1.3

	Diagnostic value			Prognostic value	Monitoring treatment
	Preclinical phase	Prodromal phase	Full-blown picture		
t1.4 AD	±	+	+	+	±
t1.5 PD	±	±	+	+	±
t1.6 Atypical parkinsonisms (4R tauopathies)	±	±	+	+	±
t1.7 DLB	±	±	±	±	±
t1.8 FTD	–	±	+	±	±
t1.9 ALS	±	±	+	±	±
t1.10 CJD	±	+	+	–	±
t1.11 HD	–	–	–	+	±
t1.12 SMA	–	–	–	±	+
t1.13 Sporadic late-onset ataxias	±	±	±	+	±
t1.14 NDDs as a whole	±	+	+	+	±

Plus sign (+), potential use, supportive data are available; plus–minus sign (±), unknown; negative sign, negative evidences are available

AD Alzheimer’s disease, ALS amyotrophic lateral sclerosis, CJD Creutzfeldt–Jakob disease, DLB dementia with Lewy body, FTD frontotemporal dementia, HD Huntington’s disease, NDD neurodegenerative diseases, PD Parkinson’s disease, SMA spinal muscular atrophy

153 dementia cases with an enormous health and economic impact  
154 [69]. The scientific and clinical research is nowadays shifting  
155 from dementia to the prodromal or even preclinical phases of  
156 AD to find effective therapeutic interventions that can delay or  
157 halt neurodegenerative progression [70, 71].

158 Biomarkers hold promise for improving early diagnosis in  
159 AD and establishing a tailored approach. The use of specific  
160 surrogate biomarkers (neuroimaging, blood [plasma/serum],  
161 and CSF) of AD pathology has been included in revised diag-  
162 nostic criteria to distinguish AD from other forms of dementia  
163 since its early disease stages. However, postmortem studies  
164 demonstrate a high degree of neuropathologic heterogeneity  
165 in patients who received a clinical diagnosis of AD [72]. The  
166 pathogenesis of AD involves interacting pathophysiological  
167 cascades in which the deposition of amyloid plaques (Aβ)  
168 and the formation of neurofibrillary tangles (NFTs) composed  
169 of hyperphosphorylated tau protein would represent only the  
170 core events. The recently established “A/T/N” scheme pro-  
171 poses three binary biomarker categories which reflect AD  
172 pathophysiology, where “A” refers to Aβ pathology, “T” to  
173 tau pathology, and N to neurodegeneration [73, 74]. However,  
174 emerging evidence stresses the existence of additional molec-  
175 ular pathophysiological pathways, such as synaptic dysfunc-  
176 tion and degeneration, innate immune response and neuroin-  
177 flammation, vascular and cell membrane dysregulation, brain  
178 metabolic dysfunction, and axonal disruption [75]. The latter  
179 is prominent in AD, and it is more closely related to cognitive  
180 decline than Aβ pathology [76], thus leading to propose CSF  
181 NFL as a non-specific biomarker to detect early AD patho-  
182 physiological alterations [77]. In addition, an increased release  
183 of NFL molecules is a consequence of aging that contributes  
184 to an axonal degeneration due to subclinical cerebrovascular  
185 changes and neuronal atrophy [78]. In this regard, a recent

186 prospective community-based study enrolling a cohort of cog-  
187 nitively intact subjects reported high variability of serum NFL  
188 levels above 60 years [79].

189 Several studies showed that CSF NFL levels are elevated in  
190 AD patients when compared with healthy controls (HCs) and  
191 higher NFL concentration is predictive of a rapid disease pro-  
192 gression along core biomarkers of AD pathology [21].  
193 Peripheral serum or plasma NFL strongly correlated with  
194 CSF NFL concentration, suggesting that it reflects the same  
195 pathological process [80]. In general, we found that the levels  
196 of NFL are higher in serum than in plasma, but the majority of  
197 studies used plasma to quantify NFL.

### NFL as Diagnostic Biomarker 198

199 Current evidence revealed that plasma NFL allows to discrim-  
200 inate AD patients from HC subjects with a good/excellent  
201 diagnostic accuracy [77, 81] (Table 2). In addition, plasma  
202 NFL levels showed to be higher in the AD dementia group  
203 than in the MCI group and in Aβ-positive MCI patients than  
204 HC [77]. More recently, this finding has been replicated in a  
205 larger study in the Dominantly Inherited Alzheimer Network  
206 (DIAN) [82]. Other studies reported higher plasma/serum  
207 NFL levels in AD and MCI patients compared with controls  
208 [76, 83, 84], but with conflicting results about the differences  
209 between MCI and cognitively normal individuals. A recent  
210 meta-analysis by Wang and colleagues [85] confirmed these  
211 findings, supporting a possible contribute of plasma NFL in  
212 the AD diagnostic workup. Moreover, plasma NFL levels  
213 could also reflect NFT pathology (as determined by  
214 NFL immunostaining) and neurodegeneration at post-  
215 mortem evaluation [76].

**Table 2** The utility of NFL in the diagnostic and progression evaluation of neurodegenerative diseases

First author (type of study)	Controls	Patients	Diagnostic/prognostic value of blood NFL	Notes
t2.1 AD				
t2.2 Preische, 2019 (longitudinal study)	HC (AD mutation non-carriers) <i>n</i> = 162	AD presymptomatic mutation carriers, <i>n</i> = 243	- Rate of change of serum NFL in differentiating non-mutation carriers vs presymptomatic mutation carriers, AUROC = 0.7 - Non-mutation carriers vs symptomatic non-mutation carriers, AUROC = 0.89 - Baseline serum NFL value in differentiating non-mutation carriers vs presymptomatic mutation carriers, AUROC = 0.49 (at the cutoff value of 37.4 pg/mL) - Non-mutation carriers vs symptomatic non-mutation carriers, AUROC = 0.85 (at the cutoff value of 27.9 pg/mL) - Plasma NFL in differentiating patients vs HC, AUROC = 0.85 (95% CI 0.772–0.934), unadjusted for other variables - AD vs HC, AUROC = 0.92 (95% CI 0.869–0.970), adjusted for age - At the cutoff maximizing Youden index, 25.7 pg/mL, the unconditional accuracy was 0.82	- Mutation carriers were subdivided into 3 groups: presymptomatic mutation carriers (individuals who scored 0 on the CDR scale across all visits), converters (CDR = 0 at baseline and CDR > 0 at subsequent visits), and symptomatic mutation carriers (CDR > 0 across all visits)
t2.3 Lewczuk, 2018 (cross-sectional study)	HC = 41	MCI-AD = 25 ADD = 33 AD = 58		- MCI-AD were AD patients at the stage of MCI. - ADD were AD patients at the stage of early dementia. - AD included MCI-AD + AD patients
t2.4 Mattsson, 2017 (longitudinal study)	HC = 193	AD = 180 MCI = 197 pMCI = 109 sMCI = 65	Plasma NFL in differentiating AD vs CNC, AUROC = 0.87 (correcting for age, sex, educational level, and <i>APOE</i> ε4 genotype). When only correcting for age, sex, and educational level, the AUROC was reduced to 0.79	- Patients were recruited from the ADNI cohort. - MCI patients were divided into stable MCI (sMCI), with no progression to dementia during ≥ 2-year follow-up) and progressive MCI (pMCI, with conversion to dementia)
t2.5 FTD				
t2.6 van der Ende, 2019 (longitudinal study)	Non-carriers of a mutation in GRN, C9orf72, or MAPT = 127	Presymptomatic carriers of a mutation in GRN, C9orf72, or MAPT = 149 FTD (symptomatic carriers of a mutation in GRN, C9orf72, or MAPT) = 59	- Serum NFL in differentiating symptomatic mutation carriers vs presymptomatic mutation carriers, AUROC = 0.93 (95% CI 0.90–0.97), at the cutoff concentration of 17 pg/mL - Symptomatic mutation carriers vs non-mutation carriers, AUROC = 0.95 (95% CI 0.92–0.98), at the cutoff concentration of 17 pg/mL - Baseline serum NFL converters vs presymptomatic mutation carriers, AUROC = 0.93 (95% CI 0.89–0.98), at the cutoff level of 15.0 pg/mL	- Multicenter cohort study on families with genetic FTD in Europe and Canada - At least 2 serum samples were taken with a time interval of 6 months or more. - 9 presymptomatic carriers became symptomatic during follow-up (converters). - Presymptomatic carriers or non-carrier mutations were healthy relatives of first degree at risk of FTD
t2.7 Katisko, 2019 (longitudinal study)		FTD = 91 (bvFTD = 66; nfvPPA = 16; svPPA = 4; FTD-MND = 5) PPD = 34	- Serum NFL in differentiating FTD vs PPD, AUROC = 0.85 (95% CI 0.776–0.923), at the cutoff level of 19.9 pg/mL	- The PPD group included patients with a severe late-onset psychiatric disorder (psychotic, mood disorders, or both).

t2.10 **Table 2** (continued)

First author (type of study)	Controls	Patients	Diagnostic/prognostic value of blood NFL	Notes
t2.10 Al Shweiki, 2019 (cross-sectional study)	HC = 27	bvFTD = 20 Schizophrenia = 11 Depression = 28 Bipolar disorder = 11	- bvFTD vs PPD, AUROC = 0.82 (95% CI 0.732–0.908), at the cutoff level of 19.9 pg/mL  - Serum NFL in differentiating bvFTD vs HC, AUROC = 0.94 (95% CI 0.87–0.99) - bvFTD vs depression, AUROC = 0.89 (95% CI 0.8–0.98), at a cutoff level above 35.7 pg/mL - bvFTD vs bipolar disorder, AUROC = 0.94 (95% CI 0.81–1.01), at a cutoff level > 26.5 pg/mL - bvFTD vs schizophrenia, AUROC = 0.9 (95% CI 0.77–1.03), at a cutoff level > 17.7 pg/mL - Serum NFL in differentiating bvFTD vs AD, AUROC = 0.67 - bvFTD vs MCI, AUROC = 0.9 (95% CI 0.84–0.97) - bvFTD vs HC, AUROC = 0.85 (95% CI 0.72–0.97) - bvFTD vs AD subgroups selected based on CSF Aβ42 levels, AUROC = 0.79, at a cutoff value of 33 pg/mL - bvFTD vs AD subgroups selected based on both CSF Aβ42 and tau/p-tau levels, AUROC = 0.77, at a cutoff value of 34.3 pg/mL	- In the FTLD group, 26 patients had a definite diagnosis due to the C9orf72 repeat expansion - 20 bvFTD consisting of 9 possible bvFTD, 5 probable bvFTD, and 6 genetic bvFTD (4 due to C9orf72 mutations, 2 MAPT mutations) - Serum NFL levels did not differ between psychiatric and control patients  - Genetic screening of 67 patients with bvFTD revealed 7 carriers for C9orf72 repeat expansion. - Analysis of MAPT and GRN in 18 patients revealed 1 carrier of the MAPT mutation and 1 carrier of the GRN mutation
t2.11 Steinacker, 2018 (longitudinal study)	HC = 15	bvFTD = 74 AD = 26 (excluding patients with normal Aβ42) AD = 11 (without the typical AD CSF biomarker pattern) MCI = 17 Subgroups were defined on the basis of both CSF Aβ42 and tau levels: - 30 bvFTD (excluding patients with decreased Aβ42) - 31 bvFTD (excluding patients with decreased Aβ42 and increased tau or p-tau) PPA = 99 (nvPPA = 40, svPPA = 38, lvPPA = 21)	- Serum NFL in differentiating FTD vs HC, AUROC = 0.81 (95% CI 0.72–0.91) at an optimal cutoff value > 36 pg/mL - ALS vs HC, AUROC = 0.99 (95% CI 0.98–1.00) - Serum NFL in differentiating FTD vs HC, AUROC = 0.97 (95% CI 0.93–1.00) - FTD vs presymptomatic carriers, AUROC = 0.93 (95% CI 0.87–0.98), at the cutoff level of 18.0 pg/mL	- Patients with PPA met only clinical diagnostic criteria without knowledge of fluid biomarker concentration  - Participants were recruited as part of GENFI or ascertained before participation in GENFI. - 4 subjects became symptomatic during follow-up (converters)
t2.12 Steinacker, 2017 (longitudinal study)	HC = 35			
t2.13 Wilke, 2016 (cross-sectional study)	HC = 46	FTD = 41 ALS = 25		
t2.14 Meeter, 2016 (longitudinal study)	HC = 71	FTD (caused by a pathogenic mutation in <i>GRN</i> , <i>MAPT</i> , or C9orf72) = 101		

**Table 2** (continued)

First author (type of study)	Controls	Patients	Diagnostic/prognostic value of blood NFL	Notes
t2.15 ALS		Presymptomatic carriers of a pathogenic mutation = 62	- Presymptomatic carriers vs HC, AUROC = 0.63 (95% CI 0.51–0.75), at the cutoff level of 8.3 pg/mL - Serum NFL was also associated with survival (HR in the higher tertile 3.10, 95% CI 1.09–8.76)	
t2.16 Thouvenot, 2019 (longitudinal study)	HC = 21	ALS = 198	- Serum NFL > 15.0 pg/mL in discriminating ALS vs HC, AUROC = 0.99 (95% CI 0.972–0.999) - Patients with serum NFL levels $\geq 71.2$ pg/mL had a higher risk of death (HR 4.7, 95% CI 3.0–7.4) - A cutoff NFL level of 49 pg/mL in differentiating ALS vs non-neurodegenerative controls, AUROC = 0.97 (95% CI 0.95 to 0.991) - A cutoff value of 62 pg/mL in differentiating ALS vs disease controls, AUROC = 0.87 (95% CI 0.81 to 0.935) - A cutoff value of 62 pg/mL in discriminating ALS vs all other categories considered together, AUROC = 0.88 (95% CI 0.849 to 0.926)	- Patients were prospectively followed up to 18.5 months - Disease controls included in the differential diagnosis of ALS
t2.17 Verde, 2019 (longitudinal study)	Non-neurodegenerative controls = 50	ALS = 124 Disease controls = 44 FTD = 20 AD = 20 PD = 19 CJD = 6	- Patients with serum NFL above the median (125 pg/mL) had a shorter survival than patients with NFL $\leq 125$ pg/mL, with an HR of 2.39 (95% CI 1.236 to 4.63) - Serum NFL in differentiating ALS vs ALS mimic, AUROC = 0.85 (95% CI 0.79–0.90), at an optimal cutoff value of 93 pg/mL - ALS fast progressor vs ALS slow progressors, AUROC = 0.87 (95% CI 0.76–0.94), at an optimal cutoff value of 159 pg/mL - ALS vs GBS + CIDP, AUROC = 0.58 (95% CI 0.51–0.64), at an optimal cutoff value of 139 pg/mL - ALS vs HSP, AUROC = 0.84 (95% CI 0.78–0.90), at an optimal cutoff value of 55 pg/mL - ALS vs PLS, AUROC = 0.89 (95% CI 0.83–0.93), at an optimal cutoff value of 88 pg/mL - ALS vs PMA, AUROC = 0.71 (95% CI 0.63–0.78), at an optimal cutoff value of 86 pg/mL - ALS patients with serum NFL in the upper tertile had a HR = 5.34 (95% CI 1.39–20.56) vs patients with serum NFL in the low tertile.	
t2.18 Gille, 2018 (longitudinal study)		ALS = 149 (C9orf72 = 15, FTD = 15, PLS = 11, PMA = 6) ALS mimic = 19 Disease controls = 82 (GBS, CIDP, HSP)	- A subset of 16 ALS repeated serum sample showing a relative stability of NFL concentrations over time	

t2.19 **Table 2** (continued)

First author (type of study)	Controls	Patients	Diagnostic/prognostic value of blood NFL	Notes
t2.19 Feneberg, 2018 (cross-sectional study)		ALS onset ≤ 6 months = 54 ALS onset > 6 months = 135 Other MNDs (PLS, SMA, Kennedy disease) = 21 ALS mimics = 27 Neurologic disease controls = 60	- ALS patients with serum NFL in the mid-tertile had a HR = 4.47 (95% CI 1.08–18.63) vs patients with serum NFL in the low tertile - Serum NFL in differentiating early ALS vs neurologic disease controls, AUROC = 0.92 (95% CI 0.85–0.99, cutoff = 128 pg/mL) - Early ALS vs MND mimics, AUROC = 0.99 (95% CI 0.97–1, cutoff = 97 pg/mL) - Late ALS vs neurologic disease controls, AUROC = 0.9 (95% CI 0.83–0.97, cutoff = 116 pg/mL) - Late ALS vs MND mimics, AUROC = 0.97 (95% CI 0.94–1, cutoff = 95 pg/mL) High NFL concentrations were associated with shorter survival (at least a 6-month follow-up) [numbers are not reported]	- A minimum 3-month follow-up - Serum NFL concentrations were not significantly different between early and later symptomatic phases
t2.20 Steinacker, 2016 (longitudinal study)	HC = 28	ALS = 125		- Patients were subgrouped according to the progression rate at first examination into slow, intermediate, and fast progressors. - Blood NFL measures were taken every 6 months up to 3 years of follow-up and were stable over time
t2.21 Lu, 2015 (longitudinal study)	HC = 78 (London cohort = 42; Oxford cohort = 36)	ALS = 167 (London cohort = 103; Oxford cohort = 64)	- Serum NFL in differentiating ALS vs HC, AUROC = 0.86, at a cutoff level of 36 pg/mL (Oxford cohort) - Plasma NFL in differentiating ALS vs HC, AUROC = 0.87, at a cutoff level of 36.2 pg/mL (London cohort) - Patients with serum NFL (Oxford cohort) in the highest tertile had a HR = 6.05 (95% CI 1.68–21.87). - Patients with plasma NFL (London cohort) in the highest tertile had a HR = 3.78 (95% CI 1.68–8.50). - Patients with serum NFL (Oxford cohort) in the middle tertile had a HR = 2.68 (95% CI 0.87–8.27). - Patients with plasma NFL (London cohort) in the middle tertile had a HR = 1.91 (95% CI 0.86–4.23). - Patients with combined blood NFL in the highest tertile had a HR = 3.82 (95% CI 1.98–7.39). - Patients with combined blood NFL in the middle tertile had a HR = 2.08 (95% CI 1.09–3.97)	- Multicenter study (2 cohorts) - Authors also conducted analyses of the 2 cohorts combined, using the corresponding NFL data (serum or plasma) from each cohort, using cohort-specific tertile cutoff levels, and adjusting Cox regression and Kaplan–Meier survival analyses by center
t2.22 Gaiotimo 2013 (cross-sectional study)	HC = 67	ALS = 46 AD = 20 GBS = 19	Serum NFL in differentiating ALS vs HC, with sensitivity of 91.3% and specificity of 91% at a cutoff level of 26.6 pg/mL	- Neurological control patients consisted of patients suffered from tension-type headache (n = 21), lower back pain (n = 7), psychiatric



t2.29 **Table 2** (continued)

First author (type of study)	Controls	Patients	Diagnostic/prognostic value of blood NFL	Notes
t2.30 Donker Kaat, 2018 (longitudinal study)	HC = 95	PSP = 131	- Serum NFL in differentiating PSP vs HC, AUROC = 0.87 (95% CI 0.83–0.92), at the cutoff value of 38.3 pg/mL - Higher NFL levels were associated with reduced survival (adjusted HR 1.5, 95% CI 1.1–1.9)	- Diagnosis of PSP was made according to NINDS-SPSP criteria (2003–2014); 23 patients had pathological confirmation
t2.31 Wilke, 2018 (cross-sectional study)	HC = 45	c-MSA = 25 SAOA = 25 SCA = 20	- Serum NFL in differentiating c-MSA vs SAOA, AUROC = 0.74 (95% CI 0.59–0.89) - SCA vs HC, AUROC = 0.91 (95% CI 0.81–1.00)	- SCA patients consisted of SCA-1 ( <i>n</i> = 6), SCA-2 ( <i>n</i> = 3), SCA-3 ( <i>n</i> = 8), and SCA-6 ( <i>n</i> = 3)
t2.32 Hansson, 2017 (longitudinal study)	- Lund cohort HC = 53 - London cohort HC = 26	- Lund cohort PD = 171 - Lund cohort AP = 54 (MSA = 30, PSP = 19, CBD = 5) - London cohort PD = 20 - London cohort AP = 71 (MSA = 30, PSP = 29, CBD = 12) - Early-stage disease cohort PD = 53 - Early-stage disease cohort AP = 56 (MSA = 28, PSP = 22, CBD = 6)	- Blood NFL in differentiating PD vs AP, AUROC = 0.91 (95% CI 0.87–0.95) (Lund cohort) - PD vs AP, AUROC = 0.85 (95% CI 0.72–0.98) (London cohort) - PD vs AP, AUROC = 0.81 (95% CI 0.73–0.90) (early-stage disease cohort)	- The study included 2 independent prospective cohorts of PD and AP patients and HC: the Lund ( <i>n</i> = 278) and London ( <i>n</i> = 117) cohorts. - The third cohort consisted of PD and AP patients with a disease duration < 3 years (early-stage disease cohort, <i>n</i> = 109)
t2.33 HD				
t2.34 Byrne, 2018 (cross-sectional study)	HC = 20	HTT mutation carriers (premanifest HD = 20; manifest HD = 40) = 60	- Plasma NFL in differentiating HTT mutation carriers vs HC, AUROC = 0.91 (95% CI 0.85–0.97) - Plasma NFL in differentiating premanifest HD vs manifest HD, AUROC = 0.93 (95% CI 0.86–0.99)	
t2.35 Byrne, 2017 (longitudinal study)	HC = 97	HTT mutation carriers (premanifest HD = 104, manifest HD = 97) = 201 They were divided into: - Early premanifest HD = 58 - Late premanifest HD = 46 - Manifest HD stage 1 = 66 - Manifest HD stage 2 = 31	Higher baseline plasma NFL levels in premanifest HD were associated with clinical disease onset during the 3-year follow-up period, adjusted HR 3.03, 95% CI 1.07–8.60	- Participants were enrolled in the TRACK-HD* study. - At enrollment, participants with <i>HTT</i> CAG expansion mutations were classified as having premanifest or manifest HD based on the UHDRS-TMS. - 18 (17%) subjects with premanifest disease at baseline were newly diagnosed as having manifest HD during the 3 years of follow-up
t2.36 CJD				
t2.37 Staffaroni et al., 2019 (longitudinal study)		sCJD = 188	Higher baseline levels of plasma NFL levels were associated with shorter survival, HR 2.08 (95% CI 1.22–3.54)	Participants included patients with probable and definite (pathology-proven) sCJD with PRNP codon 129 polymorphism data available

**Table 2** (continued)

First author (type of study)	Controls	Patients	Diagnostic/prognostic value of blood NFL	Notes
t2.39 Thompson, 2018 (cross-sectional study)	HC = 24	sCJD = 45	Serum NFL in differentiating sCJD vs HC, AUROC = 1.0	- The present study included 132 autopsy cases with rapidly progressive neurological syndromes.
t2.40 Kovacs, 2017 (cross-sectional study)	HC = 18	sCJD = 65 gCJD = 21 AD = 21 Other neurological pathologies = 25	- Plasma NFL in differentiating sCJD vs HC, AUROC = 0.99 (95% CI 0.98–1.0) - gCJD vs HC, AUROC = 1.0 (CI 1.0–1.0) - AD vs HC, AUROC = 0.99 (95% CI 0.97–1.0) - Other neurological disorders vs HC, AUROC = 0.96 (CI 0.90–1.0)	- Cases with a wide range of neuropathological alterations, including cerebrovascular disease, inflammation (meningoencephalitis), primary age-related tauopathy, non-AD tauopathies or Lewy body pathology
t2.41 Steinacker, 2016 (cross-sectional study)	HC = 40	sCJD = 33 gCJD = 9 GSS mutation carrier = 1 Demented controls (DCo) = 20	Serum NFL in differentiating sCJD + gCJD vs HC + DCo, AUROC = 0.95	- 39 CJD diagnoses were neuropathologically verified. - Patients with other diseases included AD (n = 12), MCI (n = 4), FTD (n = 3), and NPH (n = 1)

A $\beta$ 42 amyloid  $\beta$ -peptide 1–42; AD Alzheimer's disease; ADNI Alzheimer's Disease Neuroimaging Initiative; ALS amyotrophic lateral sclerosis; AP atypical parkinsonism; APOE apolipoprotein E; AUROC area under the receiver operating curve; b $\nu$ FTD behavioral variant of frontotemporal dementia; c-MSA cerebellar variant MSA; CBD corticobasal degeneration; CDR cognitive dementia rating; CI confidential interval; CIDDP chronic inflammatory demyelinating polyneuropathy; CJD Creutzfeldt–Jakob disease; CSF cerebrospinal fluid; C9ORF72 chromosome 9 open reading frame 72; DLB dementia with Lewy body; FTD frontotemporal dementia; FTD–MND frontotemporal dementia with ALS; gCJD genetic Creutzfeldt–Jakob disease; GBS Guillain-Barre syndrome; GENFI genetic FTD initiative; GSS Gerstmann–Straussler–Scheinker disease; GRN progranulin; HC healthy controls; HD Huntington's disease; HR hazard ratio; HSP hereditary spastic paraplegia; HTT huntingtin; h $\nu$ PPA logopenic variant PPA; MAPT microtubule-associated protein tau; MCI mild cognitive impairment; MCI-AD MCI converters to AD; MDS-UPDRS Movement Disorder Society-Unified Parkinson's Disease Rating Scale; MND motor neuron disease; MSA multiple system atrophy; NFL neurofilament light chain; n $\nu$ PPA non-fluent variant PPA; NINDS-SPSP National Institute of Neurological Disorders and Stroke and the Society for PSP; NPH normal-pressure hydrocephalus; PD Parkinson's disease; PLS primary lateral sclerosis; PPA primary progressive aphasia; PMA primary muscular atrophy; PPD primary psychiatric disorders; PRNP prion protein; PSP progressive supranuclear palsy; p-tau phospho-tau; sCJD sporadic Creutzfeldt–Jakob disease; SAOA sporadic adult-onset ataxia; SARA Scale for the Assessment and Rating of Ataxia; SCA spinocerebellar ataxia; SMA spinal muscular atrophy; svPPA semantic variant PPA; UHDRS Unified Huntington's Disease Rating Scale–Total Motor Score

\*TRACK-HD is a multinational prospective observational study of HD that examines clinical and biological findings of disease progression in individuals with premanifest HD and early-stage HD



216 **NFL as Staging and Prognostic Biomarker**

217 Studies in AD and MCI-AD patients found a correlation  
 218 between plasma NFL concentration and cognitive impair-  
 219 ment, MRI hippocampal volume loss and brain atrophy,  
 220 and cerebral <sup>18</sup>F-FDG-PET hypometabolism [76, 81–87].  
 221 Moreover, higher plasma NFL levels predicted faster cog-  
 222 nitive deterioration and a higher rate of brain atrophy and  
 223 hypometabolism in MCI patients over time [77]. Baseline  
 224 plasma and CSF NFL levels were similarly associated with  
 225 short-term declines in imaging measures of neurodegenera-  
 226 tion and with global cognitive worsening, but not with  
 227 change in amyloid ligand retention on PET [35], differently  
 228 from CSF t-tau concentration that critically depends on ce-  
 229 rebral Aβ burden [88]. Instead, increased plasma NFL was  
 230 related to baseline and longitudinal glucose hypometabolism,  
 231 which is an unspecific neurodegeneration marker, in AD-  
 232 related regions of MCI Aβ+ individuals [87].

233 In a longitudinal analysis of NFL plasma levels in a  
 234 large cohort of subjects enrolled in the Alzheimer’s  
 235 Disease Neuroimaging Initiative (ADNI), Mattsson and  
 236 colleagues [82] found increasing rates of NFL changes  
 237 from preclinical AD stage to frank AD dementia through  
 238 prodromal phase, suggesting NFL as a dynamic biomarker  
 239 sensitive to AD disease progression. Of note, longitudinal  
 240 NFL variations correlated with several baseline AD-related  
 241 features (CSF biomarkers, imaging measures, and cog-  
 242 nition) in the whole population, though with significant dif-  
 243 ferences regarding clinical stage. Actually, the strictest as-  
 244 sociations were reported in MCI participants. Longitudinal  
 245 NFL level was generally increased in patients who were  
 246 classified as N+ (using temporal brain atrophy as N- indi-  
 247 cator) and in those who were only T+. Therefore, NFL  
 248 might reflect a neurodegenerative process that occurred  
 249 independently from Aβ pathology. Noteworthy, the NFL  
 250 rate of change, rather than NFL absolute concentration,  
 251 was subject to a significant increase in mutation carriers  
 252 compared with non-carriers. Moreover, the NFL rate of  
 253 change strongly correlated with longitudinal precuneus  
 254 cortical thinning in both symptomatic and presymptomatic  
 255 mutation carriers [89].

256 **NFL as Risk/Screening Biomarker**

257 Blood NFL levels seem to predict the progression to AD de-  
 258 mentia in patients with subjective memory complaints [77].  
 259 An association between regional hypometabolism in the right  
 260 hippocampus and higher plasma NFL levels was reported in  
 261 cognitively normal participants from the ADNI database [87].  
 262 Hu and colleagues [90] explored the predictive role to develop  
 263 AD of plasma NFL at the preclinical stage. Interestingly, plas-  
 264 ma NFL concentrations were already abnormally high in cog-  
 265 nitively normal individuals with significant Aβ-related

pathological changes. Baseline plasma NFL levels did not  
 differ in normal elderly volunteers who remain cognitively  
 intact during the follow-up, independently from an initial amy-  
 loid PET positivity status. Instead, a trend toward elevated  
 plasma NFL concentration was observed in Aβ+ individuals  
 with subjective memory complaints compared to subjects  
 without memory complaints who were Aβ+, and plasma  
 NFL resulted to be inversely associated with cognitive perfor-  
 mance [91]. By contrast, other groups investigated the corre-  
 lation between serum NFL levels with cerebral metabolism in  
 MCI patients. Regional hypometabolism in bilateral  
 parahippocampal gyri, right fusiform, and middle temporal  
 gyri was independently predicted by plasma NFL [92].

Weston and colleagues [93] reported increased serum NFL  
 concentrations also in symptomatic and presymptomatic fam-  
 ilial AD (FAD) mutation carriers, showing a significant cor-  
 relation with the estimated years to/from symptom onset  
 across all mutation carriers as well as with cognitive decline  
 and MRI atrophy. This finding suggests that increases in se-  
 rum NFL precede the onset of AD symptoms. A large study in  
 the DIAN cohort confirmed NFL as a sensitive marker of  
 early neurodegeneration, finding significant increased serum  
 NFL levels in AD mutation carriers (Aβ precursor protein  
 (APP) or presenilin 1 (PSEN1) or presenilin 2 (PSEN2))  
 16 years before disease onset [89]. The rate of change of  
 serum NFL peaked in mutation carriers during the conversion  
 phase to clinically evident cognitive impairment and reached a  
 plateau in symptomatic carriers; absolute values of NFL  
 showed a trend toward a slow increase over time (Table 2).  
 Interestingly, the increase in plasma NFL concentration dur-  
 ing the follow-up (15–30 months) in 79 elderly participants  
 without dementia, including 15 subjects with MCI, was asso-  
 ciated with a significant decline in both attention and global  
 cognition and with an increase in cerebral amyloid PET up-  
 take [32]. More recently, baseline serum NFL was shown to  
 be a strong and independent predictor of brain volume loss  
 and subtle cognitive changes in a longitudinal study cohort of  
 neurologically intact individuals [79].

**NFL as Predictive Biomarker**

Although not yet in humans, transgenic mice models treated  
 with a β-secretase (BACE) inhibitor showed beneficial effects  
 on AD-relevant downstream markers, including reduced plas-  
 ma NFL concentrations [30].

**FTD**

The term FTD indicates a heterogeneous spectrum of NDDs  
 inexorably conveying to a dementia syndrome characterized by  
 predominant behavioral—behavioral FTD (bvFTD) [94]—or  
 language—primary progressive aphasia (PPA) [58]—

314 impairment. It is the third most common neurodegenerative  
 315 dementia after AD and DLB and is typically diagnosed in mid-  
 316 dle age [95]. bvFTD is the most prevalent phenotype (55–60%  
 317 of cases) whereas PPA (40–45% of cases) can be further clas-  
 318 sified as non-fluent/agrammatic variant (nfvFTD) and semantic  
 319 variant (svFTD) [96]. Notably, an additional clinical PPA var-  
 320 iant, named logopenic variant, can show AD pathological fea-  
 321 tures in more than half of subjects [97]. Finally, a clinical over-  
 322 lap between FTD and ALS is described and about 10–15% of  
 323 cases with ALS report a dementia syndrome in the FTD spec-  
 324 trum (ALS-FTD) [98]. The underlying pathologies in FTD,  
 325 essentially abnormal accumulations of either tau or TAR  
 326 DNA-binding protein 43 (TDP-43) proteins, may be recog-  
 327 nized only on postmortem examination. These misfolded pro-  
 328 tein aggregates lead to atrophy (structural MRI) and  
 329 hypometabolism (<sup>18</sup>F-FDG-PET) of frontal and/or anterior tem-  
 330 poral lobes depending on phenotype [41].

331 Unfortunately, with the exception of causative mutations in  
 332 genetic forms (about 10% of cases: hexanucleotide repeat ex-  
 333 pansion near the chromosome 9 open reading frame gene  
 334 (C9orf72), progranulin (GRN), and microtubule-associated  
 335 protein tau (MAPT) [99]), CSF poly(GP) detection in  
 336 C9ORF72 expansion carriers, and decreased CSF/blood  
 337 progranulin levels in GRN mutation carriers, specific patho-  
 338 physiological biomarkers for FTD are completely lacking.  
 339 However, the diagnosis of FTD remains very challenging,  
 340 especially for behavioral variant as its diagnosis is mainly  
 341 based on clinical assessment and because its symptoms show  
 342 a significant overlap with primary psychiatric disorders [100].

343 In this context, blood NFL is a promising candidate bio-  
 344 marker for FTD, especially for disease differential diagnosis,  
 345 monitoring, and prognosis [101, 102]. Recent evidence  
 346 proved that blood and CSF levels of NFL are tightly related  
 347 and significantly higher in FTD subjects than in HC [103],  
 348 without gender differences [104]. Furthermore, although  
 349 NFL levels generally increase with aging, this association  
 350 seems not present for FTD patients [16, 105–107]. Blood  
 351 NFL levels tightly correlate with CSF values in almost all  
 352 studies measuring this biomarker in both fluids, thus suggest-  
 353 ing that the peripheral concentrations of NFL substantially  
 354 reflect the pathophysiological modification within CNS lead-  
 355 ing to NFL increase in CSF.

356 **NFL as Diagnostic Biomarker**

357 Serum NFL levels distinguished FTD patients from controls  
 358 with good/optimal diagnostic accuracy (Table 2) [105,  
 359 108–110]. Interestingly, this biomarker was higher in bvFTD  
 360 individuals in comparison with psychiatric patients affected by  
 361 depression, schizophrenia, and bipolar disorders [106], in  
 362 which bvFTD misdiagnosis is common [100]. The diagnostic  
 363 accuracy to differentiate bvFTD from psychiatric disorders was

above 80% (Table 2), independently from the specific psychi- 364  
 atric condition (mood or psychotic disorders) [106, 107]. 365

366 Only one study compared both FTD and AD subjects,  
 367 reporting that serum NFL levels were higher in bvFTD patients  
 368 [109], and separated the two groups with a sensitivity and spec-  
 369 ificity of 93% and 61%, respectively (Table 2), after a prelim-  
 370 inary exclusion of bvFTD patients with an AD biomarker pro-  
 371 file and clinical AD subjects without a core biomarker confir-  
 372 mation. Moreover, serum NFL concentrations seemed to be  
 373 higher in nfvPPA and svPPA than lvPPA subjects [105],  
 374 though with only moderate accuracy. A further study showed  
 375 no significant differences between PPA subtypes [110].

376 Finally, although FTD individuals had higher serum NFL  
 377 levels than subjects with other cognitive disorders such as AD,  
 378 ALS patients reported even more elevated concentration  
 379 [108]. Notably, ALS subjects present a TDP-43 pathology in  
 380 about 95% of cases, suggesting a potential association be-  
 381 tween increased NFL concentration and TDP-43 pathology.  
 382 Further confirmation of this hypothesis is supported by ob-  
 383 serving that FTD forms expected to be TDP-43 positive  
 384 (C9ORF72 and GRN mutation carriers, or svPPA phenotype)  
 385 reported higher concentration of this biomarker than FTD  
 386 subtypes expected to be tau positive (MAPT mutation carriers  
 387 and nfvPPA) [16, 99, 104].

388 **NFL as Staging and Prognostic Biomarker**

389 In different studies, serum NFL increases overtime in FTD  
 390 subjects, independently from the phenotypes, with the excep-  
 391 tion of lvPPA [105, 109]. A longitudinal Mini-Mental State  
 392 Examination (MMSE) decline was correlated with baseline  
 393 serum NFL levels [107]. Disease duration was not associated  
 394 with NFL concentration although one study reported a poor  
 395 survival in FTD subjects in the higher tertile of serum NFL  
 396 levels (Table 2).

397 Additionally, peripheral blood NFL concentrations appeared  
 398 to reflect specific regional brain atrophy related to clinical phe-  
 399 notypes (PPAs or bvFTD). In bvFTD patients, serum NFL was  
 400 associated with a low cognitive score and a reduction in whole-  
 401 brain volume and was correlated with brain atrophy, including  
 402 frontal and subcortical regions [99, 104, 109, 110].

403 Serum NFL was correlated with baseline cognitive impair-  
 404 ment, cognitive decline overtime, and atrophy progression of  
 405 the left frontal lobe and the right middle frontal gyrus in PPA  
 406 individuals and nfvPPA/svPPA subjects, respectively [105,  
 407 107]. On the other hand, although only in one study, serum  
 408 NFL concentration was not related to specific hypometabolic  
 409 regions on <sup>18</sup>F-FDG-PET in PPA subjects [110].

410 **NFL as Risk/Screening Biomarker**

411 Noteworthy, serum NFL was higher in genetic FTD with a  
 412 full-blown clinical picture but not in presymptomatic carriers

413 compared to HC, independently from the gene mutation.  
 414 Moreover, it was consistently higher in converters than in  
 415 non-converter carriers [104] and increased overtime in con-  
 416 verters but not in symptomatic FTD as well as non-converters.  
 417 Therefore, serum NFL could differentiate genetic FTD pa-  
 418 tients from presymptomatic carriers, with an excellent diag-  
 419 nostic accuracy (Table 2) [16]. By contrast, the discriminatory  
 420 accuracy of the biomarker relative to presymptomatic genetic  
 421 carriers and controls resulted quite poor (Table 2).

## 422 ALS

423 ALS is a progressive neurological disease in which upper  
 424 motor neuron (UMN) and lower motor neuron (LMN) degener-  
 425 erate, leading to paralysis and death, typically within 3–  
 426 5 years from symptom onset. To date, there is no definitive  
 427 diagnostic test for ALS, and confirmation of diagnosis is  
 428 based on clinical findings, electromyography results, and ex-  
 429 clusion of mimics [52]. Despite efforts to increase the sensi-  
 430 tivity of diagnostic criteria, often the diagnosis is made only  
 431 after the onset of symptoms for both sporadic and familial  
 432 ALS [111, 112]. An early diagnosis would be paramount,  
 433 since it was observed that the benefit of riluzole is related to  
 434 its early administration.

435 A large body of research exists on neurochemical ALS  
 436 biomarkers [113, 114], among which phosphorylated neuro-  
 437 filament heavy chain (pNFH) and NFL have been postulated  
 438 as the most interesting candidates [115, 116]. This is not sur-  
 439 prising, given the axonal impairment that characterizes the  
 440 disease already at the early stage, with a large release of NF  
 441 in CSF [117]. Additionally, previous studies indicate cyto-  
 442 skeletal proteins as one of the key factors contributing to neu-  
 443 rodegeneration in ALS [118–123]. Other evidences provide  
 444 additional support that NFL aggregation is an early event in  
 445 motor neuron disease [124], and that NFL is involved in the  
 446 aggregation and neurotoxicity of other proteins in motor neu-  
 447 rons [125]. At the same time, elevated NFL levels in ALS may  
 448 be explained by the higher content of axonal proteins in motor  
 449 neurons compared to other neuronal populations [126].  
 450 Nonetheless, several data highlighted that NFL is able to dis-  
 451 criminate ALS patients from healthy and disease controls [14,  
 452 117, 127, 128], especially in cases with predominant UMN  
 453 signs, and correlates with clinical disability [129], disease  
 454 stage, progression, and/or prognosis [130, 131], probably  
 455 reflecting the burden of motor neuron degeneration.  
 456 Although CSF NFL remains the more robust fluid biomarker  
 457 for ALS because of its directly reflecting alterations in the  
 458 CNS, a high correlation between CSF and blood NFL concen-  
 459 trations has been reported [132]. Moreover, at odds with other  
 460 neurodegenerative diseases such AD, NFL concentrations do  
 461 not correlate with age in ALS individuals [133–135].

## NFL as Diagnostic Biomarker

462 Mounting evidence reports significantly higher blood NFL 463  
 464 levels in ALS patients when compared to controls (Table 2) 464  
 465 [40, 135–138]. The diagnostic performance of serum NFL in 465  
 466 discriminating ALS and non-neurodegenerative subjects 466  
 467 showed excellent sensitivity and specificity (Table 2) [139, 467  
 468 140]. These findings led authors to propose the introduction 468  
 469 of serum NFL measurement into clinical practice as support- 469  
 470 ive diagnostic tool. In addition, serum NFL showed signifi- 470  
 471 cantly elevated concentration in ALS even at the onset of the 471  
 472 first symptoms, confirming its potential role as a biomarker 472  
 473 for early detection of symptomatic sporadic ALS. In this re- 473  
 474 gard, serum NFL concentrations demonstrated optimal sensi- 474  
 475 tivity and specificity also in distinguishing early symptomatic 475  
 476 ALS from other neurologic diseases or motor neuron disease 476  
 477 mimics, independently whether diagnosis was definite, prob- 477  
 478 able, or possible, following the El Escorial criteria (Table 2) 478  
 479 [134]. Interestingly, Gille and colleagues [135] reported an 479  
 480 increase of serum NFL as a function of the number of regions 480  
 481 (i.e., cranial, cervical) affected by UMN degeneration. 481  
 482 Accordingly, in a MRI-based study, elevated CSF and serum 482  
 483 NFL concentrations were significantly associated with lower 483  
 484 diffusion tensor imaging (DTI) fractional anisotropy and in- 484  
 485 creased radial diffusivity in the corticospinal tract of ALS 485  
 486 patients, as well as with clinically UMN score burden [133]. 486  
 487 On the other hand, previous studies have shown that NFL 487  
 488 levels were not increased in Kennedy disease and spinal mus- 488  
 489 cular atrophy (strictly LMN diseases) [128, 134]. As a conse- 489  
 490 quence, a subclinical involvement of the UMN is likely in 490  
 491 ALS patients with isolated LMN symptoms and elevated se- 491  
 492 rum NFL concentration [117]. However, the neuroanatomical 492  
 493 correlate of NFL increase is not yet clear since Verde and 493  
 494 colleagues [139] showed a lack of association with DTI- 494  
 495 MRI measurements of the integrity of cerebral white matter 495  
 496 tracts in the brain of ALS patients. Finally, serum NFL levels 496  
 497 were reported relatively lower in patients with primary lateral 497  
 498 sclerosis (PLS) and hereditary spastic paraplegia (HSP), two 498  
 499 UMN-isolated syndromes, compared with ALS subjects, sug- 499  
 500 gesting in such patients different pathophysiological processes 500  
 501 and rates of neurodegenerative diseases [134, 135]. 501Q4

## NFL as Staging and Prognostic Biomarker

502 Several studies reported that blood NFL levels correlate with 503  
 504 disease severity parameters, such as the decline in the ALS 504  
 505 Functional Rating Scale-Revised (ALSFRS-R) score and the 505  
 506 ALS Milano-Torino Staging (MITOS) system score [135, 506  
 507 137, 140, 141]. 507

508 Furthermore, serum NFL levels at recruitment or at the 508  
 509 time of diagnosis predicted survival independently from other 509  
 510 clinical variables and were negatively associated with disease 510  
 511 duration (Table 2) [136, 138, 142]. Thouvenot and colleagues 511

512 [140] evaluated the largest series ever of serum samples taken  
 513 from ALS patients, finding that NFL concentration was the  
 514 most important parameter related to ALS survival in multivar-  
 515 iate models (Table 2). Likewise, serum NFL concentrations in  
 516 the middle and high tertile were associated with an increased  
 517 HR compared with those of patients in the lowest tertile  
 518 (Table 2) [135]. Interestingly, unlike pNFH, NFL levels seem  
 519 to change minimally throughout the course of the disease,  
 520 maintaining distinct temporal profiles from controls, and a  
 521 steady trajectory [136].

522 However, not all studies confirmed blood NFL as a robust  
 523 prognostic biomarker in ALS patients, even if all proved low-  
 524 er NFL concentration in slow disease progressors [141].  
 525 Notably, the ALSFRS-R and the ALS MITOS system better  
 526 correlated with CSF than serum at the baseline [137]. No  
 527 significant correlation has been found between blood NFL  
 528 levels and cognitive dysfunction in ALS [135].

529 **NFL as Risk/Screening Biomarker**

530 In contrast with AD, CSF and blood NFL levels are reported  
 531 normal in presymptomatic ALS mutation carriers (C9orf72,  
 532 SOD1, FUS/TLS, or TARDBP), but they increase suddenly  
 533 with symptom onset in symptomatic mutation carriers as dem-  
 534 onstrated by Weydt and colleagues [143]. Furthermore, using  
 535 the parental age of disease onset as a proxy for assumed age of  
 536 clinical onset, any trend toward an increase of NFL concen-  
 537 tration was observed in asymptomatic mutation carriers.  
 538 Despite recent longitudinal data on a large cohort of presymp-  
 539 tomatic, SOD1 mutation carriers provided evidence that an  
 540 increase in CSF and blood NFL levels occurs at least 1 year  
 541 before of any clinical manifestations of the disease [144].

542 **NFL as Predictive Biomarker**

543 It would be interesting to determine whether riluzole reduces  
 544 blood NFL levels over time given its neuroprotective effects  
 545 and, although minimally, its capacity to slow disease progres-  
 546 sion. Currently, no studies investigated blood NFL as an indi-  
 547 cator of treatment response in ALS, and no difference in blood  
 548 NFL levels between patients treated and not treated with  
 549 riluzole has been reported so far [136, 139]. On the other hand,  
 550 recent studies on spinal muscular atrophy (SMA), a group of  
 551 severe autosomal recessively inherited neurodegenerative dis-  
 552 orders characterized by degeneration of the spinal alpha motor  
 553 neurons, have highlighted an emerging role of NFL in track-  
 554 ing disease progression and response to treatment. Of note,  
 555 recent data provided evidence that CSF NFL levels normalize  
 556 and correlate with motor improvement in children with SMA  
 557 treated with nusinersen, with a greatest benefit found in chil-  
 558 dren who received treatment earliest during the course of dis-  
 559 ease [145]. Nusinersen, an antisense oligonucleotide delivered  
 560 intrathecally by a spinal tap, is the first drug clinically

approved for the treatment of all SMA types, with a rather 561  
 dramatic impact on phenotype [146]. The levels of two addi- 562  
 tional biomarkers of neurodegeneration (CSF tau and glial 563  
 fibrillary acidic protein (GFAP), an intermediate filament 564  
 present in astrocytes) decreased together with CSF NFL after 565  
 nusinersen administration, indicating that the neuronal and 566  
 astroglia damage can be restored by nusinersen treatment 567  
 [145]. Moreover, the decrease of NFL concentration was 568  
 much larger than that of tau and GFAP, suggesting NFL as 569  
 an early treatment response biomarker in SMA patients, help- 570  
 ful to select those patients will benefit to continue such an 571  
 invasive treatment. Further studies with a long follow-up are 572  
 needed, but these preliminary results in SMA indicated NFL 573  
 as a promising marker for upcoming disease-modifying ther- 574  
 apies in diseases beside SMA. Conversely, it is worth men- 575  
 tioning that the diagnostic and monitoring value of NFL in 576  
 CSF and blood has not been confirmed in adolescent and adult 577  
 SMA-type (SMA types 2 and 3) patients treated with 578  
 nusinersen [134, 147]. It was hypothesized that NFL release 579  
 is lower in late-onset SMA than in the infantile-onset subtype. 580  
 Actually, the first phenotype is characterized by a long-lasting 581  
 and chronic disease course while the foster by an acute and 582  
 highly aggressive onset. Additionally, subjects with infantile- 583  
 onset SMA report a significantly better response to nusinersen 584  
 therapy when compared to individuals with adult-onset one. 585  
 In this regard, the finding of normal blood levels of NFL in 586  
 SMA could be used in a diagnostic panel of biochemical 587  
 markers to help differentiate patients presenting with motor 588  
 neuron deficits, separating SMA from ALS. Indeed, a substan- 589  
 tial proportion of patients with SMA initially receive a diag- 590  
 nosis of ALS [148]. 591

592 **Degenerative Parkinsonisms**

593 PD is the most common degenerative parkinsonism, with ev- 593  
 idence of progressive loss of dopaminergic neurons in the pars 594  
 compacta of the substantia nigra. Diagnostic criteria have been 595  
 recently revised to improve diagnostic accuracy imaging bio- 596  
 markers as supportive features [61]. However, early diagnosis 597  
 and progression prediction remain challenging for physicians. 598  
 Of note, the differential diagnosis between PD and AP can be 599  
 difficult, mainly at the early clinical stage. Similarly, evolution 600  
 of diagnostic criteria for AP improved accuracy, but misdiag- 601  
 nosis rates are still high [149–152]. The APs that most com- 602  
 monly mimic PD are progressive supranuclear palsy (PSP) 603  
 and multiple system atrophy (MSA), whereas among APs, 604  
 the lowest diagnostic accuracy regards the corticobasal degen- 605  
 eration (CBD). 606

607 Currently, biochemical biomarkers for PD and AP are an 607  
 unmet need, but many CSF/serum molecules are under eval- 608  
 uation. CSF NFL concentration overlaps in patients with PD, 609  
 PD with dementia (PDD), and DLB and are comparable with 610

611 those in HC [31]. In contrast, it has been demonstrated that  
 612 CSF NFL levels are markedly increased in AP patients.  
 613 Accordingly, it might discriminate between PD and AP with  
 614 a high degree of diagnostic accuracy [17, 18, 153, 154]. This  
 615 is in line with the remarkable axonal degeneration of large  
 616 myelinated axons occurring in AP as well as with the rapid  
 617 neuronal loss in such conditions [155]. Furthermore, CSF  
 618 NFL concentration correlates with measures of disease sever-  
 619 ity and other clinical variables, demonstrating its capability to  
 620 reflect neurodegenerative mechanisms. However, to over-  
 621 come the well-known limits related to CSF examination,  
 622 blood-derived NFL would be a more favorable biomarker.  
 623 In this regard, the strong correlation between blood and CSF  
 624 NFL levels in parkinsonian syndromes holds potential for an  
 625 application in clinical practice [156].

626 **PD**

627 **NFL as Diagnostic Biomarker**

628 Similar to CSF results, NFL concentration in serum/plasma is  
 629 considered useful for a differential diagnosis between PD and  
 630 AP [157]. This has been tested for the first time in three inde-  
 631 pendent prospective cohorts of PD, PSP, MSA, and CBD pa-  
 632 tients, compared with HC. Blood NFL levels in AP were sig-  
 633 nificantly elevated compared with those in PD, showing a di-  
 634 agnostic accuracy ranging from good (in the early cohort with  
 635 disease duration < 3 years) to excellent (in the Lund cohort)  
 636 (Table 2) [156]. Conversely, blood NFL levels were not able  
 637 to accurately separate PD from HC. A subsequent study in  
 638 subjects with an uncertain diagnosis at the time of inclusion  
 639 confirmed similar results (Table 2) [158]. Nevertheless, recent  
 640 studies support the promise of plasma NFL as a diagnostic  
 641 biomarker also in PD, demonstrating relatively higher NFL  
 642 levels in cases vs controls [159] and a good diagnostic accuracy  
 643 in differentiating PD patients from HC (Table 2) [160].  
 644 Moreover, higher serum NFL levels were found even at early  
 645 stages of the disease and in participants at risk of disease pro-  
 646 gression (prodromal PD and symptomatic and asymptomatic  
 647 mutation carriers of known PD genetic mutations), indicating  
 648 the presence of active disease and potential for conversion to  
 649 either PD or parkinsonian syndromes [161].

650 **NFL as Staging and Prognostic Biomarker**

651 Heterogeneous results regarding possible correlations be-  
 652 tween blood NFL and PD clinical features in three indepen-  
 653 dent PD cohorts have been provided in the prospective and  
 654 longitudinal study of Hansson and colleagues [156]. In gen-  
 655 eral, higher blood NFL levels were observed in more ad-  
 656 vanced PD and, in the Lund PD cohort, a higher blood NFL  
 657 concentration was associated with disease duration and more

severe motor symptoms (measured as Hoehn and Yahr 658  
 (H&Y) stage, Unified Parkinson's Disease Rating Scale 659  
 (UPDRS) III motor score, Timed Up and Go Test, and 660  
 Tandem Gait Test). Conversely, no clinical correlations were 661  
 described in the London cohort and the early-stage disease 662  
 cohort [156]. However, further studies confirmed the positive 663  
 relation between plasma NFL levels and motor symptom se- 664  
 verity (measured as H&Y stage and UPDRS part III score) 665  
 and proved a significant correlation between plasma NFL con- 666  
 centration and cognitive dysfunction at MMSE [161, 162]. In 667  
 another study, PDD patients reported higher plasma NFL level 668  
 compared with PD subjects without dementia [84], supporting 669  
 an association between plasma NFL and cognitive function in 670  
 PD patients [84]. Furthermore, higher baseline plasma NFL 671  
 concentrations in PD patients were found to be longitudinally 672  
 associated with a higher risk of progression for both motor and 673  
 cognitive symptoms, suggesting that serum NFL may be a 674  
 biomarker of clinical progression in PD (Table 2) [160–162]. 675

**PSP** 676

**NFL as Diagnostic Biomarker** 677

Two other studies confirmed the diagnostic value of blood 678  
 NFL in PSP patients showing good capability to discriminate 679  
 between PSP and HC (Table 2) [163, 164]. In contrast, blood 680  
 NFL is not suitable to separate PSP from other forms of AP 681  
 [156] and similar levels are reported in patients with MSA and 682  
 PSP [158]. 683

**NFL as Staging and Prognostic Biomarker** 684

Greater baseline NFL levels in serum/plasma seem to correlate 685  
 with disease severity and clinical progression in PSP patients, 686  
 though with conflicting results. Such heterogeneity may re- 687  
 flect differences in study design, since PSP patients have been 688  
 evaluated as a separate group in some studies but not in others 689  
 where PSP, MSA, and CBD patients have been combined as a 690  
 whole group. Specifically, blood NFL levels positively corre- 691  
 lated with motor symptom severity, evaluated as H&Y stage 692  
 and with UPDRS III motor score, but not with disease dura- 693  
 tion or other clinical assessments, in the AP group (including 694  
 also MSA and CBD patients) [156]. Similarly, serum NFL 695  
 concentration at baseline correlated with motor performances, 696  
 measured with the International Cooperative Ataxia Rating 697  
 Scale score and Tandem Gait Test in another cohort of AP 698  
 patients including PSP [158]. 699

In studies focusing exclusively on PSP patients, higher 700  
 serum NFL levels were related to more severe motor, func- 701  
 tional, and cognitive disability as well as shorter survival but 702  
 not with age at symptom onset or disease duration (Table 2) 703  
 [163]. Notably, NFL levels in the higher tertile were 704

705 associated with worse survival (Table 2) [164]. Higher base-  
 706 line plasma NFL levels also predicted greater whole-brain  
 707 and superior cerebellar peduncle volume loss at 1-year fol-  
 708 low-up [163].

709 **MSA and Degenerative Ataxias**

710 **NFL as Diagnostic Biomarker**

711 As for PSP, studies indicate elevated blood NFL concentration  
 712 in MSA patients, suggesting its use in discrimination of MSA  
 713 from PD and HC with a good diagnostic accuracy (Table 2).  
 714 However, as aforementioned, NFL cannot discriminate  
 715 among APs [158].

716 Moreover, blood NFL was proposed to improve the differ-  
 717 ential diagnosis of degenerative ataxias. In a pilot study eval-  
 718 uating serum NFL levels in patients with a clinical diagnosis  
 719 of probable cerebellar-MSA (c-MSA) subtype, sporadic adult-  
 720 onset ataxia (SAOA), and frequent repeat-expansion  
 721 spinocerebellar ataxias (SCAs 1, 2, 3, and 6) and in HCs,  
 722 serum NFL concentration was found to be higher in SCA  
 723 patients and in the c-MSA group compared with controls.  
 724 This is probably the result of the diffuse involvement of  
 725 spinocerebellar and corticospinal tracts in these multisystemic  
 726 neurodegenerative ataxias [165]. However, the performance  
 727 of serum NFL differentiating c-MSA from SCAOA was only  
 728 moderate (Table 2), in contrast with a higher accuracy previ-  
 729 ously reported for CSF NFL (AUC = 0.93) [166]. NFL levels  
 730 were significantly lower in SCAOA and comparable with those  
 731 of HC. A further study investigated serum NFL concentration  
 732 in large cohorts of SCA-3 subjects and demonstrated higher  
 733 levels in both preclinical and manifest SCA-3 individuals  
 734 compared with HC [167]. Serum NFL levels discriminated  
 735 manifest SCA-3 from HC with excellent accuracy, and the  
 736 diagnostic performance remained good in distinguishing pre-  
 737 clinical SCA-3 subjects from HC (Table 2) [167]. Recently,  
 738 plasma NFL concentrations resulted higher also in patients  
 739 affected by Friedreich’s ataxia (FA), which is the most com-  
 740 mon autosomal recessive ataxia caused by CAG repeat expan-  
 741 sion in the ATXN3/MJD1 gene, compared with aged-  
 742 matched controls [168].

743 **NFL as Staging and Prognostic Biomarker**

744 Serum NFL in c-MSA patients does not seem to correlate with  
 745 clinical disease severity (as assessed by the Scale for the  
 746 Assessment and Rating of Ataxia (SARA)) or disease progres-  
 747 sion [165]. Similarly, a recent study in 99 patients with geneti-  
 748 cally confirmed FA did not find a correlation with disease  
 749 severity (as defined by SARA score), age at onset, or disease  
 750 duration [169]. Moreover, serum NFL concentration remains

stable in a subgroup of 14 FA patients who received a 2-year  
 follow-up evaluation [169].

Conversely, serum NFL concentration increased with dis-  
 ease severity in a large cohort of SCA-3 patients, including  
 manifest and preclinical individuals, and correlated with both  
 clinical scales (according to SARA and International  
 Cooperative Ataxia Rating Scale (ICARS) scores) and reduc-  
 tion of cerebellar and brainstem volume [167]. Preclinical  
 SCA-3 group was divided in early and late preclinical sub-  
 groups using the median predicted number of years to onset of  
 manifest disease. Serum NFL concentrations resulted higher  
 in manifest than preclinical SCA-3 subjects and in late pre-  
 clinical SCA-3 subjects compared with early preclinical SCA-  
 3 individuals. However, no differences were observed be-  
 tween early preclinical subjects and HC [167]. Despite CAG  
 repeat count is a well-known prognostic factor for SCA-3 and  
 FA, a correlation between serum NFL and CAG repeat lengths  
 has been inconsistently reported [168, 169].

**NFL as Risk/Screening Biomarker**

In their study on 133 SCA-3 patients, Li and colleagues [167]  
 demonstrated higher serum NFL concentrations in 26 preclini-  
 cal ATXN3 mutation carriers (patients with SARA score < 3)  
 compared with controls. Moreover, a correlation between mo-  
 tor symptoms, neuroimaging markers, and serum NFL was  
 found in all ATXN3 mutation carriers, suggesting that NFL  
 may serve to track neurodegeneration and disease progression  
 already in pre and prodromal SCA-3 phases.

**HD**

Among the most common neurodegenerative diseases, HD is  
 unique, since the major part (~ 99%) of individuals presenting  
 a HD phenotype have a mutation in the same gene [170].  
 Indeed, HD is an autosomal dominant inherited neurodegen-  
 erative disease with the typical manifestations of involuntary  
 movements, psychiatric symptoms, and cognitive decline.  
 The etiological basis is the deleterious expansion of polyglu-  
 tamine encoding CAG repeats in the huntingtin (HTT) gene,  
 leading to the expression of neurotoxic mutant huntingtin  
 (mHTT) and extensive degeneration of neurons primarily  
 occurring in the striatum and cortex [171]. The disease usu-  
 ally starts in midlife, with age of onset inversely correlat-  
 ing to CAG repeat number [171]. Although the cause is  
 known, disease-modifying treatments are not yet available.  
 In HD, a reliable genetic test confirms a clinical diagnosis in  
 symptomatic people or predicts disease onset in asymptomatic  
 mutation carriers [172]. As a consequence, a novel biomarker  
 should be directed to track disease progression and predict a  
 treatment response to targeted therapies. Although not conclu-  
 sive, the results of the available studies display that blood NFL

799 could have a role in this context. Previous reports on CSF  
 800 NFL indicate elevated concentrations in HD subjects [19,  
 801 173, 174]. This is not surprising because mtHTT interacts with  
 802 other proteins altering their function and finally leading to  
 803 abnormal protein aggregation and impaired axonal transport  
 804 [175]. Furthermore, the level of misfolded mtHTT protein  
 805 correlates with NFL concentration in CSF, thus suggesting a  
 806 contemporary releasing of both proteins from damaged neu-  
 807 rons [176].

808 **NFL as Staging and Prognostic Biomarker**

809 In the first retrospective study investigating NFL concentra-  
 810 tions in the blood of premanifest HD (preHD) and early-stage  
 811 HD patients enrolled in the TRACK-HD cohort, Byrne and  
 812 colleagues [177] showed higher baseline NFL levels in 201  
 813 HTT mutation carriers, including 58 with early premanifest  
 814 and 46 subjects with late premanifest disease, than in controls.  
 815 Moreover, NFL concentration reflected baseline motor and  
 816 cognitive deficits in HD patients and differed significantly  
 817 with increasing disease stage. Positive associations were  
 818 found between plasma NFL concentration, age, and CAG  
 819 triplet repeat counts, with higher CAG lengths being associat-  
 820 ed with earlier and steeper increases in plasma NFL [177].  
 821 Therefore, NFL is the first biofluid marker showing a direct  
 822 relationship with a causative gene expansion [178].

823 Of note, baseline plasma NFL predicted rates of brain at-  
 824 rophy, cognitive decline, and worsening of functional ability  
 825 and motor performance in mtHTT carriers [177].  
 826 Interestingly, it was closely associated with the rate of  
 827 whole-brain atrophy than with the rate of striatal one, suggest-  
 828 ing that plasma NFL reflects more the rate of global neuronal  
 829 degeneration than that of a specific brain area [177].  
 830 Additionally, in the same TRACK-HD cohort, Johnson and  
 831 colleagues [179] showed voxel-wise region-specific associa-  
 832 tions between plasma NFL levels and both cross-sectional and  
 833 longitudinal MRI cortical thinning and white matter volume  
 834 reduction, highlighting the value of NFL as a dynamic and  
 835 robust marker of brain atrophy. Notably, higher concentra-  
 836 tions of NFL in plasma were associated with lower volume  
 837 in regions known to be affected in HD and predicted subse-  
 838 quent occipital gray matter atrophy and widespread white mat-  
 839 ter reduction over the 3-year follow-up, independently of age  
 840 and CAG length repeats [179]. NFL increased significantly  
 841 from baseline both in individuals with premanifest HD and  
 842 in those with manifest HD [179]. Remarkably, in a subsequent  
 843 study, Byrne and colleagues [180], combining CSF/plasma  
 844 NFL and CSF mutant huntingtin protein (mHTT<sub>p</sub>) as biofluid  
 845 biomarkers, demonstrated that NFL levels were more accurate  
 846 than mHTT<sub>p</sub> to discriminate between premanifest and mani-  
 847 fested HDs and correlated with severity of symptoms better than  
 848 mHTT<sub>p</sub> in manifest HD.

**CJD**

849 CJD is the most common human prion disease. Approximately 850  
 851 85% of cases are sCJD, but in a minority of cases, CJD can be  
 852 genetically determined (gCJD) [181]. The disease is a rapidly  
 853 progressive and fatal neurodegenerative condition, whose differ-  
 854 ent phenotypes depend, at least in part, by polymorphisms on  
 855 the gene encoding prion protein (PrP) [182]. Diagnosis is fre-  
 856 quently tardive and relies on clinical World Health Organization  
 857 (WHO) criteria supported by detection of the 14-3-3 protein  
 858 and, more recently, t-tau in the CSF [66, 183, 184]. Also, CSF  
 859 NFL recently demonstrated to be a reliable biomarker in the  
 860 CJD diagnostic workup. Although few studies explored its role  
 861 as a biomarker in CJD patients so far, CSF NFL levels are  
 862 significantly increased in CJD (including those with more slow-  
 863 ly progressive and atypical disease course) compared with AD,  
 864 FTD, other NDDs (dementia), and controls, indicating a massive  
 865 synaptic degeneration and neuroaxonal damage in CJD [22, 23,  
 866 185, 186]. Additionally, it is noteworthy that NFL concentra-  
 867 tions in CSF appear highly variable among different sCJD sub-  
 868 types, with higher NFL levels in those with more rapidly  
 869 progressing disease [187]. Importantly, plasma NFL correlates  
 870 with CSF NFL concentration and recent studies suggest that  
 871 blood NFL can accurately reflect the massive neurodegeneration  
 872 in CJD patients.

**NFL as Diagnostic Biomarker**

873 Diagnostic accuracy of serum NFL for discrimination be- 874  
 875 tween CJD and controls was excellent (Table 2) [185]. 875  
 876 These findings have been independently replicated in other 876  
 877 two studies. Serum NFL distinguished patients from controls 877  
 878 with 100% sensitivity and 100% specificity in 45 sCJD pa- 878  
 879 tients enrolled in the National Prion Monitoring Cohort [188]. 879  
 880 Noteworthy, Kovacs and colleagues [189] reported high sen- 880  
 881 sitivity and specificity of plasma NFL concentration in dis- 881  
 882 criminating CJD subjects from non-CJD controls in a cohort 882  
 883 of 132 pathologically classified patients (sCJD, gCJD, and 883  
 884 AD cases) showing a rapidly progressive neurological picture. 884  
 885 However, in this study, the diagnostic value in the differenti- 885  
 886 ation between prion and other disease cases resulted lower 886  
 887 than previously reported investigations (Table 2) [189]. 887  
 888 Moreover, serum NFL values have been elevated since the 888  
 889 early phases of the disease, suggesting a possible role as a 889  
 890 screening biomarker [188]. Conversely, serum NFL concen- 890  
 891 tration overlapped between ALS and CJD patients in a recent 891  
 892 prospective study, even though the size of CJD group was 892  
 893 very small [139]. 893

**NFL as Staging and Prognostic Biomarker**

894 Longitudinal changes in serum tau and NFL levels were in- 895  
 896 vestigated in the aforementioned study of Thompson and 896

897 colleagues [188]. However, despite a trend toward increasing  
898 concentrations of both tau and NFL over the last 12 months  
899 before death, at variance with tau, no association was found  
900 between serum NFL concentration and speed of decline on the  
901 Medical Research Council (MRC) Prion Disease Rating Scale  
902 [188]. Also, a recent study strengthens the tight association of  
903 plasma tau levels with the rate of disease progression and  
904 survival time in sCJD [190].

### 905 NFL as Risk/Screening Biomarker

906 gCJD forms are linked to mutations in the prion protein gene  
907 (PRNP) inherited with an autosomal dominant pattern and  
908 variable penetrance [191]. The relationship between genotype  
909 and phenotype remains a matter of debate, and index cases do  
910 not always have a family history [192]. Under this scenario,  
911 the discovery of biomarkers serving as surrogates of outcome  
912 in clinical trials may be crucial because *PRNP* mutation carriers  
913 might benefit from a presymptomatic intervention. PrP-  
914 lowering therapeutics are now in preclinical development, and  
915 to this end, CSF total PrP has demonstrated to be a strong  
916 candidate fluid biomarker showing stable low levels in an  
917 ongoing natural history study including presymptomatic mu-  
918 tation carriers and normal controls [193].

### 919 Conclusions

920 The impact of physiological variables on blood NFL concen-  
921 tration, such as sex and age, has not been systematically in-  
922 vestigated across published studies. Other variables possibly  
923 affecting its modification in peripheral blood, including sys-  
924 temic comorbidities and concomitant drug therapies, were not  
925 taken into account. Liver and renal clearance as well as blood  
926 cell counts and plasma protein composition could affect bio-  
927 marker concentrations [194]; however, these factors were not  
928 investigated. Indeed, blood NFL concentration alterations as-  
929 sociated with renal and hepatic dysfunctions remain unknown,  
930 thus representing a potential relevant methodological bias,  
931 especially in subjects with NDDs, being generally old and  
932 frequently exhibiting vascular comorbidities. In general,  
933 blood NFL concentrations correlate with aging due to a subtle  
934 axonal degeneration and vascular changes in elderly.  
935 However, such an association was clearly absent in CJD,  
936 ALS, and AP. This suggests that the probable contribution  
937 of aging on NFL concentrations and neurodegeneration be-  
938 comes trivial in highly aggressive forms of NDDs [195].

939 Blood NFL concentrations are reported to be massively  
940 elevated in ALS patients, even in early disease stages, indicat-  
941 ing its value as an efficacious, yet unspecific, biomarker in the  
942 differential diagnosis of ALS from ALS mimics. Moreover,  
943 blood NFL concentrations can reflect disease severity and/or  
944 progression in ALS, suggesting that peripheral NFL could

945 contribute to support ALS prognosis. Other confounding var- 945  
946 variables, such as the discordant disease progression in the dif- 946  
947 ferent clinical subtypes of ALS, should be elucidated in fur- 947  
948 ther studies. Actually, blood NFL could help better stratify the 948  
949 multifaceted clinical presentation of ALS phenotypes. 949  
950 However, further studies are needed to confirm its prognostic 950  
951 value. In contrast with genetic forms of AD, blood NFL con- 951  
952 centrations are not increased in presymptomatic ALS muta- 952  
953 tion carriers appearing to tightly link to the symptomatic phase 953  
954 of the disease. 954

955 Another NDD where blood NFL increases result impres- 955  
956 sively is CJD. Because of early diagnosis remains challeng- 956  
957 ing, NFL might be a reliable screening blood-based biomarker 957  
958 with a potentially high negative predictive value for CJD sub- 958  
959 jects, ruling out more common and less aggressive neurode- 959  
960 generative dementia, such as AD. Definitely, blood NFL 960  
961 could be of usefulness as a first-step examination to promptly 961  
962 detect CJD during its prodromal phase and to start future 962  
963 disease-modifying treatments. On the other hand, preliminary 963  
964 findings do not support a potential use of NFL as a predictor of 964  
965 longitudinal disease progression in CJD, and its specificity 965  
966 should be further substantiated in comparison with other high- 966  
967 ly aggressive forms of NDD. 967

968 All reported studies showed a good or even excellent diag- 968  
969 nostic performance of blood NFL in distinguishing patients 969  
970 with neurodegenerative disorders from HC. The potential con- 970  
971 tribution of this biomarker candidate to discriminate between 971  
972 different dementia disorders remains ambiguous given the 972  
973 lack of pathognomonic specificity. However, mounting data 973  
974 suggest that blood NFL could be a useful diagnostic tool in the 974  
975 diagnostic workup of FTD, to distinguish FTD (especially 975  
976 FTD with a TDP-43 pathology) from AD patients and to iden- 976  
977 tify PPA with a likely underlying AD pathology, including 977  
978 lvPPA. Moreover, it can represent a biomarker tracking the 978  
979 disease progression and potentially identifying the transition 979  
980 phase from the presymptomatic to the symptomatic stage of 980  
981 the genetic forms of the disease. Most importantly, serum 981  
982 NFL is assumed to be a promising screening tool to rule out 982  
983 an underlying neurodegenerative disease in individuals with 983  
984 psychiatric disorders. 984

985 In AD, blood NFL may predict progression to dementia 985  
986 in individuals with MCI at high risk and identify preclin- 986  
987 ical AD before the conversion phase. Moreover, NFL cap- 987  
988 tures early neurodegenerative changes in presymptomatic 988  
989 familiar AD mutation carriers in which blood NFL con- 989  
990 centrations correlate with the predicted time to symptom 990  
991 onset. Further studies are crucial to calculate the negative 991  
992 predictive value of blood NFL as a screening tool in large 992  
993 and selected cohorts of individuals at risk of neurodegen- 993  
994 eration and AD, such as individuals with subjective mem- 994  
995 ory complaints and/or decline, late-onset psychiatric dis- 995  
996 orders, cerebrovascular disease, and diabetes, as well as in 996  
997 aging and elderly individuals in general. 997



998 Because of the high negative predictive value of elevated  
 999 blood NFL concentrations in excluding PD, this candidate  
 1000 biomarker can represent a valid screening tool for clinicians  
 1001 in the early differential diagnosis between PD and AP in cases  
 1002 with confounding clinical presentations. In contrast, blood  
 1003 NFL measurements do not suffice to differentiate PD patients  
 1004 from controls and cannot be used to separate PSP, MSA, and  
 1005 CBD from each other. Nevertheless, blood NFL may be a  
 1006 prognostic tool in clinical practice in both PD and AP patients.  
 1007 Currently, to the best of our knowledge, there are no studies  
 1008 investigating blood NFL in patients with DLB, and few stud-  
 1009 ies are available for PDD patients.

1010 Regarding other relevant clinical neurological presenta-  
 1011 tions, blood NFL may support the classification of sporadic  
 1012 late-onset ataxias, notably helping in differentiating c-MSA-C  
 1013 from SAOA. In choreic patients, blood NFL appears to be a  
 1014 robust prognostic biomarker of HD disease onset and progres-  
 1015 sion and holds potential as a predictive biomarker of response  
 1016 to disease-modifying agents in clinical trials.

1017 Finally, blood NFL seems to be a promising candidate  
 1018 predictor of the timing of clinical phenoconversion in pre-  
 1019 symptomatic mutation carriers with AD, HD, and SCA-3.  
 1020 Conversely, blood NFL concentrations are mostly normal in  
 1021 premanifest ALS and FTD mutation carriers but promptly  
 1022 increase with the onset of clinical symptoms.

1023 In general, given the rapid advances in elucidating the path-  
 1024 ophysiological mechanisms of diseases, at the molecular di-  
 1025 agnostic level, biomarkers are excellent flexible tools to im-  
 1026 prove and inform all phases of drug discovery and develop-  
 1027 ment by enabling validation of mechanisms of actions [196,  
 1028 197]. For this reason, NFL is assumed to act as an innovative  
 1029 molecular mechanistic biomarker supporting *in vivo* detection  
 1030 and the measurement of definite pathophysiological mecha-  
 1031 nisms across the spectrum of different NDDs. Together with  
 1032 other innovative molecular indicators, NFL will help establish  
 1033 panels of biomarkers—i.e., molecular signatures—  
 1034 encompassing the entire spectrum of molecular events of the  
 1035 NDD spectrum disorders. Applying these molecular signa-  
 1036 tures in longitudinal investigations will be critical to provide  
 1037 information to depict the pathophysiological processes char-  
 1038 acterizing different NDDs [198]. These innovative biomarkers  
 1039 will enable the selection of the most appropriate therapies for  
 1040 individual patients by defining which molecular pathophysio-  
 1041 logical events account for the patient’s clinical symptoms at  
 1042 different stages of the disease [199, 200]. This will establish  
 1043 the grounds to develop effective targeted treatment strate-  
 1044 gies—i.e., “molecularly” targeted therapies—for the accurate  
 1045 treatment of specific molecular pathophysiological pathways.  
 1046 Future developments in investigating NDD heterogeneity will  
 1047 allow clinicians to deliver targeted interventions that are “cus-  
 1048 tomized,” i.e., tailored, to the definite profiles of the individual  
 1049 NDD patient, according to the precision medicine paradigm.  
 1050 Such a precision medicine-based strategy is now increasingly

facing the clinical and biological/genetic complexity and het- 1051  
 erogeneity of the various forms of NDD [198]. Precision med- 1052  
 icine emphasizes the need of clinical medicine to focus on the 1053  
 pathophysiology of the individual patient, with his/her own 1054  
 distinctive, diverse, and complex matrix of multisystem fea- 1055  
 tures [200]. Concerted global efforts will pave the way for a 1056  
 future of neurology, in which drugs will timely and effectively 1057  
 support the prevention and treatment of diseases with very 1058  
 precise biomarker-guided targeted approaches for the right 1059  
 patient at the right time [201]. 1060

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 1066

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 Functional Neuromodulation, and he also participated in scientific advi- 1079  
 sory boards of Functional Neuromodulation, Axovant, Eisai, Eli Lilly and 1080  
 company, Cytox Ltd., GE Healthcare, Takeda and Zinfandel, Oryzon 1081  
 Genomics, and Roche Diagnostics. 1082

He is co-inventor in the following patents as a scientific expert and has 1083  
 received no royalties: 1084

- In Vitro Multiparameter Determination Method for the Diagnosis 1085  
 and Early Diagnosis of Neurodegenerative Disorders Patent 1086  
 Number: 8916388 1087
- In Vitro Procedure for Diagnosis and Early Diagnosis of 1088  
 Neurodegenerative Diseases Patent Number: 8298784 1089
- Neurodegenerative Markers for Psychiatric Conditions Publication 1090  
 Number: 20120196300 1091
- In Vitro Multiparameter Determination Method for the Diagnosis 1092  
 and Early Diagnosis of Neurodegenerative Disorders Publication 1093  
 Number: 20100062463 1094
- In Vitro Method for the Diagnosis and Early Diagnosis of 1095  
 Neurodegenerative Disorders Publication Number: 20100035286 1096
- In Vitro Procedure for Diagnosis and Early Diagnosis of 1097  
 Neurodegenerative Diseases Publication Number: 20090263822 1098
- In Vitro Method for the Diagnosis of Neurodegenerative Diseases 1099  
 Patent Number: 7547553 1100
- CSF Diagnostic In Vitro Method for Diagnosis of Dementias and 1101  
 Neuroinflammatory Diseases Publication Number: 20080206797 1102
- In Vitro Method for the Diagnosis of Neurodegenerative Diseases 1103  
 Publication Number: 20080199966 1104
- Neurodegenerative Markers for Psychiatric Conditions Publication 1105  
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1107 G.P., S.M., A.D.V., G.S., U.B., C.A., R.C., and F.B. declare that they  
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q5 1109 **References**

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- Q2. Please check if the section headings are assigned to appropriate levels.
- Q3. Please check the provided expanded form for the abbreviation “MMSE” if correct.
- Q4. Please check the word “diseases” inserted after the word “neurodegenerative,” which can be found in the sentence starting “Finally, serum NFL levels were reported relatively lower,” for completeness if correct.
- Q5. References [19, 174], [22, 186], [200, 203] based on original manuscript we received were identical. Hence, the latter was deleted and reference list and citations were adjusted. Please check if appropriate.
- Q6. Please provide complete bibliographic details of this reference.

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