THE IMPACT OF NEUROFILAMENT LIGHT CHAIN (NFL) QUANTIFICATION IN SERUM AND CEREBROSPINAL FLUID IN NEURODEGENERATIVE DISEASES

EDITED BY: Isabella Zanella, Helene Blasco, Massimiliano Filosto and Giorgio Biasiotto

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EDITORIAL
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Editorial: The Impact of Neurofilament Light Chain (NFL) Quantification in Serum and Cerebrospinal Fluid in Neurodegenerative Diseases

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Editorial on the Research Topic

The Impact of Neurofilament Light Chain (NFL) Quantification in Serum and Cerebrospinal Fluid in Neurodegenerative Diseases

Neurofilaments (NFs) constitute the main structural proteins of the cytoskeleton of neurons both in the central (CNS) and peripheral (PNS) nervous system and are abundantly assembled in large myelinated axons. NFs are composed of four subunits, the light (NFL), medium (NFM), and heavy (NFH) chains plus α-internexin in CNS or peripherin in PNS. Small amounts of NFs, particularly NFLs, may be released from axons into blood and cerebrospinal fluid (CSF) in healthy individuals and this release increases with age. More significant amounts are loosed upon traumatic brain injury, stroke and in several neuroinflammatory, and neurodegenerative conditions (Yuan et al., 2017). NFLs are a promising biomarker for neuronal degeneration and death, for monitoring disease progression and effectiveness of therapies and recent studies have demonstrated the potential in predicting outcome in presymptomatic subjects at risk for neurological diseases, although the major issue is that NFLs seem not specific of a particular neuropathology (Gaetani et al., 2019; Thebault et al., 2020).

The aim of this Research Topic was to provide an updated overview on the potential of serum/CSF NFL quantification in diagnosing and monitoring neurodegenerative disorders. Several researchers contributed interesting point of views, focusing on distinct diseases and covering several important technical and clinical aspects.

Yuan and Nixon summarized the neuropathological basis of NFs as biomarkers of neurological injury or neurodegeneration. The authors focused their attention on mechanisms of NF release, their trafficking between brain and blood and major determinants of NF levels in CSF and blood. They reviewed their importance as biomarkers both in human neurological diseases and injuries and in animal models of these conditions, paying attention on crucial issues: the identity and forms

of NF proteins detected by commonly used technologies, the most recent technological advances in their reliable detection and the urgent need of measurement standardization.

While CSF and blood NFLs are mostly used to monitor the severity degree of neurological diseases and the efficacy of therapies, recent evidence has highlighted the importance of their measurement in the identification of neurodegenerative diseases in their presymptomatic phases. Gaetani et al. summarized evidence in multiple sclerosis (MS), Alzheimer's disease (AD), frontotemporal dementia (FTD), and amyotrophic lateral sclerosis (ALS). This is a very important topic, considering that disease-modifying drugs are available for the treatment of diseases like MS and their efficacy often relies on early diagnosis, also bearing in mind that the study of the presymptomatic phases of neurological diseases is fundamental in understanding their early pathological mechanisms.

NFL release increases with age as well as in some pathologies, characteristic of old age, but little is known about how other comorbidities or physiological factors may affect this release. In their original article, Polymeris et al. investigated the association of estimated glomerular filtration rate (eGFR) and body mass index (BMI) with serum NFL in a large cohort of elderly patients with atrial fibrillation. In a multivariable model adjusted for all clinical variables, eGFR and BMI showed strong inverse association with NFL levels and in these associations both eGFR and BMI interacted with age. Age, eGFR and, to a lesser extent, BMI alone or all combined explained a significant proportion of NFL level variance. This study, providing crucial information on NFL homeostasis, places emphasis on how renal function and BMI contribute to NFL levels in the elderly, suggesting considering these and further physiological factors in NFL measurement interpretation.

Four manuscripts focused on NFL measurement in MS, perhaps the most popular application of this biomarker. Serum NFL levels correlate with disease activity and treatment efficacy, are predictive of poorer clinical outcomes, are elevated years before the clinical onset and in clinically isolated syndromes are predictive of conversion to clinically definite MS. In their review, Thebault et al. summarized important issues relative to analytical and clinical validity of serum NFL measurement, fundamental issues for their routine clinical use together with the currently applied tools to diagnose and monitor MS. The authors focused on preanalytical and assay standardization and data analysis methodologies and highlighted the main physiological determinants of serum NFL levels, possible confounding effects of comorbidities, the presence of anti-NFL antibodies, the lesion location, and physiologic kinetics of NFL distribution and clearance.

Ferreira-Atuesta et al. further emphasized the correlation between axonal damage and loss with progression and disability in MS, highlighting the pros and cons of NFLs as biomarker in MS. They thoroughly illustrated the correlation between NFL levels and clinical and radiological findings in MS, also considering the potential use of the biomarker in MS mimics. The authors also extensively discussed the currently used technologies, the correlation between NFL measurements in CSF and blood and the need of optimal and sensitive cut-off values. Finally, they interestingly focused on the influence of coexisting peripheral nerve and CNS diseases and antibodies raised against NFL on reliable measurements and clinical interpretation of NFL levels, also looking at other biomarkers to be examined independently or in relation to NFLs.

Pukoli et al. reported on the relationship between neuroactive metabolites, produced in the kynurenine pathway (KP) and activated by several pro-inflammatory cytokines, and the pathomechanisms of MS. The authors described how some neuroactive KP metabolites, produced by microglia and macrophages, may have a role in MS development, producing free radicals in the presence of redox active metals like Fe^{2+}; activating N-methyl-D-aspartate receptors, resulting in excitotoxicity; inhibiting the re-uptake of glutamate by astrocytes resulting in neurotoxicity; and decreasing glutamine synthetase activity so limiting the recycling of glutamate to glutamine in astrocytes. Several studies confirmed the activation of KP in MS: during the early phase of MS the production of neuroprotective kynurenine metabolites counteracts the effects of neurotoxic metabolites, while during disease progression the excess of neurotoxic metabolites contributes to the progression of MS. Interestingly, kynurenine neurotoxic metabolites play a central role in axonal damage also through the destabilization of cytoskeleton by causing hyperphosphorylation of proteins like NFL and a positive association between KP metabolites and plasma NFL levels has been demonstrated.

In their original article, Masvekar et al. tried to develop and validate a CSF-biomarker-based molecular surrogate representing MS lesional activity, mediated by immune cells migrating from the periphery to the CNS and commonly reflected by contrast-enhancing lesions (CELs) on magnetic resonance imaging (MRI). They analyzed CSF and serum samples for 20 inflammatory and axonal damage biomarkers. The authors interestingly found a significant association of some biomarkers and NFL levels in CSF with lesional activity. IL12p40 and CHI3L1 seemed reproducibly the best CSF biomarkers of MS lesional activity. Though serum NFL levels were correlated with CSF NFLs, serum NFL measurement did not differentiate between non-active and active MS lesional inflammatory activity subgroups and were weakly correlated with number of CELs and pro-inflammatory biomarkers associated with lesional inflammatory activity.

Saak et al. explored the hypothesis that elevated serum NFL may suggest nervous system involvement in patients with primary myopathies. They determined serum NFL levels in patients with myotonic dystrophy type I (DMI) and II (DMII), mitochondrial diseases or facioscapulohumeral muscular dystrophy (FSHD), also including a control group of patients with genetic defects exclusively expressed in muscle. Finding significatively elevated levels of serum NFL in DMI, DMII and mitochondrial disease patients, the authors demonstrated that serum NFL levels may be used as biomarker of neuronal damage in muscle diseases with established nervous system involvement. They interestingly further showed that serum NFL were also raised in FSHD patients, for whom the involvement of the nervous system is not usually clinically apparent,

suggesting serum NFL as a biomarker for neuronal damage in primary neuropathies.

A cohort of patients affected by movement disorders with nigrostriatal neurodegeneration were studied by Diekämper et al. using DaTscan SPECT. In these patients there was a strong correlation between NFL and plasma NFH levels and the changes of presynaptic dopamine transporter density in the pathological conditions involving putamen concomitant to nigrostriatal degeneration. Therefore, NFL concentration could help to understand the degree of impairment of motor functions also in Parkinson's disease.

The review of Zanardini et al. explored the possibility that exosomes could be correlated to NFL and the main proteins involved in FTD. NFL and exosome dosages could be important in genetic FTD and might be useful mainly before the clinical onset allowing to anticipate the therapeutic treatments.

The importance of NFL in ALS and FTD was reviewed by Verde et al. CSF NFL levels correlated positively with disease progression and negatively with survival in ALS. In FTD, NFL were more elevated than in healthy people and slightly more elevated than in other dementias. The NFL level in CSF correlates with the disease progression, but the differences seem not so important to justify a diagnostic utility. The authors underlined the importance to dose NFL in blood and described the recent advances which showed longitudinal kinetics in presymptomatic patients carrying causative mutations for ALS and FTD. Moreover, NFL could be important in ALS patients as pharmacodynamic biomarker in therapeutic clinical trials.

An interesting research was proposed by Dreger et al. to better define the NFL potential as biomarker in ALS. The authors applied the D50 progression model to overcome the heterogeneity of clinical presentation in patients. Enrolled patients were divided in three groups characterized by high, intermediate and low disease progression on the basis of D50 values. A significant difference and positive correlation between CSF NFL levels and disease activity were found comparing the groups. This model can therefore be recommended in future studies as a useful tool.

The clinical and biological implications of NFL dosage in rapidly progressive dementias were reviewed by Abu-Rumeileh and Parchi, focusing on prion and Creutzfeldt–Jakob diseases. NFL levels showed interesting prognostic and diagnostic performances and may be used as biomarker to predict clinical onset in PRNP (prion protein) mutation carriers. In addition, authors gained attention on the follow-up of cerebrovascular diseases. Then, the quantification of NFL in CSF and in blood could be a very useful tool in precision medicine also applied to rapidly progressive dementias.

The use of NFL measurement in Friedreich ataxia (FRDA) was deepened in the review of Frempong et al. Although serum NFL result higher in FRDA patients than in controls, they do not correlate with genetic and clinical severity, like GAA repeat length or disease progression and were paradoxically higher in young patients, decreasing with age as the pathology progresses. These evidences make difficult the use of NFL as a biomarker in FRDA. The authors proposed some hypotheses to explain the anomalous NFL kinetics in FRDA patients, like a relatively large early loss of peripheral axons not contributing to clinical progression or the reflection of other components of the pathophysiology of FRDA like abnormal lipid metabolism and lipid peroxidation.

The possible role of serum NFL levels in children with epileptic or febrile seizures was explored in the research article of Evers et al. NFL levels were studied comparing the results of both cohorts with those obtained in a further control cohort of children with febrile infections without convulsion. The results of the study evidenced that NFL levels did not reveal significant differences among the three cohorts of analyzed patients. Applying multivariate analysis, age was the best predictor of NFL levels followed by sex and C reactive protein. The study demonstrated an age-dependent decrease of NFL levels from early childhood until school age.

The extent of neurodegeneration is monitored by challenging clinical measures and MRI in Wolfram syndrome. The Research Topic of Eisenstein et al. tested the possibility to use NFL as biomarker especially in patients with MRI contraindications. The NFL levels were compared in children, young patients and controls in relation to the clinical severity and chosen brain region volumes. Increased NFL levels were related to worse smell identification, color vision and visual acuity. Higher NFL values were also correlated to smaller thalamic and brainstem volume and faster annual rate of decrease in thalamic volume during time. Therefore, NFL dosage could be a useful biomarker to follow the neurodegenerative process in this type of patients.

In conclusion, many questions are still open to better understanding the role of NFL levels in traumatic brain injury, stroke, neuroinflammatory and neurodegenerative conditions. This Research Topic of articles raises the most important issues to be considered in translating research findings in clinical practice.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Serum Neurofilament Levels in Children With Febrile Seizures and in Controls

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Objective: Neuroaxonal damage is reflected by serum neurofilament light chain (sNfL) values in a variety of acute and degenerative diseases of the brain. The aim of this study was to investigate the impact of febrile and epileptic seizures on sNfL, serum copeptin, and prolactin levels in children compared with children with febrile infections without convulsions.

Methods: A prospective cross-sectional study was performed in children aging 6 months to 5 years presenting with fever (controls, $n = 61$), febrile seizures (FS, $n = 78$), or epileptic seizures (ES, $n = 16$) at our emergency department. sNfL, copeptin, and prolactin were measured within a few hours after the event in addition to standard clinical, neurophysiological, and laboratory assessment. All children were followed up for at least 1 year after presentation concerning recurrent seizures.

Results: Serum copeptin values were on average 4.1-fold higher in FS and 3.2-fold higher in ES compared with controls (both $p < 0.01$). Serum prolactin values were on average 1.3-fold higher in FS compared with controls ($p < 0.01$) and without difference between ES and controls. There was no significant difference of mean sNfL values (95% CI) between all three groups, FS 21.7 pg/ml (19.6–23.9), ES 17.7 pg/ml (13.8–21.6), and controls 23.4 pg/ml (19.2–27.4). In multivariable analysis, age was the most important predictor of sNfL, followed by sex and C reactive protein. Neither the duration of seizures nor the time elapsed from seizure onset to blood sampling had an impact on sNfL. None of the three biomarkers were related to recurrent seizures.

Significance: Serum neurofilament light is not elevated during short recovery time after FS when compared with children presenting febrile infections without seizures. We demonstrate an age-dependent decrease of sNfL from early childhood until school age. In contrast to sNfL levels, copeptin and prolactin serum levels are elevated after FS.

Keywords: neuronal biomarker, convulsion, epilepsy, neurofilament, paroxysmal

INTRODUCTION

Febrile seizures (FS) are the most common convulsive events in children aged between 6 months and 5 years and arise in 2 to 5% of all children. FS are defined as seizures occurring during childhood associated with fever that is not caused by an infection of the central nervous system (Subcommittee on Febrile Seizures American Academy of Pediatrics, 2011). FS are classified as simple or complex seizures depending on age at onset, duration, short-term recurrence, and type of seizure (Livingston et al., 1979). In approximately one third of children with a first febrile seizure, a second episode, and in around 10%, three or more FS will occur (Berg et al., 1997). Especially prolonged FS may be associated with substantial long-term neurological morbidities such as temporal lobe epilepsy or mesial temporal sclerosis with possible subsequent intellectual disability (Pujar et al., 2018).

Prolactin is a polypeptide hormone secreted by the anterior pituitary gland but also in other tissues and organs such as adipose tissue, uterus, and immune cells. Apart from the production of milk, prolactin is also known to play a role in the regulation of the immune system, behavior, and metabolism. Initially, Trimble et al. debated that seizures could raise prolactin levels (Trimble, 1978). In the past decades, it has gained recognition in the support of the diagnosis of epileptic seizures in particular for the differentiation of generalized tonic–clonic or complex partial seizures from psychogenic non-epileptic seizures among adults and older children especially when the clinical setting does not provide video-EEG recording (Chen et al., 2005; Abubakr and Wambacq, 2016; Fisher, 2016).

Another hormone released by the pituitary gland is arginine vasopressin (AVP) which plays a major role not only in maintaining the fluid balance and vascular tonus but also in the regulation of the endocrine stress response. Copeptin derives from the same precursor molecule, is more stable, and is released into the periphery in the same ratio as AVP (Evers and Wellmann, 2016). Published data suggest that copeptin is involved in the thermoregulatory response to fever and convulsions and copeptin has lately been shown to have high diagnostic accuracy in FS (Kasting et al., 1980, 1981; Landgraf et al., 1990; Stocklin et al., 2015).

Neurofilaments (Nf) are highly specific major scaffolding proteins of neurons consisting of four subunits: the triplet of NfL (Nf light), Nf medium, and NfH (Nf heavy) chains and alpha-internexin in the CNS, or peripherin in the peripheral nervous system (Teunissen and Khalil, 2012). Disruption of the axonal cell membrane due to acute or chronic neuronal damage releases Nf into the interstitial fluid and eventually to the cerebrospinal fluid (CSF) and the blood compartment (Khalil et al., 2018).

Matsushige and colleagues recently determined serum pNF-H (phosphorylated form of neurofilament-heavy chain) levels in patients with prolonged and simple FS to evaluate neuronal damage and were able to show that serum pNF-H levels in children with prolonged FS were significantly higher than in children without FS (Matsushige et al., 2012). Shahim et al. (2013) demonstrated that CSF NfL levels in children were increased in status epilepticus compared with unspecified epilepsy and that NfL levels were significantly higher in lysosomal and mitochondrial disorders than in neurodegenerative disorders without known etiology. Higher NfL levels in children with suspected multiple sclerosis are predictive for clinically definite multiple sclerosis diagnosis (van der Vuurst de Vries et al., 2018; Wong et al., 2019). Furthermore, CSF NfL levels had the highest capability to distinguish opsoclonus–myoclonus syndrome from controls compared with other brain cell–specific biomarkers in a pediatric cohort (Pranzatelli et al., 2014).

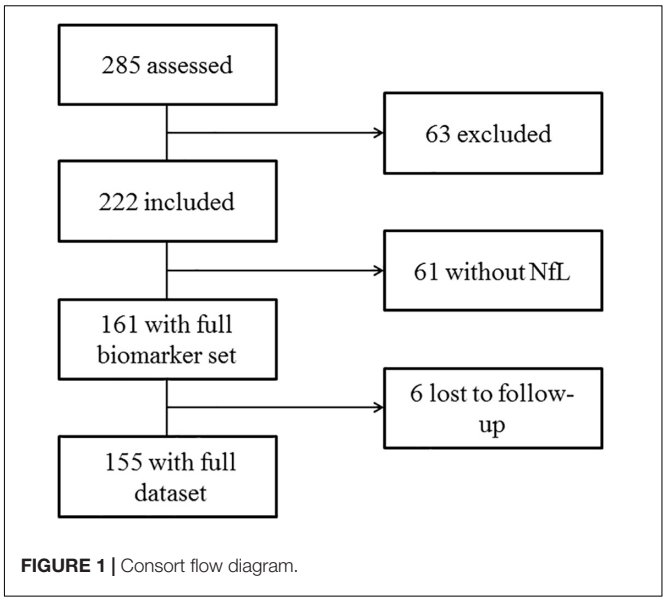

FIGURE 1 | Consort flow diagram.

The aims of this study were (1) to evaluate the short-term impact of convulsions on serum NfL (sNfL) levels in a cohort of children presenting with FS in comparison with children with febrile infections and epileptic seizures at an emergency department (ED); (2) to compare sNfL levels with other postictal serum biomarkers, namely, copeptin and prolactin; and (3) to characterize sNfL levels in the population of young children in general.

MATERIALS AND METHODS

The study was based on data and blood samples prospectively collected from a child cohort established at the University Children's Hospital of Basel (UKBB), Switzerland, between May 2013 and November 2015. The Cantonal Ethics Committee of Basel approved the study protocol (EK352/12), and written informed consent was obtained from the parents. The study was registered in the clinical trial registry ClinicalTrials.gov (No. NCT01884766). Information concerning eligibility criteria and the inclusion procedure can be obtained elsewhere (Stocklin et al., 2015). Serum concentrations of NfL were determined with a Simoa assay, which was established using the NF-light assay ELISA kit from UmanDiagnostics (Umeå, Sweden), transferred onto the Simoa platform with a homebrew kit (Quanterix, Boston, MA, United States), and has been described in detail by our group previously (Disanto et al., 2017). Calibrators (neat) and serum samples (1:4 dilution) were

TABLE 1 | Characteristics of the study groups.

	Controls (n = 61)	Febrile seizures (n = 78)	Epileptic seizures (n = 16)
Males/females	33/28	43/35	10/6
Age, months	29.4 ± 17.8 (6–72)	24.8 ± 14.5 (6–63)	53.9 ± 45.8 (9–163)*
Body weight, kg	12.9 ± 4.2 (6.8–27)	12.1 ± 3.5 (6.0–23.0)	18.4 ± 13.0 (4.4–56)†
History of seizures	NA	16 (20.5)	9 (56.3)‡
Temperature at home, °C	39.6 ± 0.7 (37.7–41.3)	39.3 ± 0.6 (38.0–41.0)	NA
Temperature at ED, °C	38.3 ± 1.0 (36.0–40.5)	38.6 ± 0.8 (36.5–40.1)	NA
Duration of event, min	NA	6.5 ± 8.1 (1–40)	5.1 ± 5.2 (1–20)
Time to presentation, min	NA	107 ± 70.7 (1–330)	96.2 ± 60.9 (7–240)§
Laboratory data at ED			
Hct, %	35.6 ± 3.7 (27.3–43.3)	37.2 ± 4.2 (28.9–56.0)	38.2 ± 3.3§§(31.8–42.3)§
WBC × 1000/mm^3	12.4 ± 7.2 (1.9–40.8)	12.9 ± 7.1 (3.4–34.2)	8.6 ± 2.7 (5.2–14.7)
Na, mmol/L	136.1 ± 3.2 (129–142)	135 ± 2.9 (118–141)	138 ± 2.1 (135–143)
Cl, mmol/L	105 ± 3.2 (98–112)	105 ± 2.6 (98–112)	106 ± 2.2 (101–110)
pH	7.37 ± 0.05 (7.20–7.40)	7.36 ± 0.06 (7.20–7.50)	7.27 ± 0.07** (7.10–7.30)**
CO_2, mmHg	31.6 ± 4.4†† (21–41)††	33 ± 5.1 (24–54)	43.2 ± 10.7 (34–70)
Bicarbonate, mmol/L	21.7 ± 2.6 (13.9–26.1)	21.6 ± 1.6 (17.5–25.3)	21.5 ± 2.6 (14.9–25.2)
Lactate, mmol/L	1.5 ± 0.8 (0.9–4.5)	1.5 ± 0.7 (0.7–4.5)	1.2 ± 0.5 (0.6–2.2)
CRP, mg/dl	50.5 ± 50.2 (0.3–220)††	12.6 ± 18.6 (0.3–91)	1.3 ± 2.4 (0.3–8.0)

*Data are presented as mean ± SD (range) unless stated otherwise. *p = 0.042 vs controls and p = 0.002 vs febrile seizures. †p = 0.043 vs. febrile seizures. ‡p = 0.010 vs febrile seizures. §p = 0.755 vs febrile seizures. §§p = 0.039 vs controls. ¶p = 0.002 vs febrile seizures. **$p < 0.001$ vs febrile seizures and controls. ††$p < 0.001$ vs febrile and epileptic seizures. Between-group comparisons were performed with Mann–Whitney U-test, Kruskal–Wallis one-way ANOVA test (with Bonferroni correction for multiple comparisons), χ^2 test, or Fisher's exact test, as appropriate. ED, emergency department; NA, not available; WBC, white blood cell.*

TABLE 2 | Differences in biomarkers among study groups.

	Controls (n = 61)	Febrile seizures (n = 78)	Epileptic seizures (n = 16)
sNfL, pg/ml	23.4 (19.2–27.4)	21.7 (19.6–23.9)	17.7 (13.8–21.6)
Prolactin, mU/L	320 (277–362)*	411 (365–458)*	429 (266–592)
Copeptin, pmol/L	9.7 (6.4–12.9)†,‡	39.9 (26.1–53.8)†	30 (13.7–46.2)‡

*Data are presented as mean (CI). *p = 0.012 for febrile seizures vs controls. †$p < 0.001$ for febrile seizures vs controls. ‡p = 0.002 for epileptic seizures vs controls. Between-group comparisons were performed with Kruskal–Wallis one-way ANOVA test (with Bonferroni correction for multiple comparisons).*

measured in duplicates. Bovine lyophilized NfL was obtained from UmanDiagnostics. Calibrators ranged from 0 to 2000 pg/ml. Batch prepared calibrators were stored at −80°C. Intra- and interassay variabilities of the assay were <10%. Repeated measuring was performed for the few samples with intra-assay coefficients of variation >20%.

Measurement of copeptin levels was done in a batch analysis with a commercial sandwich immunofluorescence assay (B·R·A·H·M·S Copeptin proAVP; Thermo Fisher Scientific, Hennigsdorf/Berlin, Germany) as described in detail elsewhere (Morgenthaler et al., 2006). The lower detection limit of the copeptin assay was 0.69 pmol/L, and the functional assay sensitivity was <1 pmol/L.

Prolactin quantification was performed using the Roche Modular E 170 (Roche Diagnostics AG, Rotkreuz, Switzerland). The lower detection limit was 1 mU/L, and the inter-assay precision <3% coefficient of variance at 102, 450, and 816 mU/L, respectively.

Statistics

Statistical analyses were performed using SPSS for Windows version 24 (IBM, United States) and included descriptive statistics, Spearman's rank-order correlation analyses, and multiple linear regressions (MLR) using sNfL as dependent variable. sNfL variables were log10 transformed for the correlations and MLR. The independent variables included for MLR were based on significant correlations and significant non-parametric univariate analyses such as the one-way ANOVA test (with Bonferroni correction for multiple comparisons), Mann–Whitney U–test (2 levels), Kruskal–Wallis test (>2 levels), χ^2 test, or Fisher's exact test. The discriminatory ability of both copeptin and prolactin was assessed by receiver operating characteristic (ROC) curve analysis and was compared by means

TABLE 3 | Ability of biomarkers to diagnose seizures.

	All seizures (FS + ES vs controls)	Febrile seizures (FS vs controls)
sNfL	0.462 (0.370–0.555)	0.494 (0.396–0.592)
Prolactin	0.620 (0.529–0.710)	0.648 (0.554–0.741)
Copeptin	0.804 (0.733–0.875)	0.807 (0.733–0.882)

Data are AUC (95% CI). ES, epileptic seizures; FS, febrile seizures; sNfL, serum neurofilament light chain.

TABLE 4 | sNfL dependencies.

	Unadjusted effect			Adjusted effect			
	R^2	Beta	*p*-value	Model 1 (R^2 0.201)		Model 2 (R^2 0.301)	
				Beta	*p*-value	Beta	*p*-value
Seizures	0.013	−0.114	0.159				
Male gender	0.001	−0.027	0.736	0.232	0.035	0.300	0.005
Age	**0.165**	**−0.406**	**<0.001**	−0.337	0.002	−0.375	0.001
Body weight	**0.139**	**−0.373**	**<0.001**				
Temperature at home	0.012	0.110	0.235				
Temperature at ED	0.007	0.086	0.288				
Hct	0.011	−0.107	0.195				
WBC	0.002	−0.050	0.566				
Na	0.005	−0.069	0.437				
Cl	0.001	0.002	0.979				
pH	**0.051**	**0.227**	**0.010**				
CO_2	0.019	−0.138	0.121				
Bicarbonate	0.013	0.115	0.199				
Lactate	0.008	−0.088	0.327				
CRP	0.008	0.089	0.310	0.278	0.012	0.241	0.023
Prolactin	0.001	0.016	0.843				
Copeptin	**0.056**	**−0.237**	**0.003**			−0.318	0.003

The unadjusted effect of each parameter was calculated by simple linear regression analysis using sNfL values (after logarithmic transformation) as the dependent variable. Significant (p-value < 0.05) parameters of the unadjusted effect are displayed in bold. The adjusted effect was calculated by stepwise linear regression analysis. CRP, C reactive protein; ED, emergency department; sNfL, serum neurofilament light chain; WBC, white blood cell.

of the area under the curve (AUC). A *p*-value of <0.05 was considered statistically significant.

RESULTS

We recruited a total of 285 children from May 2013 until November 2015. After exclusion of 63 infants, a total of 222 children were included in the final analysis. Of these, 61 did not have enough material for the analysis of sNfL, resulting in complete biomarker sets of 161 children. Six children were lost to follow-up (**Figure 1**). The children's age varied between 6 and 163 months; 44% were female. We allocated 78 children to the FS group, 16 to the ES group, and 61 febrile children without seizures were defined as controls. The characteristics of all groups are presented in **Table 1**.

There was no significant difference in age, body weight, and temperature at home or at ED when comparing the controls with FS group; the ES group had overall slightly but not significantly higher values in age and body weight than the other groups (**Table 1**). Regarding the laboratory data, pH in the ES group was significantly lower compared with the FS and control groups, whereas the controls exhibited significantly higher C reactive protein (CRP) levels than the FS and ES groups (**Table 1**). In total, 16 children (20.5%) in the FS and nine children (56.3%) in the ES group had a history of previous convulsive events. Serum values of NfL, copeptin, and prolactin in the different study groups are summarized in **Table 2**. When comparing the biomarkers in accordance to presence of fever, mean sNfL levels (95% CI) were only slightly higher in children with fever than in children without fever [fever: 22.1 pg/ml (20.1–24.1), no fever: 21.6 pg/ml (17.8–25.4), $p = 0.017$]. The evaluation of impact of seizures on biomarker levels revealed that seizures did not affect the levels of sNfL [20.8 pg/ml (18.9–22.7) vs 23.6 pg/ml (19.5–27.7)], whereas prolactin was slightly elevated in children presenting with convulsions compared with children without seizures [415 mU/L (366–464) vs 320 mU/L (277–362)] and copeptin was significantly higher in the group with seizures compared with no seizures [37.0 pmol/L (26.0–48.0) vs 9.6 pmol/L (6.4–12.8), $p < 0.001$]. Of note, no differences were found between time to presentation, which is the time elapsed from event onset to presentation at the emergency department (**Table 1**). Because blood sampling was done in all patients with FS or ES upon presentation, there was also no difference in the time to sampling.

Receiver operating characteristic curve analysis revealed that the ability to diagnose seizures differed clearly between the individual biomarkers (**Table 3**) with copeptin demonstrating the highest AUC levels compared with prolactin and sNfL [FS + ES vs controls: copeptin 0.804 (0.733–0.875) pmol/L; prolactin 0.620 (0.529–0.710) mU/L; sNfL 0.462 (0.370–0.555) pg/ml]. In consideration of the finding that sNfL levels were higher in the presence of fever, we had a closer look at the relationship between sNfL and fever and were not able to detect a correlation (**Figure 2**). With respect to the type of FS, we could not find any differences between simple and complex FS in biomarker levels [simple FS: sNfL: 20.9 (19.0–22.8) pg/ml; prolactin: 415 (366–464) mU/L, copeptin: 37.8 (26.5–49.1) pmol/L; complex FS: sNfL: 23.6 (18.9–28.4) pg/ml, prolactin: 425 (354–496) mU/L, copeptin: 38.6 (22.9–54.2) pmol/L]. When appointing sNfL as a dependent

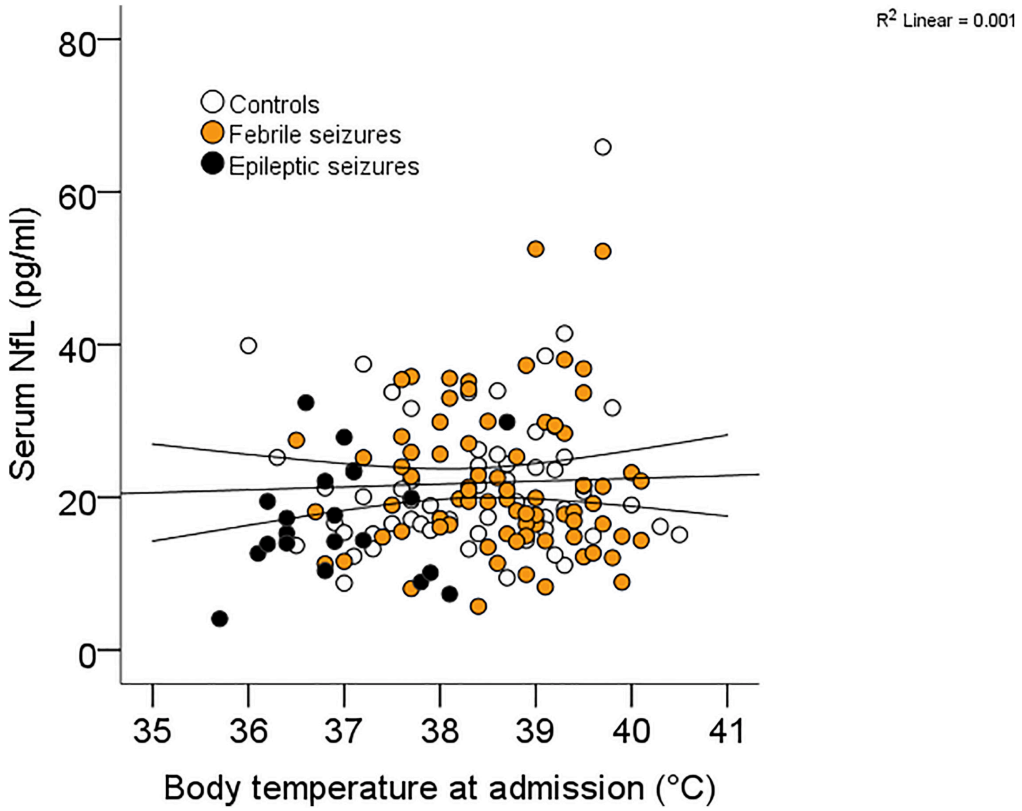

FIGURE 2 | Correlation between sNfL and body temperature.

variable in univariate models, sNfL had a significant inverse relationship with age and body weight (**Table 4**), indicating an age-dependent decrease of sNfL from early childhood until school age (**Figure 3**). MLR revealed age as the most important predictor of sNfL, followed by male sex and CRP. After including the two other biomarkers copeptin and prolactin into a model, also copeptin turned out to be a strong predictor for sNfL.

DISCUSSION

We prospectively investigated serum levels of NfL, copeptin, and prolactin in children presenting at an emergency department with FS, ES, or febrile infections without convulsions (controls). Our results provide evidence (1) that sNfL levels are not increased when measured within a few hours after convulsions in contrast to copeptin and prolactin levels; (2) that sNfL levels are higher in younger children, boys, and children with elevated CRP and elevated copeptin levels; and (3) that none of the three serum biomarkers are predictive for the recurrence of seizures.

The absent impact of convulsions on sNfL levels when measured a few hours after the events underlines the current state of evidence that simple FS are benign and do not increase the risk for the development of neurologic deficits (Steering Committee on Quality Improvement and Management, Subcommittee on Febrile Seizures American Academy of Pediatrics, 2008; Leaffer et al., 2013). Visser et al. (2012) also state that FS are not associated with problems in behavior or executive functioning in preschool children but did note an association of recurrent FS with an increased risk of expressive language delay at the age of 2.5 years which supports earlier findings about poorer language skills in school-aged children with a history of FS (Wallace, 1984). Matsushige et al. (2012) investigated the heavy chain of neurofilament (NfH) in serum of children suffering from febrile or epileptic seizures. The authors found a significant correlation between seizure duration and serum NfH levels during the first week in children with FS (Matsushige et al., 2012). Thus, whether sNfL levels may rise during recovery after febrile and epileptic convulsions warrants future studies.

Univariate analyses revealed a strong inverse relationship between sNfL and age and weight (**Table 4** and **Figure 3**). In multivariate analysis, for which weight was removed due to collinearity with age, age had the greatest impact on sNfL followed by male sex and CRP levels independently of seizures and fever. A very similar age dependency was described recently in a cohort of neurologically healthy children with decreasing sNfL in older children (Khalil et al., 2020; Reinert et al., 2020). In addition, between the age of 10 and 15 years, sNfL levels appear to mark a nadir, and beyond youth, sNfL levels increase in a linear fashion until the age of about 60 years. Afterward, sNfL levels were reported to rise much steeper

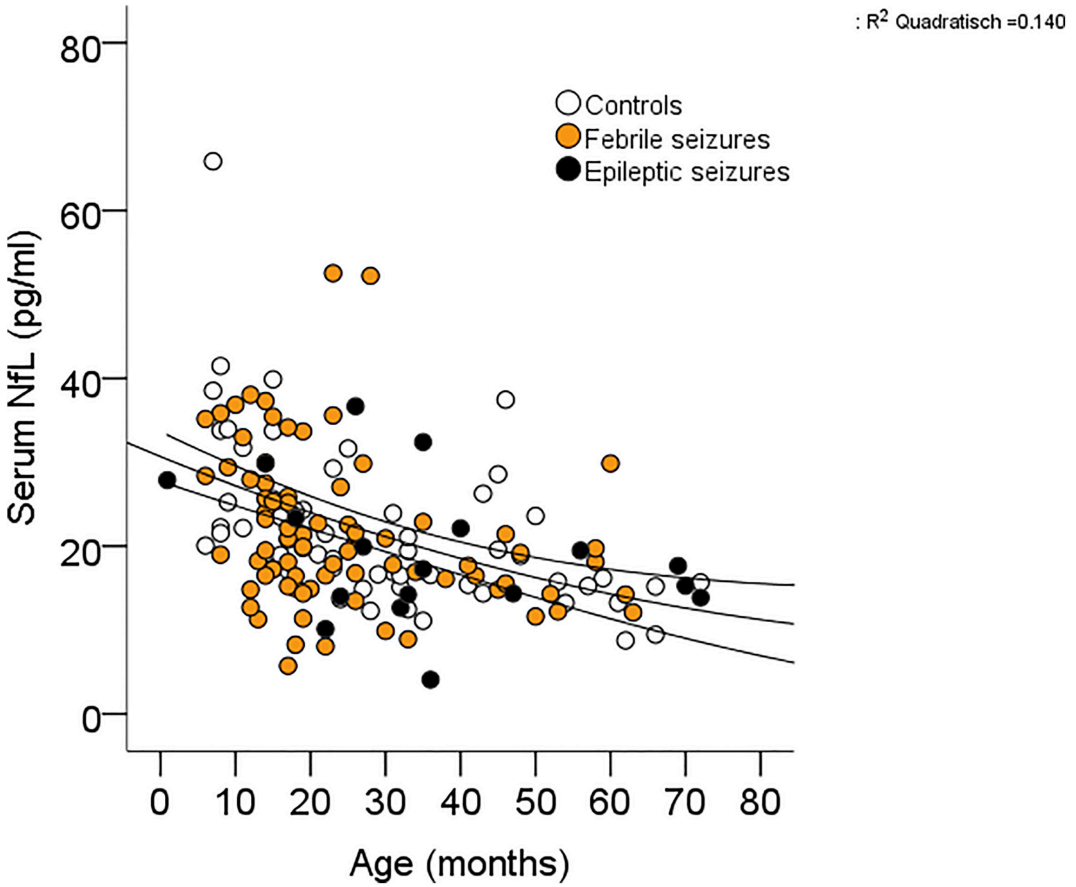

FIGURE 3 | Correlation between sNfL and age.

(Khalil et al., 2020; Reinert et al., 2020). Thus, considering sNfL level during the whole life cycle from high levels in newborn infants (Depoorter et al., 2018), decreasing until late childhood and then steadily increasing, sNfL levels represent a u-shaped curve. A possible explanation for high level in newborns is the developing brain with a high neuron turnover and a specialized system of tubulo-endoplasmic reticulum for protein transport. By the appearance of cerebral vessels being more fragile in infants than in adults, this might have the effect that the developing brain is more vulnerable (Saunders et al., 2012). In general, sNfL seems to reflect the substantial brain growth until adolescence followed by neuronal loss, which is associated with normal aging. Sexual disparity of biomarkers was described previously for copeptin in infants with higher levels in males. However, data on gender differences in sNfL are lacking (Burckhardt et al., 2014).

We observed that prolactin was elevated in the FS group when compared with the control group. The routine use of prolactin is not recommended due to limited accuracy. Moreover, copeptin levels were significantly higher in the FS group than in the control group and may be more useful for distinction of the underlying cause of the convulsive event (Stocklin et al., 2015; Pechmann et al., 2019). In contrast to these findings, our results could not provide additional support that copeptin and prolactin have the potential to predict upcoming convulsive events because none of the two biomarkers were related to recurrent seizures.

A few limitations need to be considered: the control group consisted of children presenting with febrile infections and our study revealed that sNfL levels are elevated in presence of fever alone and also correlate with CRP levels. This may lead to the suggestion that sNfL levels might be increased when compared with levels of healthy children without fever or contrariwise might only be elevated due to the rise in body temperature. We therefore propose to compare with a healthy afebrile cohort for verification of our hypothesis in potential upcoming studies. Furthermore, we must bear in mind that the diagnosis of a febrile seizure is solely based on the medical history and description of the caregivers; estimation by qualified personnel is therefore dependent on the statement of the accompanying parents. An overlap with simple shivering due to rise of temperature can therefore not be excluded. Besides, we merely analyzed blood samples at one timepoint; results of a further timepoint would give valuable information on the trend of sNfL levels and additionally might aid to assess the severity of suggested neuronal loss.

In conclusion, sNfL levels are not associated with febrile or epileptic seizures a few hours after the event, but significantly correlate with age, gender, and CRP. These findings are reassuring and indicate the benign nature of FS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Cantonal Ethics Committee of Basel. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SW, BS, KE, and JK conceived and designed the study. BS, SK, and CP were responsible for patient recruitment. JK and LB performed the biomarker measurements. SF performed the statistical analysis and prepared the tables and figures. SW, KE, MH, and JK interpreted the data. KE and SW drafted the initial manuscript. All authors critically revised the manuscript for important intellectual content, agreed on the final manuscript, and approved its submission for publication.

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OPINION
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The Role of Serum Levels of Neurofilament Light (NfL) Chain as a Biomarker in Friedreich Ataxia

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INTRODUCTION

Friedreich Ataxia (FRDA) is a progressive neurological and systemic disorder that affects about one in 50,000 people worldwide (Strawser et al., 2017). It is caused by mutations, usually GAA repeat expansions (96%) but also point mutations or deletions (4%), in the *FXN* gene, resulting in decreased production of functional frataxin protein (Babady et al., 2007; Delatycki and Bidichandani, 2019). GAA length on the shorter allele inversely correlates with disease severity (Strawser et al., 2017). Frataxin is a small mitochondrial protein that functions in iron-sulfur-cluster biosynthesis (Colin et al., 2013). Its deficiency leads to difficulties in production of cellular ATP as well as sensitivity to reactive oxygen species *in vitro* (Rötig et al., 1997; Lodi et al., 2001; Pastore and Puccio, 2013; DeBrosse et al., 2016). These properties lead to neurological injury and clinical impairment, including ataxia, dysarthria, sensory loss, and weakness in FRDA patients. While most literature has focused on neurodegeneration in FRDA, the disorder also has a large developmental component (Koeppen et al., 2017a,b). In addition, individuals with FRDA develop cardiomyopathy, scoliosis and sometimes diabetes mellitus. The cardiomyopathy of FRDA is characterized by early hypertrophy, with later progression to fibrosis and systolic dysfunction, leading to death from end-stage heart failure (Tsou et al., 2011; Lynch et al., 2012; Strawser et al., 2017). Many agents are in development for FRDA, including some designed to ameliorate mitochondrial dysfunction and others that seek to increase levels of functional frataxin (Strawser et al., 2014; Li et al., 2015; Lynch et al., 2018, 2021; Piguet et al., 2018; Zesiewicz et al., 2018a,b; Belbella et al., 2019; Rodríguez-Pascau et al., 2021).

NfL AS A BIOMARKER OF FRDA AND OTHER DISEASES

In many neurodegenerative diseases, the need for disease-modifying treatments is facilitated by identification of biomarkers to track disease progression. Such markers can capture subclinical changes in a rapid manner and show evidence of target engagement in clinical trials in slowly progressive neurological disorders. In other neurological disorders, including Multiple Sclerosis (MS), Alzheimer's disease (AD), and Parkinson's disease (PD), neurofilament light chain levels (NfL) in body fluids such as serum, plasma or CSF may provide a biomarker for tracking disease activity including progression (Bridel et al., 2019; Forgrave et al., 2019; Aktas et al., 2020; Del Prete et al., 2020; Milo et al., 2020; Thebault et al., 2020; Wang et al., 2020). Neurofilaments are cytoskeletal proteins located in both the peripheral and central nervous system, particularly in larger myelinated axons. They play a significant role in axonal growth and the determination of axonal caliber (Hsieh et al., 1994; Kurochkina et al., 2018; Bott and Winckler, 2020). Logically, as axons are damaged and die in neurodegenerative processes, NfL should leak into the interstitial

space, then into CSF and plasma. Thus, concentrations of NfL should generally increase as neurodegenerative diseases progress and should reflect disease activity. For example, in progressive MS, NfL appears to track with neuronal and axonal death, the stage of disease, and treatment response. NfL concentration in the CSF of MS patients parallels T2 lesion changes on MRI. Similarly, plasma NfL concentration is higher in AD patients than in controls and is associated with greater cognitive deficit. Such findings suggest that NfL is a promising biomarker for determining the stage of disease, tracking progression and aiding in identification of disease-modifying treatments in neurological disorders. However, in stable MS, NfL levels may not track with clinical dysfunction, providing a reminder that changes in biomarkers must be interpreted in the context of clinical changes (Aktas et al., 2020).

In FRDA, data on NfL levels in serum is more complex. Most features of FRDA depend on genetic severity (GAA repeat length) and worsen over the course of time, thus correlating positively with disease duration or age (Strawser et al., 2017). Overall, the two main determinants of clinical severity in FRDA are genetic severity and disease duration. In the three studies evaluating serum NfL in FRDA patients, serum NfL is elevated in patients with FRDA when compared to controls and carriers (Zeitlberger et al., 2018; Clay et al., 2020; Hayer et al., 2020). This shows that serum NfL levels reflect a pathological process in FRDA. However, in these three studies NfL levels generally do not correlate with markers of clinical or genetic severity. Moreover, while NfL levels correlate positively with age in non-FRDA patients (controls and carriers) in cross sectional analysis, in FRDA patients, NfL levels are highest in young children and *decrease* with age as the disease progresses (Clay et al., 2020). Thus, serum NfL is paradoxically high in young individuals and far lower in older individuals with more severe disease. At later ages it even overlaps with control values. A greater genetic severity in early onset individuals could explain some of this paradox; however, NfL levels overall do not correlate with GAA repeat length after accounting for age, and they even appear to correlate inversely with GAA length in some of these studies. Accounting for age, individuals who are more severe genetically have lower levels of NfL. Interestingly, Nfl levels are relatively stable over 1–2 years; consequently, NfL levels could be used as an assessment of therapeutic response over the time used in most clinical trials. Still, while NfL may provide a biomarker of FRDA in some manner, the relationship of NfL to disease progression is complex suggesting its utility may be limited to certain situations.

DISCUSSION

Understanding the exact meaning of NfL levels in serum and how they reflect disease activity in FRDA would facilitate their use as a marker of FRDA. In most other disorders, NfL is viewed as a marker of neurodegeneration of either axons or other neuronal regions. Degeneration in FRDA, though, is complex, including both peripheral nerve degeneration (including very early degeneration of proprioceptive afferents) with later degeneration of central nervous system axons; loss of central nervous system elements controls most of the clinical progression of the disease (Selvadurai et al., 2016; Koeppen et al., 2017a; Strawser et al., 2017; Marty et al., 2019; Rezende et al., 2019; Harding et al., 2020; Naeije et al., 2020). Brain imaging studies are typically normal early in disease, with the exception of atrophy of the cervical spinal cord, with progressive loss of CNS pathways later (Selvadurai et al., 2016; Koeppen et al., 2017b; Marty et al., 2019; Rezende et al., 2019; Harding et al., 2020; Naeije et al., 2020). Thus, serum NfL levels in FRDA, with high values early in disease, are discrepant from the tangible loss of CNS axons by MRI and the loss of specific functional clinical systems (**Figure 1**). The present data on serum NfL levels could be explained by a relatively large early loss of peripheral axons that does not contribute to clinical progression. Similarly, the inverse correlation with GAA repeat length in early disease might lead to a large developmental deficit at presentation. This in turn might lead to lower serum NfL levels during neurodegeneration. This interpretation would be consistent with the prevailing concept of NfL levels reflecting a relatively passive leakage from dying neurons into surrounding fluids and eventually to the serum.

Alternatively, increased levels of NfL could reflect other components of the pathophysiology of FRDA in a manner not directly associated with cell death. FRDA is associated with abnormalities in lipid metabolism as well as lipid peroxidation (Navarro et al., 2010; Obis et al., 2014; Abeti et al., 2016; Chen et al., 2016; Tamarit et al., 2016; Cotticelli et al., 2019; Turchi et al., 2020). Both could lead to membranes that are inherently more permeable than normal, with consequent loss of NfL from the cell. Why these processes would decrease with age, however, is unclear.

Still, other processes might contribute to the paradox of elevated NfL levels early in FRDA. NfL levels must to some degree reflect its synthesis, as increased synthesis leads to higher levels of soluble NfL (before it is incorporated into neurofilaments) that should more readily efflux from neurons cells than NfL assembled into intact neurofilaments. Increased synthesis of structural proteins in axons occurs in response to injury and during neuronal regeneration (Pearson et al., 1988; Havton and Kellerth, 2001; Toth et al., 2008; Balaratnasingam et al., 2011; Yin et al., 2014; Liu et al., 2016), and neurofilaments play different roles in development than simply structural maintenance. The very high levels of NfL early in FRDA could reflect attempts at regeneration that become more impaired as the disease progresses, leading to falling levels of NfL later in the course of the disorder. In general, the plasticity of the nervous system decreases with aging, matching the falls in serum NfL over time in FRDA (Bouchard and Villeda, 2015). Thus, elevated levels of serum NfL early in the course of FRDA could be driven by enhanced synthesis of NFL during regeneration superimposed on increased membrane fragility.

Interestingly, cardiac troponin levels are elevated in FRDA serum during the period of hypertrophic disease, long before cardiomyocytes die and cardiac fibrosis develops (Friedman et al., 2013; Plehn et al., 2018; Legrand et al., 2020). Such elevated levels

Abbreviations: FRDA, Friedreich Ataxia; NfL, Neurofilament light chain; GAA, Guanine-adenine-adenine; MS, Multiple sclerosis; PD, Parkinson's disease; CSF, Cerebrospinal fluid; AD, Alzheimer's disease; CNS, central nervous system.

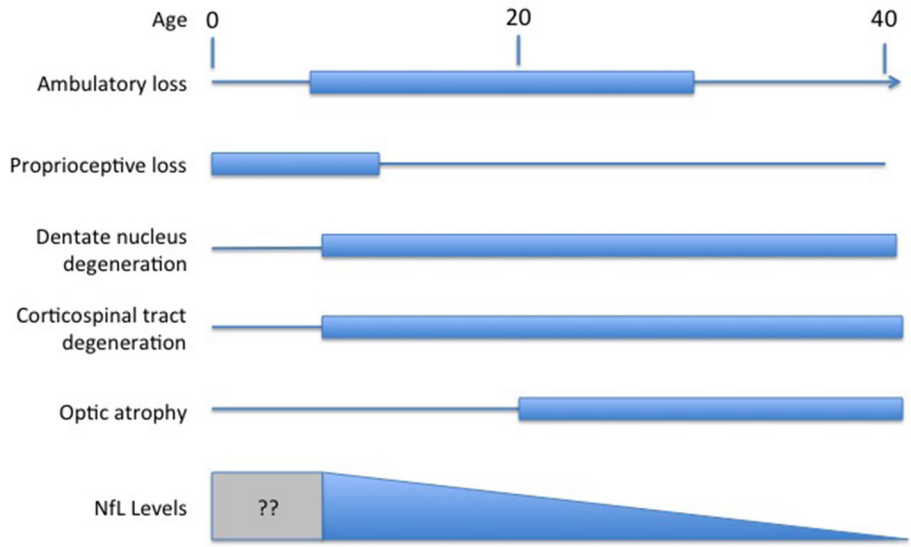

FIGURE 1 | Temporal course of changes in FRDA. Diagram illustrating the contrasting temporal course of clinical changes in FRDA along with serum NfL levels over time (amalgamated from Bridel et al., 2019; Del Prete et al., 2020; Wang et al., 2020). Clinical changes are presented based on the clinical course of an early onset individual (onset between ages 5–10). At present, no study has measured NfL in the presymptomatic period before age five.

of troponin in early FRDA cardiomyopathy might result from similar mechanisms to the elevated levels of NfL early in FRDA (Thebault et al., 2020).

A final possibility is that both cell-loss and cell-repair mechanisms—and possibly still other mechanisms—mediate the changes in NfL in FRDA. Such interpretations may only be distinguishable over time with collection of long-term serial data, and with collection of data during the presymptomatic period. Furthermore, a more complete characterization of the features of immunoreactive NfL in FRDA serum may be helpful. While the assays used are specific for NfL, they do not assess whether it represents full-length protein. This does not change the observation that Nfl levels can serve as biomarkers of disease in FRDA. However, without understanding the reason for the unusual distribution of NfL values, it is difficult to provide precise interpretations for clinical trial results. Normalization of biomarker levels can provide evidence for benefit in the correct circumstances, but can also reflect impairment of compensatory mechanisms, thus being associated with deleterious effects. A deeper understanding of the mechanisms of NfL elevation in serum in FRDA is needed to make it a useful biomarker in FRDA.

AUTHOR CONTRIBUTIONS

BF created the first draft and edited the final work. RW and KS provided ideas, editing, and critical review. DL contributed to the first draft, performed critical review, ideas for the project, and designed the figure. All authors contributed to the article and approved the submitted version.

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Cerebrospinal Fluid and Blood Neurofilament Light Chain Protein in Prion Disease and Other Rapidly Progressive Dementias: Current State of the Art

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Rapidly progressive dementia (RPD) is an umbrella term referring to several conditions causing a rapid neurological deterioration associated with cognitive decline and short disease duration. They comprise Creutzfeldt–Jakob disease (CJD), the archetypal RPD, rapidly progressive variants of the most common neurodegenerative dementias (NDs), and potentially treatable conditions such as infectious or autoimmune encephalitis and cerebrovascular disease. Given the significant clinical and, sometimes, neuroradiological overlap between these different disorders, biofluid markers also contribute significantly to the differential diagnosis. Among them, the neurofilament light chain protein (NfL) has attracted growing attention in recent years as a biofluid marker of neurodegeneration due to its sensitivity to axonal damage and the reliability of its measurement in both cerebrospinal fluid (CSF) and blood. Here, we summarize current knowledge regarding biological and clinical implications of NfL evaluation in biofluids across RPDs, emphasizing CJD, and other prion diseases. In the latter, NfL demonstrated a good diagnostic and prognostic accuracy and a potential value as a marker of proximity to clinical onset in pre-symptomatic *PRNP* mutation carriers. Similarly, in Alzheimer's disease and other NDs, higher NfL concentrations seem to predict a faster disease progression. While increasing evidence indicates a potential clinical value of NfL in monitoring cerebrovascular disease, the association between NfL and prediction of outcome and/or disease activity in autoimmune encephalitis and infectious diseases has only been investigated in few cohorts and deserves confirmatory studies. In the era of precision medicine and evolving therapeutic options, CSF and blood NfL might aid the diagnostic and prognostic assessment of RPDs and the stratification and management of patients according to disease progression in clinical trials.

Keywords: Creutzfeldt-Jakob disease, prion disease, neurofilament light chain, Alzheimer's disease, frontotemporal dementia, encephalitis, autoimmune, biofluid biomarkers

INTRODUCTION

Rapidly progressive dementia (RPD) is an umbrella term that comprehends several heterogenous diseases characterized by a rapidly progressive cognitive decline and a short disease duration, typically 1–2 years between onset and occurrence of dementia and/or of 2–3 years from onset to death (Cohen et al., 2015; Grau-Rivera et al., 2015; Geschwind and Murray, 2018; Rudge et al., 2018; Zerr and Hermann, 2018). Evidence from epidemiological studies indicates that RPD is not a rare condition. However, data concerning the actual prevalence are lacking. The most frequent causes of RPDs comprise prion disease and other neurodegenerative dementias (NDs), autoimmune and infectious encephalitis, vascular and metabolic encephalopathies, and malignancies (Geschwind and Murray, 2018; Zerr and Hermann, 2018).

Sporadic Creutzfeldt–Jakob's disease (CJD) represents the prototype of RPDs and is often referred to as the "great mimicker" given its wide phenotypic heterogeneity (Baiardi et al., 2018; Geschwind and Murray, 2018). The extensive clinical overlaps between RPDs, the urgency and severity of the clinical scenario, and the existence of treatable forms make the availability of reliable diagnostic and prognostic biomarkers of primary importance. Brain magnetic resonance imaging (MRI), cerebrospinal fluid (CSF) surrogate markers such as proteins total (t)-tau and 14-3-3, and electroencephalographic (EEG) examination, support the clinical diagnosis of probable CJD according to current criteria (Zerr and Parchi, 2018; Rhoads et al., 2020). However, the overall sensitivity and specificity of these diagnostic investigations are not optimal. Indeed, a significant proportion of non-prion RPDs may show positivity in one or more of these tests, whereas, on the other hand, atypical disease subtypes with slower progression may escape recognition (Hamlin et al., 2012; Lattanzio et al., 2017; Rudge et al., 2018). In this regard, the prion real-time quacking-induced conversion (RT-QuIC) assay with its virtually full specificity for prion disease significantly contributed to the improvement of the diagnostic assessment of RPDs (Candelise et al., 2020). However, the assay is still not fully available and represents in many laboratories a second-step diagnostic assay to be applied when screening tests are suggestive for prion disease (Abu-Rumeileh et al., 2019a). Thus, the availability of sensitive and readily accessible analytes for large and rapid screening remains of critical importance. Besides, there is also urgent need for markers to use as surrogate end points in ongoing clinical trials showing positive associations with prognostic variables, such as overall survival, disease severity, and progression rate.

The assessment of neurofilament light chain protein (NfL) has attracted growing attention in recent years due to its sensitivity to neuronal damage and reliability of its measurement in both CSF and blood (Gaetani et al., 2019; Barro et al., 2020). NfL is a subunit of neurofilaments, which are cytoskeletal proteins mainly located in large myelinated axons where they play an important role in maintaining neuronal structure. After neuronal damage, NfL reaches the interstitial fluid, which communicates freely with the CSF and the blood (Gaetani et al., 2019). Several studies have explored the added values of this biomarker in the diagnostic and prognostic assessment of suspected CJD cases, with promising results. Moreover, given the high awareness of clinical neurologists for the treatable forms of RPD, particularly for autoimmune encephalitis (AE), attempts to evaluate the diagnostic and prognostic role of NfL have also been recently extended to this field.

In the present review, we summarize the current knowledge regarding the biological and clinical implications of CSF and blood NfL across the most prevalent etiologies of RPD.

PRION DISEASE

Prion diseases or transmissible spongiform encephalopathies are rare, phenotypically heterogeneous, rapidly progressive neurodegenerative diseases (Parchi and Saverioni, 2012). The pathogenesis of prion diseases relies on the templated seeded conversion of the cellular prion protein (PrP^{C}), encoded by the prion protein (*PRNP*) gene, into a pathological isoform with abnormal conformation (PrP^{Sc}), which shows a tendency to aggregate and forms amyloid fibrils (Parchi and Saverioni, 2012; Puoti et al., 2012; Baiardi et al., 2019).

Sporadic CJD (sCJD) represents the most common form of human prion disease and accounts for about 85–90% of cases. The second most common variant (10–15% of cases) is genetic prion disease, which is related to pathogenic mutations in *PRNP* and encompasses three distinct clinical–pathological phenotypes, the genetic form of CJD (gCJD), fatal familial insomnia (FFI), and Gerstmann–Sträussler–Scheinker syndrome (GSS). Finally, a small proportion of cases (1–2%) is acquired (Baiardi et al., 2019). In turn, sCJD includes six major clinicopathological subtypes that are mainly determined by the genotype at the methionine (M)/valine (V) polymorphic codon 129 of the *PRNP* gene and the type (1 or 2) of PrP^{Sc} accumulating in the brain. The subtypes are named and classified according to these two main molecular variables into MM(V)1, MM2 cortical (MM2C), MM2 thalamic (MM2T), MV2 kuru (MV2K), VV1, and VV2 subtypes (Parchi et al., 1999, 2012; Baiardi et al., 2019).

Diagnostic Value and Distribution Across Prion Disease Subtypes

Several studies (**Table 1**) evaluated CSF NfL in the prion disease spectrum. They showed significantly increased mean levels in most disease subtypes compared to non-neurodegenerative controls and other NDs (Steinacker et al., 2016; Kovacs et al., 2017; Abu-Rumeileh et al., 2018b, 2019a, 2020a; Zerr et al., 2018; Kanata et al., 2019; Vallabh et al., 2020). Interestingly, CSF NfL concentration varied significantly among sCJD subtypes and only partially correlated with t-tau levels (Abu-Rumeileh et al., 2018b; Zerr et al., 2018), suggesting that the two markers reflect distinct pathophysiological mechanisms. Both t-tau and NfL levels seem to be influenced by the neuronal degeneration occurring in a given period (i.e., speed of disease progression). Still, NfL raises more significantly than t-tau in the diseases with more widespread subcortical pathology (i.e., in deep nuclei, brainstem, cerebellum, and spinal cord), likely because of a more significant involvement of myelinated axons in white matter

tracts in these regions compared to the cerebral cortex (Abu-Rumeileh et al., 2018b). Indeed, sCJD subtypes VV2 and MV2K showed significantly higher CSF NfL levels in comparison to the MM(V)1 group (Abu-Rumeileh et al., 2018b, 2020a; Zerr et al., 2018), which correlates with the more widespread and severe subcortical pathology and the higher amount of PrP^{Sc} accumulation and microglial activation in subcortical white matter in the former subtypes (Parchi et al., 2012; Baiardi et al., 2017; Franceschini et al., 2018). The divergent behavior of NfL and t-tau is especially evident in the MV2K subtype, which shows significantly lower concentrations of 14-3-3 and t-tau in CSF (Lattanzio et al., 2017) and a slower disease progression than the VV2. This might also explain the very high sensitivity of NfL for other slowly progressive prion disease subtypes showing low CSF values of t-tau and protein 14-3-3 [e.g., sCJD MM2C, gCJD E200K, GSS, FFI, and variable protease-sensitive prionopathy (VPSPr)] (Abu-Rumeileh et al., 2018b, 2019a; Zerr et al., 2018).

CSF NfL showed good diagnostic value across different studies on prion disease and full discrimination between CJD patients and controls (AUC 0.949–1.000) (Steinacker et al., 2016; Kovacs et al., 2017; Abu-Rumeileh et al., 2018b; Zerr et al., 2018). Interestingly, CSF NfL, either alone or in combination with other biomarkers, yielded a performance similar to t-tau in the distinction of prion disease from other NDs (AUC 0.926 vs. 0.939) and showed even a higher diagnostic value than t-tau in the specific comparisons between atypical prion disease and other rpNDs (AUC 0.839 vs. 0.722) (Abu-Rumeileh et al., 2018b). However, the biomarker accuracy for an early clinical diagnosis should be ideally assessed in a clinically or neuropathological based cohort of patients with heterogeneous RPD etiologies raising the clinical suspicion of prion disease, but only a few studies considered this approach (Kovacs et al., 2017; Abu-Rumeileh et al., 2019a, 2020a; Kanata et al., 2019). In this type of studies, CSF NfL diagnostic accuracy (AUC 0.451–0.890) was significantly lower than that of CSF t-tau (AUC 0.849–0.918) and/or protein 14-3-3 (AUC 0.711–0.908) (Kovacs et al., 2017; Abu-Rumeileh et al., 2018b, 2020a; Kanata et al., 2019). Indeed, increased levels of NfL are detected in most vascular, and neuroinflammatory diseases, and even in neurodegenerative disorders commonly associated with a slower progressive course than RPDs. However, NfL levels overlapping significantly with those seen in CJD mainly characterize frontotemporal dementia, amyotrophic lateral sclerosis, and atypical parkinsonism (Gaetani et al., 2019). Therefore, given its low specificity, CSF NfL has a limited role as an isolated test in the differential diagnosis of RPDs (Abu-Rumeileh et al., 2019a, 2020a; Kanata et al., 2019). Nevertheless, when specific tests such as RT-QuIC are also adopted in series, CSF NfL might be useful as a first step screening assay for suspected CJD cases as an alternative to t-tau or 14-3-3.

More recently, blood NfL levels were also found significantly higher in patients with prion disease belonging to all CJD forms and subtypes than both controls and other NDs (**Table 1**; Steinacker et al., 2016; Kovacs et al., 2017; Thompson et al., 2018, 2020; Abu-Rumeileh et al., 2020a). However, in contrast to the CSF analyte, blood NfL levels did not significantly differ among the most prevalent sCJD subtypes (Kovacs et al., 2017; Thompson et al., 2018; Abu-Rumeileh et al., 2020a). Interestingly, sCJD VV2, typically characterized by the highest CSF NfL and t-tau values among sCJD subtypes (Lattanzio et al., 2017; Abu-Rumeileh et al., 2018b, 2020a; Zerr et al., 2018), showed similar blood NfL and reduced blood tau levels compared with the MM(V)1 type (Kovacs et al., 2017; Abu-Rumeileh et al., 2020a). These data suggest that the different regional lesion profiles between the two CJD subtypes (Parchi et al., 1999; Baiardi et al., 2017), particularly the early cortical neuronal damage featuring MM(V)1 but not VV2, might be responsible for a higher spill over of these molecules in the blood compared to other brain regions (Abu-Rumeileh et al., 2020a). An overview of the distribution of CSF and blood NfL and a comparison with other classic and new biofluid markers across distinct prion disease forms and subtypes are provided in **Table 2**.

Regarding the diagnostic value of blood NfL, only three studies to date evaluated the marker in clinically and/or neuropathological heterogeneous cohorts of RPDs (Kovacs et al., 2017; Abu-Rumeileh et al., 2020a; Thompson et al., 2020). They obtained similar results, namely, the lower value of blood NfL compared to blood tau in the discrimination between prion (or sCJD) and non-prion RPDs (NfL: AUC 0.497–0.724 vs. tau: AUC 0.722–0.837) (Kovacs et al., 2017; Abu-Rumeileh et al., 2020a; Thompson et al., 2020). Notably, the performance of classic CSF markers such as CSF t-tau and 14-3-3 appeared significantly superior to both plasma tau and NfL, raising the question of the real utility of blood surrogate markers in the diagnostic assessment of RPDs (Abu-Rumeileh et al., 2020a).

In conclusion, the very high negative predictive value of NfL for prion disease, due to its high sensitivity for neural damage, may justify its use as an early screening marker. Indeed, low or normal CSF and/or blood levels of NfL in a patient with RPD might exclude with very high certainty the diagnosis of prion disease and induce clinicians to consider other etiologies and to introduce an *ex adiuvantibus* therapy. In contrast, a significant increase of its levels in at least one biofluid should prompt a second more specific test such as the RT-QuIC.

Prognostic Value

Different methods have been adopted to assess the rate of clinical progression in prion disease. The MRC Prion Disease Rating Scale, a validated scale of functional impairment, has been used to model linear slopes of functional decline as a measure of the rate of clinical progression (Thompson et al., 2013). Despite the lack of a significant association between NfL values and the rate of disease progression (MRC slope), Thompson et al. (2018, 2020) found a slight correlation between blood NfL values and the degree of functional impairment, along with a tendency toward blood NfL levels raising over time in serial samples of symptomatic patients with increasingly functional impairment. Moreover, they found elevated blood NfL concentrations in all disease stages, with no overlapping values with controls in every sample tested longitudinally, suggesting a potential role of the marker as an outcome measure in clinical trials (Thompson et al., 2020). Similarly, in another study, CSF and blood NfL levels significantly correlated with another scale (i.e., Barthel Index) of functional impairment (Staffaroni et al., 2019).

TABLE 1 | Main findings of studies evaluating CSF and blood NfL in prion disease.

	Country of studied population	Examined biofluid	Assay	*N* according to prion disease form	*N* according to non-prion disease	AUC (cutoff)* sCJD vs. Controls	AUC (cutoff)* sCJD vs. non-prion	Other significant findings
Steinacker et al. (2016)	Germany	CSF, Blood	CSF: ELISA (IBL) Blood: Simoa	42 CJD (39 definite): 33 sCJD, 9 gCJD, 1 GSS (pre-symp)	20 dementia patients	CSF: 0.949 (>2,156 pg/ml) Blood: 0.959 (>44.7 pg/ml)		Elevated CSF NfL in one pre-symptomatic GSS case
Kovacs et al. (2017)	Austria	CSF, Blood	CSF: ELISA (Uman Dia.) Blood: Simoa	86 definite CJD: 65 sCJD, 21 gCJD	46 non-prion RPDs (all definite)	CSF: 0.979 Blood: 0.992	CSF: 0.451, vs. AD 0.768. Blood: 0.497, vs. AD 0.657.	No marked differences in blood and CSF NfL among sCJD subtypes
Abu-Rumeileh et al. (2018b)	Italy	CSF	CSF: ELISA (IBL)	141 prion (115 definite): 123 sCJD, 1 VPSPr 16 gCJD, 1 GSS	73 AD (37 rp), 35 DLB (11 rp), 44 FTLD (9 rp)	CSF: 1.00	0.926 vs. all. Atypical prion vs. rpNDs 0.839.	Significantly higher CSF levels in the sCJD subtypes VV2 and MV2K compared to the MM(V)1 group
Thompson et al. (2018)	United Kingdom	Blood	Blood: Simoa	45 sCJD (40 definite) 6 with serial samples	–	Blood: 1.00 (>44.7 pg/ml)	–	No correlation between blood NfL and rate of disease progression. Trend toward increased NfL in serial samples taken within 12 months from death
Zerr et al. (2018)	Germany, Poland, Spain, Portugal, Italy	CSF	CSF: ELISA (Uman Dia.)	314 prion (257 definite) Cohort 1: 112 sCJD Cohort 2: 20 sCJD 182 gCJD	Cohort 1: 11 MCI, 88 AD, 41 DLB/PDD, 36 VaD, 11 FTD. Cohort 2: 37 MCI, 20 AD, 12 DLB/PDD, 10 VaD, 30 FTD	CSF: 0.99 (>7,000 pg/ml)	0.90 overall (>10,500 pg/ml) >0.9 vs. AD, >0.9 vs. DLB/PDD, 0.83 vs. FTD; 0.76 vs. VaD	Prion disease: Significantly higher CSF levels in VV than in MM or MV codon 129 *PRNP* genotypes. Increase in NFL levels in consecutive LPs in cases with duration > 6 months. CSF NfL as a moderate prognostic marker.
Abu-Rumeileh et al. (2019a)	Italy	CSF	CSF: ELISA (IBL)	103 prion (103 definite): 80 sCJD, 1 VPSPr, 22 gCJD	109 non-prion RPDs	–	0.693 (>1,847 pg/ml)	CSF NfL most sensitive surrogate marker in virtually all prion diseases
Kanata et al. (2019)	Poland, Greece	CSF	CSF: ELISA (Uman Dia.)	Cohort 1: 43 CJD Cohort 2: 21 CJD	Cohort 1: 34. Cohort 2: 29	–	CSF: Cohort 1: 0.89 (>4,200 pg/ml), Cohort 2: 0.86 (>4,200 pg/ml)	
Staffaroni et al. (2019)	United States	CSF, Blood	CSF: ELISA (IBL) Blood: Simoa	188 CJD (147 definite)	–	–	–	CSF and blood NfL associated with survival (before adjusting for covariates)
Abu-Rumeileh et al. (2020a)	Italy	CSF, Blood	CSF: ELISA (IBL) Blood: Simoa	336 prion (254 definite): 275 sCJD, 1 VPSPr, 28 gCJD, 3 FFI, 3 GSS	106 non-prion-RPDs	–	CSF: 0.646 (>1,846 pg/ml). Blood: 0.616 (>87.9 pg/ml)	CSF and blood NfL associated with survival (even after adjusting for covariates). Blood NfL as strong prognostic factor in slowly progressive prion disease
Thompson et al. (2020)	United Kingdom	Blood	Blood: Simoa	231 sCJD, 14 iCJD, 17 vCJD 23 pre-symptomatic, 9 converting, 83 symptomatic *PRNP* mutation carriers	31 AD, 33 FTD, 24 non-prion RPDs	Blood: 1.00 (>25.87 pg/ml)	Blood: 0.912 vs. all (>60.65 pg/ml), 0.724 vs. non-prion RPDs (>60.65 pg/ml)	Blood NfL higher in sCJD compared to genetic prion disease and vCJD. Blood NfL correlates with severity of functional impairment but not with rate of disease progression. Higher blood NfL up to 2 years before onset but not earlier in pre-symptomatic *PRNP* mutation carriers
Vallabh et al. (2020)	United States, Australia	CSF, Blood	CSF: ELISA (Uman Dia., in-house) Blood: Simoa	27 pre-symptomatic *PRNP* mutation carriers 26 definite CJD: 24 sCJD, 2 gCJD	–	–	–	No difference in CSF and blood NfL between pre-symptomatic carriers and controls. No temporal increase in blood and CSF NfL in longitudinal assessments

**Cutoffs were reported in parentheses when available.*

TABLE 2 | Distribution of CSF and blood markers across prion disease subtypes.

	Sporadic CJD				Genetic prion disease			
Matrix–Marker (reference)	**MM(V)1**	**VV2**	**MV2K**	**MM2C**	**gCJD E200K**	**gCJD V210I**	**FFI**	**GSS**
Neuronal/axonal damage								
CSF NfL (Abu-Rumeileh et al., 2018b*, 2019a*; Kovacs et al., 2017; Zerr et al., 2018)	↑↑	↑↑↑	↑↑(↑)	↑↑	↑↑	↑↑	↑(↑)	↑↑
Blood NfL (Kovacs et al., 2017; Abu-Rumeileh et al., 2020a)	↑↑	↑↑	↑↑	↑	↑↑	↑↑	↑	↑
CSF t-tau (Lattanzio et al., 2017*; Abu-Rumeileh et al., 2018b*, 2019a*, 2020a*)	↑↑(↑)	↑↑↑	↑(↑)	↑(↑)	↑↑	↑↑(↑)	(↑)	↑(↑)
Blood tau (Kovacs et al., 2017; Abu-Rumeileh et al., 2020a)	↑↑↑	↑(↑)	↑(↑)	↑	↑↑(↑)	↑↑(↑)	(↑)	=
CSF 14-3-3 (Lattanzio et al., 2017*; Abu-Rumeileh et al., 2019a*, 2020a*)	↑↑(↑)	↑↑↑	↑	↑	↑↑	↑↑(↑)	(↑)	NA
Neuroinflammation								
CSF YKL-40 (Llorens et al., 2017b; Abu-Rumeileh et al., 2019b*)	↑↑	↑↑↑	↑↑	↑(↑)	↑↑	↑↑	↑(↑)	↑↑
CSF GFAP (Abu-Rumeileh et al., 2019b*)	↑	↑↑	↑	↑	↑	(↑)	(↑)	↑
CSF CHIT1 (Abu-Rumeileh et al., 2019b*)	↑↑	↑↑↑	↑↑↑	↑(↑)	↑(↑)	↑	↑↑	↑↑(↑)
Synaptic damage								
CSF α-synuclein (Llorens et al., 2017a, 2018)	↑↑(↑)**	↑↑(↑)**	NA	NA	↑↑	↑↑	=	=
CSF neurogranin (Blennow et al., 2019)	↑↑	↑	NA	NA	NA	NA	NA	NA
Other mechanisms								
CSF t-PrP (Villar-Piqué et al., 2019)	↓↓	↓↓	↓↓	NA	↓↓	↓	↓↓	=
Blood t-PrP (Llorens et al., 2020b)	↑↑	↑↑	↑	↑↑	↑↑	↑↑(↑)	↑↑	NA
CSF p-tau (Lattanzio et al., 2017*, Abu-Rumeileh et al., 2018b*)	(↑)	↑↑	↑	=	(↑)	(↑)	NA	NA
CSF ubiquitin (Abu-Rumeileh et al., 2020b)	↑↑	↑↑↑	↑	↑	↑	↑↑	=	↑

*We considered only recent studies (2017–2020) and prion disease subtypes, in which at least one of the reported fluid biomarkers was analyzed in at least 10 patients. ↑ indicates increased values in comparison to controls. The number of arrows expresses the degree of increase (↑: mild, ↑↑: moderate, ↑↑↑: severe), the bracket indicates intermediate levels between two degrees. ↓ decreased values in comparison to controls with the same semiquantitative scale. =, values within the normal range. NA: data not available. *CSF t-tau, NfL, 14-3-3 and p-tau data from some patients were included in multiple studies. **Only data on MM and VV genotypes are available. However, >95% of VV cases are VV2 and >95% of MM cases are MM1 (or MM1+2C).*

To date, only three studies explored the association between NfL in CSF and/or blood and survival in prion disease (Zerr et al., 2018; Staffaroni et al., 2019; Abu-Rumeileh et al., 2020a). Zerr and colleagues originally reported that CSF NfL performs only moderately as a prognostic marker (Zerr et al., 2018). However, two studies later showed that both CSF and blood NfL were significantly associated with survival in prion disease/CJD (Staffaroni et al., 2019; Abu-Rumeileh et al., 2020a). However, only Abu-rumeileh et al. stratified the analysis according to the disease subtype, a strong independent factor in prion disease. The collected data showed that while blood and/or CSF t-tau best predicted survival in sCJD MM(V)1 and VV2 subgroups, blood NfL (but not CSF NfL) was significantly associated with survival in the slowly progressive prion diseases. Although a test for *in vivo* PrP^{Sc} typing is currently unavailable, codon 129 genotyping allows partial patient stratification since early disease onset. In this regard, multivariate analyses adjusted for age and codon 129 genotype showed that CSF and blood NfL, CSF tau, and CSF 14-3-3 were still predictive of survival (Staffaroni et al., 2019; Abu-Rumeileh et al., 2020a; Llorens et al., 2020a). Therefore, current evidence strongly recommends a stratification according to disease subtype or at least codon 129 genotype (in the clinical setting) to select outcomes and endpoints in future clinical trials (Abu-Rumeileh et al., 2020a).

Biomarker Values Throughout the Disease Course of Genetic Prion Disease

Any future treatment aimed to prevent prion disease cannot prescind from the identification and longitudinal evaluation of pre-symptomatic *PRNP* mutation carriers. The significant heterogeneity of the age at onset in the prion disease spectrum combined with the rarity of the disease strongly limits the use of clinical onset as an outcome in preventive clinical trials. In this regard, identifying a biomarker of proximity to disease onset might be critical to overcome the issue (Minikel et al., 2019, 2020; Vallabh et al., 2020).

To date, only two studies explored neurofilament dynamics in cross-sectional and/or longitudinal samples from pre-symptomatic and symptomatic *PRNP* mutation carriers. One reported no difference in CSF and/or blood NfL levels between pre-symptomatic *PRNP* mutation carriers and healthy controls and no temporal increase in longitudinal blood and CSF samples in pre-symptomatic cases (Vallabh et al., 2020). Similarly, in the study of Thompson et al. (2020), NfL concentrations did not differ between pre-symptomatic *PRNP* mutation carriers with samples collected more than 2 years before symptom onset and those of controls. However, the marker increased progressively in the longitudinal evaluation from 2 years from onset to the symptomatic phase. In summary, the available data on this aspect are, to some extent, controversial. They potentially reflect a common selection bias, namely, the inclusion of subjects with several different *PRNP* mutations and heterogeneous clinical progression rates.

Nevertheless, recent evidence indicates that blood NfL may perform well as a surrogate endpoint of response to antisense oligonucleotides (ASOs) therapy in a prion disease mouse model (Minikel et al., 2020), where a significant reduction of the biomarker level was documented after drug administration. These findings resembled those obtained in the II phase trial

for ASOs in SOD1 ALS (Miller et al., 2020) and open new possible horizons for implementing blood NfL as a reliable and accessible marker of disease monitoring in clinical trials for neurodegenerative diseases.

OTHER NEURODEGENERATIVE DEMENTIAS

NDs represent the most common etiology in non-prion RPD patients' cohorts and a frequent source of CJD misdiagnosis (Geschwind and Murray, 2018; Zerr and Hermann, 2018). In this regard, rapidly progressive variants of AD (rpAD), dementia with Lewy bodies (DLB), and frontotemporal dementia (FTD) may represent distinct subtypes of these disorders characterized by rapid cognitive decline, short disease duration (1–3 years), and subtle distinctive neuropathological and/or biochemical features (Cohen et al., 2015; Drummond et al., 2017; Lee et al., 2017; Abu-Rumeileh et al., 2018a; Geut et al., 2019; Zerr and Hermann, 2018).

The differential diagnosis between CJD and rp-NDs is still challenging, given the partial overlap in clinical features and CSF levels of the surrogate protein markers 14-3-3 and total-tau (Abu-Rumeileh et al., 2018b; Geschwind and Murray, 2018). For this purpose, other CSF biomarkers, including NfL (Abu-Rumeileh et al., 2018b), were tested, especially in the comparison between prion disease and rpAD. However, the evaluation of NfL levels in slowly and rp-ND forms failed to reveal a significant difference between the two groups, apart from FTD (Abu-Rumeileh et al., 2018b).

Another crucial open issue concerns the *a priori* identification of subjects with faster cognitive decline in AD and FTD cohorts to improve patient stratification for future clinical trials. In this regard, few studies showed an association between high CSF NfL levels and rapid cognitive decline in AD's mild cognitive impairment stage (Zetterberg et al., 2016; Pillai et al., 2020). Similarly, in the FTD spectrum, higher basal CSF and/or blood NfL values have been related to faster disease progression (in some clinical syndromes) (Ljubenkov et al., 2018; Rojas et al., 2018) and shorter survival (Meeter et al., 2018; van der Ende et al., 2019; Benussi et al., 2020; Cajanus et al., 2020), suggesting the utility of an early NfL assessment in the prediction of future disease aggressiveness.

AUTOIMMUNE ENCEPHALITIS AND OTHER POTENTIALLY TREATABLE CAUSES OF RPD

AE represents an increasingly recognized common cause of RPD (Geschwind and Murray, 2018).

Apart from the relevant diagnostic issue of the exclusion of prion disease (Abu-Rumeileh et al., 2019a), CSF and blood NfL have been explored in AE as easily accessible tools for disease monitoring, prognosis, and response to therapy. Although data are scanty and inconsistent, some findings deserve to be mentioned. In analogy with other RPDs, patients with AE typically showed higher CSF and/or blood NfL values than controls or reference intervals (Constantinescu et al., 2016, 2017; Li et al., 2019; Mariotto et al., 2019). However, concentrations in the normal range have also been reported (Körtvelyessy et al., 2018).

Interestingly, patients on active disease/progression, those on recovery and/or after disease treatment, and those with a stable clinical picture seem to show increased, decreased, or steady levels, respectively (Constantinescu et al., 2016; Li et al., 2019; Mariotto et al., 2019). Given the good correlation between CSF NfL and the disability grade (Constantinescu et al., 2016; Li et al., 2019), these data suggest a role for NfL as a marker of disease activity in AE.

CSF NfL concentration also predicted long-term outcomes in patients with AE (Constantinescu et al., 2016, 2017; Macher et al., 2020). However, most studies showed no associations between NfL values, CSF parameters, and MRI variables (Constantinescu et al., 2016; Mariotto et al., 2019; Macher et al., 2020), reflecting a possible discrepancy between clinical severity and radiological or biochemical features in these conditions (Mariotto et al., 2019).

On a significant issue, all the studies mentioned above merged patients with several subtypes of AE, raising concerns about a possible selection bias that may have influenced the results discussed above.

In RPDs secondary to infectious etiologies, CSF NfL has been reported to predict long-term neurocognitive status in herpes simplex encephalitis (Westman et al., 2020). In contrast, the measurement of NfL concentrations in patients with varicella-zoster encephalitis showed discordant results between studies (Eckerström et al., 2020; Tyrberg et al., 2020). Moreover, despite an inconsistent correlation between NfL levels and degree of cognitive impairment, NfL concentrations have been reported to increase 2 years before the appearance of cognitive decline in patients with HIV–dementia complex, suggesting a possible prognostic role for the marker (Gisslén et al., 2007; McLaurin et al., 2019).

Interestingly, NfL data have also been recently obtained in cerebrovascular disease forms that are occasionally associated with a rapid course, such as small vessel disease. Here, blood NfL concentrations have been associated with disability and neurological symptoms, including future cognitive impairment (Gattringer et al., 2017; Duering et al., 2018; Peters et al., 2020). Moreover, the marker correlated with disease severity, disease progression, and survival in CADASIL patients (Gravesteijn et al., 2018).

DISCUSSION AND CONCLUDING REMARKS

Here, we have provided an overview of currently available CSF and blood NfL data on RPDs. Evidence suggests the potential value of NfL as a first-step diagnostic test in the assessment of RPD patients to quickly screen for the presence of ongoing neuronal damage before performing more specific and/or expensive investigations such as RT-QuIC and/or MRI. Moreover, NfL measurement in biofluids might be useful

to track the disease trajectory in prion disease and other neurodegenerative dementias. In this regard, higher levels of CSF and/or blood protein seemed to predict a faster disease progression, higher grade of functional impairment, and/or shorter survival, which are all relevant variables for the stratification and management of patients in ongoing clinical trials. Furthermore, preliminary evidence suggests that blood NfL may have a role as a marker of proximity to clinical onset and, possibly, as the surrogate endpoint of response to ASOs in pre-symptomatic *PRNP* mutation carriers, which are currently the best candidates for new therapeutic options. While in cerebrovascular disease, the evidence of an association between NfL, disease severity, and/or outcome is growing, the role of the marker in AE and infectious encephalitis is not well-clarified, given that current data sets include small cohorts of heterogeneous etiologies and/or insufficient data. In the era of precision medicine, the imminent application of biofluid markers in the routine flow chart may significantly improve the diagnostic and prognostic assessment of patients with RPD.

AUTHOR CONTRIBUTIONS

SA-R and PP assembled all the data and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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frontiers in Neuroscience

MINI REVIEW
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Serum Neurofilament Light Chain Measurement in MS: Hurdles to Clinical Translation

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Measurement of serum neurofilament light chain concentration (sNfL) promises to become a convenient, cost effective and meaningful adjunct for multiple sclerosis (MS) prognostication as well as monitoring disease activity in response to treatment. Despite the remarkable progress and an ever-increasing literature supporting the potential role of sNfL in MS over the last 5 years, a number of hurdles remain before this test can be integrated into routine clinical practice. In this review we highlight these hurdles, broadly classified by concerns relating to clinical validity and analytical validity. After setting out an aspirational roadmap as to how many of these issues can be overcome, we conclude by sharing our vision of the current and future role of sNfL assays in MS clinical practice.

Keywords: multiple sclerosis, translation, neurofilament light, blood, biomarker

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INTRODUCTION

The spectrum of multiple sclerosis (MS) disease severity is broad, encompassing mild or even benign forms of the disease that may not require treatment at all (Sartori et al., 2017) to rapid progressors who accumulate irreversible worsening early-on unless drastic interventions are made (Atkins et al., 2016). Due to advancements in disease modifying therapies (DMT) for MS over the past two decades, a range of treatment options are now available, and "no evidence of disease activity" on clinical and MRI measures is a realistic treatment goal for many patients (Lublin, 2012). Following assessment of demographic, clinical and MRI features, patients identified as having more severe disease are increasingly treated with higher efficacy therapies up-front, with a lower threshold for treatment escalation upon disease breakthrough on less effective treatments (Giovannoni, 2018). However, a double-edged sword, the expanding range of higher efficacy immunosuppressive therapies is often accompanied by toxicity and iatrogenic morbidity. As such, neurologists aspire to closely titrate the minimal treatment intensity required to achieve disease control, and quickly react to breakthrough activity thereafter (Freedman et al., 2020). However, the current *status quo* of disease prognostication and monitoring in MS in routine clinical practice leaves much to be desired, lagging behind the therapeutic advancements. Clinical decision-making is still dependent on a synthesis of incomplete clinical and MRI information. Initial treatment selection remains a vaguely informed decision, based on our best assessment and patient preferences, but conflated by financial/insurance considerations and clinician preference. Once on a given therapy, subsequent escalation of therapy often lags behind the damaging disease activity that neurologists and their patients seek to prevent. Fluid biomarkers that conveniently and accurately

measure and track subclinical disease activity have been long sought to fill this knowledge and practice gap (Comabella and Montalban, 2014). After decades of searching, serum concentrations of neurofilament light chain (sNfL) have emerged over the past few years as a promising candidate.

Neurofilament light chain (NfL) is the most abundant of a family of highly conserved neuron-specific structural neurofilament proteins (Fuchs and Cleveland, 1998). Although our understanding of the physiology, pathophysiology and kinetics remains incomplete, it has been known for some time using conventional assays that CSF NfL is elevated in neurological conditions that cause neuroaxonal damage, such as MS (Eikelenboom et al., 2003). In the last 5 years, with the advent of ultrasensitive single molecule detection technologies, reliable blood measurements which correlate with CSF concentrations has become possible (Kuhle et al., 2016a; Wilson et al., 2016). Although concentrations in serum and plasma are closely related, they are not interchangeable; as the majority of current evidence relates to NfL concentrations in serum (sNfL), this is the focus of this review. Analogous to the cardiologist's troponin, the promise of a serum test of neuroaxonal damage has driven remarkable interest (Thebault et al., 2020b). Although sNfL has broader applicability, MS has become the test case for sNfL clinical utility due the unmet need for biomarkers and facilitated by extensive availability of specimens from retrospective cohorts (Thebault et al., 2021). In MS, sNfL is primarily a surrogates of inflammatory disease activity, the highest concentrations seen around the time of relapses and new MRI lesions, where sNfL trends up and then down for several months before and after (Kuhle et al., 2016b; Akgün et al., 2019). In pragmatic clinical settings outside of clinical trials, sNfL decreases broadly in line with demonstrated treatment efficacies (Bittner et al., 2020; Delcoigne et al., 2020). Furthermore, higher sNfL is predictive of poorer future clinical outcomes at every stage. NfL concentrations are elevated 6 years prior to the clinical onset of MS (Bjornevik et al., 2020). In clinically isolated syndromes, higher sNfL independently predicted faster conversion to clinically definite MS (Disanto et al., 2016). Following diagnosis, higher sNfL has been associated with short and long term poorer outcomes, including relapses, EDSS score progression including progression independent of relapse-activity, clinical conversion to a progressive phenotype, poorer cognitive measures as well as both MRI lesion activity and atrophy (Disanto et al., 2017; Barro et al., 2018; Chitnis et al., 2018; Siller et al., 2018; Cantó et al., 2019; Lorscheider and Benkert, 2020; Thebault et al., 2020a). Some experts consider this test to be on the cusp of widespread clinical adoption (Leppert and Kuhle, 2019), while others remain skeptical (Javed and Stankiewicz, 2020). As early adopters of sNfL testing in MS, in this review, we summarize what we see are key unknowns before the test could and should be deployed in routine clinical practice.

Hurdles to Widespread Clinical Translation of sNfL

Despite showing great promise, there are a number of issues relating to sNfL that must be either overcome or at least better appreciated before it can be considered part of the routine armamentarium of MS care (**Figure 1**). Challenges can be summarized in terms of analytical validity (test performance for sNfL) and clinical validity (sNfL performance as a surrogate of MS-related clinical outcomes of interest). Regulatory approvals of the test are contingent on both components being satisfactorily being met.

CLINICAL VALIDITY

sNfL is not specific for MS pathology. While there is an elegance to this, as both inflammatory and neurodegenerative activity in MS are summarized in a single marker, this means sNfL is not a diagnostic marker in MS. Unlike MRI, where new lesions have a characteristic appearance and can be specially correlated with clinical signs and symptoms, sNfL is agnostic of the underlying pathological process causing neuronal loss. Thus, if an individual patient's concentration is found to be high or rising, neurologists need to carefully consider confounders such as age and comorbidities (discussed in detail below) and may need to order additional testing such as MRI to clarify the situation.

Furthermore, there is significant overlap in sNfL in MS patients and healthy controls, even including cohorts of patients with the most aggressive forms of MS (Thebault et al., 2019). Owing to the relapsing remitting nature of the condition clinically in many, at any given time, the majority of MS patients might be expected to have similar sNfL to healthy controls (Barro et al., 2018). Concentrations frequently fall in an intermediate/gray zone. Thus, while the correlation between sNfL and important MS outcomes in cross-sectional studies is remarkable, interpretation of individual sNfL concentrations in clinical decision making remains challenging. This again highlights the need for careful consideration or even adjustment for the principal clinical confounders.

Age

Age is the principal physiologic determinant of sNfL. This is likely attributable to the cumulative effects of subclinical pathologies, such as white matter disease causing accelerated neuronal loss (Khalil et al., 2020). There is a moderate association between sNfL and age in both healthy controls and MS patients alike, with typical r values ranging from 0.6 to 0.7 (Khalil et al., 2020) and an increase in adult control sNfL levels of 2.2% per yer of age (Disanto et al., 2017; Barro et al., 2018). In healthy controls, there is an inflection around the age of 60, after which subsequent age-related sNfL increases accelerate, as does inter-individual variability within a given age cohort (Khalil et al., 2020).

There are two possible solutions to this problem. Firstly, patients could serve as their own baseline, using concentrations obtained during a stable period of remission as the comparator for subsequent serial measurements. This could not only account for age but also other commodities which we outline in the sections below. Using such a technique in a prospective observational cohort of 15 MS patients sampled during alemtuzumab treatment, one study found that sNfL "peaks" (>3 standard deviations above steady state concentrations) were

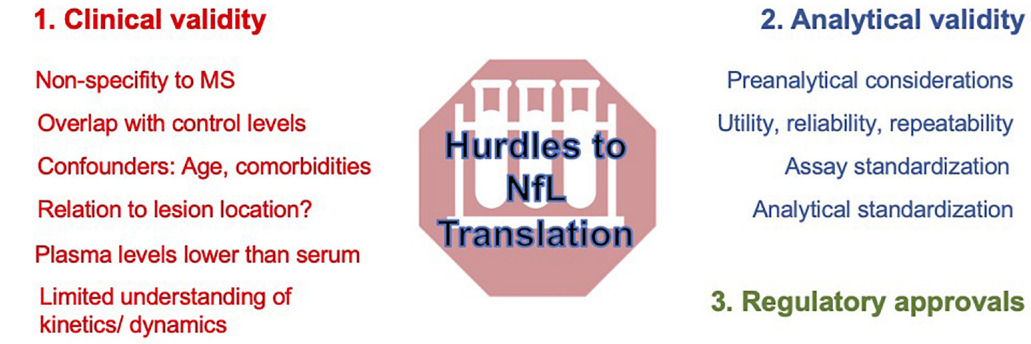


FIGURE 1 | Barriers to the clinical translation of sNfL in 2021.

associated with clinical and MRI activity in the majority of cases (Akgün et al., 2019). The downside to this approach is that it requires baseline measurement(s) during a preceding period of stability to serve as a subsequent longitudinal benchmark: this is contentious to define and difficult or impossible to obtain early on in the most active patients who would benefit the most from close monitoring. In this situation, a lack of reduction in sNfL following treatment initiation is itself meaningful (Huss et al., 2020) and could be used to guide escalation.

Alternatively, or perhaps in conjunction, others have adjusted for age by comparison to normative datasets from healthy controls. This approach is principally limited by the availability of large biobanks of healthy control sera required to generate such data. The Swiss group based in Basel has been particularly successful, initially presenting patient data in relation to percentiles of healthy control concentrations (Barro et al., 2018), but more recently and statistically rigorously as *z*-scores of log-normalized sNfL (Yaldizli et al., 2020). The availability of such normative datasets as well as ability of local laboratories to apply an age-adjustment factor is undetermined. Nonetheless, a relatively simple age adjustment which can be calculated for both a single measurement as well as serial measurement means is a significant step toward being able to use sNfL measurement to follow individual patents.

Confounding Effects of Other Neurological and Non-neurological Comorbidities

Extensively reviewed elsewhere (Khalil et al., 2018; Barro et al., 2020), higher sNfL is seen in many central and peripheral nervous system diseases that involve neuroaxonal injury including neurodegenerative conditions (Forgrave et al., 2019), stroke (Nielsen et al., 2020- plasma concentrations), and peripheral neuropathies (Altmann et al., 2020). Analogous to troponin in cardiac disease, clinical context is required. Fortunately, many alternate explanations of an elevated sNfL are usually clinically apparent or uncommon in the demographic of MS patients requiring active surveillance. More troubling however is the increase in sNfL seen following even mild traumatic brain injury. Here concentrations increase acutely, are predictive of the severity of injury, and remain elevated for several years after the injury (Shahim et al., 2020). High risk groups include military personnel (Boutté et al., 2019) and athletes (Shahim et al., 2018- plasma concentrations). In the context of MS and superimposed head injury, it may be difficult to attribute concentrations or dynamic changes to one pathology or the other.

Iatrogenic causes of sNfL elevation have also been identified. In a cohort of patients over 60 years old serially sampled after non-neurological surgeries requiring general anesthesia (mostly arthroscopies), concentrations increased by 67% and remained elevated beyond 48 h (Evered et al., 2018). An important consideration for MS patients is the possible effects of lumbar puncture: in Macaque monkeys, a lumbar puncture in the preceding 2–3 weeks increased median sNfL by 162% (Boehnke et al., 2020). Although lumbar punctures are generally infrequent events for most MS patients, much of our current understanding of sNfL is derived from intensively investigated cohorts of patients undergoing treatments, many of which underwent frequent lumbar puncture, a possible confounder of concern. Thus, appropriate timing of blood collection is exceedingly important for correct interpretation and can mitigate the potential for misinterpretation of results.

MS patients can also be at risk for other neurological complications that cause sNfL concentrations to rise. For instance, a 10-fold increase was noted at the time of onset of natalizumab-induced progressive multifocal leukoencephalopathy (Dalla Costa et al., 2019). In a cohort of patients undergoing ablative hemopoietic stem cell transplantation for aggressive MS, transient increases in the first year after the treatment reflected chemotherapeutic toxicity (Thebault et al., 2020c). Nonetheless, we feel that the identification of a rapidly rising NfL in these situations could be a useful warning signal to trigger a reassessment and additional investigations to identify the cause, or switch therapy.

Non-neurological conditions are also known to affect sNfL and need to be considered in any comorbid patient. BMI has been shown to have an effect on the sNfL, likely due to an increase in volume of distribution, where every 1 kg/m^2 rise in body mass index, sNfL decreases by 0.02 pg/ml (Manouchehrinia et al., 2020). Data from the stroke literature suggests that cardiovascular

risk factors including hypertension and poor glycemic control and perhaps renal function are also associated with higher sNfL (Korley et al., 2019). Similar to the proposed explanations for increased concentrations with age, these associations could be driven by comorbid but clinically silent white matter disease (Khalil et al., 2020). Renal function may also be important for NfL clearance as a cause for higher measurements in these patients (Akamine et al., 2020).

Other Physiologic Considerations

Our understanding of the pathophysiologic processes surrounding NfL release, distribution and metabolism are incomplete. This is illustrated by the correlation of serum and CSF concentrations, which typically has an *r*-value of 0.7–0.8 (e.g., Thebault et al., 2019). This equates to about 50% of sNfL being directly attributable to CSF NfL concentrations. Once NfL leaves axons and enters the extracellular space, it is not known what proportion is drained by lymphatic routes vs. direct drainage into CSF. Both individual and dynamic differences in these routes vary in a manner that could impact sNfL. In the CSF, there could be regional variation in NfL correlation, for example in the cul-de-sac of the lumbar cistern where CSF sampling occurs. Blood brain barrier permeability itself may be a confounder; NfL quotient in serum compared to CSF could be selectively increased following periods of inflammation such as that seen in MS relapse, positively skewing serum measures. However recent studies on this topic in MS patients present conflicting results (Kalm et al., 2017; Engel et al., 2020; Uher et al., 2020a). The diurnal timing of blood collection may also be an important consideration; in a study of 15 healthy males, one group found a more than 10% increase in plasma concentrations of NfL in the morning compared to the evening, although were surprised to find that elevation was not seen following acute sleep deprivation (Benedict et al., 2020). A hypothetical explanation for this diurnal variation proposed by the authors is that synaptic pruning in sleep may alter NfL kinetics.

Once NfL enters the blood, there are other physiologic considerations. One such possible confounder is existence of anti-NfL antibodies found in many MS patients (Silber et al., 2002). While the pathogenic potential of these antibodies is debatable, the presence or absence of these antibodies could alter peripheral NfL clearance.

Related to the physiologic kinetics of NfL distribution and clearance, the half-life of sNfL is a key consideration with implications on the frequency of disease activity monitoring. In a longitudinal study of NfL before and after intrathecal catheter insertion, NfL in both CSF and serum peaked at 1 month post-surgery, returning to baseline after 6–9 months (Bergman et al., 2016). In longitudinally sampled MS patients around the time of relapse, sNfL increased 5 months before, peaked at clinical onset, and recovered within 4–5 months (Akgün et al., 2019). In another observational cohort of 94 patients enrolled in the Comprehensive Longitudinal Investigation of Multiple Sclerosis at the Brigham and Women's Hospital (CLIMB) study, sNfL was elevated by one third in a 3 month window around gadolinium (Gd) enhancing lesions compared to remission samples (Rosso et al., 2020). Thus, while some possible individual influences of sNfL kinetics remain ill-defined, many groups are now selecting testing frequencies in the 3–6 month range for MS disease activity monitoring.

Possible Importance of MS Lesion Location

It is the authors' opinion that lesion location may be an important consideration in the interpretation of sNfL. To date, all studies have compared sNfL concentrations to total whole brain lesion volumes on MRI, and identified this to be one of the most consistent associations of sNfL. However, a large lesion in the right frontal lobe would likely result in a very significant elevation in sNfL conceivably with minimal appreciable disability. Conversely a small lesion affecting key brainstem structures may result in a smaller sNfL rise but significant long-term disability. Additionally, we speculate that other factors such as axon density in different brain and spine regions could be important determinants of the quantitively rise in sNfL in response to a given lesion.

ANALYTICAL VALIDITY

Preanalytical Considerations

Variations in sample acquisition, transport, processing and storage prior to protein quantification are important preanalytical confounders for many blood biomarkers. Although serum and plasma neurofilament levels are very strongly correlated ($r = 0.96$, Sejbaek et al., 2019), plasma concentrations are around 25% lower than paired serum concentrations, highlighting the need to standardize blood measurements to a single specimen type. For this reason in this review we have chosen to focus on serum as the more prevalent and studied blood biofluid to promote comparability and utility. Otherwise, sNfL has shown good stability over multiple freeze-thaw cycles and prolonged exposure to room temperature (e.g., Hviid et al., 2020, reviewed by Table 1 in Barro et al., 2020).

Assay Standardization

Much of the focus on sNfL in recent years is directly attributable to development of a clinical immunoassay platform capable of detecting the low concentrations in blood. The Single Molecule Array (SiMoA) has transformed NfL from a CSF-only research-marker of merit to its current status on the verge of clinical translation in blood (Kuhle et al., 2016a). Comparison of traditional ELISA with electrochemiluminescence and SiMoA demonstrated the superiority of SiMoA with an analytical sensitivity of 0.62 pg/mL compared with 15.6 pg/mL electrochemiluminescence and 78.0 pg/mL for enzyme linked immunosorbent assay (Kuhle et al., 2016a). This increased sensitivity of SiMoA is able to detect sNfL in 100% of healthy individuals. The SiMoA assay uses a unique ELISA method of detecting very low concentration analytes (Rissin et al., 2010). Briefly, antibodies are linked to a solid surface as in a traditional ELISA, however the SiMoA assay utilizes microbeads

2.7 μm in size that individually fit into a microwell array. When measuring very low concentration analytes (subfemtomolar concentrations), the antigen-bead ratio is approximately 1:1 and follow a Poisson distribution. This distribution suggests that beads carry either a single immunocomplex or none, and with very low analyte concentrations only 1–2% of beads carry an immunocomplex. Detection of such low concentrations is not possible through routine enzymatic methods. To accomplish detection, each individual bead is loaded into a single microwell which can be "digitally" counted. Detection is through fluorescent labeling of immunocomplexes which is sensitive enough to measure a single immunocomplex on a single bead. In this way, the number of beads are counted and quantitated against a standard curve, allowing extremely low analyte concentrations to be reliably measured.

Use of the SiMoA assay has facilitated the measurement of NfL in blood and allowed much of the research in MS. The initial NfL assay developed for the SiMoA assay used a home-brew method developed by the Basel group (Kuhle et al., 2016a). They used monoclonal NfL antibodies developed by Umam Diagnostics (47:3 and 2:1, subsequently purchased by Quanterix) along with bovine NfL calibrators. The majority of the early studies were completed using the home-brew assay. More recent studies use the commercially available Quanterix NF-light™ assay kit which uses recombinant human (rhuman) NfL calibrators. It is important for investigators and clinicians to recognize which assay has been used, as that there is a significant positive bias (5:1) of the home-brew assay relative to the commercial NF-light™ assay (Hendricks et al., 2019).

These assay differences highlights the need for assay standardization, and the role of multi-site validation to inform reproducibility and create standardization protocols. In one such international validation effort of the Quanterix NF-light™ assay that sought to assess a variety of analytical outcomes including instrument qualification, precision, level of detection and level of quantification, parallelism and proficiency, the assay performed well across 17 sites with intra- and inter-assay coefficients varying less than 6 and 9%, respectively (Kuhle et al., 2018). However, as interest in this biomarker increases, several other assay platforms are now showing near-equivalent dynamic range and level of detectability. While this competition will drive down setup and testing costs to increase availability, careful work will be needed to confirm inter-platform equivalence.

Data Analysis and Clinical Reporting

Of the published data available, there is significant variation in data analysis methodologies, limiting inter-study comparability or subsequent meta-analyses. Groups have variably reported measures of central tendency with the mean, median, and geomean. Subsequently reported statistics have included a mixture of parametric and non-parametric techniques, sometimes inappropriately deployed. In our experience, sNfL concentrations are distributed logarithmically. Therefore, comparisons of raw conentrations are constrained to non-parametric techniques, whereas more powerful parametric statistics are possible following Ln/log transformation. Preliminary data from the Basel group (Yaldizli et al., 2020) takes this one step further as they generated age-adjusted z-scores of log transformed data. Although use of *z*-scores may be the most appropriate technique for dealing with age-related increases in sNfL, it may pose challenges for reporting from clinical diagnostic laboratories. Similarly, future data analysis challenges are to determine the most meaningful and clinically deployable measure of sNfL change when trending values. Currently, it is unclear if the raw number alone, reported with reference to an age adjusted population, is important or if an absolute or relative increase is most clinically relevant. Regardless, with every statistical manipulation beyond simple reporting of raw values and cut-offs, may cause implementation hurdles in the clinical diagnostic laboratory and may become practically challenging in a real-world clinical setting.

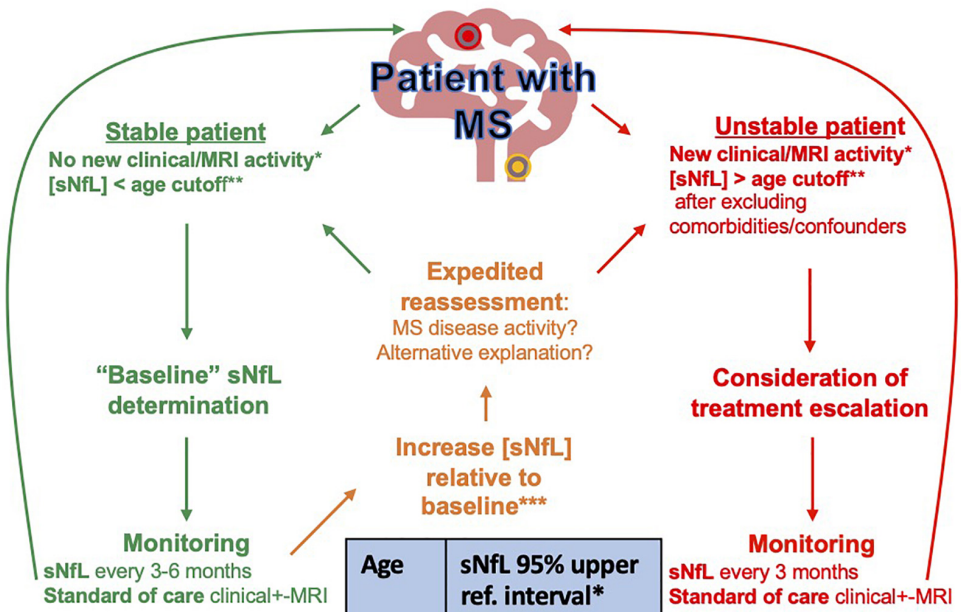
A CURRENT ROLE FOR sNfL IN MS?

We already know that as an adjunctive measure in MS, high or increasing concentrations of sNfL are associated with relapses, EDSS worsening, lesions on MRI scans and atrophy of both the brain and spinal cord. Conversely, serially low sNfL is reassuring. Yet many see the limitations and unknowns relating to the precise interpretation of individual sNfL concentrations so problematic that the marker is "not ready for prime time" (Javed and Stankiewicz, 2020). However, it is the authors' opinion that the clinical translation of sNfL need not be so black or white. To demand stringent criteria for clinical translation not only ignores the rapidly accumulating body of evidence that already indicates utility for the marker but also seems like a double standard. Neurologists have long tried to use the accurate, often machine generated changes on serial MRI scanning in clinical trials to estimate disease change in their MS patients, only to be challenged with inaccuracies in real life (as opposed to carefully regimented clinical trial MRI studies) due to malalignment, different MRI sequences, different scanners or simply differences in the quality of imaging. Despite this, MRI has become the gold standard non-clinical means for measuring disease in MS. This has not stopped clinicians from making interpretations from serial scans to inform on treatment decisions.

It is our opinion that with a good appreciation of the shortcomings and pitfalls, individual patient sNfL concentrations are already a helpful adjunct to clinical practice. In **Figure 2** we propose how sNfL can be incorporated into clinical practice as it currently stands: an imperfect marker, that should never be interpreted in isolation. We find it a helpful adjunctive tool and a useful trigger for expedited reassessment when unexpectedly high or rising. Better age dependent based on parametric *z*-score cutoffs (rather than non-parametric percentile cutoffs) are imminently and eagerly awaited. As concerns of clinical validity are better understood if not accounted for and the analytical validity is further established, we hope this marker will be further incorporated into the standard of care. Enabled by less constraining approval processes for clinical use in some jurisdictions, some centers such as our own are already using this test routinely in the MS clinic.

sNfL, A FUTURE STANDARD OF MS CARE?

Analogous to the implementation of MRI in routine disease activity monitoring in MS, neurologists will require some education on how to correctly interpret sNfL and incorporate it into routine clinical practice. Similar to MRI, using sNfL may require the establishment of a "baseline" from whence future changes can be referenced and size changes be interpreted. The establishment of better of age-adjusted normative datasets (reference intervals) and biological variation (reference change values) will be a vital step in further individualizing sNfL group level associations. As with many new technologies, the cost of NfL testing itself remains high; competition will help reduce these costs but also present new issues relating to inter-assay comparability (**Figure 3**).



Age	sNfL 95% upper ref. interval*
18-40	9.7 pg/mL
41-65	21.4 pg/mL
>65	53.8 pg/mL

FIGURE 2 | Proposed algorithm for NfL monitoring in MS. *Clinical or MRI disease activity: New relapses, EDSS worsening, New/enlarging MRI lesion. **sNfL 95% age-dependent upper reference interval calculated on SiMOA HD1 instrument using Quanterix NF-light™ (Hviid et al., 2020). ***The increase in sNfL from baseline that best denotes impending disease activity that should prompt further action is still to be determined. Preliminary data from 58 patients with MS followed every 3 months over 1 year suggests that a doubling of sNfL from baseline is associated with a 2.2 × relative risk of relapse (Thebault, Unpublished observation).

FIGURE 3 | Aspirational predictions of sNfL in the next 5–10 years.

sNfL in patients with early-stage disease could be incorporated into prognostic models and aid initial treatment selection. Serial measurements, for instance every 3 months, could be useful to monitor for subclinical disease activity both on or off treatment. Here, increasing sNfL would be an objective trigger for neurologists to consider expedited clinical and MRI reassessment, and serially low or stable sNfL would be reassuring (Uher et al., 2020b). There is already substantial evidence for sNfL to be included in future definitions of "no evidence of disease activity." Thus, through more refined initial treatment selection and closer disease activity monitoring, we think sNfL could have the power to modify the trajectory of MS for the better and improve outcomes. Furthermore, sNfL could reduce current costs by optimizing utilization of MRI, where annual scans for all clinically stable patients is not only expensive but also unfeasible in many settings and could be perhaps better targeted to patients with high or rising sNfL. While the role of sNfL as a clinically useful marker in progressive MS is less clear, this remains a key area of need where clinical responsiveness can be more difficult to quantify. Finally, the potential of sNfL may be augmented by the inclusion of additional markers into combinatorial metrics. While sNfL represents an important first step in a biomarker-driven personalization of MS care, it certainly will not be the last.

AUTHOR CONTRIBUTIONS

ST conceived the study, performed the literature review drafted, and edited the manuscript. RB, CR, and HM drafted the manuscript. MF conceived the study, drafted, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Cerebrospinal Fluid Biomarkers of Myeloid and Glial Cell Activation Are Correlated With Multiple Sclerosis Lesional Inflammatory Activity

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Multiple sclerosis (MS)-related inflammation can be divided into lesional activity, mediated by immune cells migrating from the periphery to the central nervous system (CNS) and non-lesional activity, mediated by inflammation compartmentalized to CNS tissue. Lesional inflammatory activity, reflected by contrast-enhancing lesions (CELs) on the magnetic resonance imaging (MRI), is effectively inhibited by current disease modifying therapies (DMTs). While, the effect of DMTs on non-lesional inflammatory activity is currently unknown. Reliable and simultaneous measurements of both lesional and non-lesional MS activity is necessary to understand their contribution to CNS tissue destruction in individual patients. We previously demonstrated that CNS compartmentalized inflammation can be measured by combined quantification of cerebrospinal fluid (CSF) immune cells and cell-specific soluble markers. The goal of this study is to develop and validate a CSF-biomarker-based molecular surrogate of MS lesional activity. The training cohort was dichotomized into active (CELs $>$ 1 or clinical relapse) and inactive lesional activity (no CELs or relapse) groups. Matched CSF and serum samples were analyzed for 20 inflammatory and axonal damage biomarkers in a blinded fashion. Only the findings from the training cohort with less than 0.1% probability of false positive (i.e., $p < 0.001$) were validated in an independent validation cohort. MS patients with lesional activity have elevated IL-12p40, CHI3L1, TNFα, TNFβ, and IL-10, with the first two having the strongest effects and validated statistically-significant association with lesional activity in an independent validation cohort. Marker of axonal damage, neurofilament light (NfL), measured in CSF (cNfL) was also significantly elevated in MS patients with active lesions. NfL measured in serum (sNfL) did not differentiate the two MS subgroups with pre-determined significance, ($p = 0.0690$) even though cCSF and sNfL correlated (Rho = 0.66, $p < 0.0001$). Finally, the additive model of IL12p40 and CHI3L1 outperforms any biomarker discretely. IL12p40 and CHI3L1, released predominantly by immune cells of myeloid lineage are reproducibly the best CSF biomarkers of MS lesional activity. The residuals from the IL12p40/CHI3L1-cNfL correlations may identify MS patients with more destructive inflammation or contributing neurodegeneration.

Keywords: multiple sclerosis, cerebrospinal fluid biomarkers, contrast-enhancing lesions, lesional inflammatory activity, axonal damage

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INTRODUCTION

The multiple sclerosis (MS) lesional inflammatory activity is associated with blood brain barrier (BBB) breakdown and transmigration of immune cells from periphery to central nervous system (CNS) (Lassmann, 2018). The lesional inflammatory activity can be measured by contrast-enhancing lesions (CELs) on brain magnetic resonance imaging (MRI). The measurement of lesional activity via CELs is non-invasive and convenient, and thus utilized as an outcome in most phase II trials of immunomodulatory treatments for MS (Sormani and Bruzzi, 2013; Filippi et al., 2019). However, the broad use of CELs in the MS field led to a false generalization that CELs represent all MS-related inflammatory activities in the CNS. CELs only reflect the perivascular inflammation associated with the opening of the BBB and influx of immune cells from blood to form MS lesion. We call this "lesional" inflammation. But there are other inflammatory processes in MS that are not captured by CELs, such as formation of cortical lesions not typically associated with BBB opening and more diffuse inflammation compartmentalized to CNS tissue, often seen in progressive MS. We call this "non-lesional" inflammation (Frischer et al., 2009; Androdias et al., 2010; Komori et al., 2015; Milstein et al., 2019).

The current disease modifying therapies (DMTs) effectively inhibit formation of focal lesions (Buron et al., 2020), but their effect on non-lesional inflammatory activities is unknown. Understanding contributions of lesional versus non-lesional MS inflammatory activity to CNS tissue destruction requires reliable and simultaneous measurements of both processes in the same patients. While measuring lesional activity via CELs using MRI is non-invasive and convenient, there are limitations to this measurement: (a) Most often, only brain CELs are measured, but inflammation and BBB breakdown occurs in the spinal cord too (Moccia et al., 2019); (b) Most common CEL measurement is only semi-quantitative, representing the number of unique CELs, but not their volume; and (c) CEL detection is also dependent on the dose of the contrast administered and the length of time that elapsed between contrast administration and the image acquisition. CSF biomarkers can't compete with the convenience of repeated MRIs for monitoring MS lesional activity; however, they can be used in research settings or in diagnostic lumbar puncture (LP) to measure contributions of lesional versus non-lesional MS inflammation to axonal damage in individual patients.

We have previously devised methodology that allows measurement of CNS compartmentalized inflammation in living human subjects without a need for CNS tissue biopsy, using combination of CSF cellular, and molecular biomarkers (Komori et al., 2015). This method relies on soluble biomarkers that are exclusively, or predominantly released from a specific immune cell type, such as sCD27 (mostly released by T cells, CD8 > CD4), sCD21 (released by B cells, naive > memory) and sCD14 (released by monocytes and possibly microglia). The ratio of these biomarkers to the cells of their origin (measured in CSF via flow cytometry) in healthy volunteers who have no compartmentalized inflammation, represents release of these cell-specific markers by CSF immune cells. If there is excess of cell-specific biomarkers in comparison to the CSF cells of their origin, then there must be other sources of immune cells, that are not in the CSF, but can release their biomarkers into CSF. Brain pathology demonstrated that these additional cells are in CNS tissue, representing compartmentalized inflammation, or non-lesional MS inflammatory activity.

Thus, the next step is to analyze multiple candidate CSF biomarkers of MS lesional activity to determine which of them is most accurate and whether they can be combined into a model that outperforms the single best CSF biomarker. We then thought to assess the strength of correlation between the winning CSF surrogate of MS lesional activity and neurofilament light chain (NfL), a validated marker of axonal damage (Norgren et al., 2004; Teunissen and Khalil, 2012; Gaetani et al., 2019; Alirezaei et al., 2020). Such CSF-biomarker-based differentiation of lesional and non-lesional inflammation from the identical CSF samples will allow for assessing the contribution of these two processes to CNS tissue destruction in MS.

Neurofilaments are important cytoskeletal proteins of axons; in contrast to other cytoskeletal proteins neurofilaments are specific to neurons. Damage of axons during various neurodegenerative diseases leads to releases of neurofilaments into interstitial fluid and finally into CSF and blood. As NfL has the lowest molecular weight of the three neurofilament subunits (light, medium, and heavy), it diffuses more easily from parenchyma to CSF after axonal damage or neuronal death (Fuchs and Cleveland, 1998; Scherling et al., 2014; Alirezaei et al., 2020). Thus, NfL is a reliable biomarker of axonal damage.

NfL concentration in CSF is approximately 100-fold higher than in the blood. But with advent of highly sensitive assays (e.g., single molecular array, SIMOA) (Rissin et al., 2010; Kan et al., 2012). NfL concentration in blood also can be measured reliably. As collection of blood is easier and less invasive compared to CSF, blood NfL is being commonly examined as a biomarker for axonal damage in neurological diseases. Thus, to measure the relationship between lesional MS activity and axonal damage, we measured NfL concentrations both in CSF (cNfL) and blood/serum (sNfL).

MATERIALS AND METHODS

Research Subjects

Subjects were prospectively recruited to the NIH Institutional Review Board (IRB)-approved protocol "Comprehensive Multimodal Analysis of Neuroimmunological Diseases of the CNS" (ClinicalTrials.gov Identifier: NCT00794352), between 07/2003 to 05/2019.

The inclusion criteria for healthy donors (HD): (1) at least 18 years old at time of enrollment, (2) vital signs within normal range at time of screening visit, and (3) able to give informed consent and undergo required research procedures. The exclusion criteria for HD: (1) previous or current history of alcohol or substance abuse, (2) inflammatory

or non-inflammatory neurological diseases, (3) medical contraindications with required research procedures, and (4) pregnancy or current breastfeeding.

The inclusion criteria for patients: (1) at least 12 years old at time of enrollment, (2) presentation with a clinical syndrome consistent with immune-mediated CNS disorder and/or neuroimaging evidence of inflammatory and/or demyelinating CNS disease, (3) adults able to give informed consent on their own or via legally authorized representative and minors willing to assent and able to give informed consent via parents or legal guardian, and (4) able to undergo required research procedures. The exclusion criteria for patients: (1) significant medical conditions that would make participation in diagnostic or research part of evaluation impossible or risky, (2) unable or unwilling to give informed consent, and (3) medical contraindications with required research procedures.

All subjects underwent neurological examination to derive the measures of neurological disability Expanded Disability Status Scale (EDSS) (Kurtzke, 1983). Composite MRI scale of CNS tissue destruction (COMRIS-CTD) was calculated from 3T brain MRI images as described (Kosa et al., 2015). MS diagnostic subgroups (relapsing remitting MS [RR-MS], secondary progressive MS [SP-MS] and primary progressive MS [PP-MS]) were classified using McDonald's criteria, 2010 and 2017 revisions (Polman et al., 2011; Thompson et al., 2018). MS patients were divided into two subgroups based on presence or absence of lesional inflammatory activity determined by recognition of CELs on MRI using clinical-grade structural MRI images of the brain collected under a published protocol (Kosa et al., 2015). The CELs were recognized on co-registered images as hyperintense on T2WI/FLAIR, hypo-or iso-intense on T1WI and hyperintense on post-contrast T1WI (3D-GRE or 3D-FSPGE-BRAVO).

The findings from the training cohort (70 MS patients) that had less than 0.1% probability of false positive (i.e., unadjusted $p < 0.001$) were then validated in an independent validation cohort (130 MS patients); **Table 1** represents demographic details of subjects from both cohorts.

CSF and Blood Sample Collection and Processing

CSF and blood samples were collected according to standard operating procedures as described (Masvekar et al., 2019). CSF samples were collected by LP and stored on ice until further processing. Research CSF aliquots were centrifuged at 1,200 rpm for 10 min at 4°C to pellet out cells. Then cell-free CSF supernatants were aliquoted (0.5 ml/vial) and stored at −80°C until further use. Blood samples were collected in serum separation tubes (SST^{TM}, BD Vacutainer^{TM}, Thermo Fisher Scientific, Waltham, MA, United States) and centrifuged at 3000 rpm for 10 min at 4°C. Then serum supernatants were aliquoted (0.5 ml/vial) and stored at −80°C until further use. Frozen CSF and serum samples were thawed on ice and used for biomarker analyses; repeated freezing and thawing of biological samples was avoided.

Biomarker Analyses

CSF and serum samples were analyzed using single molecule arrays (Simoa^{TM}) assay kits (Quanterix^{TM}, Billerica, MA, United States) or spectrophotometric ELISA kits (UmanDiagnostics, Umea, Sweden) or homebrew electrochemiluminescence (ECL) ELISAs using the Meso Scale Discovery detection system (MSD; Rockville, MD, United States) (**Table 2**).

Assay kits were used as per manufacturer's instructions. For homebrew assay development, we used a published protocol (Komori et al., 2015). Briefly, MSD bare plates (MULTI-ARRAY 96-Well Plate; Catalog# L15XA-3) were coated with working concentrations of capture antibody overnight at 4°C. Coating solution was aspirated, plates were washed with phosphate buffer saline (PBS)-Tween 20 (PBS-T) and then incubated with 1% bovine serum albumin (BSA) in PBS for 2 h at room temperature. After washing, working dilutions of standards and samples were added to the plate and incubated for 2 h at room temperature. Plates were washed and incubated with working concentrations of biotinylated-detection antibody for 2 h at room temperature. After washing, plates were incubated with 0.25 μg/ml of SULFO-TAG^{TM} streptavidin (MSD, Catalog# R32AD-1) in 1% BSA/PBS for 1 h at room temperature. Finally, plates were washed and added 2X Read Buffer (MSD, Catalog# R92TC-1), and then ECL was analyzed using QuickPlex SQ 120 (MSD).

All samples were analyzed in blinded fashion and in duplicates, results were accepted only when coefficient of variance (CV) across the sample duplicates was <20%. Samples were analyzed on multiple plates, location of samples on each plate were randomized. On each plate a control sample was analyzed in duplicate; The CV for the control sample across the plates is <20%, confirming the assay precision and reproducibly.

Adjustment for Effect of Healthy Aging

Some biomarkers analyzed in this study are known to change with age in healthy subjects. Specifically, cNfL (Vågberg et al., 2015), sNfL (Disanto et al., 2017) and CHI3L1 (Bonneh-Barkay et al., 2010) have shown to be correlated with age within healthy subjects. As in this study, age of MS subjects across lesional activity subgroups is significantly different (**Table 1**; inactive versus active), it is essential to adjust for the effect of healthy aging. To derive adjustment equations, we pooled all cNfL, sNfL, and CHI3L1 HD subjects' data available in our research database (**Table 3** and **Supplementary Data File 1**). As described previously (Barbour et al., 2020), linear regression models for the logarithmic value of biomarker concentrations with age as an independent variable were used to predict the healthy, age-related levels of these biomarkers for all MS patients according to their age at time of sample collection; Then to exclude the effect of healthy aging, these predicted biomarker levels due to healthy aging were subtracted from true, measured biomarker levels.

Data Transformation and Statistical Analyses

Data were organized using Microsoft Excel (Microsoft, Redmond, WA, United States); all biomarker concentration

TABLE 1 | Demographic details of training and validation cohorts. Across MS lesional activity subgroups (inactive versus active) categorical variables (gender and MS disease type distributions) were compared using Chi-square test (*) and continuous variables (age, disease duration, and EDSS) were compared using *t*-test (#).

				MS Lesional Activity		
			HD	Inactive	Active	*p*
Training cohort	N		5	35	35	
	Gender	(Female/male)	4/1	16/19	22/13	0.1500*
	MS type	(RR-MS/SP-MS/PP-MS)		7/15/13	28/7/0	<0.0001*
	Age	Mean (SD)	47.5 (14.4)	52.8 (11.3)	37.6 (10.8)	<0.0001#
	Disease duration	Mean (SD)		13.7 (10.8)	5.1 (7.4)	0.0002#
	EDSS	Mean (SD)		5.1 (1.9)	2.7 (2.1)	<0.0001#
Validation cohort	N		13	96	34	
	Gender	(Female/male)	6/7	50/46	19/15	0.7029*
	MS type	(RR-MS/SP-MS/PP-MS)		42/26/28	24/5/5	0.0267*
	Age	Mean (SD)	37.5 (10.8)	49.4 (10.4)	41.0 (13.2)	0.0003#
	Disease duration	Mean (SD)		12.5 (9.5)	6.3 (8.6)	0.0009#
	EDSS	Mean (SD)		3.7 (2.2)	2.7 (2.1)	0.0229#

TABLE 2 | List of kits and antibodies used for biomarker analyses using either Simoa (Quanterix) or spectrophotometric or homebrew MSD-ECL ELISAs; working concentrations or dilutions of coating and detection antibodies for homebrew assays and lower limit of detection (LLoD) of all assays is provided here. IL-12p40 in training (*) and validation (#) cohort was analyzed using Simoa and MSD-ECL ELISA, respectively.

Biomarkers	ELISA Type	Kit or antibodies source	Working concentrations/dilutions of coating and detection antibodies	LLoD
TNFα	Simoa™	Quanterix (101580)		0.016 pg/ml
IL-1β	Simoa™	Quanterix (101605)		0.016 pg/ml
TNFβ	Simoa™	Quanterix (102091)		0.052 pg/ml
LIF	Simoa™	Quanterix (102394)		0.015 pg/ml
TRAIL	Simoa™	Quanterix (100906)		0.0083 pg/ml
GM-CSF	Simoa™	Quanterix (102329)		0.0019 pg/ml
IL-10	Simoa™	Quanterix (101643)		0.0038 pg/ml
TGFβ	Simoa™	Quanterix (101984)		0.137 pg/ml
IL-17F	Simoa™	Quanterix (102082)		1.08 pg/ml
IL-12p40*	Simoa™	Quanterix (101871)		0.02 pg/ml
IL-12p40#	MSD-ECL	Mesoscale Diagnostics (K15050D)		4.6 pg/ml
sNfL	Simoa™	Quanterix (103186)		0.038 pg/ml
cNfL	Spectrophotometric	UmanDiagnostics (10-7002)		100 pg/ml
SERPINA3	Homebrew MSD-ECL	R&D Systems (MAB1295 and BAF1295)	1 μg/ml and 250 ng/ml	125 ng/ml
CXCL13	Homebrew MSD-ECL	R&D Systems (DY801)	1 μg/ml and 100 ng/ml	17.4 pg/ml
CD27	Homebrew MSD-ECL	Sanquin (M1960)	1:100 and 1:100	0.4 U/ml
CD14	Homebrew MSD-ECL	R&D Systems (DY383)	2 μg/ml and 100 ng/ml	0.49 ng/ml
BAFF	Homebrew MSD-ECL	R&D Systems (DY124-05)	500 ng/ml and 50 ng/ml	7 pg/ml
CD21	Homebrew MSD-ECL	R&D Systems (DY4909-05)	250 ng/ml and 100 ng/ml	5.8 pg/ml
BCMA	Homebrew MSD-ECL	R&D Systems (DY193)	400 ng/ml and 400 ng/ml	7 pg/ml
CHI3L1	Homebrew MSD-ECL	R&D Systems (DY2599)	4 μg/ml and 100 ng/ml	41 pg/ml

data for both cohorts (training and validation) is provided in **Supplementary Data File 1**. All biomarkers were compared across MS lesional activity subgroups (inactive versus active) using unpaired *t*-test.

Correlations between biomarkers and number of CELs were evaluated using Spearman coefficient, and multiple linear regression analyses. For normality assumption of regression analysis, natural logarithm transformation was applied to some biomarkers (cNfL, sNfL, IL-12p40, CHI3L1, and CXCL13). As concentrations of cNfL, sNfL, and CHI3L1 were adjusted for effect of healthy aging some patients may have negative adjusted values; So, before applying natural logarithm transformation to age-adjusted concentrations these values were mathematically transformed to positive by adding "minimum value +1."

Principal component analysis was performed using the biomarkers showing significant correlation with number of CELs. The first component (a linear combination of the biomarkers) was used to predict the lesional activity.

TABLE 3 | Demographic details of healthy cohort, used to derive linear regression models for the biomarker concentrations (cNfL, sNfL, and CHI3L1) with age as an independent variable.

		cNfL	sNfL	CHI3L1
N		78	26	68
Gender	(Female/male)	39/39	14/12	37/31
Age	Mean (SD)	40.8 (12.6)	38.9 (15.7)	41.3 (12.8)
	Range	19.4–71.3	19.4–71.3	19.4–71.3

GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, United States) and SAS 9.4 (SAS Institute Inc.) were used for the data analyses.

RESULTS

Effect of Healthy Aging on Biomarker Concentrations

Out of 20 biomarkers analyzed, only 3 biomarkers showed statistically significant correlations with age in HD subjects' data available in our research database: cNfL (n = 78, R^2 = 0.44, 95% CI = 0.018–0.031, and $p < 0.0001$), sNfL (n = 26, R^2 = 0.52, 95% CI = 0.010–0.025, and $p < 0.0001$) and CHI3L1 (n = 68, R^2 = 0.22, 95% CI = 0.009–0.025, and $p < 0.0001$) (**Figure 1**).

Linear regression models of cNfL (ln[cNfL] = 0.0243*Age + 5.2043), sNfL (ln[sNfL] = 0.0177*Age + 0.9696) and CHI3L1 (ln[CHI3L1] = 0.0172*Age + 3.8088) with age as an independent variable were used to predict the healthy, age-related levels of these biomarkers for all MS patients according to their age at time of sample collection.

Analysis of Proinflammatory Biomarkers Across MS Lesional Activity Subgroups

Biomarkers were analyzed in subjects' CSF samples in blinded manner. After unblinding categorization of MS patients into active versus inactive groups, TNFα, TNFβ, IL-10, IL-12p40, and CHI3L1 (unadjusted p = 0.0096, 0.0032, 0.0072, 0.0004, and <0.0001, respectively) were significantly elevated in CSF of MS patients with active lesional activity (CELs > 1 or in clinical relapse) compared to patients with inactive lesional activity (CELs = 0 or not in clinical relapse). While, TGFβ (unadjusted p = 0.0050) was significantly downregulated in active MS subjects (**Table 4** and **Figure 2**).

In the training cohort, MS disease type (RR-MS and P-MS, representing both primary[PP-MS]- and secondary [SP-MS]-progressive MS) distribution across lesional activity subgroups (active vs inactive) is significantly different (**Table 1**; Chi-square test, $p < 0.0001$). Thus, significantly ($p < 0.01$) different biomarkers within lesional activity subgroups were compared across MS disease type (RR-MS vs P-MS) within respective lesional activity subgroups (active and inactive) using unpaired t-test (**Supplementary Figure 1**). None of these biomarkers were statistically significantly ($p < 0.05$) different between MS disease types within respective lesional activity subgroup. These findings suggest that observed variability in biomarkers across lesional activity subgroups (active vs inactive) is not driven by variability in MS disease type distribution.

Analysis of Axonal Damage Biomarkers Across MS Lesional Activity Subgroups

NfL a marker of axonal damage (Norgren et al., 2004; Teunissen and Khalil, 2012; Gaetani et al., 2019; Alirezaei et al., 2020), was analyzed in patients' CSF and serum samples, and then compared across MS lesional activity subgroups. In MS patients with active lesional activity CSF NfL (cNfL) level was significantly elevated compared to patients without lesional inflammatory activity (unadjusted p = 0.0043; **Table 4** and **Figure 3A**). Though there is a strong correlation between NfL levels in serum and CSF (Spearman Rho = 0.60, R^2 = 0.34 and $p < 0.0001$; **Figure 3B** and **Supplementary Table 1**), sNfL is not statistically significantly different between active and inactive MS subgroups (unadjusted p = 0.0690; **Table 4**, and **Figure 3A**).

Correlations between cNfL and other biomarkers were analyzed using Spearman analysis; for statistically significantly ($p < 0.01$) correlated biomarkers linear regression with cNfL as a dependent variable was analyzed: sNfL (Rho = 0.60, R^2 = 0.34, and $p < 0.0001$), TNFα (Rho = 0.46, R^2 = 0.06, and p = 0.0493), IL-12p40 (Rho = 0.55, R^2 = 0.24, and $p < 0.0001$) and CHI3L1 (Rho = 0.59, R^2 = 0.25, and $p < 0.0001$) (**Figure 3B** and **Supplementary Table 1**).

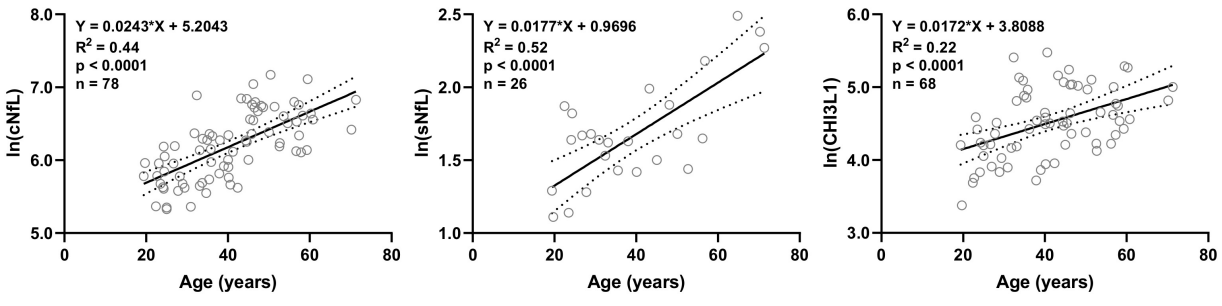

FIGURE 1 | In HD subjects' research database, linear regression of CSF and serum NfL (cNfL and sNfL) and CHI3L1 with age as an independent variable was analyzed; All three biomarkers were significantly (R^2 = 0.44, 0.52 and 0.22, respectively, and $p < 0.0001$) correlated with age.

TABLE 4 | In the training cohort, biomarker concentrations across HD (n = 5) and MS lesional activity subgroups (Inactive and Active, n = 35 each subgroup) are represented as median (range).

			MS Lesional Activity		
Biomarkers	**Unit**	**HD**	**Inactive**	**Active**	***p***
TNFα	pg/ml	0.09 (0.08–0.14)	0.14 (0.06–0.40)	0.22 (0.06–0.78)	0.0096
IL-1β	pg/ml	0.05 (0.03–0.05)	0.05 (0.03–0.12)	0.56 (0.02–0.19)	0.2925
TNFβ	pg/ml	0.15 (0.06–0.29)	0.12 (0.02–0.30)	0.21 (0.01–2.10)	0.0032
LIF	pg/ml	0.02 (0.02–0.03)	0.02 (0.00–0.07)	0.02 (0.00–0.11)	0.6073
TRAIL	pg/ml	0.59 (0.38–0.90)	0.39 (0.19–0.93)	0.44 (0.22–0.74)	0.6025
GM-CSF	pg/ml	0.03 (0.02–0.05)	0.03 (0.01–0.07)	0.03 (0.01–0.036)	0.0234
IL-10	pg/ml	0.08 (0.05–0.10)	0.16 (0.04–0.93)	0.27 (0.07–1.69)	0.0072
TGFβ	pg/ml	5.58 (5.12–7.19)	6.05 (3.72–11.63)	5.12 (0.93–8.37)	0.0050
IL-17F	pg/ml	1.34 (1.27–2.32)	1.10 (0.18–25.45)	0.94 (0.17–3.17)	0.233
ILIL-12p40	pg/ml	2.74 (1.53–3.78)	3.45 (1.86–21.20)	12.64 (3.93–147.43)	0.0004
sNfL	pg/ml	0.62 (−2.67–4.28)	8.48 (0.33–33.62)	12.37 (1.71–432.25)	0.0690
cNfL	pg/ml	−17.1 (−166.2–158.5)	248.3 (−212.0–24895.0)	3917.4 (179.5–29799.9)	0.0043
SERPINA3	ng/ml	134.0 (113.6–240.4)	364.8 (9.3–1741.4)	470.7 (11.1–1412.5)	0.5138
CXCL13	pg/ml	21.4 (10.4–32.5)	0.0 (0.0–795.6)	14.4 (0.0–1342.9)	0.5453
CD27	U/ml	5.9 (3.6–15.0)	29.0 (8.0–162.6)	48.1 (11.3–151.5)	0.0455
CD14	ng/ml	96.6 (62.7–104.2)	76.4 (25.5–173.1)	66.9 (20.7–187.6)	0.1235
BAFF	pg/ml	72.1 (69.5–74.8)	61.9 (21.9–175.7)	50.1 (17.3–164.7)	0.2755
CD21	pg/ml	88.4 (63.0–154.7)	142.2 (59.5–334.2)	154.4 (53.6–365.5)	0.4104
BCMA	pg/ml	309.1 (272.1–346.1)	530.4 (135.9–1766.7)	562.6 (110.5–2162.3)	0.4705
CHI3L1	ng/ml	13.9 (−7.9–80.6)	78.2 (−48.3–272.7)	195.0 (18.6–607.0)	<0.0001

Biomarker concentrations were compared between inactive and active subgroups using unpaired t-test; Only biomarkers that are statistically significantly different ($p < 0.01$) between these subgroups are highlighted.

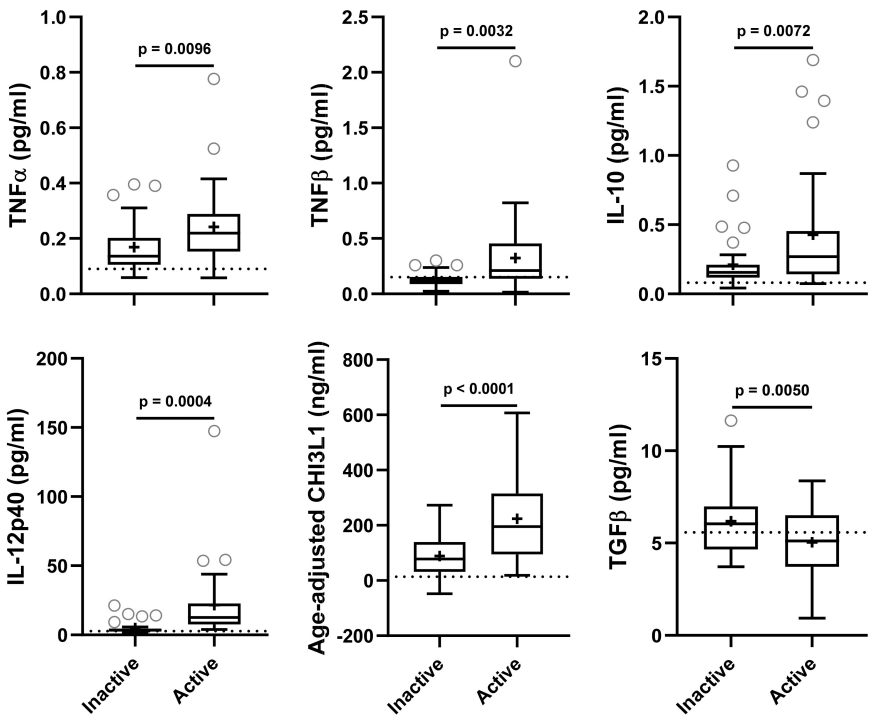

FIGURE 2 | In the training cohort, biomarker concentrations were compared across lesional activity subgroups (inactive versus active, n = 35 each subgroup). Dotted line represents median of HDs and "+" sign represents mean of respective subgroup. Data only for biomarkers which are statistically significantly different ($p < 0.01$; unpaired t-test) between two subgroups is depicted here.

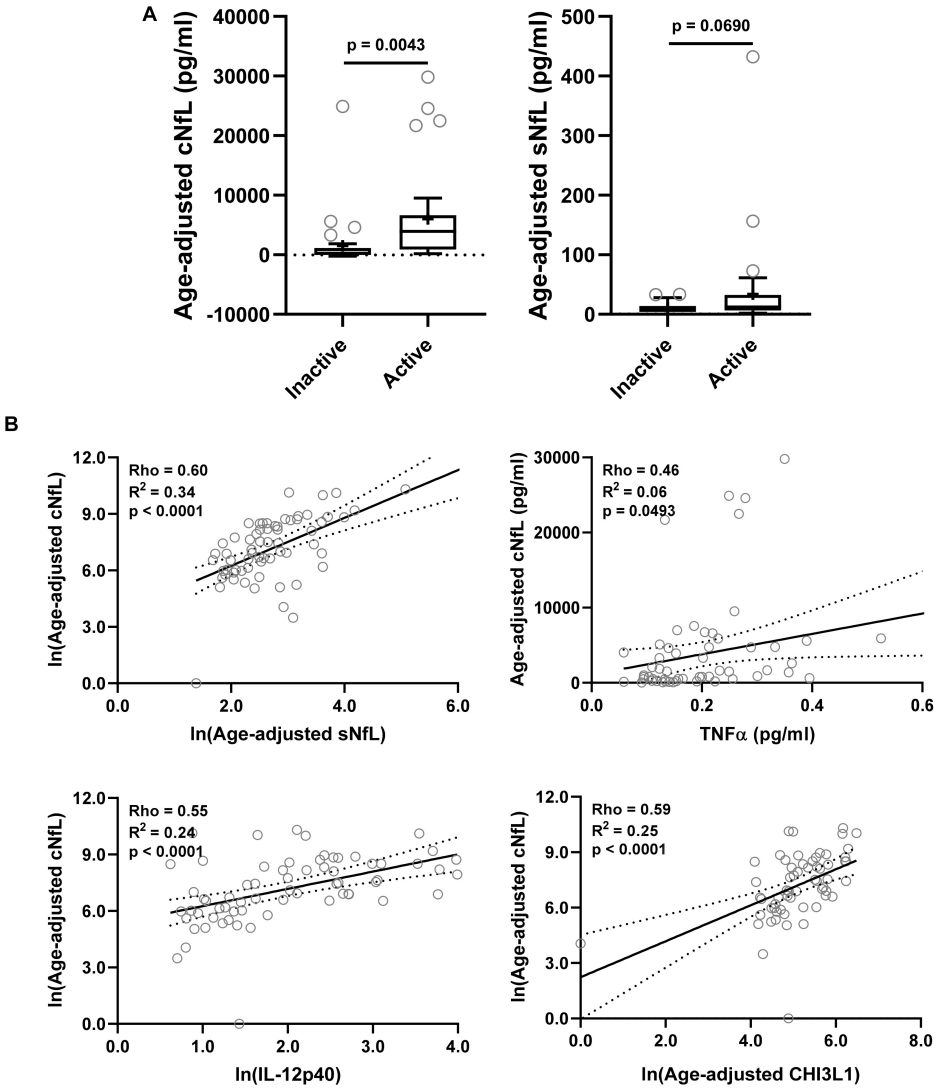

FIGURE 3 | In the training cohort, **(A)** CSF and serum NfL (cNfL and sNfL) concentrations were compared across MS lesional activity subgroups (n = 35 each subgroup). The median concentration of HDs is represented with a dotted line, and the mean for respective subgroups is represented with "+" sign. cNfL was significantly elevated in MS patients with active lesional activity ($p < 0.0043$; unpaired t-test). However, though sNfL was elevated in active MS patients it did not reach predefined statistical significance ($p = 0.0690$). **(B)** Within MS patients (n = 70) correlations between cNfL and all other biomarkers were analyzed; Only for statistically significantly ($p < 0.01$; Spearman correlation analysis) correlated biomarkers linear regression with cNfL as a dependent variable was analyzed. Solid line represents "line of best fit," and dotted line represents 95% confidence intervals.

Correlations Between Biomarkers and Number of CELs

Within both (inactive and active) type of MS patients, correlations between number of CELs and all other biomarkers were analyzed using Spearman analysis; And then only for statistically significantly ($p < 0.01$) correlated biomarkers linear regression with number of CELs as a dependent variable was analyzed: IL-12p40 (Rho = 0.66, R^2 = 0.36, and $p < 0.0001$), cNfL (Rho = 0.62, R^2 = 0.31, and $p < 0.0001$), CXCL13 (Rho = 0.42, R^2 = 0.12, and $p = 0.0067$) and CHI3L1 (Rho = 0.54, R^2 = 0.22, and $p = 0.0001$) (**Figure 4** and **Supplementary Table 2**).

Combined Model of Biomarkers

We tried to develop a stronger molecular model to predict lesional activity via combining several biomarkers together using the first principal component. But none of the principal component scores outperformed individual IL-12p40 and CHI3L1 in differentiating between active versus inactive subgroups.

In order to get a combined model of biomarkers, for two strongly correlated lesional inflammatory biomarkers with cNfL, IL-12p40, and CHI3L1, the multiple linear regression analysis with cNfL as a dependent variable was performed. The multiple linear regression analysis yields a model

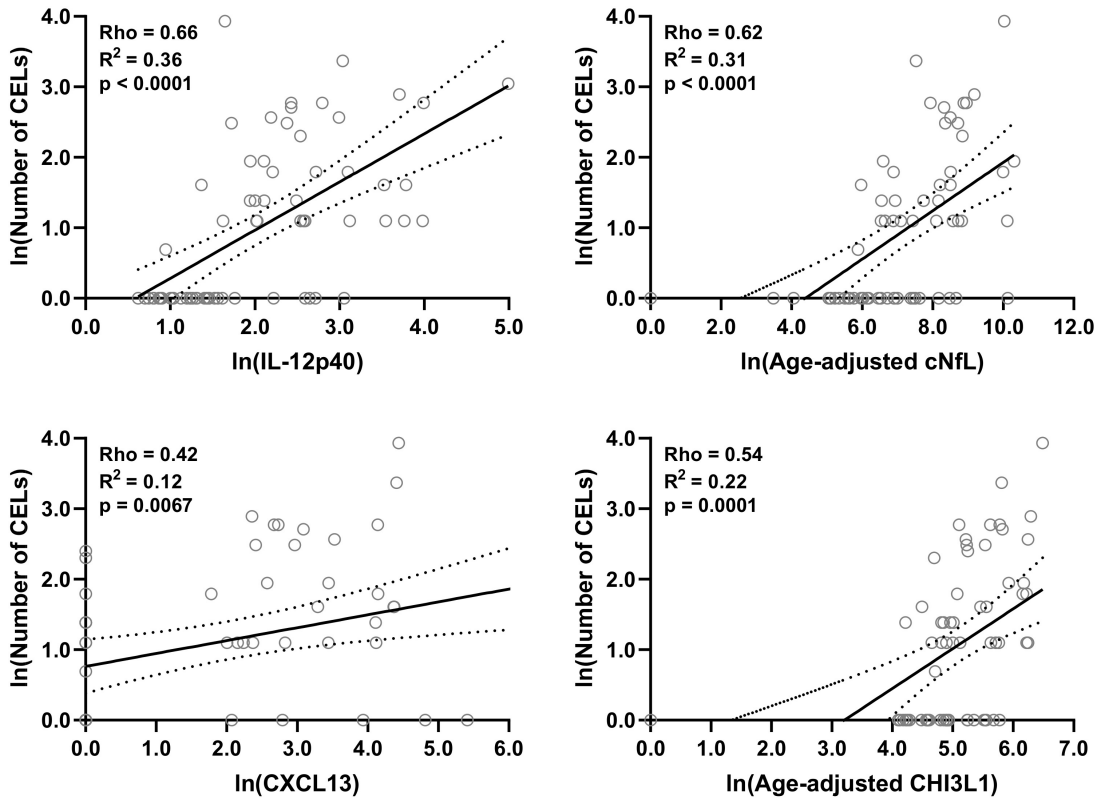

FIGURE 4 | In the training cohort, within MS patients (n = 70) correlations between number of CELs and all biomarkers were analyzed; Only for statistically significantly ($p < 0.01$; Spearman correlation analysis) correlated biomarkers linear regression with number of CELs as a dependent variable was analyzed. Solid line represents "line of best fit," and dotted line represents 95% confidence intervals.

(ln[cNfL] = 2.403 + 0.6643*ln[IL-12p40] + 0.6747*ln[CHI3L1]) which outperforms (R^2 = 0.35 and $p < 0.0001$) any biomarker individually.

Similarly, multiple linear regression analysis of IL-12p40, CHI3L1, and cNfL with the number of CELs as a dependent variable was performed (ln[Number of CELs] = −2.161 + 0.3865*ln[IL-12p40] + 0.1972*ln[CHI3L1] + 0.1938*ln[cNfL]). This model performs (R^2 = 0.45 and $p < 0.0001$) better than any single biomarker.

Analysis of Correlations Between Biomarkers

Correlations between all biomarkers were analyzed using Spearman analysis (**Figure 5**). In a correlation matrix a strong cluster of four proinflammatory biomarkers (CHI3L1, IL-12p40, TNFα, and TNFβ) can be identified; these four proinflammatory biomarkers were significantly elevated in active MS patients, and they were strongly correlated with axonal damage (cNfL) and number of CELs.

Validation Cohort

The findings from the training cohort with a stronger statistical effect ($p < 0.001$; IL-12p40, CHI3L1, and cNfL) were then validated in an independent validation cohort (**Figure 6**). All three biomarkers were significantly elevated in MS patients with active lesional activity compared to inactive patients, but with a less strong statistical effect (IL-12p40, CHI3L1, and cNfL unadjusted p = 0.0145, 0.0319, and 0.0335, respectively; **Figure 6A**).

IL-12p40 (Rho = 0.44, R^2 = 0.14, and $p < 0.0001$) and CHI3L1 (Rho = 0.46, R^2 = 0.14, and $p < 0.0001$) were moderately but significantly correlated with cNfL (**Figure 6B**). While only IL-12p40 (Rho = 0.29, R^2 = 0.05, and p = 0.0097) and cNfL (Rho = 0.25, R^2 = 0.06, and p = 0.0048) were weakly but significantly correlated with number of CELs (**Figure 6C**).

Within validation cohort, the cNfL concentrations predicted using combined linear regression model of IL-12p40 and CHI3L1 (from training cohort) outperforms (Rho = 0.53, R^2 = 0.21, and $p < 0.0001$) any biomarker discretely. Similarly, the number of CELs predicted using combined linear regression model of IL-12p40, CHI3L1 and cNfL is marginally better (Rho = 0.31, R^2 = 0.08, and p = 0.0010) than any biomarker alone (**Figure 6D**).

DISCUSSION

We observed moderate but reproducible positive associations of IL-12p40 and CHI3L1 with MRI CELs and with cNfL, the

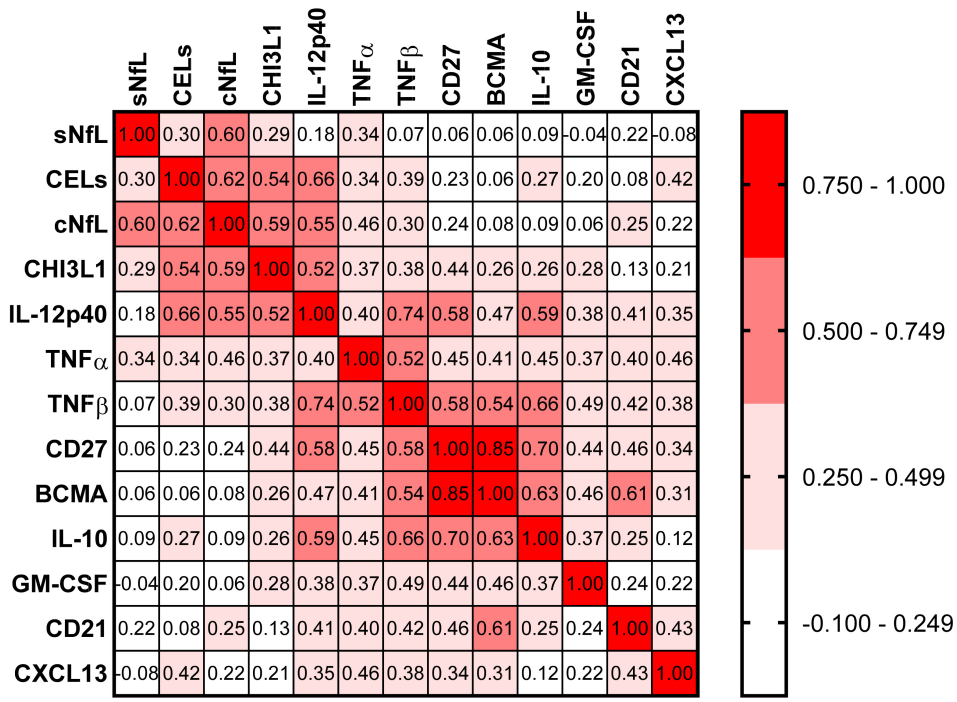

FIGURE 5 | In the training cohort, correlations between all biomarkers were analyzed using Spearman analysis. In correlogram, the color intensity of each cell denotes the correlation coefficient (Spearman Rho) for correlation between respective biomarkers. All biomarkers were analyzed, but only biomarkers which have at least one statistically significant ($p < 0.01$) correlation are depicted here.

marker of axonal damage. IL-12p40 (encoded by IL12B gene) is mostly produced by activated cells of myeloid lineage, such as monocytes, macrophages, microglia, neutrophils and myeloid dendritic cells, and induces Th1 polarization of T cells (Kanangat et al., 1996; Kichian et al., 1996; Cooper and Khader, 2007), while CHI3L1 is mostly produced by neutrophils and activated astrocytes in the CNS and plays a role in Th2 T cell polarization (Boesen et al., 2018). CELs exhibited a weaker positive correlation with pro-inflammatory mediators, TNFα (mostly produced by activated macrophages but also by T cells) and TNFβ (also called Lymphotoxin-α and secreted mostly by activated lymphocytes), and also with immunoregulatory cytokine, IL10 (secreted mostly by monocytes). Collectively these results point to a strong CNS activation of innate immunity at the time when CELs are visible. We can't make any determination of causality from our study, although the indirect evidence for pathogenicity of CHI3L1 does exist in the literature: subjects with clinically isolated syndrome (CIS) who had high CSF levels of CHI3L1 had greater and faster transition to clinically definite MS and a four-fold increased risk for the development of neurological disability compared to CIS subjects with low CSF CHI3L1 levels (Comabella et al., 2010; Canto et al., 2015). Additionally, CHI3L1, in concentrations analogous to those measured in the active MS group in this study was mildly neurotoxic to primary cultured neurons *in vitro* (Matute-Blanch et al., 2020). The possibility of direct or indirect neurotoxicity of CSF biomarkers associated with CELs is supported by the positive correlation of CHI3L1 (as well as IL-12p40 and TNFα) with cNfL. In contrast, CSF concentrations of immunoregulatory cytokine TGFβ, secreted in the CNS mostly by resting microglia, was significantly, although mildly decreased in MS patients with CELs. This is likely due to activation of microglia and their phenotypical switch from an immunoregulatory toward pro-inflammatory phenotype. The strong validation of cNfL prediction using combined model of IL-12p40 and CHI3L1 suggests that MS lesional activity is major contributor to MS-associated axonal damage. This conclusion is supported by validity of combined model of IL-12p40, CHI3L1 and cNfL to predict MS lesional activity measured by number of CELs.

In the introduction, we alluded to the limitations of quantifying MS lesional inflammatory activity via MRI CELs: CEL numbers do not capture CEL volume and the visibility of CELs is dependent on dose of the contrast administered and the delay between contrast administration and image acquisition. From that standpoint, it is intriguing to observe that MS patients who lacked CELs still had elevated CSF levels of CEL-associated biomarkers: IL-12p40 (1.3-fold), CHI3L1 (5.6-fold), TNFα (1.6-fold), and IL10 (2.0-fold) in comparison to HD medians. This suggests presence of MS lesional activity that is not visible by CELs, either because it's located in the CNS tissue not imaged (e.g., spinal cord), reflects formation of demyelinating cortical MS lesions which do not enhance, or is below the CEL detection threshold using a standard contrast dose.

Surprisingly, we found that other inflammatory CSF markers, especially those associated with adaptive immunity are elevated in both MS subgroups (i.e., with CELs or without) comparatively:

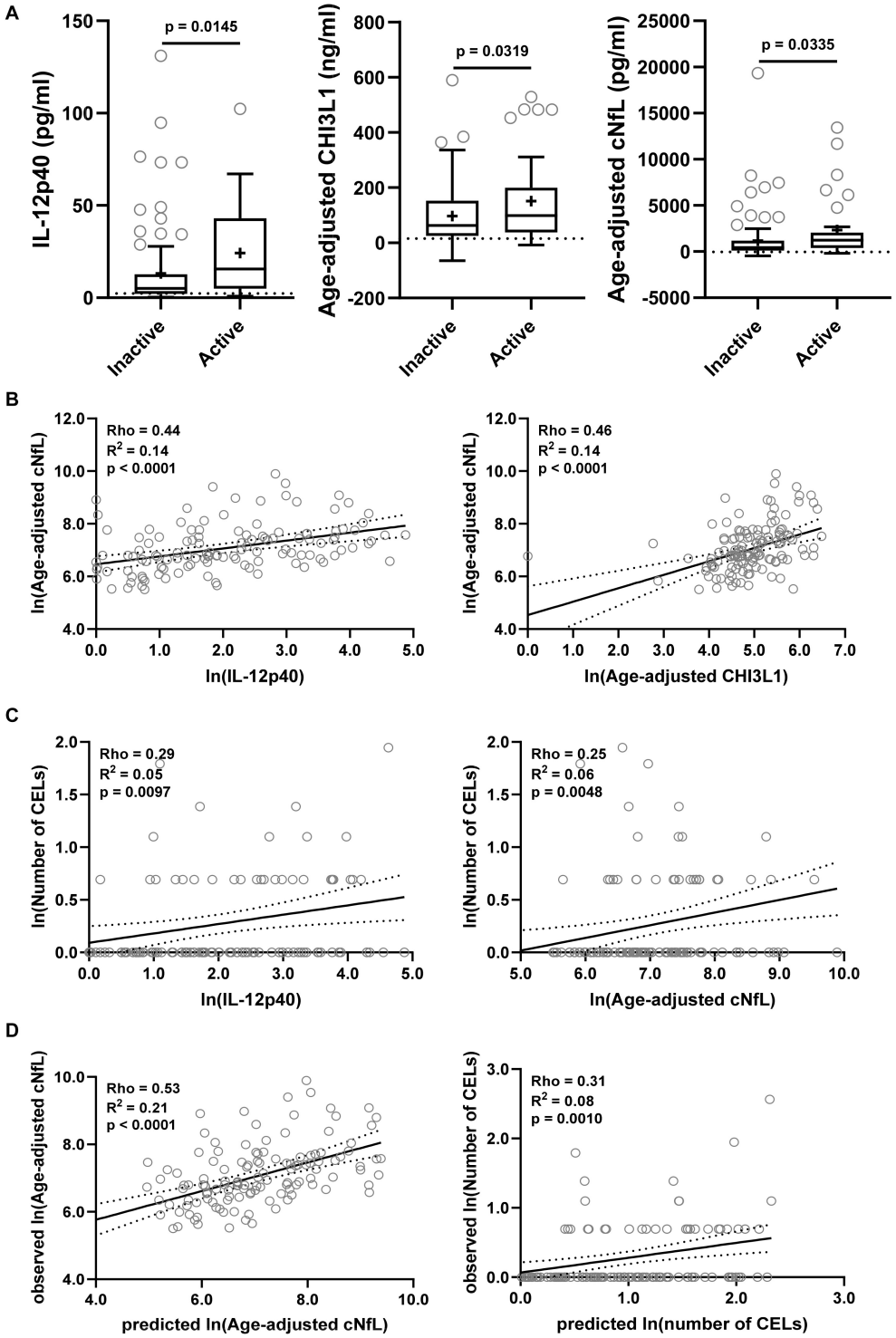

FIGURE 6 | The findings from the training cohort with a stronger statistical effect ($p < 0.01$; IL-12p40, CHI3L1, and cNfL) were then validated in an independent validation cohort. **(A)** Biomarker concentrations were compared across lesional activity subgroups (inactive versus active, $n = 96$ and 34, respectively). Dotted line represents median of HDs and "+" sign represents mean of respective subgroup. **(B)** In MS patients ($n = 130$) correlations between cNfL and IL-12p40, CHI3L1 were analyzed using Spearman analysis. Linear regression of IL-12p40 and CHI3L1 with cNfL as a dependent variable was analyzed. Solid line represents "line of best fit," and dotted line represents 95% confidence intervals. **(C)** In MS patients ($n = 130$) correlations between number of CELs and all biomarkers were analyzed using Spearman analysis; Only for statistically significantly (IL-12p40 and cNfL; $p < 0.01$) correlated biomarkers linear regression with number of CELs as a dependent variable was analyzed. **(D)** In validation cohort, the cNfL concentrations were predicted using combined linear regression model of IL-12p40 and CHI3L1 (from training cohort), and number of CELs were predicted using combined linear regression model of IL-12p40, CHI3L1 and cNfL. And then linear regression between predicted and observed values were analyzed.

CD27 (mostly secreted by T cells, CD8 > CD4), CD21 (secreted by naïve B cells), BCMA (secreted by plasma cells), and CXCL13 (chemokine secreted by follicular helper T cells and follicular dendritic cells that is B cell attractant). This observation was further supported by an analogous decrease in CSF BAFF, a fundamental survival factor consumed by B cells, found in both active and inactive MS groups. This was also true for a marker of toxic astrocytes, SERPINA3.

While the simplest interpretation of these observations is that cells of adaptive immunity do not play an essential role in the formation of MS lesions, this interpretation is clearly incorrect. Although pathological studies of active MS lesions have shown that in some instances the loss of oligodendrocytes and demyelination can occur in absence of adaptive immune cells, B and T lymphocytes (Barnett and Prineas, 2004; Henderson et al., 2009; Hernández-Pedro et al., 2013), and other studies have shown a predominance of activated myeloid and glial cells (monocytes, macrophages, microglia, and astrocytes) in acute MS lesions (Lucchinetti et al., 2000; Prineas et al., 2001; Howell et al., 2010; Mishra and Wee Yong, 2016; Moliné-Velázquez et al., 2016) the high therapeutic effect of B cell-depleting or lymphocyte-depleting treatments provides proof for the essential role of lymphocytes, especially B, cells in the formation of MS CELs.

So how can we explain this apparent discrepancy? We believe that the answer lies in the different topological distribution of cells of adaptive immunity in the early versus later stages of the disease: at the beginning of the MS process, when no lymphoid tissue has been formed in CNS, all cells of adaptive immunity concentrate in the active MS lesions. Once tertiary lymphoid follicles are formed in the meninges, then many cells of adaptive immunity reside in these lymphoid organs or are distributed across CNS tissue behind closed BBB. An alternative explanation is that cells of adaptive immunity become activated first and secrete factors that open BBB and recruit cells of myeloid lineage; by the time CELs are visible, the lymphocytes pass their expansion/activation status. This hypothesis is contradicted by observations stemming from a clinical trial of altered peptide ligand (APL) of myelin-basic protein (MBP) in MS, which demonstrated clear expansion of T cells in the CSF at the time of APL-induced CELs (Bielekova et al., 2000).

Finally, the last group of CSF biomarkers, comprised of IL1β, IL17F, GM-CSF, CD14, TRAIL, and LIF were not significantly elevated in either MS group. We note that first three of these are linked to Th17 T cells, which play a pathogenic role in animal model Experimental Autoimmune Encephalomyelitis (EAE). However, our data do not necessarily rule out the pathogenic role these cytokines may play in MS, because CSF levels of some were close to the detection limit of the applied assays (i.e., IL17F, LIF, and IL1β), while those with CSF levels clearly above the detection limits (i.e., GM-CSF and TRAIL) could have been consumed by activated immune cells.

Finally, we want to address differences between cNfL and sNfL as less invasive blood collection is preferred over spinal tap and advent of highly sensitive assay (Simoa^{TM}) (Rissin et al., 2010; Kan et al., 2012) allows reproducible measurement of sNfL (Kuhle et al., 2016, 2017; Disanto et al., 2017; Siller et al., 2019). But multiple studies, including this one demonstrated some loss of accuracy and clinical utility of sNfL as compared to cNfL (Kuhle et al., 2016; Disanto et al., 2017).

Though sNfL levels were correlated with cNfL (Rho = 0.60, R^2 = 0.34, and $p < 0.0001$) sNfL measurement did not differentiate between non-active and active MS lesional inflammatory activity subgroups with pre-defined statistical significance (cNfL: $p = 0.0043$ and sNfL: $p = 0.0690$). Similarly, sNfL levels were only moderately or weakly correlated with number of CELs (Rho = 0.30 and $p = 0.011$) and pro-inflammatory biomarkers associated with lesional inflammatory activity: IL-12p40 (Rho = 0.18 and $p = 0.129$) and CHI3L (Rho = 0.29 and $p = 0.021$). Whereas, cNfL levels were strongly correlated with both number of CELs (Rho = 0.62 and $p < 0.0001$) and biomarkers of lesional inflammatory activity: IL-12p40 (Rho = 0.55 and $p < 0.0001$) and CHI3L (Rho = 0.59 and $p < 0.0001$).

Correlation analysis between cNfL and sNfL explains just 34% of variance (R^2 = 0.34); that leaves 66% of variance unexplained. To make sNfL more accurate, we need to identify possible confounding factors that will need to be mathematically adjusted for to strengthen correlation between cNfL and (adjusted) sNfL and thus enhance the clinical utility of the latter.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National Institutes of Health (NIH) and Institutional Review Board (IRB). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BB and RM designed the study. RM, JP, and MK performed the experiments. BB, RM, and TW analyzed the data. RM wrote the first draft of the manuscript. BB supervised all aspects of the study. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.649876/full#supplementary-material

Supplementary Figure 1 | In the training cohort biomarker concentrations were compared across MS disease type (RR-MS vs P-MS) within respective lesional activity subgroups (inactive and active) using unpaired *t*-test (Inactive: RR-MS and P-MS, $n = 7$ and 28, respectively; Active: RR-MS and P-MS, $n = 28$ and 7, respectively). "+" sign represents mean of respective group.

Supplementary Table 1 | In the training cohort, within MS patients ($n = 70$) correlations between cNfL and other biomarkers were analyzed using Spearman analysis; Only statistically significant correlations ($p < 0.01$) are highlighted.

Supplementary Table 2 | In the training cohort, within MS patients ($n = 70$) correlations between number of CELs and all analyzed biomarkers were analyzed using Spearman analysis; Only statistically significant correlations ($p < 0.01$) are highlighted.

Supplementary Data File 1 | Sheet 1: biomarker concentrations (cNfL, sNfL, and CHI3L1) with their respective sample collection date, age, and gender for healthy cohort. Sheet 2 and 3: all analyzed biomarkers' concentrations for all subjects with their respective sample collection and MRI dates, age, disease duration, gender, EDSS, MS disease type, lesional activity (Inactive or Active), and number of CELs, for both (training: sheet 2 and validation: sheet 3) cohorts.

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REVIEW
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The Evolution of Neurofilament Light Chain in Multiple Sclerosis

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Multiple sclerosis (MS) is an autoimmune, inflammatory neurodegenerative disease of the central nervous system characterized by demyelination and axonal damage. Diagnosis and prognosis are mainly assessed through clinical examination and neuroimaging. However, more sensitive biomarkers are needed to measure disease activity and guide treatment decisions in MS. Prompt and individualized management can reduce inflammatory activity and delay disease progression. Neurofilament Light chain (NfL), a neuron-specific cytoskeletal protein that is released into the extracellular fluid following axonal injury, has been identified as a biomarker of disease activity in MS. Measurement of NfL levels can capture the extent of neuroaxonal damage, especially in early stages of the disease. A growing body of evidence has shown that NfL in cerebrospinal fluid (CSF) and serum can be used as reliable indicators of prognosis and treatment response. More recently, NfL has been shown to facilitate individualized treatment decisions for individuals with MS. In this review, we discuss the characteristics that make NfL a highly informative biomarker and depict the available technologies used for its measurement. We further discuss the growing role of serum and CSF NfL in MS research and clinical settings. Finally, we address some of the current topics of debate regarding the use of NfL in clinical practice and examine the possible directions that this biomarker may take in the future.

Keywords: multiple sclerosis, biomarkers, individualized medicine, prediciton, neurofilament light, demyelination

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INTRODUCTION

The clinical use of biomarkers in the diagnostic work-up of neurodegenerative diseases has significantly increased in the last decades (Beart et al., 2017). Screening, drug development, early diagnosis, individualized therapy, and accurate prognosis are some of the key factors driving the need of identifying objective and quantifiable markers and developing sensible biomedical tools for their measurement.

Multiple sclerosis (MS) is an autoimmune, inflammatory neurodegenerative disease characterized by demyelination and neurodegeneration of the central nervous system (CNS; Reich et al., 2018). The pathomechanisms vary greatly from person to person, resulting in different pathological phenotypes, clinical presentations, progression trajectories, and treatment responses (Lassmann et al., 2001; Gnanapavan and Giovannoni, 2015; Gafson et al., 2017; Reich et al., 2018;

Dobson and Giovannoni, 2019; Ziemssen et al., 2019). Current diagnostic criteria and clinical management depend on radiological markers (i.e., MRI), clinical status (i.e., disease activity and disability), and immunological parameters suggestive of inflammation (i.e., oligoclonal IgG bands; Thompson et al., 2018). However, these markers often fail to predict individual relapse rates, disability progression and therapy response (Håkansson et al., 2017). A meta-analysis showed that the number and volume of T2 lesions on MRI correlated poorly with clinical presentation and disease progression (Li et al., 2006). Similarly, the MAGNIMS study group concluded that clinical and MRI activity in isolation was not enough to evaluate treatment response (Gasperini et al., 2019). There is, therefore, an increased need to identify and validate biomarkers that could be used as surrogate measures for clinical endpoints in a more individualized manner (Giovannoni, 2006; Gnanapavan et al., 2013; Pachner et al., 2019; Gaetani et al., 2019a; Ehrenberg et al., 2020).

One of the hallmark features of MS, and which correlates highly with disability, is axonal damage, and loss (Trapp et al., 1998; de Stefano et al., 2002; Filippi et al., 2003; Pascual et al., 2007). Recent reports have demonstrated that people with MS (pwMS) have up to 60% reduction in axons at all spinal levels involving all fibers regardless of their diameter (Tallantyre et al., 2009; Petrova et al., 2018). This axonal loss has been associated with brain and cervical atrophy (Bjartmar and Trapp, 2001; Lee et al., 2014; Petzold, 2015; Barro et al., 2018), cortical thinning (Popescu et al., 2015), disability (Siffrin et al., 2010; Domingues et al., 2019), fatigue (Tartaglia et al., 2004), cognitive dysfunction (Gadea et al., 2004), and suboptimal response to therapy. Quantifying and monitoring axonal loss could be a reliable marker of MS progression, disability and treatment response (Novakova et al., 2017a, 2018).

NEUROFILAMENTS AS BIOMARKERS IN MS

Neurofilaments (Nf) are structural scaffolding proteins of the axonal cytoskeleton. Nf are essential for stability, radial growth and maintenance of axonal caliber and electrical-impulse transmission (Yuan et al., 2012; Gnanapavan and Giovannoni, 2015; Ziemssen et al., 2019). Given that Nf are involved in axonal radial growth, larger myelinated axons express significantly more Nf (Yin et al., 1998). Nf are composed of four subunits: neurofilament heavy, median and light polypeptides [NfH, NfM, and Neurofilament Light chain (NfL), respectively], as well as α-internexin (Int). Each subunit possesses a particular molecular mass (68 kDa for NfL, 150 kDa for NfM, and 190 to 210 kDA for NfH) and their relative concentration is uneven, however, NfL is the most abundant and soluble of the subunits. Under normal conditions and in a non-linear, sex-, and age-dependent manner (Gisslén et al., 2016), Nf are constantly released from axons (Disanto et al., 2017; Bridel et al., 2019), reflecting normal aging (Khalil et al., 2020). However, during axonal damage, Nf are released in larger quantities into the extracellular space, the cerebrospinal fluid (CSF), and eventually into the blood, where concentrations are 40-fold lower than in the CSF (Gaetani et al., 2019a). Overall, measurement of Nf levels indicate the extent of axonal damage, and therefore, is a bulk marker of disease activity (Lycke et al., 1998; Bergman et al., 2016; Disanto et al., 2017; Novakova et al., 2017a; Håkansson et al., 2018; Khalil et al., 2018; Cantó et al., 2019; Domingues et al., 2019; Varhaug et al., 2019; Gaetani et al., 2019b).

Several characteristics make Nf, and particularly NfL, a good biomarker of neurodegeneration. Firstly, NfL can be objectively measured and quantified, it is highly sensitive to neurodegenerative processes and its concentration changes as the disease worsens or improves (Disanto et al., 2017). Numerous studies have shown that NfL levels increase during MS relapses and correlate with MRI lesion development (Disanto et al., 2016, 2017; Novakova et al., 2017b; Barro et al., 2018), disease activity, disability and disease progression (Thebault et al., 2020). Secondly, NfL measurement is safe for the patient and NfL levels are relatively easy to detect. Emerging technologies allow for rapid, simple and minimally invasive quantification methods. This allows periodic measurements, and easier sampling acquisition and storage. Last but not least, several clinical trials have included NfL as an outcome measure and have shown that disease modifying therapies (DMTs) significantly reduce NfL levels compared with placebo (Gunnarsson et al., 2011; Axelsson et al., 2014; Christensen et al., 2014; Kuhle et al., 2015; Romme Christensen et al., 2019). This finding makes NfL a valuable outcome measure in clinical trials (Axelsson et al., 2014).

Yet, there are some important caveats that should be considered when quantifying and using NfL measurements in clinical practice. Importantly, NfL is not specific for MS. Neurodegenerative diseases such as prion diseases, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, Hungtington's disease, and traumatic brain injury have all demonstrated increased levels of NfL (Bridel et al., 2019; Gaetani et al., 2019a). Other studies have shown that different subunits might reflect different neurodegenerative processes (Zucchi et al., 2020). For example, NfH, which undergoes extensive phosphorylation and influences the transportation dynamics along axons, is particularly specific for ALS (Xu et al., 2016). Additionally, NfL levels do not provide information on the specific location of axonal damage (Disanto et al., 2017), and a growing body of evidence has shown that NfL is also elevated in individuals with peripheral nerve disease (Sandelius et al., 2018; Hyun et al., 2020), which further limits its use as a diagnostic biomarker. Moreover, standardized normal cut-off values are still lacking, and despite longitudinal measurements being preferred for clinical decision-making, optimal sampling frequency and thresholds are yet to be defined (Domingues et al., 2019; Bittner et al., 2020). It has been documented that NfL levels depend on age, sex and, presumably, body mass index and blood volume (Manouchehrinia et al., 2020a). But as yet, no normative values accounting for confounders have been established.

In the case of individuals with established MS, some authors have reported elevated NfL as the sole indicator of disease activity in people with progressive MS (PMS) vs relapsing remitting MS (RRMS) (Reyes et al., 2020), while others have found greater levels of NfL in RRMS vs PMS (Martin et al., 2019), and

that NfL levels at time of diagnosis correlated with long- term progression from RRMS to PMS (Bhan et al., 2018). A recent systematic review concluded that associations with current or future disability are inconsistent, and that there is no evidence of NfL being a responsive marker of purportedly neuroprotective treatments (Williams et al., 2020). More and longer studies are needed examining the evolution and significance of NfL as the disease progresses.

CORRELATION OF NfL LEVELS WITH RADIOLOGICAL AND CLINICAL FINDINGS

The correlation between NfL levels and clinical and radiological findings has been widely reported in the literature. Clinically, it has been shown that NfL levels peak at 3–4 weeks after a clinical relapse and remain elevated for the next 6–12 months (Novakova et al., 2017b; Damasceno et al., 2019). It has also been shown that the cumulative number of relapses in the past 12 months are the main predictor of high NfL levels (Bergman et al., 2016; Kuhle et al., 2019a). In addition, high levels of NfL correlate with brain atrophy and spinal cord volume loss, even in the absence of MRI activity (Arrambide et al., 2015; Kuhle et al., 2016b, 2019a; Disanto et al., 2017; Novakova et al., 2017b; Barro et al., 2018; Chitnis et al., 2018; Piehl et al., 2018; Siller et al., 2019; Khalil et al., 2020).

The predictive value of NfL levels as an independent predictor of conversion from clinical isolated syndrome (CIS) or radiological isolated syndrome (RIS) to MS has been studied. Hakason and colleagues compared the predictive role of NfL and other molecules for MS conversion in individuals presenting with CIS and found that CSF NfL (cNfL) at baseline was the best predictive biomarker (Håkansson et al., 2017). Gaetani et al. (2019b) concluded that a cNFL cut off value of 500 pg/mL was able to predict conversion to MS in individuals presenting with isolated clinical events. Importantly, cNfL levels have been found to be an independent predictor of conversion from RIS to MS (hazard ratio = 1.03, P = 0.003; Matute-Blanch et al., 2018), as well as when combined with oligoclonal bands (OCBs; Fyfe, 2018). Moreover, a recent nested case-control examining NfL levels in blood (bNfL) samples from asymptomatic United States military personnel that later developed MS found an association between baseline or presymptomatic levels and long-term risk of developing MS (p = 0.008; Bjornevik et al., 2020). Even though similar results have been found elsewhere (Martínez et al., 2015; van der Vuurst de Vries et al., 2019; Dalla Costa et al., 2019a), some studies have reported only a weak predictive value (Arrambide et al., 2016; Disanto et al., 2016).

Axonal damage resulting in brain volume loss plays a key role in long term disability (Furby et al., 2008; Domingues et al., 2019; Marciniewicz et al., 2019). As expected, bNfL levels have been used as potential predictors of long-term progression according to the Expanded Disability Status Scale (EDSS; Häring et al., 2020), however, findings are inconsistent. A study that included 607 individuals with MS followed up for 12 years, showed a significant increase of 80% in bNfL levels per increase in EDSS score (Cantó et al., 2019). Yet, they did not observe an association with long term disability progression. Disanto and colleagues reported increased worsening of EDSS and increased relapse rates at 2 years in individuals with CIS and RMS with bNfL levels above the 80th percentile compared to healthy controls (Disanto et al., 2017). Later on, they reproduced their findings adjusting for other predictors such as T2 lesion load and observed a modest association (OR 2.79) for bNfL above the 90th percentile (Barro et al., 2018). Likewise, Anderson et al. (2020) found a non-significant association between bNfL and EDSS at 5 years in a cohort of 164 pwMS. Moreover, Chitnis et al. (2018) did not find any association between 10-year EDSS scores and bNfL levels collected within 5 years of disease onset. Interestingly, they reported that bNfL correlated with 10-year MRI markers including T2-weighted lesion volume and atrophy. A composite model including bNfL and T2 lesion load was, therefore, deemed robust for predicting long term disability (Chitnis et al., 2018). A similar conclusion was reached by Häring et al. (2020) and Bittner et al. (2020), who measured bNfL and MRI lesion load in 814 individuals with CIS or newly diagnosed MS from 22 centers across Germany.

The correlation between disability and cNfL has also been studied. A randomized controlled trial extension study including 235 pwMS reported that cNfL levels measured at 2 years and bNfL levels measured at 3 years were associated with EDSS scores at 8 years (Kuhle et al., 2019b). However, these levels were measured after beginning of treatment with intramuscular interferon β-1a. Similar results were found elsewhere (Manouchehrinia et al., 2020b). Similar to findings on bNfL, a group reported a correlation between cNfH levels and brain and spinal cord atrophy after 15 years of follow up, but not with EDSS (Petzold et al., 2016). A possible explanation for the modest association between Nf and disability progression is the fact that most studies have not controlled for these potential confounders, such as treatment with DMTs (Anderson et al., 2020).

The association of NfL and non-motor symptoms of MS (e.g., cognition, psychological disorders, and fatigue) has been examined. While some studies reported a significant inverse association between NFL levels and cognitive function (Tortorella et al., 2015; Gaetani et al., 2019c) and long-term fatigue (Chitnis et al., 2018), others did not find any association (Håkansson et al., 2019; Aktas et al., 2020). Cognitive symptoms and fatigue in MS are strongly associated with sleep quality, depression, DMT, disease duration and severity, and lesion localization (Rocca et al., 2014; Berard et al., 2019; Palotai et al., 2019). Therefore, future studies examining the association between NfL and non-motor symptoms should control for these confounders.

NfL AND MS MIMICS

Neuromyelitis optica spectrum disorder (NMOSD) and myelin oligodendrocyte glycoprotein antibodies (MOG-Ab) associated disorders (MOGAD), have clinical features that overlap with MS which makes misdiagnosis possible (Alkhasova et al., 2020). Around one third of individuals with NMSDO and

MOGAD are seronegative for the highly specific pathognomonic antibodies: aquaporin-4 (AQP-4) and MOG-Ab, which further increases the potential for misdiagnosis and wrong treatments (Fujihara, 2019). Recent studies have analyzed NfL levels in these individuals to see whether threshold values could help differentiate MS from other idiopathic inflammatory demyelinating disorders. A study from China found no significant differences between bNfL levels in pwMS vs CIS vs NMOSD (Peng et al., 2019). In addition, and in line with the underlying pathophysiology of NMOSD vs MS (astrocytic vs axonal involvement, respectively), they found no correlation between bNFL levels and serum AQP-4 (Peng et al., 2019). Importantly, some have reported that high bNfL levels in seropositive NMOSD and MOGAD are associated with a more malignant course of the disease ($p < 0.05$), presumably reflecting concomitant axonal damage (Mariotto et al., 2017). This association between bNfL levels and disability in NMOSD (Watanabe et al., 2019; Kim et al., 2020) and MOGAD (Mariotto et al., 2019) has been found elsewhere. Recently, Watanabe et al. (2019) reported that higher sGFAP/bNfL ratio at relapse was sensitive (73%) and specific (75.8%) to differentiated NMOSD from MS. However, more studies are needed for this to be translated to the clinical setting. Moreover, Lee and colleagues found that NFL levels in NMOSD vs MS vary more significantly according to age (Lee et al., 2020), which emphasizes the need for age-controlled normative values.

BIOMARKER TECHNOLOGY

The first assay to measure NfL was developed by Rosengren et al. (1996). This was an ELISA assay based on polyclonal antisera, which was later upgraded to a highly specific assay based on monoclonal antibodies (47:3 and 2:1) against NfL epitopes (Norgren et al., 2002). More recently, Gaetani et al. (2018) generated two novel monoclonal antibodies (NfL21 and NfL23) and a new ELISA assay, which has expanded the currently available methods to measure NfL. NfL ELISA, which is commercially available as NF-light® ELISA kit; UmanDiagnostics AB, Umeå, Sweden, allows for a fast quantification of cNfL with a low sample volume (50 μL). Additionally, it shows good stability after handling and storage (Norgren et al., 2002). The main disadvantage is its low sensitivity for quantifying bNFL. In comparison to CSF sampling, blood sampling is less invasive. Methods such as electrochemiluminescence (ECL)-based immunoassays, which use the luminescence produced during electrochemical reactions of specific antibody combinations, are more sensitive ways of measuring NfL in blood (Li and Mielke, 2019). They are also affordable and require smaller sample volumes (Limberg et al., 2015; Kuhle et al., 2016a). Nevertheless, it has been shown that ECL-based methods are not sufficiently sensitive to detect the lowest concentrations in MS (Hendricks et al., 2019; Li and Mielke, 2019), which limits its utility.

Quantification of bNfL and cNfL has become optimized with the development of the ultrasensitive Simoa® (Quanterix; Hendricks et al., 2019; Gaetani et al., 2019a). Simoa is 125- and 25-fold more sensitive than conventional ELISA and ECL-based assays, respectively. Notably, it can detect a concentration as low as 0.1 pg/mL of protein (Kuhle et al., 2016a). Kuhle et al. (2016a) quantified and compared bNfL levels across the mentioned technologies and found that 55% of ELISA serum measurements and 60% of ECL measurements were below sensitivity when compared to Simoa. A number of studies have shown that Simoa bNFL has good correlations with clinical and radiological findings (Disanto et al., 2017; Novakova et al., 2017b; Piehl et al., 2018), which supports its potential role as a surrogate biomarker in MS. Additionally, Simoa can also detect tau and other proteins associated with neurodegeneration to similar sensitivities, which widens its utility (de Wolf et al., 2020).

Several studies have examined the correlation between cNfL and bNfL levels between the different technologies. Kuhle et al. (2016a) compared the three mentioned technologies using matched CSF and blood samples from individuals with various neurodegenerative conditions. When comparing paired cNfL and bNfL, they found a strong correlation for Simoa and ECL, but weaker for ELISA (**Figure 1**). They showed that cNfL were well correlated between technologies, whereas bNfL levels were only similar between ECL assay and Simoa, but not between ELISA and ECL, or and ELISA-Simoa (**Figure 2**). Similar findings were found by Gisslén et al. (2016). Generally, is it expected to find

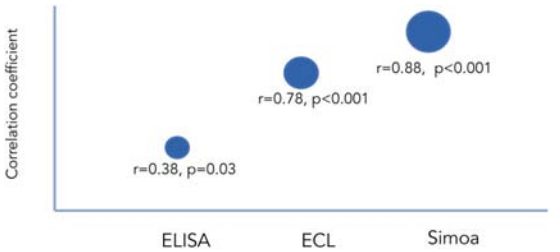

FIGURE 1 | Correlation between cNfL and bNfL across different technologies as reported by Kuhle et al. (2016a). Correlation coefficients between cNfl and bNfL levels measured with ELISA, ECL, and Simoa.

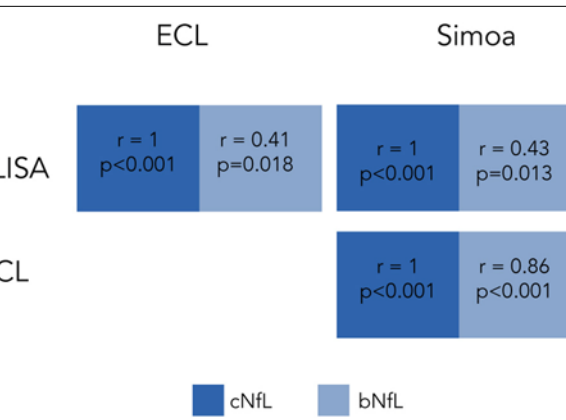

FIGURE 2 | Correlation of cNfL and bNfL across different technologies as reported by Kuhle et al. (2016a). Correlation coefficients of cNfl and bNfL levels across ELISA, ECL and Simoa.

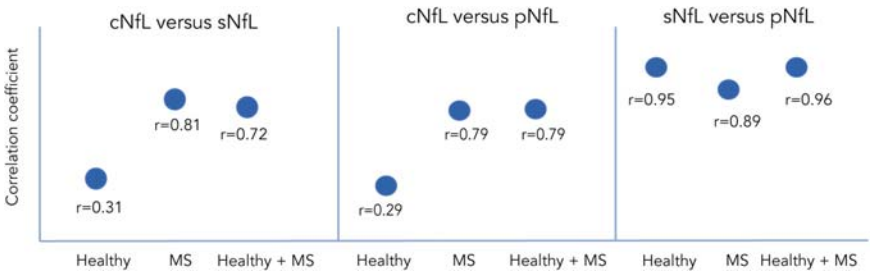

FIGURE 3 | Correlation of NfL between CSF, serum and plasma in different population as reported by Harp et al. (2019), Hendricks et al. (2019), and Sejbaek et al. (2019). Correlation coefficients between NfL measured in CSF vs serum or vs plasma across different populations.

higher NfL levels when using Simoa, given its ultra-sensitivity. All together, these findings highlight the need of calibration and validation of cut-off levels across technologies (Kuhle et al., 2016a; Varhaug et al., 2019). Overall, Simoa is the preferred bNfL assay due to its low detection limit, simple and fast sampling, storage and handling, and feasibility of longitudinal sampling. Ongoing projects, led by Siemens Healthineers, are developing bNfL immunoassays using the Quanterix NfL antibodies. After reporting high correlation of bNfL levels between Simoa and the assay, they plan to adapt the Simoa assay onto a routine analyzer platform, which will accelerate the availability of NfL tests for patients around the world (Didner, 2019).

NfL IN CSF AND BLOOD

High variability between CSF and serum NfL (sNfL) levels have been found in pwMS (**Figure 3**). Harp and colleagues used the Simoa platform to compare levels of NfL in plasma, serum, and CSF samples obtained from the same individual, with and without brain pathology. Their results showed a strong correlation between serum and plasma NfL levels within the same person, but a weaker correlation between CSF and serum or plasma levels (Harp et al., 2019). Hendricks et al. (2019) analyzed NfL levels in 112 MS individuals and found a good correlation between CSF, serum, and plasma levels. Other group analyzed NfL levels in the CSF, plasma and serum of 52 untreated RRMS individuals, 23 healthy controls, and 52 MS individuals treated with placebo, matched by age, sex, and NfL (Sejbaek et al., 2019). They found that NfL levels were approximately 200 times higher in the CSF compared to plasma or serum. Additionally, even though the plasma and serum levels were highly correlated, plasma levels were 23% lower than in serum. Similar findings have been found in murine models of neurodegeneration (Gaiottino et al., 2013; Bacioglu et al., 2016; Disanto et al., 2017; Bianchi et al., 2019). Other authors examining treatment effects showed that both CSF and sNfL levels decrease with DMTs (Piehl et al., 2018; Sejbaek et al., 2019) suggested that serum levels might be more useful than plasma levels when evaluating treatment effects, given that sNfL levels show a relatively greater reduction compared to plasma levels.

It has been hypothesized that the differences between CSF, plasma/serum are probably due to the fact that the CSF compartment is closer to the damaged site, and to the integrity of the blood brain barrier, whilst concurrent peripheral inflammatory or infectious processes might affect the increase of blood NfL levels (Bacioglu et al., 2016; Sejbaek et al., 2019; Dalla Costa et al., 2019b). Despite the high variability found between compartments, most evidence seems to suggest there is a good correlation, which will likely shift the balance toward blood sampling given its practical advantages.

DETERMINING WHAT IS ABNORMAL

A key point to validate NfL levels as surrogates of disease activity is establishing optimal and sensitive cut-off values. Currently, there is large heterogeneity on optimal values to establish what is pathological, to estimate the risk of conversion from RIS/CIS to clinically definite MS, to accurately predict disease progression, and to determine adequate treatment response (i.e., what would be considered a significant drop in NfL to reflect treatment response). Furthermore, gender and age stratification are lacking. Generally, cNfL cut-off levels range between 400–1,000 ng/l (Arrambide et al., 2016; Novakova et al., 2017b; Gaetani et al., 2019a), and corresponding sNfL cut-off levels between 3–30 ng/L (Norgren et al., 2004; Håkansson et al., 2018; Dalla Costa et al., 2019a; Thebault et al., 2020). Others have instead used percentiles across different ages (Disanto et al., 2017). More recently, authors have been calling for longitudinal measurements of sNfL rather than absolute cut-off values (Bittner et al., 2020; Ferraro et al., 2020; Häring et al., 2020).

This variability reflects the lack of consensus on measuring techniques between centres, no fixed criteria for setting abnormal values, and in determining the rate or percent change that would be clinically meaningful over time.

UTILITY OF NfL MEASUREMENT IN CLINICAL PRACTICE

Monitoring and predicting disease activity and treatment response is crucial for the individualized management of pwMS (Kalincik et al., 2017). MRI is a reliable tool to assess therapeutic monitoring, however, it is costly and is not always available owing to geographic reasons or lack of local resources. Recent

TABLE 1 | Reported reduction in NfL levels after treatment with DMTs.

DMTs	Reduction at last follow up
Natalizumab	37%, $p = 0.03$ (Christensen et al., 2014)
	20%, $p < 0.001$ (Kapoor et al., 2018)
	30%, $p < 0.001$ (Gunnarsson et al., 2011)
Fingolimod vs IFNβ1a	38%, $p < 0.001$ (Kuhle et al., 2019a)
Alemtuzumab and disease activity	DMT associated with no disease activity $p < 0.001$ (Hyun et al., 2020)
IFN or glatiramer acetate to rituximab	21%, $p = 0.01$ (de Flon et al., 2016)
Injectable therapies to fingolimod	33%, $p < 0.001$ (Piehl et al., 2018)
Ocrelizumab vs placebo	43%, $p < 0.001$ (Bar -Or et al., 2019)
Ibudilast vs placebo	no difference (Fox et al., 2021)

studies have examined the effects of DMTs on NfL levels and evaluated the potential role as a therapy monitoring tool (**Table 1**). Kuhle et al. (2019a) measured sNfL in individuals with RRMS that were part of two ongoing clinical trials. Those receiving fingolimod had lower NfL levels throughout the course of the disease compared to those receiving IFNβ1a, which is congruent with clinical observations. Likewise, a multicenter Swedish study showed that both cNfL and sNfL levels correlated with DMT response, regardless of whether they were receiving DMTs or not (Novakova et al., 2017b). They observed that NfL levels were reduced in those initiating DMT for the first time or after switching from first- to second line DMTs. Similar results were found in studies analyzing the effect of dimethyl fumarate in individuals without previous therapy (Sejbaek et al., 2019), individuals treated with natalizumab (Gunnarsson et al., 2011) or alemtuzumab (Hyun et al., 2020), and individuals switching from IFN or glatiramer acetate to rituximab (de Flon et al., 2016), or from injectable therapies to fingolimod (Piehl et al., 2018). Interestingly, Dalla Costa et al. (2019a) analyzed the role of NfL as markers of progressive multifocal leukoencephalopathy (PML) secondary to natalizumab therapy. They showed that sNfL had a tenfold increase with PML onset, which was higher than those having an MS relapse.

Individualized management is a key goal in MS management. Current treatment strategies are based on clinical and radiological findings; however, these often fail to capture the full extent of the disease and have a limited correlation with disability and prognosis. With the recent findings on the correlation of NfL levels and disease activity, disability and therapy monitoring, the next step is to examine whether including NfL measurements into day-to-day clinical practice is beneficial. A recent observational study examined the role of NfL measurement in treatment decisions and as a surrogate marker of clinical or radiological activity (Reyes et al., 2020). They found that NfL levels were closely associated with clinical and radiological activity, and in a significant proportion of individuals with PMS, elevated NfL was the only evidence of disease activity used in the treatment decision-making process. In line with the available evidence, individuals with elevated NfL levels were more likely to have treatment escalation as a means to reduce axonal damage and neurodegeneration. Importantly, they emphasized the need of age-related cut-offs and predefined time intervals for measurements as indispensable prior to introducing NfL measurements in clinical decision making.

ROLE OF NfL IN RESEARCH

To date, more than 2000 MS clinical trials are active[1]. Of these, more than 150 correspond to phase 2 trials, of which one third are testing new drugs. A large percentage of these trials use MRI measures to evaluate outcomes. Even though there is substantial evidence on the association between MRI findings, disease progression and treatment response, subclinical activity and neuroaxonal damage markers cannot be fully evaluated with available neuroimaging techniques (Gajofatto et al., 2013). In addition, long-term (i.e., 24 months or more) MRI-based endpoints do not correlate as well as short term imaging endpoints, and therefore frequent scans are usually needed. Repeated scans increase costs, are burdensome for many individuals, and do not usually cover the spinal cord. Given the advantages of NfL measurement and the good correlation it has shown with MRI findings of disease activity and treatment response, it has gained significant attention as a potential end point marker for clinical trials (Sormani et al., 2019; Kuhle et al., 2019a). In addition, the findings from the nested case-control study of United States military personnel by Bjornevik et al. (2020) suggest that NfL levels in asymptomatic individuals could be used to select candidates that would benefit from disease prevention trials.

THE KNOWN UNKNOWNS

How Much NfL From Peripheral Nerve Disease Will Affect Serum/CSF NfL?

The effect of peripheral nerve disease on NfL levels has not been widely studied but will be highly relevant when incorporating NfL measurements in the clinical management of pwMS. Mariotto et al. (2018) studied a cohort of 25 individuals with acquired peripheral neurological disease and observed a significant increase between cNfL and sNfL and disease activity, severity and outcome, although only sNFL remain significant. Interestingly, they noticed a correlation between NfL only in individuals with possible brain-nerve barrier damage, suggesting that disrupted brain-nerve barrier could contribute to the increase in cNfL seen in patients with peripheral nerve disorders. Similar results were found in a study that included individuals with Charcot Marie Tooth disease (Sandelius et al., 2018), vascular neuropathy (Bischof et al., 2018), progressive axonal sensorimotor polyneuropathy (Louwsma et al., 2020), and prediabetic neuropathy (Celikbilek et al., 2014). More studies will be needed to characterize the impact of peripheral nerve disease in NfL levels in individuals with concomitant CNS disease.

[1] clinicaltrials.gov

Can Anti-NfL Antibodies Reduce the Circulating NfL Levels?

The generation of antibodies against neuronal antigens, such as neurofilament light, has been reported (Bartoš et al., 2007). There is some evidence on the relationship between anti-NfL antibodies and circulating NfL levels. Fialová et al. (2013) demonstrated increased NfL levels and intrathecal synthesis of anti-NfL and anti-NfH autoantibodies in early MS, but not in those with long standing disease. They suggested that this observation was in line with the clinical observation that autoimmune mechanisms predominate at initial phases of the disease. Interestingly, they also observed an association between anti-NfL levels and early conversion to clinically definite MS in individuals with CIS. They concluded that CSF anti-NfL antibodies and CSF anti-NfL/serum anti-NfL antibody ratio could help differentiate individuals with CIS with a higher risk of conversion. Moreover, Silber et al. (2002) observed that anti-Nfl and anti-NfH correlated with disease duration and EDSS, and Amor et al. (2014) observed a reduction in anti-NfL levels after natalizumab therapy. Despite these findings, the clinical usefulness of anti-Nf measurement is not straightforward. Other groups have reported presence of anti-NfM antibodies in individuals with non-immune neurological disorders, such as migraine and chronic fatigue syndrome (Bartoš et al., 2007). Also, discrepancy between CSF and serum levels for these antibodies have been documented (Ehling et al., 2004; Bartoš et al., 2007), which limits its utility as an isolated measurement (Dubuisson et al., 2017). Further studies are required to draw solid conclusions about the impact of anti-Nf antibodies on circulating NfL and their clinical utility.

Other Biomarkers, Where do They Stand?

Even though there is a higher volume of evidence and increased clinical and research interest in NfL as a biomarker in MS, there are other biomarkers that have been examined independently and in relation to NfL.

Oligoclonal bands are detected in the CSF of about 95% of pwMS and are considered the best diagnostic element supportive of MS diagnosis (Deisenhammer et al., 2019). CSF OCBs are produced by B cells and plasma cells and can be found in other neuroinflammatory diseases. OCBs positivity, independent of MRI lesion load, strongly predicts conversion from clinically isolated syndromes (CIS) to CDMS (Dobson and Giovannoni, 2019). The predicate risk increases significantly (adjusted hazard ratio 11.3 (6.7–19.3) when OCBs are found together with 10 or more lesions on MRI (Ignacio et al., 2010; Tintore et al., 2015). Similarly, studies examining the predictive role of OCBs and cNfL have shown that individuals with RIS presenting with positive OCBs and/or high levels of cNfL have a shorter time to conversion to CIS and MS compared to those with negative OCB and/or low cNfL levels (Matute-Blanch et al., 2018). Interestingly, the association between cNfL and time to conversion was restricted to individuals older than 37 years, whereas the one with OCBs was present regardless of age. Among pwMS, a low number of OCBs at diagnosis may be associated with a better prognosis and treatment response (Avasarala et al., 2001; Joseph et al., 2009), however, no clear prognostic value has been established (Becker et al., 2015).

Osteopontin (OPN) is an extracellular matrix protein widely expressed in immune cells and involved in T-mediated inflammatory response (Brown, 2012). OPN expression has been found in MS lesions (Chabas et al., 2001) and specific OPN genotypes have been associated with an increased risk of developing MS (Chiocchetti et al., 2005). Previous studies have shown that OPN levels are increased in secondary progressive MS (SPMS; Comabella et al., 2005; Romme Christensen et al., 2013; Shimizu et al., 2013), PPMS and RRMS (Vogt et al., 2003; Romme Christensen et al., 2013). They have also been associated with cognitive impairment and treatment response (Iaffaldano et al., 2014). A recent meta-analysis concluded that elevated levels of OPN in CSF and in the peripheral blood of pwMS are suggestive of active inflammation (Agah et al., 2018). A study by Tortorella and colleagues found that OPN was inversely correlated with the volume of the corpus callosum, whereas NfL was associated with gray matter volume in a cohort of individuals with CIS (Direnzo et al., 2015). They suggested that cNfL and OPN levels during CIS might reflect different patterns of early neurodegeneration. A study examining the stepwise predictive value of 18 biomarkers including cNfL and cOPN and MRI lesion load concluded that baseline cNfL combined with OPN and CLL2 correctly predicted the clinical activity status of 91% of the individuals with CIS or MS. In contrast, cOPN + cNfL, cNfl alone, T2 lesion load + cNfL or alone, showed lower percentages of correct prediction (86, 83, 81, and 71%, respectively; Håkansson et al., 2017).

C-X-C motif chemokine-13 (CXCL13) is a B cell chemoattractant that has been shown to be involved in the recruitment of B cells into the CNS during neuroinflammatory conditions (Cui et al., 2020). Unlike NFL, CXCL13 is not commonly produced in the absence of neuroinflammatory conditions. Recent evidence indicates that CXCL13 is associated with prognosis and disease activity, and is reduced with corticosteroids, fingolimod, natalizumab and B-cell depletion therapies (Lycke and Zetterberg, 2017; Matute-Blanch et al., 2017). CXCL13 levels alone or in combination with NFL might also predict CIS conversion to MS (Brettschneider et al., 2010). Interestingly, a recent study examining the clinical utility of combined cNfL and CXCL13 measurements concluded that these biomarkers continue to be evaluated in individuals with no clinical or radiological activity, which could complement the assessment of disease activity in pwMS (Novakova et al., 2020). Remarkably, DiSano and collegues recently showed that CXCL13 combined with NfL had excellent sensitivity (100%), specificity (72%), positive predictive value (71%), and negative predictive value (100%) compared to either CXCL13 or NfL alone to predictive future disease activity (DiSano et al., 2020). Additionally, they showed that CXCL13 + NfL had better predictive value compared to NfL + OCBs, CXCL13 + OCBs, and CXCL13 + OCBs + NfL to predictive disease activity in pwMS.

Chitinase-3-like protein 1 (CHI3L1) is a glycosidase secreted by monocytes, astrocytes and microglia and it is thought to modulate CNS inflammation. There is evidence that CHI3L1 levels are associated with CIS conversion to MS, disability progression (Cantó et al., 2011; Hinsinger et al., 2015;

Håkansson et al., 2018; Huss et al., 2020), and presumably treatment response (Matute-Blanch et al., 2018). Similar to other biomarkers of neuroinflammation and tissue damage, CHI3L1 is not specific for MS and has been found to be elevated in cancer and rheumatoid arthritis (Tsuruha et al., 2002). A recent study examining the value of cNfL and CHI3L1 concluded that CHI3L1 levels were associated with spinal cord volume loss but not with brain gray matter atrophy. In contrast, cNfL was associated with brain but not with spinal cord volumes (Schneider et al., 2021). The combined measurement of CHI3L1 + cNfL could therefore provide complementary information on the location of atrophy in pwMS. Gil-Perotin et al. also provided evidence of the utility of CHI3L1 levels over NfL to discriminate different MS phenotypes (Gil-Perotin et al., 2019).

FUTURE DIRECTIONS

Standardization and Guidelines

There is now a large pool of data supporting the use of NfL in MS, directing it toward the adoption of NfL in clinical trial protocols (Kapoor et al., 2020). Evidence for use of NfL in routine clinical practice is also mounting suggesting that cNfL can complement established markers of disease activity to guide treatment strategies in MS (Reyes et al., 2020), adding further weight to the argument that NfL can be included in clinical guidelines. However, important gaps remain, particularly concerning validity, which stems from the lack (and need) of standardized values across the world. These include. standardization of NfL measurement techniques (i.e., sample collection and assay methods) and a well-defined diagnostic and prognostic cut-off levels, for both healthy individuals and MS.

Multicenter studies have reported a low variation of NfL and NfH measurements across sites and between assays (Oeckl et al., 2016; Kuhle et al., 2018), however, more is needed to evaluate variation across batches (Sharma et al., 2018). There is also a pressing need to establish ideal sampling time windows (e.g., timing with a relapse) and assessing their impact on the clinical predictive value of NfL levels in the long-term. This will enable comparisons across individuals and the optimization of resources, both in the clinical and research setting. Multicenter collaborations are therefore needed to address these gaps, with formulation of guidelines that address use and limitations of this test.

Isobaric Tags and Dried Plasma Spots

Large scale biomarker studies and routine clinical measurement of biomarkers come at a great cost (Collinson, 2015). Isobaric tags and dried plasma spot (DPS) have emerged as potential measurement substrates that could reduce costs, processing times, and the need of specialized infrastructure. A recent study analyzed NfL levels in 17 individuals using DPS and Simoa (Lombardi et al., 2020). They observed a good discrimination of ALS from healthy controls, which was comparable to the discrimination obtained using sNfL measures. However, biological interaction with other blood components may interfere with quantitative and qualitative measurements. Leoni et al. (2019) observed good sensitivity of isobaric tags to proteins linked to ALS, including neurofilament light. To our knowledge, no studies have evaluated the use of DPS to measure NfL in MS.

CONCLUSION

To date, diagnosis, management, and prognosis of MS relays on neuroimaging and clinical findings. However, the discovery of biomarkers such as NfL is turning the page toward a much desired and needed individualized medicine. NfL, a biological surrogate of CNS axonal damage, has consistently shown to reflect both clinical and subclinical changes in the activity and short-term burden of the disease. It has also proven to be an excellent indicator of treatment response and even as predictor of MS in presymptomatic individuals, which adds value to its utility as a clinical trial endpoint. The technological developments seen in the past years, and the ones yet to come, are widening the access to minimally invasive, fast, and low-cost measurements of bNfL levels. Additionally, this will permit larger and more longitudinal studies to be carried out, which in turn, will help determine and validate cut-off values according to the individual's characteristics, current treatment status, and neurological comorbidities. In the upcoming years, we may see NfL being included in best clinical practice guidelines and it being routinely and longitudinally evaluates in MS. The inclusion of NfL measures into the clinical decision making will allow for more individualized and prompt management of MS, with accurate prognosis and optimized follow-up of patients presenting with MS and those with established MS.

AUTHOR CONTRIBUTIONS

CF-A contributed to the literature review and the preparation of the manuscript. CF-A, SR, GG, and SG contributed to the design of the manuscript and critically revised the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

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Cerebrospinal Fluid Neurofilament Light Chain (NfL) Predicts Disease Aggressiveness in Amyotrophic Lateral Sclerosis: An Application of the D50 Disease Progression Model

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Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive neurodegenerative disorder. As previous therapeutic trials in ALS have been severely hampered by patients' heterogeneity, the identification of biomarkers that reliably reflect disease progression represents a priority in ALS research. Here, we used the D50 disease progression model to investigate correlations between cerebrospinal fluid (CSF) neurofilament light chain (NfL) levels and disease aggressiveness. The D50 model quantifies individual disease trajectories for each ALS patient. The value D50 provides a unified measure of a patient's overall disease aggressiveness (defined as time taken in months to lose 50% of functionality). The relative D50 (rD50) reflects the individual disease covered and can be calculated for any time point in the disease course. We analyzed clinical data from a well-defined cohort of 156 patients with ALS. The concentration of NfL in CSF samples was measured at two different laboratories using the same procedure. Based on patients' individual D50 values, we defined subgroups with high (<20), intermediate (20–40), or low (>40) disease aggressiveness. NfL levels were compared between these subgroups via analysis of covariance, using an array of confounding factors: age, gender, clinical phenotype, frontotemporal dementia, rD50-derived disease phase, and analyzing laboratory. We found highly significant differences in NfL concentrations between all three D50 subgroups ($p < 0.001$), representing an increase of NfL levels with increasing disease aggressiveness. The conducted analysis of covariance showed that this correlation was independent of gender, disease phenotype, and phase; however, age, analyzing laboratory, and dementia significantly influenced NfL concentration. We could show that CSF NfL is independent of patients' disease covered at the time of sampling. The present study provides strong evidence for the potential of

NfL to reflect disease aggressiveness in ALS and in addition proofed to remain at stable levels throughout the disease course. Implementation of CSF NfL as a potential read-out for future therapeutic trials in ALS is currently constrained by its demonstrated susceptibility to (pre-)analytical variations. Here we show that the D50 model enables the discovery of correlations between clinical characteristics and CSF analytes and can be recommended for future studies evaluating potential biomarkers.

Keywords: amyotrophic lateral sclerosis, neurofilaments, NfL, cerebrospinal fluid, prognostic biomarker

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that is predominately characterized by the progressive loss of motor neuron function. The clinical presentation of the disease varies significantly among patients, with atrophy and weakness as well as spasticity and fasciculations in limb, bulbar, and thoracic muscles. Despite constant efforts to develop new disease-modifying therapies, survival for most patients with ALS is still restricted to 2–5 years after symptom onset (Paganoni et al., 2014).

As phenotypic variability and disease course variability represent major constraints to clinical management and therapeutic trials in ALS, the search for biomarkers that can accurately predict progression is a current research priority. Previous therapeutic trials predominantly employed clinical measures such as long-term survival rates and linearly approximated declines of the ALS Functional Rating Scale-Revised (ALSFRS-R) as outcome measures (Petrov et al., 2017). The detection of significant treatment effects in these trials requires large sample sizes and consumes time and resources, which could be improved by specific pharmacodynamic or prognostic/predictive biomarkers. The importance of such biomarkers has been underlined in the recently revised Airlie House consensus criteria for clinical trial development in ALS (Van Den Berg et al., 2019).

Cerebrospinal fluid (CSF) neurofilaments are promising candidate biomarkers with prognostic implications in ALS. Neurofilaments constitute the main structural components of motor axons. Following neuroaxonal damage, increased concentrations of neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH) have been reported in both CSF and blood in various neurologic disorders (Khalil et al., 2018). While CSF pNfH has demonstrated greater diagnostic accuracy (Poesen et al., 2017), the concentration of NfL in the CSF of ALS patients reportedly correlates with both survival (Zetterberg et al., 2007; Pijnenburg et al., 2015; Gaiani et al., 2017; Gong et al., 2018; Illán-Gala et al., 2018; Rossi et al., 2018; Scarafino et al., 2018; Schreiber et al., 2018; Kasai et al., 2019; Abu-Rumeileh et al., 2020) and the disease progression rate (Tortelli et al., 2012; Lu et al., 2015; Menke et al., 2015; Steinacker et al., 2016, 2018b; Gaiani et al., 2017; Poesen et al., 2017; Andres-Benito et al., 2018; Gong et al., 2018; Rossi et al., 2018; Scarafino et al., 2018; Schreiber et al., 2018; Abu-Rumeileh et al., 2020). These findings suggest that CSF NfL concentrations at baseline may allow early stratification of patients in clinical trials according to anticipated progressiveness, thereby reducing clinical heterogeneity and enabling the detection of significant treatment effects even in smaller ALS patient cohorts.

However, the exact role of NfL in ALS is not yet entirely understood, and several challenges hamper its routine use as a biomarker in clinical trials. CSF NfL has been reported to correlate not only with the rate of disease progression but also with the clinical status at the time of lumbar puncture, as assessed by clinical scores or imaging measures of disease severity (Tortelli et al., 2012; Steinacker et al., 2016, 2018b; Gong et al., 2018; Scarafino et al., 2018). This raises the question of whether CSF NfL reflects cumulative neuroaxonal damage rather than the rate of neuroaxonal breakdown. As patients with faster disease courses have typically reached a more advanced disease stage at the time of sampling (sampling shift), these factors are inextricably intertwined in ALS patient cohorts. The temporal profile of CSF NfL throughout the disease course remains to be more precisely elucidated. The few available longitudinal studies on CSF NfL in patients with ALS comprised rather small sample sizes and reported inconsistent results (Lu et al., 2015; Steinacker et al., 2016; Poesen et al., 2017; Skillbäck et al., 2017; Benatar et al., 2018; Huang et al., 2020). Furthermore, the concentration of NfL in the CSF may be influenced by several other factors, including the presence of frontotemporal dementia (FTD) (Illán-Gala et al., 2018; Steinacker et al., 2018a), different ALS genotypes (Zetterberg et al., 2007; Huang et al., 2020), or the predominant affection of upper motor neurons (UMNs) rather than lower motor neurons (LMNs) (Rosengren et al., 1996; Gaiani et al., 2017; Schreiber et al., 2018).

An additional concern is the interlaboratory variation of CSF NfL measurements (Oeckl et al., 2016; Gray et al., 2020), as validation of biomarkers and translation into clinical trials require multicenter confirmation.

In an attempt to address the mentioned uncertainties regarding the prognostic role of CSF NfL in ALS, we applied the D50 disease progression model (Poesen et al., 2017; Prell et al., 2019; Steinbach et al., 2020) in a large-scale cross-sectional cohort. As the model addresses the phenotypic heterogeneity inherent to the disease and reduces the noise associated with the ALSFRS-R, this approach may help uncover the effect of

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised; ANCOVA, analysis of covariance; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; FTD, frontotemporal dementia; LMN, lower motor neuron; MiToS, Milano–Torino Staging System; NfL, neurofilament light chain; pNfH, phosphorylated neurofilament heavy chain; rD50, relative D50; SD, standard deviation; UMN, upper motor neuron.

disease aggressiveness on CSF neurofilament levels in a clinically diverse ALS patient cohort, while simultaneously controlling for the potential influence of disease accumulation at the time of sampling.

MATERIALS AND METHODS

Participants

All participants were recruited from the neuromuscular center at the University Hospital of Jena, Germany, between 2013 and 2020. The participants provided written and informed consent prior to study commencement, and the study was approved by the local ethics committee (Nr 3633-11/12). Two hundred seventy-three participants with available CSF NfL measurements were identified from our local specialized neuromuscular disease database. Based on clinical disease histories, a total of 238 participants could be allocated to one of the four following condition groups: (a) non-neurological controls (n = 15); (b) disease controls (n = 56), suffering from neurologic disorders other than ALS; (c) ALS mimics (n = 11), with other conditions that shared symptomatology with an ALS disease course; and (d) patients with ALS (n = 156) (**Supplementary Table 1**). Of the initial 185 ALS patients, 29 patients were excluded, either because fewer than two ALSFRS-R assessments were available (n = 16), or because the Gold Coast criteria for the diagnosis of ALS (Shefner et al., 2020) were not fulfilled (n = 13). From a total of 62 disease controls, six were excluded because of an uncertain diagnosis (n = 5) or acute intracranial bleedings (n = 1).

Diagnosis and Phenotypic Characterization of Patients With ALS

One hundred fifty-six patients fulfilled the recently defined Gold Coast criteria for the diagnosis of ALS at the time of CSF sampling (Shefner et al., 2020) and had a minimum of two ALSFRS-R scores obtained throughout the disease course. According to the revised El Escorial criteria at the time of lumbar puncture, ALS patients had either suspected, possible, laboratory-supported probable, probable, or definite ALS (Brooks et al., 2000). According to the evaluation of the entire disease history of these patients, they presented with one of the following clinical phenotypes: classic, bulbar, pyramidal, flail arm, flail leg, or respiratory or pure LMN, according to the classification by Chió et al. in 2011 (Chiò et al., 2011). The diagnosis of clinically overt frontotemporal dementia (FTD) was made by experienced neurologists at the University Hospital Jena based on clinical observations. All 6 patients diagnosed with FTD fulfilled the original Strong diagnostic criteria for the diagnosis of FTD (Strong et al., 2009, 2017).

We also estimated the number of regions (bulbar, cervical, thoracic, or lumbar) with UMN and/or LMN involvement at the time of CSF sampling. The four regions were evaluated clinically and electromyographically according to the revised El Escorial and Awaji criteria (Brooks et al., 2000; de Carvalho et al., 2008). Hence, ALS patients were divided into categories of one (none or one region), two (two regions), or three (three or four regions) regions affected by UMN and/or LMN degeneration. ALS patients were also classified according to (a) the King's staging system (Roche et al., 2012) and (b) the Milano–Torino Staging System (MiToS) (Chio et al., 2015), both calculated using the ALSFRS-R closest to the time of CSF sampling. The King's staging system allocates patients to stages I (involvement of one clinical region) to IV (respiratory or nutritional failure), whereas the MiToS System describes stages 0 (functional involvement) to IV (loss of independence in four domains) (Roche et al., 2012; Chio et al., 2015).

The D50 Disease Progression Model

To assess the impact of clinical characteristics of patient's ALS disease course on CSF NfL, the D50 disease progression model was applied (Poesen et al., 2017; Prell et al., 2020; Steinbach et al., 2020). The D50 model was chosen because it provides quantitative measures of disease aggressiveness, distinct from parameters of disease accumulation, and thus provides a framework to interpret associations with any biomarker (**Figure 1**). The model uses regularly assessed ALSFRS-R scores of each individual patient to calculate a sigmoidal state transition from full health to functional loss. Here, we applied an adaptation of the model that allows a variable presymptomatic phase of supratotal functionality up to 6 months prior to symptom onset. This approach accounts for the known uncertainties in the exact time point of first symptoms as reported by the patients, as well as a presymptomatic breakdown of motoric functional reserves. The resulting sigmoidal curve can be characterized by (a) the value D50, which describes the time taken in months from symptom onset to reach halved functionality, and (b) the dx, the time constant of functional decline. Because dx and D50 correlate linearly (**Figure 1C**), the D50 value alone provides a meaningful descriptor of patients' overall disease aggressiveness.

The ALS patient cohort could thus be divided into three groups of (a) high ($0 \leq D50 < 20$), (b) intermediate ($20 \leq D50 < 40$), and (c) low ($40 \leq D50$) disease aggressiveness. A normalization of patient's real-time sigmoidal disease trajectory to D50 yields the parameter relative D50 (rD50), an open-ended reference scale where 0 signifies disease onset and 0.5 the time point of halved functionality (**Figure 1B**). The rD50 provides an individualized time scale of accumulated disease (independent of disease aggressiveness) and was calculated for the individual time point of lumbar puncture. Patients with ALS could thus also be grouped into one of the following three phases: the early semistable phase I ($0 \leq rD50 < 0.25$), the early progressive phase II ($0.25 \leq rD50 < 0.5$), and the late progressive and stable phase (III/IV) ($0.5 \leq rD50$).

For comparability with former studies, we also calculated the more traditionally used linear disease progression rate at the time of CSF sampling, defined as (48 - ALSFRS-R at sampling)/disease duration in months **(Figure 1E)**.

CSF Collection and Analysis

All CSF samples were collected via lumbar puncture at the Department of Neurology, Jena University Hospital. The samples were centrifuged, aliquoted, and stored at −80°C within 2 h after lumbar puncture. NfL concentration was assessed using the commercially validated IBL International enzyme-linked

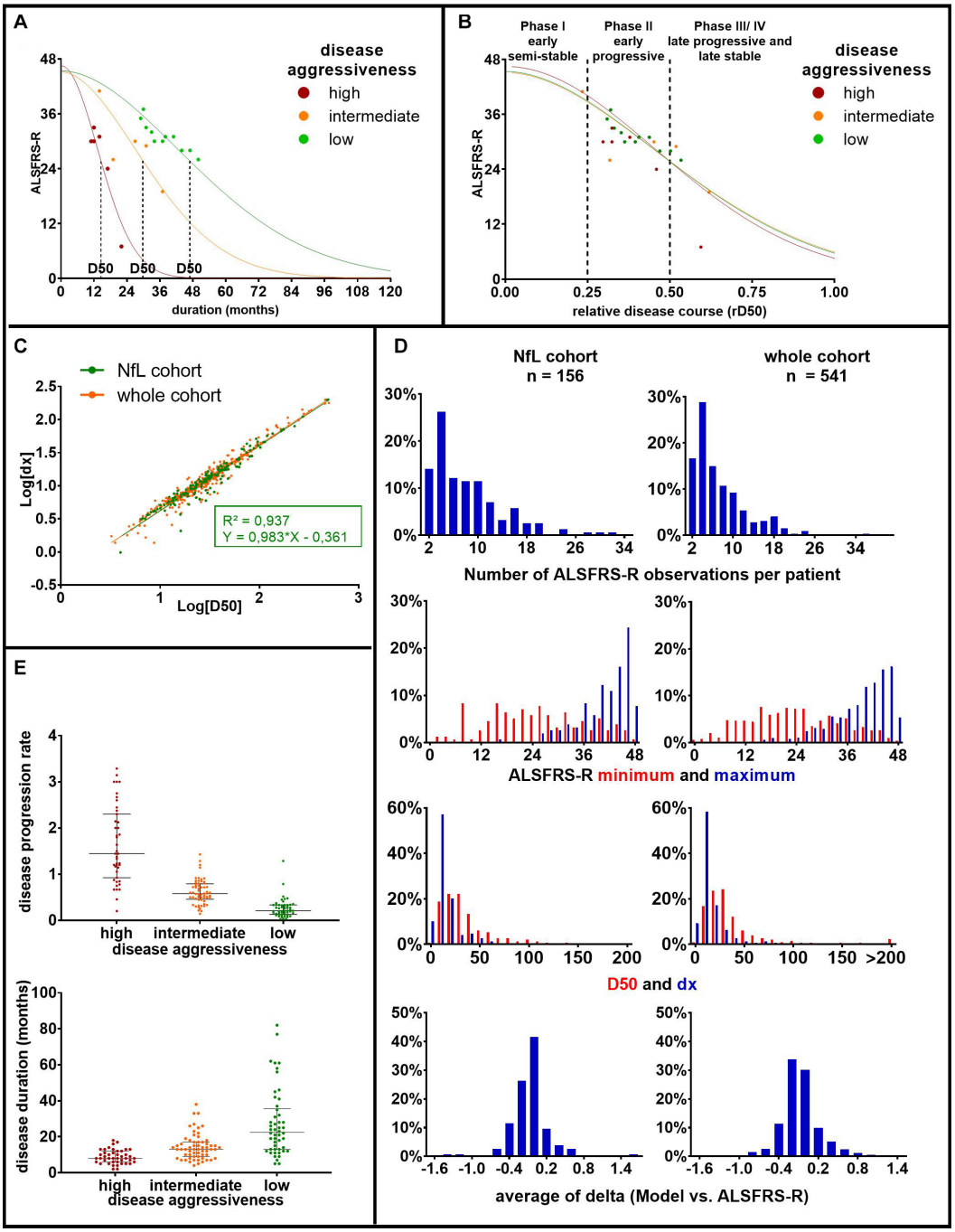

FIGURE 1 | Principles and parameters of the D50 model: **(A)** based on consecutively obtained ALSFRS-R scores (dots), a sigmoidal functional decline curve is calculated. The value D50 depicts the individual time in months since symptom onset until halved functionality, indicating the overall disease aggressiveness of each individual patient. The curves represent three example patients with either high (D50 = 14.56 months, in red), intermediate (D50 = 29.88 months, in orange), or low disease aggressiveness (D50 = 46.84 months, in green). **(B)** Normalization of patient's individual sigmoidal curves by D50 yields the parameter relative D50 (rD50). rD50 describes the individual disease covered and facilitates the comparison of vastly differing progression types. **(C)** The parameter D50 linearly correlates with the time constant of ALSFRS-R decline (dx) in this, as well as in other ALS cohorts. Thus, D50 alone can be used to describe patients' disease aggressiveness. **(D)** Histograms of pertinent disease variables for the patients of the current cohort (NfL cohort), as well as all ALS patient data available in our center (whole cohort). It illustrates that the current cohort well coincides with the entire ALS patient cohort treated at our center. **(E)** Scatterplots of patients' disease progression rate and disease duration at the time of sampling, subdivided by the three D50 subgroups in our cohort: (a) high ($0 \leq D50 < 20$, in red), (b) intermediate ($20 \leq D50 < 40$, in orange), and (c) low ($40 \leq D50$, in green) disease aggressiveness. It illustrates large variations of the disease progression rate, especially within the high aggressive subgroup. Bars indicate median and interquartile range. ALS, amyotrophic lateral sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised; rD50, relative D50; NfL, neurofilament light chain.

immunosorbent assay (ELISA) kit at two different European laboratories: (a) in Germany (n = 140) and (b) in Belgium (n = 99). All samples and standards were assayed in duplicate and in accordance with manufacturer instructions; intra-assay and interassay variations were ≤10%, and ≤20%, respectively.

Statistical Analysis

Statistical analyses were performed using the SPSS® Statistics software program (v27.0.0.0 IBM®, Chicago, IL, United States). For graphical representation of data, GraphPad Prism was used (v9.0.0 for Windows, GraphPad Software, San Diego, CA, United States). Normal distribution of variables was assessed with the Shapiro–Wilk test. Normal distribution of NfL concentration was achieved via log transformation, and log[NfL] was used for parametric testing. Two-sample t-tests were used for comparison of Log[NfL] concentrations between ALS patients and control groups. Receiver operating characteristic curves were used to calculate the sensitivity and specificity of CSF NfL for differentiating ALS from the control groups. The optimal cutoff was calculated with the Youden Index.

To evaluate differences in NfL concentrations between different ALS subgroups, a one-way analysis of covariance (ANCOVA) was applied, followed by pairwise *post hoc* tests with Bonferroni correction. For the comparison of low, intermediate, and high disease aggressiveness subgroups, the following covariates were applied: age, sex, FTD, laboratory of NfL measurement, clinical phenotype, and disease phase.

In our ALS cohort, a significant sampling shift occurred, which was previously observed in other cohorts analyzed using the D50 model (**Table 1**): patients with slow and intermediate progression were still in the earlier phases of the disease at the time of sampling, whereas patients with fast progression had already reached later disease phases by the time they were referred to our center, and lumbar puncture was performed. Therefore, the covariate disease phase did not meet the assumption for ANCOVA of homogenous distribution over the three subgroups. We therefore conducted an additional ANCOVA in a filtered ALS cohort, in which patients of all disease phases were equally distributed throughout the three aggressiveness subgroups (**Supplementary Table 2**).

A one-way ANCOVA was conducted to compare CSF Log[NfL] concentration of the three disease phases, while controlling for disease aggressiveness, FTD, clinical phenotype, age, gender, and laboratory of measurement.

Linear regression analysis and Spearman correlation was used to assess correlations between NfL, D50, and rD50 at the time of sampling. Pearson correlation was used to assess correlation between paired Log[NfL] measurements from the two centers in Germany and Belgium. Differences between CSF NfL concentrations of ALS patients with and without FTD were tested with a Mann–Whitney U test.

For survival analyses, ALS patients were divided into three groups with low (Log[NfL] < 3.651), intermediate (3.651 ≤ Log[NfL] < 4.149), and high (4.149 ≤ Log[NfL]) CSF NfL concentrations, with cutoffs derived from the estimated marginal means of our previously described ANCOVA (comparing disease aggressiveness subgroups). The Kaplan–Meier method was used for survival analyses, and subgroups were compared with a log–rank test. 97 patients (13 with low, 51 with intermediate, and 33 with high CSF NfL levels) reached the endpoint death or tracheostomy, whereas the remaining 59 patients were censored. Statistical significance was defined as $p < 0.05$.

RESULTS

Diagnostic Performance of CSF NfL in ALS

Cerebrospinal fluid Log[NfL] levels were significantly higher in the ALS group (mean = 3.87, SD = 0.37) as compared to the non-neurological control (mean = 2.72, SD = 0.27, $p < 0.001$), disease control (mean = 3.18, SD = 0.38, $p < 0.001$), and ALS mimic groups (mean = 3.20, SD = 0.19, $p < 0.001$). When distinguishing ALS from disease controls, the area under the curve (AUC) was 0.895 (0.849–0.9413), sensitivity was 87.8%, and specificity was 78.6% at a cutoff of 2,946.00 pg/mL. For the differentiation between ALS and ALS mimics, the AUC was 0.941 (0.897–0.985), sensitivity was 91.0%, and specificity was 90.9% at a cutoff of 2,259.55 pg/mL. A cutoff of 1,620.5 pg/mL distinguished ALS patients from non-neurological controls with a sensitivity of 96.15% and specificity of 100% [AUC = 0.993 (0.984–1.002)] (**Figure 2**).

Cerebrospinal fluid NfL levels did not significantly differ between different ALS phenotypes [$F(6,149) = 0.925$, $p = 0.479$]. Patients with FTD had significantly higher CSF NfL levels relative to those without FTD ($U = 208.0$, $Z = -2.23$, $p < 0.05$).

Cohort of Patients With ALS

Detailed demographic and clinical data of ALS patients are shown in **Table 1**. Age, gender, and laboratory of analysis did not significantly differ between patients with high, intermediate, or low disease aggressiveness. The rD50 at the time of lumbar puncture, as well as the rD50-derived disease phase, showed significant differences between these three subgroups, as patients with lower disease aggressiveness were still in the earlier phases of the disease due to the sampling shift. Accordingly, the more traditionally used disease metrics, namely, the ALSFRS-R, the King's and MiToS stages, the stage of diagnostic certainty according to the revised El Escorial criteria (Brooks et al., 2000), the disease duration (time between symptom onset and lumbar puncture), and the disease progression rate, differed significantly between the three subgroups. Other disease characteristics, such as ALS phenotype, presence of FTD, or Riluzole intake, were homogenously distributed throughout the three subgroups.

CSF NfL Predicts Disease Aggressiveness

The ANCOVA showed a significant main effect for CSF Log[NfL] (pg/mL) concentrations of the three disease aggressiveness subgroups [$F(2,147) = 30.055$, $p < 0.001$]. *Post hoc* pairwise comparisons of the estimated marginal means showed that CSF Log[NfL] was highest in the highly aggressive disease subgroup

TABLE 1 | Demographic and clinical data for patients with ALS (n = 156).

		Disease aggressiveness			*p*
		High (D50 < 20)	**Intermediate (20 ≤ D50 < 40)**	**Low (D50 ≥ 40)**	
n		43	61	52	
Neurofilament light chain (NfL) measurements					
NfL (pg/mL)[$]		14,500.0 (7,883.0–24,680.0)	8,959.67 (4,410.5–12,157.5)	4,426.69 (2,879.5–7,333.5)	<0.001*
Laboratory: Germany/Belgium		29/14 67.4%/32.6%	35/26 57.4%/42.6%	40/12 76.9%/23.1%	0.253
Demographics					
Age at lumbar puncture		64.3 ± 9.54	63.33 ± 10.47	61.42 ± 10.95	0.384
Gender:Male/female		23/20 53.5%/46.5%	37/24 60.7%/39.3%	34/18 65.4%/34.6%	0.514
D50 disease progression model parameters					
D50[$]		13.62 (9.40–16.14)	28.81 (23.07–31.73)	62.58 (46.12–96.61)	–
rD50[$]		0.37 (0.23–0.45)	0.23 (0.17–0.32)	0.18 (0.10–0.32)	<0.001*
Phase	I (rD50 < 0.25)	11 (25.6%)	32 (52.2%)	33 (63.5%)	0.001*
	II (0.25 ≤ rD50 < 0.5)	27 (62.8%)	27 (44.3%)	19 (36.5%)	
	III/IV (rD50 ≥ 0.5)	5 (11.6%)	2 (3.3%)	0 (0%)	
Traditional disease metrics					
ALSFRS-R at lumbar puncture[$]		35 (29–40)	41 (38.50–44)	42 (39.25–45.75)	<0.001*
Disease progression rate[$]		1.64 (1.05–2.30)	0.60 (0.46–0.74)	0.21 (0.13–0.33)	<0.001*
Disease duration at lumbar puncture (mo)[$]		8 (2–18)	13 (4–38)	23.50 (5–212)	<0.001*
King's stage	I	10 (23.3%)	20 (32.8%)	21 (40.4%)	0.008*
	II	11 (25.6%)	24 (39.9%)	24 (46.2%)	
	III	17 (39.5%)	12 (19.7%)	7 (13.5%)	
	IV a	3 (7%)	1 (1.6%)	0 (0%)	
	IV b	2 (4.7%)	4 (6.6%)	0 (0%)	
	V	0 (0%)	0 (0%)	0 (0%)	
MiToS stage	0	21 (48.8%)	52 (85.2%)	46 (88.5%)	<0.001*
	I	18 (41.9%)	7 (7%)	6 (11.5%)	
	II	4 (9.3%)	2 (3.3%)	0 (0%)	
	III–V	0 (0%)	0 (0%)	0 (0%)	
ALS phenotype	Classic	21 (48.8%)	38 (62.3%)	33 (63.5%)	0.058
	Bulbar	18 (41.9%)	19 (31.1%)	9 (17.3%)	
	Pyramidal	3 (7%)	4 (6.6%)	5 (9.6%)	
	Respiratory	1 (2.3%)	0 (0%)	0 (0%)	
	Flail arm	0 (0%)	0 (0%)	3 (5.8%)	
	Flail leg	0 (0%)	0 (0%)	1 (1.9%)	
	Pure LMN	0 (0%)	0 (0%)	1 (1.9%)	
Revised El Escorial criteria	Definitive	10 (23.3%)	3 (4.9%)	1 (1.9%)	<0.001*
	Probable	22 (51.2%)	33 (54.1%)	19 (36.5%)	
	Laboratory-supported probable	8 (18.6%)	20 (32.8%)	18 (34.6%)	
	Possible	3 (7%)	3 (4.9%)	9 (17.3%)	
	Suspected	0 (0%)	2 (3.3%)	5 (9.6%)	
Presence of FTD: yes/no		2/41 4.7%/95.3%	3/58 4.9%/95.1%	1/51 1.9%/98.1%	0.671

(Continued)

TABLE 1 | Continued

	Disease aggressiveness			*p*
	High (D50 < 20)	**Intermediate (20 ≤ D50 < 40)**	**Low (D50 ≥ 40)**	
Riluzole treatment: yes/no	42/1 97.7%/2.1%	60/1 98.4%/1.6%	49/3 94.2%/5.8%	0.671

Continuous variables with normal distribution are expressed as mean with standard deviation. Categorical variables are expressed as number and percentage. For the comparison of demographic and clinical variables among the three aggressiveness subgroups, analyses of variance, Kruskal–Wallis tests, χ^2 tests, or Fisher–Freeman–Halton exact tests were applied where appropriate.

$Non-parametric nominal variables, represented as median and interquartile range.

**Statistical significance at $p < 0.05$.*

ALS, amyotrophic lateral sclerosis; ALSFRS-R, Revised ALS Functional Rating Scale; FTD, frontotemporal dementia; LMN, lower motor neuron; MiToS, Milano–Torino Staging System; NfL, neurofilament light chain; rD50, relative D50.

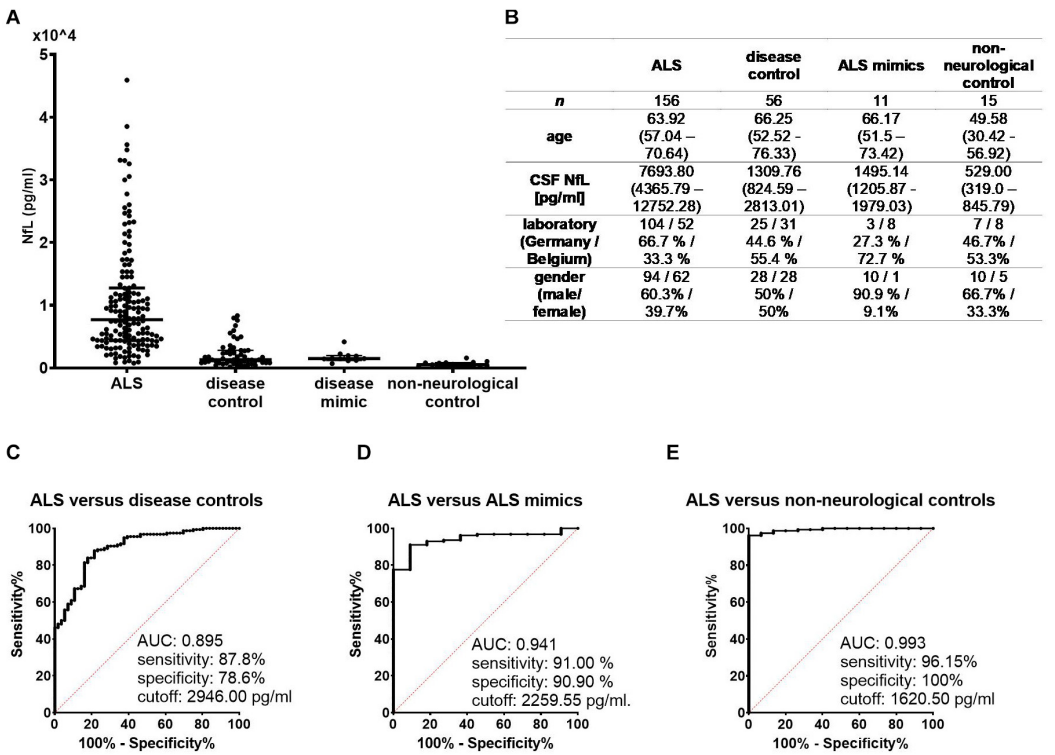

FIGURE 2 | (A) NfL concentrations in cerebrospinal fluid were significantly higher in the ALS group compared to each control group ($p < 0.001$ for each pairwise comparison). **(B)** Demographic and clinical data of the four condition groups are expressed as either median with interquartile range or as number and percentages. Receiver operating characteristic curves illustrate the diagnostic performance of NfL in distinguishing ALS from disease controls **(C)**, ALS mimics **(D)**, and non-neurological controls **(E)**. ALS, amyotrophic lateral sclerosis; AUC, area under the curve; NfL, neurofilament light chain.

(mean = 4.149), lower in the intermediate aggressiveness subgroup (mean = 3.857) and lowest in patients with low disease aggressiveness (mean = 3.651; $p < 0.001$ for all pairwise comparisons) **(Figure 3)**. The covariates age, [$F(1,147) = 12.451$, $p < 0.001$], laboratory of analysis [$F(1,147) = 13.748$, $p < 0.001$], and FTD [$F(1,147) = 6.176$, $p = 0.014$] were also significantly related to CSF Log[NfL], whereas gender, disease phenotype, and phase showed no impact.

The main effect of disease aggressiveness on Log[NfL] remained in a similar ANCOVA for the filtered cohort (with homogenous distribution of disease phases over the three aggressiveness subgroups). Most importantly, the disease phase did not have a significant effect on Log[NfL] concentrations (**Supplementary Table 3**).

There was a negative correlation between the D50 parameter and CSF NfL ($p < 0.001$, $\rho = -0.553$) (**Figure 4A**). The linear regression analysis showed that 31.3% of the variation in CSF NfL can be explained by the D50 parameter ($R^2 = 0.313$, Log[NfL] = 4.734 - 0.581 × Log[D50], $p < 0.001$). This correlation remained significant when analyzing patients in disease phases I and II separately (phase I: n = 76, $p < 0.001$, $\rho = -0.528$, phase II: $n = 73$, $p < 0.001$, $\rho = -0.521$). Patients in

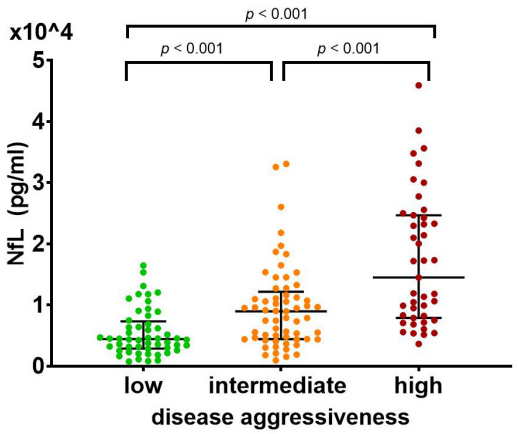

FIGURE 3 | CSF NfL differs significantly between patients with (a) high ($0 \leq$ D50 < 20, in red), (b) intermediate ($20 \leq$ D50 < 40, in orange), and (c) low ($40 \leq$ D50, in green) disease aggressiveness. This effect remained significant after controlling for clinical phenotype, presence of frontotemporal dementia, age, gender, disease phase, and laboratory of measurement in an ANCOVA ($p < 0.001$). *Post hoc* pairwise comparisons of the estimated marginal means confirmed an increase of NfL levels with increasing disease aggressiveness (low: 4,477.13, intermediate: 7,194.49, high: 14,092.89; $p < 0.001$ for all pairwise comparisons). Bars indicate median and interquartile range. ANCOVA, analysis of covariance; NfL, neurofilament light chain.

phase III/IV showed a similar tendency of negative correlation, but did not reach statistical significance, most likely due to the small sample size ($n = 7$, $p < 0.337$, $\rho = -0.429$).

CSF NfL Is Independent of Disease Phase and Number of Affected Regions

There was no significant main effect of disease phase on Log[NfL] concentrations [$F(2,147) = 1.692$, $p = 0.188$] in the respective ANCOVA, but the covariates disease aggressiveness $F(1,147) = 61.032$, $p < 0.001$), age [$F(1,147) = 13.603$, $p < 0.001$], laboratory of analysis [$F(1,147) = 13.927$, $p < 0.001$], and FTD [$F(1,147) = 6.284$, $p = 0.013$] showed a significant impact.

For the whole ALS patient cohort, a correlation between CSF NfL and rD50 was noted ($p = 0.005$, $\rho = 0.224$); however, this did not retain significance when stratifying patients into the three D50 subgroups (**Figure 4B**). This calculated correlation of CSF NfL with rD50 is thus likely attributable to the aforementioned cohort-specific intercorrelation between the parameters rD50 and D50, resulting from the sampling shift ($p < 0.001$, $\rho = -0.432$) (**Supplementary Figure 1**).

There were no significant differences in the CSF Log[NfL] concentration when stratifying patients according to the number of regions with UMN [$F(2,153) = 2.858$, $p = 0.060$] or LMN [$F(2,153) = 0.659$, $p = 0.519$] involvement at the time of sampling. Also, in combination, the number of regions with UMN and/or LMN affection did not have a significant effect on the CSF Log[NfL] concentrations [$F(2,153) = 1.403$, $p = 0.249$] (**Supplementary Table 4**).

CSF NfL Predicts Survival in Patients With ALS

Kaplan–Meier survival curves and log–rank tests showed significant differences in survival [$\chi^2(2) = 56.505$, $p < 0{,}001$], when trichotomizing the ALS patients into groups with high ($n = 36$), intermediate ($n = 77$), and low ($n = 43$) CSF Log[NfL] concentrations based on disease aggressiveness–adjusted marginal means (**Figure 5**).

Interlaboratory Variation and Paired Sample Comparison

Cerebrospinal fluid samples from 57 patients with ALS were pairwise analyzed in both laboratories. The mean coefficient of variation of CSF NfL measurements between laboratories was 21.19% (SD = 24.75) for these 57 samples. There was a strong positive correlation between paired CSF Log[NfL]

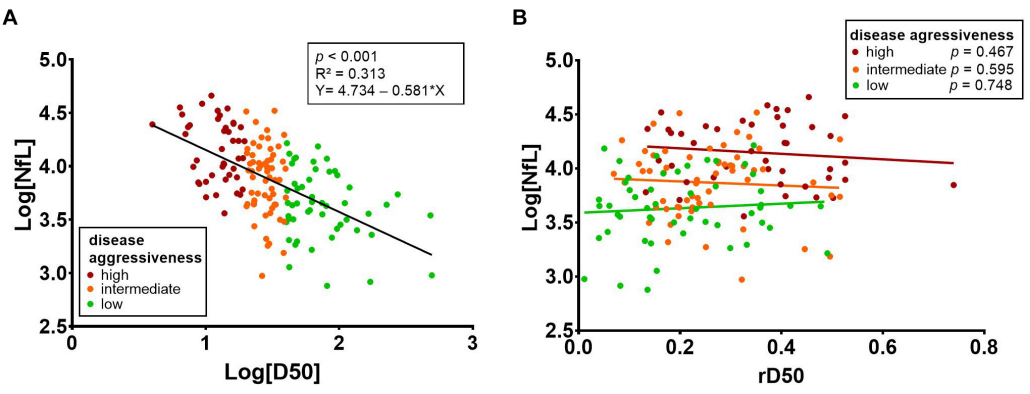

FIGURE 4 | (A) There was a negative correlation between the D50 parameter and CSF NfL ($p < 0.001$, $\rho = -0.553$). Linear regression analysis showed that 31.3% of the variation in CSF NfL can be explained by the D50 parameter ($R^2 = 0.313$, Log[NfL] = 4.734 - 0.581 × Log[D50]; $p < 0.001$). **(B)** Stratification of patients into the three aggressiveness subgroups (based on D50) reveals that there is no significant correlation of CSF NfL with rD50. CSF, cerebrospinal fluid; rD50, relative D50; NfL, neurofilament light chain.

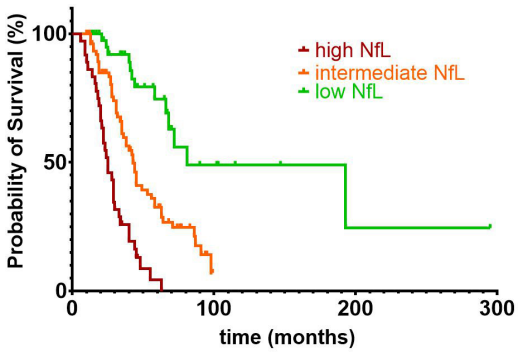

FIGURE 5 | Kaplan–Meier survival curves and log-rank test showed significant differences in survival [$\chi^2(2) = 56.505$, $p < 0.001$], when trichotomizing the ALS patients into groups with high ($n = 36$), intermediate ($n = 77$), and low ($n = 43$) CSF NfL concentrations. Estimated marginal means of the previously described analysis of covariance were used for the subdivision of ALS patients into groups with high (Log[NfL] > 4.149), intermediate (3.651 < Log[NfL] < 4.149), and low (Log[NfL] < 3.651) CSF NfL concentrations. Of the 156 ALS patients included in the survival analysis, 97 patients (13 with low, 51 with intermediate, and 33 with high CSF NfL levels) reached the endpoint death or tracheostomy, whereas the remaining 59 patients were censored. ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; NfL, neurofilament light chain.

measurements from both laboratories ($r = 0.918$, $p < 0.001$) (**Supplementary Figure 2**).

DISCUSSION

In the present study we showed that CSF NfL levels in ALS patients significantly differ between patients according to their D50-derived disease aggressiveness. In addition to interlaboratory variation, significant effects for age and FTD on CSF NfL concentrations were also noted. However, the rD50 value and the derived disease phase did not influence NfL levels.

Associations between CSF NfL and the disease progression rate in ALS have been previously proposed (Tortelli et al., 2012; Lu et al., 2015; Menke et al., 2015; Steinacker et al., 2016, 2018b; Poesen et al., 2017; Andres-Benito et al., 2018; Gong et al., 2018; Scarafino et al., 2018; Schreiber et al., 2018; Abu-Rumeileh et al., 2020). However, the interpretation of these analyses remained constrained, because of the incomplete evaluation of confounding factors that influence NfL levels and/or the lack of longitudinal validation studies. Moreover, former results were limited to correlations with single disease metrics, such as the disease progression rate or the ALSFRS-R. This neglects the huge interindividual heterogeneity of disease courses in ALS, requiring a quantifiable framework within which to interpret patients' individualized disease trajectories and putative biomarkers.

We therefore applied the D50 model that provides quantifications for both measures of disease aggressiveness (D50), as well as the amount of disease covered (rD50, phase) at the time of CSF sampling to generate a large-scale pseudo–longitudinal analysis. This allowed us to demonstrate that CSF NfL is increased in patients with higher disease aggressiveness, even after adjustment for interlaboratory variation, age, gender, ALS phenotype, presence of FTD, and disease phase at the time of sampling. Former studies showed correlations between CSF NfL and linearly approximated progression rates. Most of these studies calculated the decline in ALSFRS-R from symptom onset until CSF sampling (Tortelli et al., 2012; Lu et al., 2015; Menke et al., 2015; Steinacker et al., 2016, 2018b; Poesen et al., 2017; Andres-Benito et al., 2018; Gong et al., 2018; Scarafino et al., 2018; Schreiber et al., 2018) or from symptom onset until disease diagnosis (Gaiani et al., 2017; Rossi et al., 2018). However, linear mixed-effects models using consecutively obtained ALSFRS-R scores have also been used to demonstrate associations with CSF NfL levels (Huang et al., 2020).

All these studies presume a linear decline of the ALSFRS-R score over time, despite prior observations that the rate of decline varies with disease progression and follows a curvilinear course (Gordon et al., 2010). Moreover, the calculation of progression rates based on a single score is highly susceptible to the known intrarater and interrater variability associated with ALSFRS-R assessments (Bakker et al., 2020). We therefore propose that the D50 model provides a more accurate representation of clinical progression, as it calculates an individualized sigmoidal curve of functional deterioration for each patient (Poesen et al., 2017; Prell et al., 2020; Steinbach et al., 2020).

The association between CSF NfL and survival in our ALS cohort further substantiates the ability of this biomarker to reflect prognosis in these patients and is in accordance with previous studies on CSF NfL and survival in ALS (Zetterberg et al., 2007; Pijnenburg et al., 2015; Gaiani et al., 2017; Gong et al., 2018; Illán-Gala et al., 2018; Rossi et al., 2018; Scarafino et al., 2018; Schreiber et al., 2018; Kasai et al., 2019).

The lack of a significant effect of the disease phase on NfL levels indicates that CSF concentrations remain longitudinally stable throughout the disease course. This suggests that any baseline NfL measurement is able to predict patients' disease aggressiveness, independent of the time point of CSF sampling. While longitudinal studies on CSF NfL concentrations in ALS would be best suited to support this observation, these are scarce and mostly comprise small numbers of patients. Some longitudinal studies reported rather stable levels throughout the disease course (Benatar et al., 2018; Huang et al., 2020), but slightly decreasing (Steinacker et al., 2016) and increasing concentrations in specific subpopulations of ALS patients (Lu et al., 2015; Poesen et al., 2017; Skillbäck et al., 2017) have been reported as well.

Several longitudinal studies following presymptomatic ALS-causing mutation carriers until the occurrence of manifest disease have aided in the understanding of the temporal profile of CSF NfL concentrations (Benatar et al., 2018, 2019). In these studies, while asymptomatic patients initially had CSF NfL concentrations similar to controls, increases were observed more than a year prior to phenoconversion (defining a presymptomatic stage) (Benatar et al., 2018, 2019). Recent findings also suggest that the duration of this presymptomatic stage may differ in accordance to the patient's survival (Benatar et al., 2019).

Studies have also reported correlations between CSF NfL and the ALSFRS-R at the time of sampling (Tortelli et al., 2012; Steinacker et al., 2016, 2018b; Gong et al., 2018; Scarafino et al., 2018), suggesting that NfL reflects cumulative neuroaxonal damage (disease accumulation) rather than the rate of neuronal breakdown (i.e., aggressiveness). We would like to emphasize that both aspects (disease accumulation and aggressiveness) are inherently interdependent in ALS cohorts, as patients with higher disease aggressiveness typically have reached a more advanced disease phase at the time of referral to ALS centers (sampling shift).

Moreover, most studies on neurofilaments performed univariate analyses to assess associations between clinical metrics and CSF NfL concentrations and neglected possible confounders. In one multivariate study by Gaiani et al., a repeated-measures ANCOVA was performed to investigate the effects of CSF NfL, ALS subtype, age, disease progression rate, gender, and cognitive impairment on longitudinal ALSFRS-R and MiToS scores. It was shown that all covariates, except cognitive impairment, exhibited significant effects on the functional-impairment scores (Gaiani et al., 2017). Another recent study investigated the effect of several clinical predictors of prognosis (including age, sex, C9ORF72 status, site of onset, baseline ALSFRS-R, and disease progression rate) on the ALSFRS-R slope in a multivariate model and demonstrated that serum NfL adds prognostic value to the model, but a comparable analysis on CSF NfL was lacking (Benatar et al., 2020). However, to the best of our knowledge, no former study has used multivariate analysis to probe the impact of several disease-specific variables on CSF NfL levels in ALS.

The present study provides strong evidence that CSF NfL reflects overall disease aggressiveness in ALS, independent of disease accumulation. This supports the concept that NfL and, more broadly, neurofilament proteins reflect disease activity. They might be directly linked to the pathophysiological process itself rather than being a collateral by-product of neuronal degeneration (Julien, 2001; Petzold, 2005). NfL may thus be used to directly monitor the therapeutic effects of neuroprotective or other disease-modifying drugs in clinical trials, where a positive therapeutic effect may be reflected by a reduction in the rate of release of NfL into the CSF. There is currently a growing momentum for the implementation of neurofilaments as secondary endpoints in such trials, with first promising findings in ALS (Miller et al., 2020), as well as spinal muscular atrophy patients (Olsson et al., 2019) under disease-modifying treatments. Our data suggest that CSF NfL represents a suitable monitoring biomarker for ALS that might be sensitive to therapeutic regimens aimed at decreasing disease aggressiveness. However, future longitudinal studies would be needed to assess its potential as an outcome measure for long-term treatment in ALS.

Besides disease aggressiveness, three covariates exhibited statistically significant effects on CSF NfL levels of ALS patients. In accordance with previous studies, age showed a positive association with CSF NfL (Vågberg et al., 2015; Gong et al., 2018; Steinacker et al., 2018b; Sun et al., 2020). This most likely reflects the degenerative process in the brain associated with normal aging, which leads to a slowly progressive rise of neurofilaments in the CSF. The ELISAs were performed in two different laboratories, and the site of analysis showed a statistically significant impact on NfL concentrations in the CSF. Stability issues of NfL measurements have been reported in previous multicentric studies on NfL and have been related, *inter alia*, to differences in perianalytical procedures (Petzold et al., 2010; Oeckl et al., 2016; Gray et al., 2020). This underlines the necessity for the implementation of standard operating procedures and round-robin tests. However, the coefficient of variation between measurements of both participating laboratories in this study was lower than previously reported for the same ELISA kit (Petzold et al., 2010; Miller et al., 2016; Gray et al., 2020), and the interlaboratory variations did not obscure the highly significant effect of disease aggressiveness on CSF NfL. Higher NfL levels in ALS patients with a concomitant diagnosis of FTD in our study are also in accordance with previous reports (Illán-Gala et al., 2018; Steinacker et al., 2018a).

We did not find a significant association between CSF NfL and the number of regions affected by UMN or LMN degeneration at the time of CSF collection. This further corroborates the notion that NfL levels are independent of disease accumulation. Previous studies, however, have reported conflicting results. CSF NfL was reported to increase with increasing number of regions affected by both UMN and LMN degeneration (Poesen et al., 2017); several studies also showed that NfL correlated with UMN burden (defined clinically or by neuroimaging) but not with the extent of LMN damage (Menke et al., 2015; Gong et al., 2018; Schreiber et al., 2018). Conversely, a recent study identified a significant association of NfL with the number of regions affected by LMN degeneration, but not UMN damage (Abu-Rumeileh et al., 2020).

This study is not without limitations. Comprehensive genetic profiles were not available for the entire ALS cohort. Given that CSF NfL levels are reported to be higher in patients with *C9orf72* mutations (Huang et al., 2020) and lower in those with *SOD1* mutations (Zetterberg et al., 2007), this may also represent a confounding factor. Further studies are needed to clarify if genotype-specific differences exist independent of disease aggressiveness, as, for example, *C9orf72* expansion carriers are known to have a worse prognosis relative to patients with sporadic ALS or other familial mutations (Miltenberger-Miltenyi et al., 2019; Rooney et al., 2019). The presence of clinically overt FTD was assessed, but this should be examined in more detail in future studies, as previous data have indicated links between cognitive deterioration and NfL levels (Illán-Gala et al., 2018; Delaby et al., 2020). Furthermore, this study is limited to the analysis of NfL concentrations in the CSF. Owing to recent technical advances, assessment of serum NfL is becoming increasingly available and holds promise as a prognostic biomarker for ALS (Benatar et al., 2020). However, future large-scale studies with matched assessments in both serum and CSF are necessary to adequately compare the prognostic potential of NfL in both biofluids. While serum and CSF levels of NfL are known to correlate well (Gille et al., 2019; Benatar et al., 2020), the considerably less invasive manner of collection speaks in favor of using blood biomarkers. However,

taking into consideration the proximity of CSF to the key pathological processes in ALS, we posit that CSF analyses should still play an important role in future research, as relevance has been demonstrated in this and other studies. Furthermore, a baseline lumbar puncture constitutes an essential step in the diagnostic workup of any patient with (suspected) ALS. Future studies should also incorporate pNfH and multicenter data, in order to fully explore the biomarker potential of neurofilaments.

Given the number of pseudolongitudinal CSF NfL data points analyzed in this study, our findings provide strong evidence for the ability of CSF NfL to reflect the rate of neuroaxonal degeneration in ALS and its potential to serve as a biomarker in future clinical trials. We show that the D50 progression model is an easily applicable and precise tool for investigating associations between biomarkers and clinical parameters in a heterogeneous ALS cohort. We recommend the use of this model for future ALS biomarker studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Jena University Hospital Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MD, RS, NG, and JG contributed to conception and design of the study. JG developed the D50 model and the database. JG, BS, MD, RS, NG, and KM performed the data curation. NG and KM conducted the laboratory analyses. MD and RS performed the statistical analysis. MD wrote the first draft of the manuscript. MD, NG, and RS wrote sections of the manuscript. JG and OW provided the funding acquisition. JG conducted the Project administration and supervision. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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Tracing Neurological Diseases in the Presymptomatic Phase: Insights From Neurofilament Light Chain

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The identification of neurological diseases in their presymptomatic phase will be a fundamental aim in the coming years. This step is necessary both to optimize early diagnostics and to verify the effectiveness of experimental disease modifying drugs in the early stages of diseases. Among the biomarkers that can detect neurological diseases already in their preclinical phase, neurofilament light chain (NfL) has given the most promising results. Recently, its measurement in serum has enabled the identification of neurodegeneration in diseases such as multiple sclerosis (MS) and Alzheimer's disease (AD) up to 6–10 years before the onset of symptoms. Similar results have been obtained in conditions such as frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS), up to 2 years before clinical onset. Study of the longitudinal dynamics of serum NfL has also revealed interesting aspects of the pathophysiology of these diseases in the preclinical phase. This review sought to discuss these very recent findings on serum NfL in the presymptomatic phase of neurological diseases.

Keywords: neurofilament light chain, multiple sclerosis, Alzheimer's disease, frontotemporal lobar degeneration, amyotrophic lateral sclerosis, presymptomatic

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INTRODUCTION

Reliable cerebrospinal fluid (CSF) and blood biomarkers allow for the *in vivo* measurement of axonal damage in neurological diseases. The detection of axonal damage has a variety of potentially useful experimental and clinical implications. Among them, it might serve as a sort of "alarm system" that can reveal the presence of a central nervous system (CNS) disease before its clinical onset. The identification of a neurological disease in its presymptomatic phase may have different benefits, including the expansion of the window of therapeutic opportunities. For such an "alarm system" to be efficient, the "sensor" of axonal damage has to be easily measurable, sensitive, reliable, and reproducible. In the case of a fluid biomarker, it should have robust evidence of validity, and it should be measurable through easy-to-perform longitudinal sampling.

Among axonal damage biomarkers, neurofilament light chain (NfL) is the best in terms of meeting these requirements (Gaetani et al., 2019). As a subunit of neurofilaments, it is released in CNS interstitial space upon axonal injury. From there, it reaches CSF and blood through dynamics

that are still not completely understood (Gafson et al., 2020), but its concentrations in these biofluids are strongly correlated (Disanto et al., 2017).

Two enzyme-linked immunosorbent assays (ELISA) are currently available for measuring NfL in the CSF (Norgren et al., 2003; Gaetani et al., 2018), and ultrasensitive methods (such as electrochemiluminescence and single molecule array) have been developed to measure it at a lower concentration in blood (Rissin et al., 2010; Kuhle et al., 2015).

NfL has been demonstrated to reflect the severity of neurological diseases, as it would be expected from a biologically valid marker of axonal damage. From degenerative to inflammatory CNS diseases, NfL correlates with the severity of the clinical pictures in different functional systems, ranging from motor to cognition, and has clear predictive properties (Gaetani et al., 2019). The recent availability of assays that are able to measure NfL in the blood led to the investigation of its variations over time in individuals at high risk for neurological diseases. Studies have demonstrated that blood NfL can trace the presymptomatic course of different CNS disorders (Parnetti et al., 2019).

In this review, we summarize evidence on NfL in the presymptomatic phase of neurological diseases. For each disease, we will discuss its potential in implementing clinical research. Finally, we will provide insights on the contribution of NfL in understanding the early pathophysiology of CNS diseases.

NfL IN PRESYMPTOMATIC MULTIPLE SCLEROSIS

The natural history of multiple sclerosis (MS), the commonest chronic inflammatory disease of the CNS, is characterized by a long course, with clinical onset in early adulthood (Filippi et al., 2018). Both the possibility of a pediatric onset and the frequent detection at the time of diagnosis of inactive demyelinating lesions on brain magnetic resonance imaging (MRI) (Rovira et al., 2009) suggest that MS may have a long presymptomatic phase. Different disease-modifying drugs (DMDs) are available for MS treatment, and their efficacy often relies on an early diagnosis (Comi et al., 2017). Ideally, the detection of the disease in its presymptomatic phase would allow for the earliest treatment possible.

The potential of NfL in presymptomatic MS has been retrospectively tested in a case control study performed on United States military personnel who had serum samples stored in government repositories. Serum NfL was found to be higher in healthy soldiers who later developed MS compared to those who did not, with a median time between serum collection and MS onset of 6 years (range: 4–10). Of interest, the difference in serum NfL tended to increase as the onset of the disease approached, with a marked increase at the time of the first clinical manifestation. Intraindividual variations of serum NfL were also associated with a higher risk of subsequent MS development (Bjornevik et al., 2019). This suggests that the presymptomatic phase of MS can last up to 6 years and that, during this phase, serum NfL tends to increase, with a peak preceding the first clinical manifestation.

Similar findings have been found with CSF NfL in radiologically isolated syndrome (RIS), i.e., that condition characterized by the incidental finding of brain MRI abnormalities highly suggestive of MS in individuals who are asymptomatic or have non-MS specific symptoms (Okuda et al., 2009). RIS may be considered a sort of presymptomatic MS, given that 30–45% of these patients develop MS within around 2–5 years (Lebrun, 2015). In this specific group of patients, CSF NfL demonstrated to be an independent, though weak, predictor of the development of a first clinical episode suggestive of MS [hazard ratio (HR) = 1.02, 95% confidence interval (CI) 1.00–1.04, $p = 0.019$], or even of MS (HR = 1.03, 95% CI 1.01–1.05, $p = 0.003$). Higher CSF NfL values have also been associated with a shorter time to MS development (Matute-Blanch et al., 2018).

Taken together, these findings on blood and CSF NfL in presymptomatic MS may have several repercussions. On an experimental level, the possibility of verifying the effectiveness of pharmacological intervention in the presymptomatic phase of the disease requires a screening test to identify the subjects to be enrolled in a hypothetical clinical trial. The measurement of serum NfL could be that screening test, to be followed by more specific investigations, such as MRI and CSF analysis. Clinically, serum NfL could again serve as a screening test in individuals at high risk of developing MS, for example in people with a strong familiarity with the disease. These individuals could benefit from lifestyle interventions aimed at minimizing exposure to known environmental risk factors for MS. Additionally, a prodromal phase of MS lasting up to 5 years has been identified, and it is characterized by more frequent use of health care services for unspecific or minor symptoms in individuals who will later develop MS, compared to controls (Wijnands et al., 2017). It could therefore be hypothesized that, in subjects at high risk of MS and who begin to frequently use health care services for any reason, serum NfL could provide a screening tool to identify the presymptomatic or prodromal phase of the disease at an early stage.

NfL IN PRESYMPTOMATIC ALZHEIMER'S DISEASE

Neurodegenerative diseases, though distinct from each other in terms of clinical manifestations, share different common features, such as the presence of a presymptomatic phase (Dickson et al., 2008; Eisen et al., 2014; Dubois et al., 2016). During this phase, the most characterizing pathophysiological mechanisms, e.g., amyloidosis and tauopathy in Alzheimer's disease (AD)—the commonest neurodegenerative disease, have already taken place (Jack et al., 2013). As a consequence, neuronal loss begins before clinical symptoms, and remains below the clinical threshold the longer, the higher the neuronal functional reserve (Stern, 2012). In this presymptomatic phase, neuronal loss is still limited, and a therapeutic intervention could theoretically have the best chance to provide its maximum

effectiveness. Once already in the clinical stages, potential DMDs could be ineffective or demonstrate a biological, but not clinical efficacy.

Another common aspect of neurodegenerative diseases is the presence of familial forms due to genetic mutations (Dion et al., 2009; Van Cauwenberghe et al., 2016; Kim and Alcalay, 2017). These familial forms share many pathophysiological and clinical features with the more common sporadic forms. Additionally, mutation carriers will develop the disease, often with a predictable age at onset, and therefore they represent a valid presymptomatic model of neurodegenerative diseases.

In a cross-sectional study performed on presymptomatic mutation carriers for familial forms of AD, namely carriers of pathogenic mutations in the genes coding for presenilin 1 (*PSEN1)* and amyloid precursor protein (*APP)*, serum NfL was found to be higher compared to non-carriers, suggesting ongoing quantifiable neurodegeneration already in the presymptomatic phase. Of interest, the mean estimated years from symptom onset was around 10 years, and serum NfL correlated with the distance with the estimated onset of the disease (Spearman $\rho = 0.81$, $p > 0.0001$). Specifically, individuals at a disease stage closer to the estimated clinical onset had higher NfL concentrations. Symptomatic carriers also had the highest value of serum NfL compared to presymptomatic carriers and non-carriers (Weston et al., 2017). In a similar cohort of patients with *PSEN1* and *APP* pathogenic mutations, serum NfL was confirmed to be significantly higher in mutation carriers compared to non-carriers up to 15 years before estimated symptom onset (Weston et al., 2019).

Of interest, when measuring serum NfL longitudinally, it has been found that the temporal dynamics of this biomarker differed between *PSEN1*, presenilin 2 (*PSEN2*), and *APP* mutation carriers and controls. The rate of change over time of serum NfL was able to discriminate between carriers and non-carriers a decade earlier than the single time-point measurement, i.e., around 16 years before the estimated onset of the disease. As noted in MS, in presymptomatic AD subjects, serum NfL peaked at the time of symptoms appearance, suggesting an acceleration in neuronal loss at the border zone between presymptomatic and symptomatic stages (Preische et al., 2019).

Taken together, these data suggest that AD has a long, gradually progressive presymptomatic phase that can be tracked by serum NfL changes over time. Therefore, the window for early detection of the risk of conversion from the asymptomatic to the clinical phase of AD might be particularly long and it should be longitudinally monitored. In AD, a marker of downstream neurodegeneration, such as NfL, might be useful as a tool for patients' recruitment in clinical trials on presymptomatic subjects, as well as outcome measures to verify the potential in disease course modification along with the presymptomatic phase. However, the length of the preclinical phase of AD raises the complexity in the set-up of a clinical trial. In clinical practice, a blood test for the ongoing neuronal loss might be used to identify those individuals with subjective cognitive decline or minimal cognitive deficits to be prioritized for more detailed investigations in the suspicion of AD, such as CSF analysis or PET imaging for amyloid or tau-pathologies biomarkers.

NfL IN PRESYMPTOMATIC FRONTOTEMPORAL DEMENTIA

Similar to other neurodegenerative diseases, frontotemporal dementia (FTD) may have familial forms, which are associated with mutations in the genes coding for progranulin (*GRN*), chromosome 9 open reading frame 72 (*C9orf72*), or microtubule-associated protein tau (*MAPT*) (Lashley et al., 2015). As for AD, these genetic forms represent a good model to understand the pathophysiology of the disease early in the presymptomatic phase.

In a multicenter cross-sectional study on symptomatic and presymptomatic carriers of *GRN, C9orf72,* and *MAPT* mutations, CSF and serum NfL were significantly higher in symptomatic compared to presymptomatic carriers, while no significant difference at the group level was found between presymptomatic carriers and controls (Meeter et al., 2016). In the same study, longitudinal CSF samples were available for five individuals, showing a three- to fourfold increase in NfL levels over conversion into the symptomatic stage in two mutation carriers who converted to manifest disease (Meeter et al., 2016). In line with this finding, in another study, the longitudinal rate of change of serum NfL was found to be similar between non-carriers and presymptomatic carriers, while it was significantly higher in converter patients, around 1–2 years before symptom onset (van der Ende et al., 2019). However, when modeled by age, a significant difference in serum NfL emerged between presymptomatic mutation carriers and non-carriers from the age of 48 years (van der Ende et al., 2019). This highlights the need for further longitudinal studies to better define the real extension of the preclinical phase of FTD-related neurodegeneration.

Overall, these preliminary results seem to suggest a shorter duration of the preclinical phase of FTD if compared with AD. Thus, the window for early detection of the risk of conversion from the asymptomatic to the clinical phase of FTD could be particularly short. This phase, therefore, should be closely monitored to identify those at-risk individuals who deserve more detailed investigations in the suspicion of FTD. A blood NfL increase in presymptomatic gene carriers could be a good biomarker to include these individuals in clinical trials, and a non-interventional study as preparation for pivotal clinical trials is ongoing, with the aim of qualifying blood NfL as an endpoint for the prevention of familial forms of FTD (ClinicalTrials.gov identifier: NCT04516499). Since the conversion to the symptomatic phase of FTD is shorter than in AD, clinical trials in these individuals might be easier to design.

NfL IN PRESYMPTOMATIC AMYOTROPHIC LATERAL SCLEROSIS

As for AD and FTD, asymptomatic carriers of amyotrophic lateral sclerosis (ALS) gene mutations represent an opportunity to investigate the preclinical phase of the disease. The most frequent causative mutations involve the genes *C9orf72,* CuZn-superoxide dismutase (*SOD1*), fused in sarcoma (*FUS*), and TAR DNA binding protein (*TARDBP*) (Dion et al., 2009). Different studies

on sporadic ALS have demonstrated that elevated CSF and serum NfL, as well as elevated CSF phosphorylated neurofilament heavy chain (pNfH)—a different neurofilament subunit, are valid markers of the neurodegeneration taking place in ALS with potential implications in the differential diagnosis (Oeckl et al., 2016; Steinacker et al., 2016). Over the last 5 years, more attention has been directed toward the preclinical phase of ALS, with findings that are in line with what was observed in other neurodegenerative diseases, especially for FTD.

CSF and serum NfL and CSF pNfH were initially found to be not significantly different between controls and asymptomatic gene mutation carriers, suggesting the possibility of a very short presymptomatic phase of the disease (Weydt et al., 2016). At the cross-sectional level, this finding was confirmed on independent populations (Benatar et al., 2018). However, when measuring serum NfL longitudinally, presymptomatic mutation carriers showed a progressive increase in serum NfL in contrast to the substantial stability of the biomarker over time in symptomatic ALS patients. Moreover, among individuals moving from the presymptomatic to the symptomatic phase, elevated serum NfL levels were observed as back as around 12 months before symptom onset with an increase lasting for the first 6 months after clinical conversion (Benatar et al., 2018). These results were obtained in a cohort of patients who, for the most part, were carriers of the *SOD1* mutation. When looking at the different mutations responsible for ALS, a longer presymptomatic phase was detected in *FUS* (2 years) and *C9orf72* (3.5 years) mutation carriers (Benatar et al., 2019).

Compared to other neurodegenerative diseases, ALS seems to have a short preclinical phase, especially in carriers of *SOD1* mutations, with serum NfL dynamics similar to that observed in FTD patients. These results open to the possibility of selecting asymptomatic patients at genetic risk for ALS who are most likely to develop manifest disease within a relatively short period of time. A presymptomatic increase in serum NfL might therefore become an eligibility criterion for a clinical trial on a potential DMD for ALS.

EARLY PATHOPHYSIOLOGY OF CNS DISEASES: THE LESSON LEARNED FROM NfL STUDIES

The study of the presymptomatic phase of neurological diseases represents a unique opportunity to understand the early pathophysiological mechanisms underlying neurodegeneration and neuroinflammation. Serum NfL has been shown to sensitively identify the neuronal damage that takes place in the presymptomatic phase of different neurological diseases. Interestingly, the duration of the preclinical phase could be a characteristic that depends on both the neurological reserve of the subject and the specific pathological processes taking place in the CNS (**Figure 1**). MS could have a long presymptomatic phase that can be caught by serum NfL up to 6 years before the clinical onset of the disease. Similarly, the presymptomatic stage of AD seems to be detected by serum NfL up to 10 years before clinical onset. On the contrary, diseases such as FTD and ALS could have a faster preclinical course, with a duration of the presymptomatic phase that can last around 2 years. This could be the consequence of faster neurodegenerative processes taking place in FTD and ALS compared to MS and AD. However, methodological differences dealing with the study designs should be considered when comparing the dynamics of NfL in the presymptomatic phase of different diseases. Further studies comparing CNS disorders with the same methodology are needed to confirm these preliminary findings.

Interesting insights on the transition from the presymptomatic to the symptomatic phase of neurological diseases have been provided by serum NfL studies. Indeed, its longitudinal dynamics seem to demonstrate that neurodegeneration progressively increases along time in the preclinical phases, with a fast acceleration as symptoms approached. The pathophysiological model that NfL trajectories suggest is that the transition of neurological diseases from the preclinical to the clinical phase is not only the consequence of progressive neuronal damage that, at a given time, exceeds the clinical threshold. In MS, AD, FTD, and ALS, the onset of the clinical picture is associated with a spike in the increase of serum NfL that suggests the possibility of a flare in the ongoing pathophysiology, which associates with the appearance of clinical symptoms.

This hypothesis deserves further investigation, such as a more detailed prospective analysis of the interaction between serum NfL and other pathophysiology-related biomarkers at multiple time points in the presymptomatic phase of neurological diseases. These studies might allow for a better understanding of the biological changes that take place in proximity with the appearance of the first clinical manifestations, hopefully identifying early therapeutic targets for neurodegenerative and neuroinflammatory diseases.

From a therapeutic point of view, the identification of neurodegeneration in the preclinical phase of CNS diseases could allow for the study of new and already established therapies before neurodegeneration flares up, with the goal of delaying or preventing the onset of clinically manifest disease in the population at risk for developing diseases such as MS, AD, FTD or ALS.

CURRENT LIMITATIONS OF NfL AND FUTURE DIRECTIONS

There are several limitations to the potential use of blood NfL as a biomarker in clinical practice. From an analytical point of view, reference materials must be standardized and the methods for NfL measurement in blood must be accessible to many laboratories. Another critical point is related to the lack of universal normative values. Recently, a population-based cohort study provided age-dependent cut-off levels for serum NfL, but the investigated population aged between 38 and 85 years and, therefore, information regarding younger adults was lacking. Additionally, for individuals aged > 60 years, a substantial variability of serum NfL was found, probably reflecting the contribution of subclinical brain tissue damage beyond the

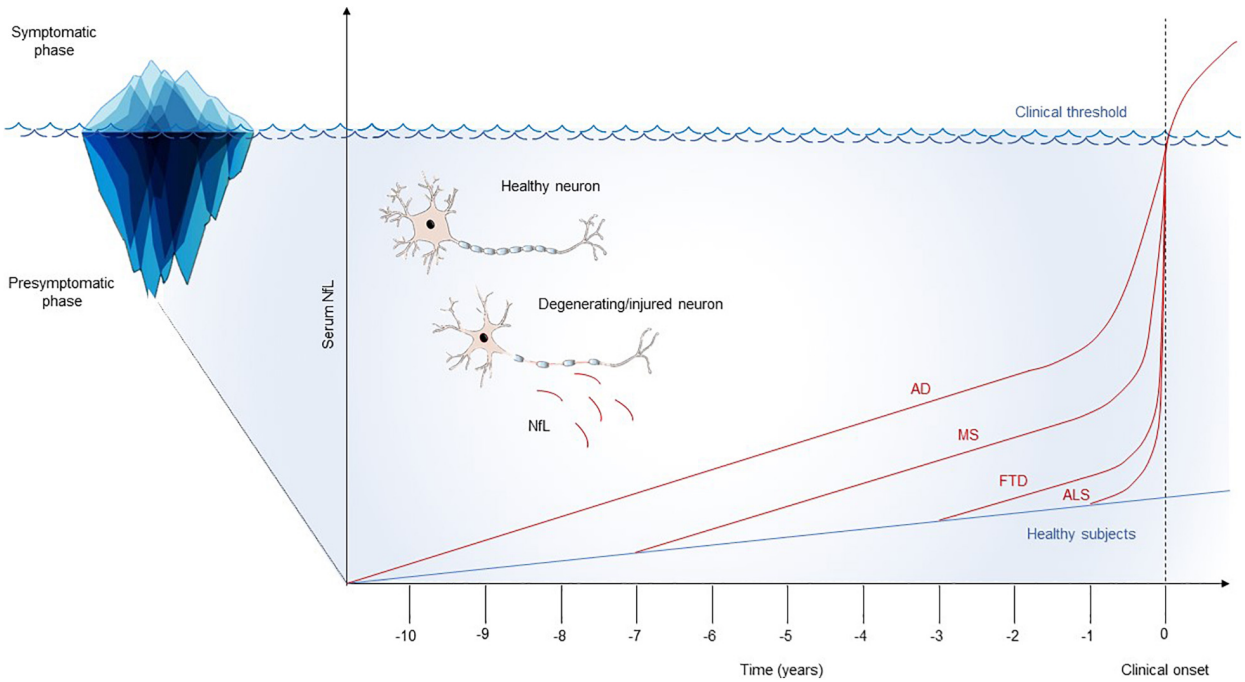

FIGURE 1 | Schematic model of the longitudinal trajectories of serum NfL. Serum NfL studies highlight possible differences in the natural history of neurological diseases, with variable durations of the presymptomatic phase, probably reflecting the rate and overall burden of the underlying neurodegenerative processes. For all the diseases, an acceleration on serum NfL raise in proximity with the appearance of the first clinical manifestations has been documented. This longitudinal trend could suggest a flare in the pathophysiology of neurological diseases at the border zone between preclinical and clinical phases. AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia; MS, multiple sclerosis; NfL, neurofilament light chain.

normal process of aging (Khalil et al., 2020). Furthermore, other potential sources of variability of blood NfL normal values (e.g., ethnicity) have not been considered yet. Population studies on multicenter cohorts are therefore needed both to verify the dynamics of serum NfL independent of CNS diseases and to have reliable normative values for all age groups.

Data obtained so far are at the group level and require deeper analysis and further longitudinal studies to interpret NfL concentrations at the individual level. Future studies should address the level of change in the concentration of NfL, which equates to a threshold change that can be accepted as clinically meaningful.

In the specific context of the use of serum NfL in the preclinical phase of neurological diseases, other limitations must be overcome. Data reviewed and discussed here have been retrospectively obtained for the most part. Prospective studies should confirm the estimated dynamics of this biomarker along the preclinical course of CNS diseases. Moreover, as far as neurodegenerative diseases are concerned, the data presented derive from studies on carriers of genetic mutations responsible for the familial forms of these diseases. Although genetic and sporadic forms share different common pathophysiological and clinical aspects, differences exist that require studies on serum NfL in presymptomatic sporadic neurodegenerative diseases. Such studies, however, are difficult to realize, given the need to include large populations of healthy individuals to be longitudinally followed-up for a long time. Additionally, blood NfL might temporarily increase because of acute CNS diseases, such as stroke, transient ischemic attack, and traumatic brain injury (Shahim et al., 2016; De Marchis et al., 2018), which could be a confounding factor in monitoring the trajectories of this biomarker. Therefore, longitudinal measurements of serum NfL should always be coupled with a thorough clinical assessment, and the exact timing for serum samplings in monitoring patients at risk for neurological diseases must be defined. Finally, the sensitivity of blood NfL in detecting CNS diseases even in the symptomatic phase spans between roughly 45% for MS (Sejbaek et al., 2019), to 80–90% for AD, FTD, and ALS (Lewczuk et al., 2018; Verde et al., 2018; Katisko et al., 2020), meaning that some symptomatic patients have NfL levels within the range of controls. Therefore, the use of blood NfL alone as a screening test could miss the remaining presymptomatic cases. Once overcome these issues, the cost-effectiveness of a screening test with longitudinal serum NfL measurements applied to large populations should be demonstrated.

CONCLUSION

Serum NfL is an excellent tool to early detect neurodegeneration in CNS diseases and to investigate the dynamics of neuronal damage over time in their preclinical phase. These findings open to the possibility of improving patient selection in clinical

trials to test the real disease-modifying potential of experimental therapies. The next studies in this field should focus on large and unselected cohorts of healthy individuals to be followed-up to the appearance of suspected clinical manifestation of CNS diseases. Converter patients should then undergo deeper investigations with other more disease-specific biomarkers to better characterize the transition from the submerged to the visible part of the iceberg of neurological diseases.

AUTHOR CONTRIBUTIONS

LG critically read, analyzed, and discussed the literature. LG and MDF conceived the outline of the manuscript and wrote the manuscript. PC and LP edited the manuscript and provided valuable discussion and criticism. All authors contributed to the article and approved the submitted version.

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REVIEW
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Kynurenines and Neurofilament Light Chain in Multiple Sclerosis

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Multiple sclerosis is an autoimmune, demyelinating, and neurodegenerative disease of the central nervous system. In recent years, it has been proven that the kynurenine system plays a significant role in the development of several nervous system disorders, including multiple sclerosis. Kynurenine pathway metabolites have both neurotoxic and neuroprotective effects. Moreover, the enzymes of the kynurenine pathway play an important role in immunomodulation processes, among others, as well as interacting with neuronal energy balance and various redox reactions. Dysregulation of many of the enzymatic steps in kynurenine pathway and upregulated levels of these metabolites locally in the central nervous system, contribute to the progression of multiple sclerosis pathology. This process can initiate a pathogenic cascade, including microglia activation, glutamate excitotoxicity, chronic oxidative stress or accumulated mitochondrial damage in the axons, that finally disrupt the homeostasis of neurons, leads to destabilization of neuronal cell cytoskeleton, contributes to neuro-axonal damage and neurodegeneration. Neurofilaments are good biomarkers of the neuro-axonal damage and their level reliably indicates the severity of multiple sclerosis and the treatment response. There is increasing evidence that connections exist between the molecules generated in the kynurenine metabolic pathway and the change in neurofilament concentrations. Thus the alterations in the kynurenine pathway may be an important biomarker of the course of multiple sclerosis. In our present review, we report the possible relationship and connection between neurofilaments and the kynurenine system in multiple sclerosis based on the available evidences.

Keywords: multiple sclerosis, kynurenine, neurofilaments, quinolinic acid, glutamate excitotoxicity, mitochondrial dysfunction, neuro-axonal damage, oxidative stress

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INTRODUCTION

Multiple sclerosis (MS) is an immune-mediated, chronic inflammatory and demyelinating disease of the central nervous system (CNS), which affects both gray and white matter (Bo et al., 2006). Clinically MS appears in different courses, such as relapsing-remitting MS (RRMS) and the progressive form, which can be primary progressive MS (PPMS) or secondary progressive MS (SPMS) (Polman et al., 2011). Characteristic pathological changes of the disease are perivenular inflammatory lesions, which lead to the formation of demyelinating plaques. These inflammatory

infiltrates comprise a large number of T-lymphocytes, in addition fewer B- and plasma cells and autoantibodies were also found (Lassmann, 2013; Karussis, 2014). As a consequence of the inflammatory infiltration and oligodendrocytes damage severe demyelination occurs. Axons are usually less affected at the onset of MS, however, irreversible axonal damage occurs as the disease progresses (Trapp et al., 1998). As the pathomechanism of MS is heterogeneous, the inflammatory process can initiate a pathogenic cascade, including microglia activation, chronic oxidative stress or accumulated mitochondrial damage in the axons, that finally leads to neurodegeneration. Moreover the altered mitochondrial function due to chronic cell stress and disruption of electrolyte homeostasis at the end causes neuronal death (Rajda et al., 2015).

Many studies have described the relationship between several points in the kynurenine pathway (KP) during tryptophan (TRP) degradation and the pathomechanism of neurodegenerative diseases, such as MS. TRP is an essential amino acid with complex metabolism and more than 95% is degraded via the KP, while the remaining 5% is metabolized by the serotonin pathway. During the KP (which may be activated by different pro-inflammatory cytokines) several neuroactive and neuroprotective metabolites are formed. This pathway is responsible for the production of nicotinamide adenine dinucleotide (NAD^+) (Vécsei et al., 2013). Many of the kynurenines such as the *N*-methyl-D-aspartate (NMDA) receptor agonist excitotoxin quinolinic acid (QUIN), the free radical generators 3-hydroxykynurenine (3-HK), 3-hydroxyantranilic acid (3-HAA), and the neuroprotective kynurenic acid (KYNA), picolinic acid (PIC) display neuroactive properties (Chen et al., 2010; Sundaram et al., 2014). This process can initiate a pathogenic cascade, including microglia activation, glutamate excitotoxicity, chronic oxidative stress or accumulated mitochondrial damage in the axons, that finally disrupt the homeostasis of the neuron, destabilizes the neural cell cytoskeleton, contributing to neuro-axonal damage and neurodegeneration [see review (Lovelace et al., 2016)].

Neurofilaments (NFs) are released in the cerebrospinal fluid (CSF) and blood after cell damage, thus they can be considered as an indicator of neuro-axonal damage (Yabe et al., 2001; Fitzner et al., 2015). This protein serves as a good prognostic biomarker for different neurodegenerative disorders including MS, as its level is proportionally elevated with extended neuro-axonal damage (Khalil et al., 2018). There is some evidence that the KP metabolite, QUIN, causes the hyperphosphorylation of neurofilament in neurons and astrocytes, thereby destabilizing their cytoskeleton (Pierozan et al., 2010, 2014, 2015). In our previous study we have found, that the levels of QUIN is associated with the levels of the neuronal cytoskeleton protein, neurofilament light chain (NfL) (Rajda et al., 2020). These results suggest, that on one hand, the kynurenines are highly relevant contributors to neurodegeneration, on the other hand the metabolic profiling of the KP and NfL could potentially serve as useful biomarkers in the MS progression in the future.

In this review we describe how the dysregulation of the KP and their neurotoxic metabolites contribute to the pathological processes in MS. We focus on the cells which produce these metabolites and the mechanism of action how they cause the neuro-axonal damage leading to neurodegeneration.

KYNURENINE PATHWAY

Previous studies have suggested a role of the altered metabolism of the TRP system in MS (Rejdak et al., 2002; Hartai et al., 2005; Vécsei et al., 2013). In human brain the majority of TRP is metabolized via the KP, however, not all cells of the CNS contain the complete enzymatic pathway. Astrocytes lack the kynurenine-3-monooxygenase (KMO), and oligodendrocytes do not express indolamine-2,3-dioxygenase (IDO-1/IDO-2) and tryptophan 2,3-dioxygenase (TDO), hence these cells do not synthesize neurotoxic QUIN. The complete pathway is present in infiltrating macrophages, activated microglia cells and neurons (Guillemin et al., 2000; Lovelace et al., 2016).

The first and rate-limiting step in this pathway, the conversion of TRP into L-kynurenine (L-KYN), this process is catalyzed by the TDO, which is mainly localized in liver cells, or the IDO enzyme, which is diffused in most of the human tissues (Sforzini et al., 2019). The KP can be divided into three branches at the L-KYN level, one branch leads to KYNA in an irreversible transamination by kynurenine aminotransferases (KATs), the second route leads to the metabolite that will be metabolized to anthranilic acid or as a third branch it can be degraded to neurotoxic kynurenines in several enzymatic steps. In the human brain, from the four KAT isoforms mainly KAT-II is responsible for KYNA synthesis, which is expressed by astrocytes. In the second branch of the KP, anthranilic acid may be formed from L-KYN by kynureninase. In the third branch of this pathway, L-KYN is transformed to 3-HK by KMO. Further steps in the KP produce 3-HAA from both anthranilic acid by anthranilate 3-monooxygenase and 3-HK by kynureninase. 3-HAA is also capable of autooxidation, it produces cinabarinic acid and generates H_2O_2 and superoxide radicals. 3-HAA can be converted via 2-amino-3-carboxymuconate semialdehyde intermediate to PIC by picolinic carboxylase or it can be metabolized by non-enzymatic cyclization to QUIN. Finally, QUIN is metabolized for the synthesis of NAD^+, via quinolinate phosphoribosyltransferase (QPRT) enzyme [see **Figure 1**; for review see Bohár et al. (2015)].

NEUROACTIVE METABOLITES

Numerous metabolites of the KP are neuroactive compounds. This section discusses the metabolites with a potential role in the development of MS, including the free radical forming 3-HK, the pro-oxidant, glutamate excitotoxicity inducing, neurotoxic QUIN, and the potentially neuroprotective KYNA.

3-HK

3-hydroxykynurenine is formed directly from L-KYN in a reaction catalyzed by KMO enzyme exclusively produced by microglia. Its neurotoxic effect is connected with it's free radical formation and which increases oxidative

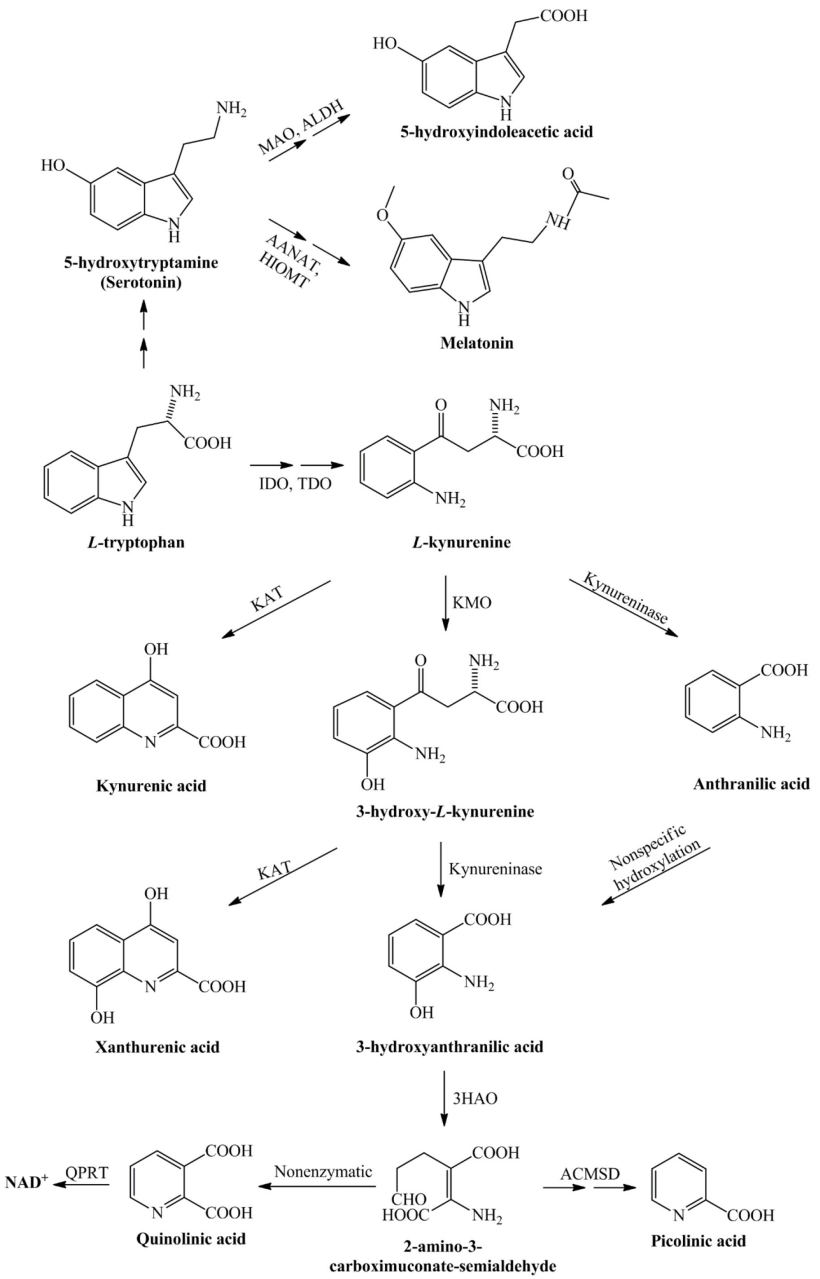

FIGURE 1 | Detailed representation of the kynurenine pathway. Tryptophan, as an essential amino acid, is a precursor of the serotonin- and kynurenine pathway. During the serotonin pathway, melatonin and 5-hydroxyindoleacetic acid are formed, while the kynurenine pathway of tryptophan metabolism leads to the production of nicotinamide adenine dinucleotide. The conversion of tryptophan to L-kynurenine is performed by IDO/TDO. From L-kynurenine various neuroactive metabolites are formed during this pathway, which eventually leads to the nicotinamide adenine dinucleotide, that plays a significant role in the production of cellular energy. 3-HAO, 3-hydroxyanthranilate oxidase; AANAT, arylalkylamine *N*-acetyltransferase; ACMSD, α-amino-β-carboxymuconate-semialdehyde-decarboxylase; ALDH, aldehyde dehydrogenase; HIOMT, hydroxyindole-*O*-methyltransferase; IDO, indoleamine 2,3-dioxygenase; KAT, kynurenine aminotransferase; KMO, kynurenine 3-monooxygenase; MAO, monoamine oxidase; NAD^+, nicotinamide adenine dinucleotide; QPRT, quinolinate phosphoribosyltransferase; TDO, tryptophan 2,3-dioxygenase.

stress (Németh et al., 2005). Its concentration is elevated in pathological states [e.g., Parkinson's disease (PD), Huntington's disease (HD), and MS] (Vécsei et al., 2013). Its auto-oxidation leads to the formation of hydrogen peroxide and hydroxyl free radicals. However, this process requires the presence of redox active metals (Cu^{2+} and Fe^{2+}) and no significant cellular toxicity is expected under physiological conditions (Goldstein et al., 2000). Numerous studies have confirmed that 3-HK has a pro-oxidant effect and generates reactive molecules that induce apoptosis [see review (Colín-González et al., 2013)].

Its oxidation leads to the generation of reactive oxygen species (ROS), resulting in lipid oxidation, protein modification, modulation of inflammatory response and DNA damage and ultimately to cell death. Changes in its concentration can indirectly alter gene expression, DNA repair and intracellular calcium levels (Okuda et al., 1996, 1998). Multiple lines of evidence show that 3-HK is a neurotoxic metabolite and accordingly it may play an important role in the development of neurodegeneration in MS (Vamos et al., 2009). Studies performed in rats with experimental autoimmune encephalomyelitis (EAE) an animal model for MS, demonstrated elevated plasma, brain, and spinal cord 3-HK levels (Chiarugi et al., 2001). Another study evaluated the potential synergistic effect of 3-HK and QUIN in neurotoxicity. In studies of rat brain, the intrastriatal injection of either 3-HK (5 nM) or QUIN (15 nM) caused no or mild damage, while in combination they induced a significant increase in the size of lesions (Colín-González et al., 2013).

QUIN

Under normal conditions, the brain contains nanomolar concentrations of QUIN (Bohár et al., 2015). In neurodegenerative conditions accompanied by chronic inflammation (e.g., MS) the brain levels of extracellular QUIN increase significantly, primarily due to activated microglia and to a lesser extent due to macrophages penetrating the CNS (Guillemin et al., 2003). Its stimulating effects are exerted via the selective stimulation of the NMDA receptor which can be fully inhibited by NMDA receptor antagonists (Stone and Perkins, 1981). This stimulating effect is not particularly efficient (ED50 > 100 μM) but specific to NR2A and NR2B subunit containing NMDA receptors primarily expressed in the forebrain (striatum and hippocampus) (de Carvalho et al., 1996; Guillemin, 2012). QUIN accumulated in the CNS has a neurotoxic effect acting through multiple mechanisms. In pathological concentration QUIN can activate NMDA receptors resulting in excitotoxicity (Monaghan and Beaton, 1991). Additionally, it can inhibit the re-uptake of glutamate by astrocytes which results in strong neurotoxicity in its microenvironment (Tavares et al., 2002). Thirdly, it can further enhance the toxicity of itself and those of other excitotoxins (e.g., NMDA, glutamate) (Schurr and Rigor, 1993; Guillemin et al., 2005a). Finally, it can decrease glutamine synthetase activity and through this pathway limits the recycling of glutamate to glutamine in astrocytes (Ting et al., 2009). Furthermore, it is known that the complex formation of iron (II) ions with QUIN leads to intense free radical formation (Guillemin et al., 2005b). Its neurotoxic effect contributes to the pathomechanism of MS (Lim et al., 2017).

KYNA

The neuroprotective kynurenic acid is present in nanomolar concentration in mammalian brain (Moroni et al., 1988). KYNA is an endogenous antagonist of ionotropic glutamate receptors with significant neuroprotective properties (Han et al., 2010). It can affect the glutamatergic transmission in different ways (Zádori et al., 2011): it can be a competitive NMDA receptor antagonist (Kessler et al., 1989) or it behaves as a weak antagonist on the kainate- and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Birch et al., 1988). Furthermore, KYNA can inhibit presynaptic α7 nicotinic acetylcholine receptors resulting in regulation of presynaptic glutamate release, which effect contributes greatly to the neuroprotective effect of KYNA (Rajda et al., 2015). Although recent reports suggest this role is failed to repeat to same result (Stone, 2020). KYNA may function as a neuroprotective agent in potential neuroprotective process [for review see Rajda et al. (2015)].

EVIDENCE OF DYSREGULATION OF KP METABOLITES IN MS

The development and progression of numerous diseases affecting the CNS (including MS) are connected to an enzymatic imbalance of the KP and chronic changes in the physiological concentration of certain kynurenine metabolites (Platten et al., 2005; Lim et al., 2010; Mancuso et al., 2015). The first study, completed 10 years ago, on the potential role of kynurenines in the pathogenesis of MS confirmed lower TRP levels in the blood and CSF of patients with MS (Monaco et al., 1979). Although these results were not supported by a subsequent research, recent studies have demonstrated low blood and CSF TRP concentrations during the chronic phase of MS (Rudzite et al., 1996; Sandyk, 1996). Numerous other studies on changes in KP metabolites have confirmed the activation of KP in MS: Mancuso et al. (2015) verified an increased expression of IDO-1 (the first enzyme of the KP) in patients with RRMS. Furthermore, one of our own study has demonstrated that interferon- β (IFN-β) used to treat MS increases the KYN/TRP ratio, indirectly confirming IDO-1 enzyme mediated activation of the KP in MS (Amirkhani et al., 2005). Rejdak et al. (2002) have confirmed low KYNA level in the CSF of patients with MS. However, two additional studies contradicted these results: Kepplinger et al. (2005) found increased KYNA levels in the CSF of MS patients. In a later study, Rejdak et al. (2007) were found elevated KYNA levels of MS patients in acute relapse. Apart from differences in methodology, these results can be explained by using samples from different phases of disease activity. In particular, the study by Rejdak et al. (2002) was likely carried out in the chronic, inactive phase of the disease, while a more recent study (Kepplinger et al.) analysed samples taken during the patients' relapse. Hartai et al. (2005) were found elevated KYNA levels in the plasma of MS patients. One study found lower KYNA and PIC levels and increased QUIN levels in MS patients. This neurotoxic level of QUIN is associated with cellular damage in astrocytes, oligodendrocytes and neurons (Vécsei et al., 2013), i.e., disease progression. A comprehensive study examining the connection between KP metabolites and neuro-cognitive symptoms of MS subtypes has detected small differences in KP metabolite levels. RRMS patients demonstrated elevated QUIN levels and higher QUIN/kynurenine ratio during relapse and lower TRP and KYNA levels have been measured in SPMS patients (Aeinehband et al., 2016). These results suggest that the KP is induced during the active phase of MS and leads to

increased KYNA production, while during the progressive phase of the disease QUIN levels increase and KYNA levels decrease indicating a change in the KP profile during progression (see **Figure 2**) (Rejdak et al., 2002, 2007; Hartai et al., 2005). This is further supported by results published by Lim et al., namely that significant aberrations in kynurenine metabolism (divergent levels of two key KP metabolites, KYNA and QUIN) were identified in the blood of MS patients. According to their results, the increased KYNA levels observed in RRMS may constitute a compensatory mechanism working in the initial phase of the disease to counteract neurotoxicity caused by QUIN, and furthermore a moderate correlation can be confirmed between the QUIN/KYNA ratio and the severity of MS. It is the opinion of the authors that changes in kynurenine metabolism may be an important biomarker of the transition from the early, relatively mild form to the progressive form of the disease (Lim et al., 2017).

In summary, alterations of the KP can be found in all phases of the disease (see **Table 1**). These results suggest that during the early phase of MS (dominated by neuroinflammation) the production of neuroprotective kynurenine metabolites (KYNA and PIC) is the primary mechanism that in all likeliness works to counteract the effects of neurotoxic metabolites. Disease progression brings about a change in KP profile and the chronic activation of the enzymes involved in the pathway enhances the production of neurotoxic metabolites and thus contributes to the emergence of progression in MS (Lim et al., 2010, 2017; Lovelace et al., 2016).

KYNURENINE PATHWAY AND IMMUNOREGULATION OF MS

In MS the autoimmune process is mediated by CD4+ Th1 and Th17 cells (Aranami and Yamamura, 2008). At the onset of the disease or in case of a relapse proinflammatory monocytes/macrophages migrate across the damaged blood-brain barrier into the CNS. During the process of neuroinflammation the Th1 cells secrete a variety of proinflammatory cytokines (tumor necrosis factor alpha or TNF-α, interleukin-1, and interleukin-6, etc.) and interferon gamma (IFN-γ) (Sundaram et al., 2020). In the CNS, IFN-γ acts as the primary activating factor for macrophages/microglia cells and enhances their antimicrobial effects by enhancing their production of ROS and nitrogen oxide (NO) and the secretion of various cytokines (interleukin-1 and TNF-α, etc.); and on the other hand, IFN-γ is one of the most potent activators of IDO-1, the primary enzyme of the KP [see review (Braidy and Grant, 2017)]. IDO-1 is present in various immune cells, including monocytes, macrophages, microglia, and may take part in the immunoregulation through TRP depletion and the production of kynurenines (such as KYNA and QUIN) (Mándi and Vécsei, 2012). It's known, that increased IDO-1 activity suppress the T-cell mediated immune response in MS (Mellor and Munn, 2004; Mancuso et al., 2015), via activation of aryl hydrocarbon receptor (AhR) (Bessede et al., 2014). This has been demonstrated by multiple studies. 3-HAA and QUIN induce the selective apoptosis of Th1 cells (Fallarino et al., 2002). In EAE the pharmacological inhibition of IDO-1 (by the administration of 1-methyl-tryptophan) enhanced the Th1 and Th17 immune response accompanied by decreased regulatory T cells (Treg) response, and the inhibition of IDO-1 exacerbated disease progression. Also, in EAE, 3-HAA suppressed Th1 and Th17 cell activity resulting in enhanced Treg cell formation (Sakurai et al., 2002; Kwidzinski et al., 2005; Yan et al., 2010). As demonstrated, the upregulation of IDO-1 can inhibit the proliferation of autoreactive T cells and promote their selective (Th1 and Th17 cells) apoptosis (Munn et al., 1999; Frumento et al., 2002; Terness et al., 2002). In addition it is capable to induce the formation of immunosuppressive FoxP3 + Treg cells (Fallarino et al., 2006) which inhibit both Th1 and Th2 cells and "assist" in the restoration of a balanced immune

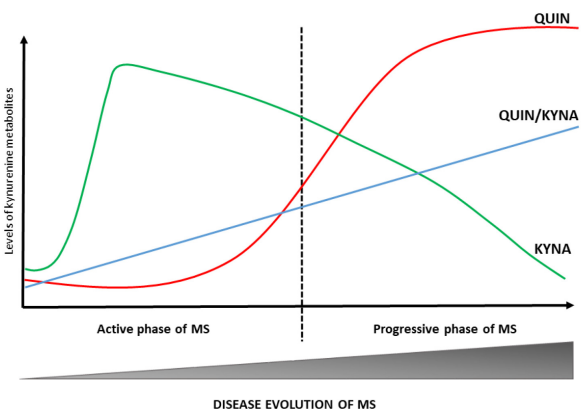

FIGURE 2 | Changes in KP profile during disease course of MS. The KP metabolic profile changes at different stages of MS. In the active phase of MS, the KYNA levels are increased. During the progressive phase of the disease QUIN levels and QUIN/KYNA ratio are increased. These changes in metabolic profile of the KP have a strong association with disease activity. KYNA, kynurenic acid; MS, multiple sclerosis; QUIN, quinolinic acid.

TABLE 1 | The role of kynurenine metabolites of MS.

Alterations of the KP in all phases of MS	References
Low KYNA level in the CSF of MS patients in remission	Rejdak et al., 2002
Increased KYNA levels in the CSF of MS patients with acute relapse	Kepplinger et al., 2005; Rejdak et al., 2007
Elevated KYNA levels in the serum of RRMS patients	Lim et al., 2017
Elevated QUIN levels in the CSF of RRMS patients in relapse	Aeinehband et al., 2016
Decreased TRP and KYNA levels in the CSF of SPMS patients	Aeinehband et al., 2016
Elevated QUIN levels in the serum of PPMS patients	Lim et al., 2017

CSF, cerebrospinal fluid; KP, kynurenine pathway; KYNA, kynurenic acid; MS, multiple sclerosis; PPMS, primary progressive multiple sclerosis; QUIN, quinolinic acid; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; TRP, tryptophan.

system (Mándi and Vécsei, 2012). Several studies explored that the increased kynurenine (by-product of IDO-1) activate the AhR and inhibits the inflammatory response in EAE (Quintana et al., 2010; Mondanelli et al., 2020).

We hypothesize, that in the initial phase of MS–dominated by neuroinflammation–the activation of IDO-1 can be actually beneficial in the short term since it can suppress autoimmune processes via modulation of the T cell mediated immune response (as a negative feedback loop) and promote the development of immune tolerance (Mellor and Munn, 2004; Mancuso et al., 2015). However, in the long term–in the presence of continuous neuroinflammation–chronic IDO-1 activation becomes harmful since the consequential release of chronic neuroactive metabolites contributes to the neurodegeneration observed during MS progression (Lim et al., 2010, 2017).

EFFECTS OF GLIAL CELLS SPECIFICITY OF KP METABOLITES

The primary immune cells of the CNS, microglia cells are highly significant in maintaining the homeostasis of nerve cells. When activated they express multiple pro- and anti-inflammatory cytokines and therefore play an important role in inflammatory and immune response [see review (Luo et al., 2017)]. They also have a significant role in the pathogenesis of MS since during neuroinflammation they damage the myelin sheath and/or oligodendrocytes, activate T cells, and lead to tissue damage, demyelination and cell death, thus contribute to disease progression and neurodegeneration [see review (Luo et al., 2017; Chu et al., 2018)]. During neuroinflammation, pro-inflammatory cytokines induce microglia cells to activate the KP leading to the production of neurotoxic metabolites (QUIN), which play a central role in neuro-axonal damage through various effects such as stimulation of oxidative stress inducers such as ROS, nitric oxide synthase (iNOS) and enhancement of glutamate-mediated neurotoxicity, which can induce neuronal and glial cell death. These effects of the microglia cells can exacerbate neurodegeneration in MS (Lovelace et al., 2017).

Astrocytes are the most abundant cell type in the CNS and play a critical role in the regulation of the blood-brain barrier, providing neuron-glia contact, cellular homeostasis, and glutamate recycling (Guillemin et al., 2001). These cells favor KYNA synthesis as they do not express KMO and therefore they are unable to synthetize neurotoxic metabolites (Guillemin et al., 2001). Rejdak et al. (2007) demonstrated enhanced astrocyte activation in the CSF of MS patients which showed a good correlation with enhanced KYNA production. These results suggest that astrocytes may play a neuroprotective role in MS.

As a result of inflammation in MS, the oligodendrocytes generating the myelin sheath sustain damage which in time leads to axonal degeneration and later to neurodegeneration (Itoh, 2015). Human oligodendrocytes may be important in neuroprotection. During neuroinflammation they provide neuroprotection by generating KYNA and they are capable of taking up exogenous KYN as a substrate for production of downstream KP metabolites causing increased NAD^+ synthesis. Oligodendrocytes express multiple types of glutamate receptors, such as NMDA, AMPA, and kainate, and are constitutively involved in glutamate clearance (Lim et al., 2010). It is known that during the active phase of MS, the large amounts of neurotoxic QUIN produced by activated macrophages and microglia cells damage oligodendrocytes in the inflammatory microenvironment (Lim et al., 2017), and lead to demyelination followed by neuro-axonal damage.

MECHANISM OF NEURO-AXONAL DAMAGE OF QUINOLINIC ACID IN MS

The pro-inflammatory cytokines produced during neuroinflammation can influence IDO-1 activation and thus significantly modify kynurenine metabolism. The neurotoxic metabolites produced during this process (primarily QUIN) can act through multiple pathways to induce neuro-axonal damage (see **Figure 3**) and ultimately contribute to the process of neurodegeneration observed in MS.

In the next paragraphs, we give insight in some of the most important mechanism of toxicity of QUIN in MS, which process finally lead to neuro-axonal damage.

Glutamate Excitotoxicity

Excitotoxicity is a pathological process during which the overactivation of excitatory amino acid receptors leads to neuronal damage and death. Excitatory amino acids are the primary excitatory neurotransmitters of the hippocampus and the cerebral cortex and therefore play an essential role in the physiological function of neurons. When it comes to neuronal excitotoxicity it is primarily due to overexposure to glutamate which is the most important excitatory neurotransmitter of the CNS (Mehta et al., 2013).

Glutamate excitotoxicity observed at all stages of MS and might have a substantial role in the neuro-axonal damage, also include progression of MS [see review (Rajda et al., 2017)]. The most important sources of extracellular glutamate are activated microglia/macrophage cells and leukocytes (Stojanovic et al., 2014). Inflammation is triggered by the release of a large amount of glutamate by activated microglia cells [which are activated in all subtypes of MS (Sriram and Rodriguez, 1997)], ultimately leading to nerve cell excitotoxicity and death (Piani et al., 1991; Stojanovic et al., 2014). Glutamate activates NMDA, AMPA, and kainate receptors as well as metabotropic glutamate receptors (Lau and Tymianski, 2010). The NMDA receptor is the central mediator of glutamate excitotoxicity (Fuchs et al., 2012) which is found on the surface of neurons, astrocytes, oligodendrocytes, and microglia cells in the CNS (Kaindl et al., 2012). Its activation or in other words the opening of the channel requires not only glutamate binding but glycine or D-serine binding as well (Papouin et al., 2012).

It is known that QUIN is a selective agonist of NMDA receptors, specifically their NR2A and NR2B subunits (de Carvalho et al., 1996), and can also be considered an endogenous excitotoxin (Stone and Perkins, 1981). Several

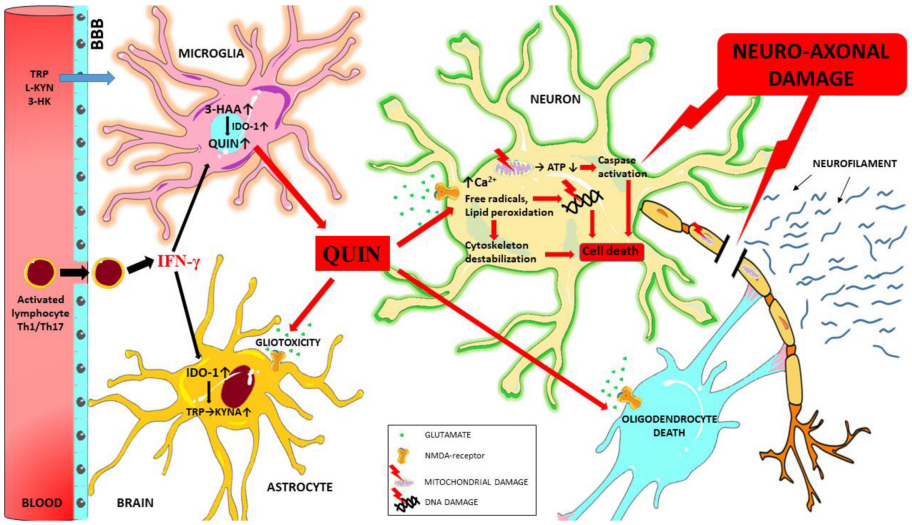

FIGURE 3 | Schematic overview of the mechanisms of QUIN toxicity in MS. During neuroinflammation autoreactive T cells (Th1 and Th17) and activated monocytes/macrophages migrate to the central nervous system through the damaged blood-brain barrier, and these cells secrete numerous pro-inflammatory cytokines (such as interferon gamma). Interferon gamma is one of the most potent activators of IDO-1, the primary enzyme of the KP. In case of an enzymatic imbalance in the KP and also through the chronic alteration of the physiological concentration of kynurenine metabolites in the microenvironment the balance between neuroprotective and neurotoxic processes is no longer ensured. Neurotoxic QUIN secreted in abnormal quantities by activated macrophages and microglia cells in an inflammatory microenvironment can damage oligodendrocytes, astrocytes and neuron through multiple mechanisms. QUIN induces caspase activation, mitochondrial impairment, oxidative stress, lipid peroxidation and energy deficit through the overactivation of NMDA receptors (and also independently of overactivation). These factors combine to result in the destabilization of the cytoskeleton, leading to DNA damage, cell death and ultimately neuro-axonal damage. Neurofilaments are released as a result of neuro-axonal injury. 3-HAA, 3-hydroxyantranilic acid; 3-HK, 3-hydroxykynurenine; ATP, adenosine triphosphate; BBB, blood-brain barrier; IFN-γ, interferon gamma; IDO-1, indoleamine 2,3-dioxygenase; L-KYN, L-kynurenine; KYNA, kynurenic acid; QUIN, quinolinic acid; TRP, tryptophan.

studies have already proven the role of QUIN in the pathogenesis of MS. Pathologically high levels of QUIN were measured in EAE and in MS patients (Sundaram et al., 2014). Moreover, locally elevated QUIN levels may contribute to demyelination in MS and in EAE (Füvesi et al., 2012). In EAE, exposure to QUIN led to the apoptosis of oligodendrocytes, microglia cells, astrocytes, and neurons (Cammer, 2001). According to the results of Flanagan et al. (1995) a causal relationship can be demonstrated between the degree of clinical severity of the EAE model and QUIN levels measured in the spinal cord. Studies using hippocampus and astrocyte cultures showed that QUIN enhances glutamate release in the synapses, inhibits reuptake and reduces glutamate to glutamine conversion by inhibiting glutamine synthetase. All these effects lead to an increase of synaptic glutamate concentration and eventually to excitotoxicity via the further overstimulation of NMDA receptors (Tavares et al., 2000, 2002; Ting et al., 2009), resulting in an increase of intracellular Ca^{2+} levels. Elevated intracellular Ca^{2+} levels in turn lead to increased neurotransmitter release and the activation of various enzymes. Ca^{2+} ions activate calpains and lead to proteolysis in the cells. Calpain induced proteolysis primarily affects cytoskeletal and signal transduction proteins and transcription factors (Goll et al., 2003). Additionally, phospholipase A2 (PLA2) and cyclooxygenase activation are also enhanced leading to free radical formation followed by lipid peroxidation (Farooqui et al., 1997), and compromised mitochondrial function which in turn causes the formation of ROS and leads to oxidative stress and ultimately to apoptotic cell death (Hardingham, 2009; Sekine et al., 2015). This induced cell death has been observed under *in vitro* conditions in rat oligodendrocytes treated with pathological concentrations of QUIN (1 mM), primary human neurons and astrocytes (at 150 nM QUIN) and motor neurons (at 100 nM QUIN) (Cammer, 2001, 2002; Braidy et al., 2009; Chen et al., 2011).

Mitochondrial Dysfunction and Oxidative Stress

It has been firmly established that mitochondrial damage and free radicals, such as ROS, and reactive nitrogen species (RNS) play an important role in the pathomechanism and progression of neurodegenerative and inflammatory conditions including MS. Several studies have confirmed that during MS relapse the elevation of free radical concentrations are accompanied by significant changes in the blood concentrations of antioxidant enzymes/reducing agents (Karg et al., 1999; Rajda et al., 2017). Furthermore, free radicals are involved in the maintenance of chronic neuroinflammation, damage the blood-brain barrier and thus promote the migration of immune cells to the CNS, and promote the secretion of pro-inflammatory cytokines. Activated microglia and the pro-inflammatory cytokines, ROS and reactive NO released by macrophages lead to extensive tissue damage,

demyelination and cell death, and thus contribute to the mechanism of neurodegeneration in MS (Ohl et al., 2016; Chu et al., 2018).

Through the respiratory chain, mitochondria play important role in cellular energy supply (ATP synthesis), fatty acid metabolism and programmed cell death (apoptosis). Impairment of mitochondrial function causes ATP deficit followed by energy deficit which impairs the function of Na^+/K^+-ATPase and Na^+/Ca^{2+} transporter and consequently leads to membrane depolarization. As a consequence of abnormal membrane potential, cells are more prone to excitotoxic and oxidative damage (Novelli et al., 1988; Sas et al., 2007), and mitochondrial dysfunction results in uncontrolled release of free radicals (ROS and RNS) (Holmström and Finkel, 2014).

We have described above that QUIN can lead to mitochondrial dysfunction via overactivation of NMDA receptors. Recent evidence shows that metabolic impairment in mitochondria is an important mechanism of the manifestation of QUIN toxicity. It has been demonstrated that QUIN can inhibit monoamine oxidase B (MAO-B) in human brain synaptosomal mitochondria (Naoi et al., 1987). QUIN can potentiate its own toxicity and that of other excitotoxins, such as NMDA and glutamate, and thus produce progressive mitochondrial dysfunction (Bordelon et al., 1997). Various studies have shown that the intrastriatal injection of QUIN impairs cellular respiration and induces a reduction of ATP levels (Bordelon et al., 1997, 1998). Ribeiro et al. (2006) have observed that the injection of QUIN in nanomolar concentrations also inhibited creatine kinase activity, an important enzyme involved in intracellular energy transfer. Additionally, QUIN induced a significant reduction of the activity of respiratory chain complexes II (50%), II–III (35%), and III (46%) in the striatum homogenates of juvenile rats. This effect occurred 12 h after QUIN administration. It is possible that these results are primarily due to the activation of glutamate receptors and secondarily to the effect of free radicals induced by QUIN on energy production (Pérez-Severiano et al., 1998; Cabrera et al., 2000; Ganzella et al., 2006). Furthermore, QUIN inhibits around 35% succinate dehydrogenase (SDH), an enzyme involved in the citric acid cycle and in the respiratory chain. Additionally, this effect is independent on the NMDA receptor since MK-801 and KYNA (two NMDA receptor antagonists) and L-NG-nitroarginine methyl ester (L-NAME), a NOS inhibitor, did not prevent the inhibitory effect, but preincubation with superoxide dismutase and catalase did (Schuck et al., 2007). This evidence suggests that the neurotoxic effects of QUIN may be independent of NMDA receptor activity and mitochondrial impairment may be caused by an alternative mechanism of QUIN induced toxicity.

Under physiological conditions (during oxidative phosphorylation) significant amounts of free radicals are produced (ROS and RNS) and they are kept under control by both enzymatic and non-enzymatic antioxidant systems, thus ensuring the redox balance of the body in cells and tissues. The most important enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and catalase (Sindhu et al., 2005). This process is in an exquisitely delicate balance and its imbalance leads to oxidative stress and ultimately to cell death. Neurons are especially sensitive to oxidative damage due to their high energy requirements supporting the maintenance of membrane potential, the restoration of ion gradients after action potentials, the release of neurotransmitters and the re-uptake of neurotransmitters from the synaptic gap (Bohár et al., 2015). It is also important to note with respect to neurons that axonal and synaptic mitochondria are more sensitive to oxidative damage than those in dendrites and the cell body (Borrás et al., 2010; Lores-Arnaiz and Bustamante, 2011), suggesting that oxidative damage may also play a role in neuro-axonal damage.

Quinolinic acid induced toxicity includes the generations of free radicals and oxidative stress. In connection with this, it has been shown that QUIN can produce oxidative damage independent of its effects exerted via the NMDA receptor; this mechanism involves the formation of a complex of QUIN and Fe^{2+}. The QUIN-Fe^{2+} complex has been shown to induce intensive free radical formation likely responsible for *in vitro* lipid peroxidation and DNA damage (Goda et al., 1996). Additionally, there is evidence that QUIN can enhance free radical production through the induction of NOS activity in astrocytes and neurons which also leads to oxidative stress (Braidy et al., 2010): during neuroinflammation, activated microglia and QUIN produced by macrophages can enhance–via the activation of the NMDA receptor–the activity of NOS enzyme activity in astrocytes and neurons resulting in enhanced nitric oxide (NO) production [see review (Lugo-Huitrón et al., 2013)]. It is important to note that the activated microglia and macrophages themselves can enhance NOS activity (Su et al., 2009). High NO concentration is toxic for cells, since the reaction between NO and oxygen radicals results in the formation of peroxynitrite (ONOO-). NO and ONOO- damage cellular components, inhibit the mitochondrial respiratory chain, cause DNA damage through the overactivation of poly(ADP-ribose) polymerase (PARP) enzymes and enhances extracellular lactate dehydrogenase (LDH) activity and oxidative stress (Sas et al., 2007; Pérez-De La Cruz et al., 2010; Lugo-Huitrón et al., 2013). Overactivation of the PARP enzymes causes free radical accumulation which in turn ultimately leads to cell death through NAD^+ depletion [see review (Braidy and Grant, 2017)].

Furthermore, it has been shown that in rat brain QUIN is able to modify the profile of certain endogenous antioxidants, for example, it lowers reduced glutathione content and copper and zinc dependent SOD activity (Cu^{2+} and Zn-SOD) (Rodríguez-Martínez et al., 2000). In rat brain the intracerebral injection of QUIN induced a significant decrease in nerve cell numbers and a marked increase in SOD1 expression (Noack et al., 1998). The increase in SOD1 expression was likely a neuroprotective effect limiting oxidative damage caused by QUIN. Based on the above evidence and taking into account that oxidative stress is caused by an imbalance between antioxidant protection and reactive free radicals, we can conclude that there are multiple mechanisms of QUIN neurotoxicity. Far from excluding each other, all these factors are somehow closely related and act synergistically to induce axonal damage.

Axonal Damage

Axonal damage is a major hallmark of MS (Haines et al., 2011). The mechanism of axonal damage in MS is not well understood, but certainly the mitochondrial dysfunction (energy deficit, disrupt of cell homeostasis, etc.) is a key player in it. During the acute inflammatory attack in MS the excitotoxic glutamate and NO produced by activated microglia can damage axonal mitochondria, leading to an imbalance in the energy demands, which can potentiate persistent Na^+ influx within the axon. To compensate for this redistribution, the Na^+/Ca^{2+} exchanger instead functions of reverse mode to offset the rising levels of Na^+, causing increased levels of intracellular Ca^{2+}. Dysregulation of Ca^{2+} homeostasis can activate several enzymes, which process leads to axonal damage (Lee et al., 2014). These results suggest that the glutamate excitotoxicity and oxidative/nitrosative stress results in mitochondrial dysfunction, which cause abnormalities of the axonal transport [see review (Su et al., 2009)].

Cytoskeleton Destabilization

The cytoskeleton plays a key role in maintaining the shape the of neuronal cell and is crucial for its normal functions such as neurite outgrowth, synapse formation, and internal transport of various molecules. Impairment of the cytoskeletal system leads to cell death. There is abundant evidence that QUIN damages the cytoskeletal system, axons, and dendrites (Kerr et al., 1998; Kells et al., 2008). In connection with this, recently published studies have demonstrated that QUIN toxicity can lead to phosphorylation of structural proteins and consequent destabilization of the cytoskeleton (Rahman et al., 2009; Pierozan et al., 2010). During acute intrastriatal injection of QUIN into human primary fetal neurons, NMDA mediated calcium inflow and oxidative stress leading to the phosphorylation of intermediate filaments of striatal nerve cells has been observed (Rahman et al., 2009; Lugo-Huitrón et al., 2013). In rat striatum slices, 100 μM QUIN altered cytoskeleton homeostasis in both astrocytes and neurons. In astrocytes the toxic effects of QUIN manifested in calcium inflow via L-type voltage dependent calcium channels (L-VDCC) and via NMDA receptors, while in neurons they manifested via intracellular calcium channels and metabotropic glutamate receptors. As a result, both cell types show a similar cascade of events: caspase-3 activity is increased, domains in the neurofilament subunits and GFAP are phosphorylated and damage to intermediate filaments develop in both glia cells and nerve cells (Pierozan et al., 2010, 2014, 2015), causing apoptotic cell death (Ishidoh et al., 2010). Rahman et al. (2009) show that mechanisms induced by QUIN cause hyperphosphorylation of the tau protein, also leading to structural changes inside the neuron and they have detected a decrease in the serine/threonine protein phosphatase expression and activity as the culprit of increased tau phosphorylation (Rahman et al., 2009). Their results also suggest, that tau hyperphosphorylation caused by QUIN is also a NMDA receptor-mediated process, as glutamate antagonists had an inhibitory effect on tau hyperphosphorylation. Normally, tau promotes the assembly and maintenance of microtubules, but hyperphosphorylated tau has a decreased affinity to microtubules, and also separates normal tau proteins from microtubules, while forming neurofibrillary tangles inside neurons, leading to structural changes in neurons, and quite possibly being one of the key mechanisms leading to neurodegenerative disorders like Alzheimer's disease (AD) (Rahman et al., 2009). Abnormal tau phosphorylation and accumulation of tau proteins are associated with loss of axons and nerve cells with parallel proportionate axonal damage and MS progression (Anderson et al., 2008; Petzold et al., 2008). These findings suggest that QUIN exerts a toxic effect on the cytoskeleton mainly through NMDA receptor cascade and is closely related to neurodegeneration. Furthermore, the results of another study suggest, that in addition to QUIN, other KP metabolites may be associated with disturbance of the neuronal cytoskeletal proteins (NfL) (Chatterjee et al., 2019).

NEUROFILAMENTS AS A TARGET OF QUIN TOXICITY IN MS

Kynurenine pathway is a main route of TRP degradation. Pro-inflammatory cytokines can influence IDO-1 activation and thus significantly modify this process. The previous sections provided detailed descriptions of how this process affects the cells of the immune system, and additionally that neurotoxic metabolites produced in the process can inflict direct damage on the cells of the CNS (microglia cells, astrocytes, and oligodendrocytes) and cause glutamate excitotoxicity, leading to mitochondrial dysfunction, free radical formation, destabilization of the cytoskeletal system and ultimately to axonal damage [see review (Lovelace et al., 2016)].

Neuro-axonal damage is a main pathogenic factor in many neurological disorders, therefore a biomarker that is specific for axonal damage could be invaluable in the diagnosis of neurodegenerative conditions, determining the extent of the disease, and monitoring treatment efficacy. NFs could be adequate candidates for this purpose, as they are expressed exclusively in neurons and their level closely mirror the extent of axonal damage and neuronal cell death.

Neurofilaments are intermediate filaments (classified by their diameter, ∼10 nm) specific for neurons, and they establish the neuronal cytoskeleton together with microfilaments and microtubules, thereby providing a structural role in axons and axonal transport (Khalil et al., 2018). NFs are usually composed of the following three subunits: the neurofilament light chain (NfL, 68 kDa), medium chain (NfM, 160 kDa) and heavy chain (NfH, 205 kDa), and they can also include α-internexin (66 kDa) and peripherin (58 kDa) (Gentil et al., 2015; Laser-Azogui et al., 2015; Khalil et al., 2018). NF proteins have a structure that is characteristic for intermediate filaments: they consist of an N-terminal head domain, that is a short, variable region; a central α-helical rod domain, that is relatively conserved; and a C-terminal tail domain of highly variable length (Gentil et al., 2015; Khalil et al., 2018). The head domain contains serine and threonine residues and has multiple phosphorylation and glycosylation sites, while the dominant amino acids in the tail domain are lysine and serine, which

also provide multiple phosphorilation sites, and the length of the tail domain is highly characteristic of the neurofilament protein subunit (Khalil et al., 2018). The central rod domain contains hydrophobic heptad repeats, that facilitate the head-to-tail alignment of NF proteins forming coil-to-coil dimers (Gentil et al., 2015; Khalil et al., 2018). This is the first step of the formation of neurofilament heteropolymers: after the formation of dimers, they form tetramers by antiparallel aggregation, then eight tetramers join laterally, creating the cylindrical unit-length filament (ULF) structure (Khalil et al., 2018). The next step is the longitudinal elongation of the NF by the annealing of ULFs, which is followed by radial compaction to form the final NF structure with the diameter of 10 nm (Khalil et al., 2018). The core of the NF consists of NfL subunits, while the NfM and NfH subunits are arranged peripherally, their tails containing multiple phosphorylation sites projecting out radially from the filament structure (Pierozan and Pessoa-Pureur, 2018). NFs are synthesized in the cell body and they are phosphorylated after being transported to the axon.

The exact function of NFs is not yet fully understood, but it is established, that NFs are structural proteins, therefore they have an important role in neuronal organization. NFs provide an intracellular network that protects neurons from mechanical stress, and together with other cytoskeletal elements takes part in creating the intracellular environment and the positioning of organelles, such as the nucleus, axonal mitochondria and the endoplasmic reticulum (ER) (Gentil et al., 2015). NFs are also integral in the radial growth and stability of axons, the maintenance of the axonal diameter and the myelinization, which are the main determinants for efficient, rapid and high–speed nerve conduction. The functions associated with NFs seem to be dependent upon the formation of filamentous structures, however, it is yet undetermined, if they have any functions independent of this process, and neurofilament proteins may have individual functions besides being constituents of NFs (Gentil et al., 2015).

As mentioned earlier, NFs have multiple phosphorylation sites, with the tail domains of NfH and NfM proteins containing many of these sites protruding from the surface of the filament structure. Phosphorylation-dephosphorylation of proteins are an important regulatory mechanism, where negatively charged phosphate groups are added to or removed from Ser, Thr, and Tyr amino acids, thereby changing the function of the protein (Pierozan and Pessoa-Pureur, 2018). The phosphorylation process is executed by protein kinases, while the dephosphorylation is catalyzed by phosphatases. The phosphorylation sites of NfM and NfH subunits are found at repeating Lys-Ser-Pro (KSP) regions at their C-termini (Gentil et al., 2015; Pierozan and Pessoa-Pureur, 2018). The phosphorylation of NFs is a highly specific process catalyzed by mitogen-activated protein kinases (MAPK), such as ERK1/2, JNK, p38MAPK, and proline-directed kinases (e.g., Cdk5), and glycogen synthase kinase 3 (GSK3). The phosphorylation of the NfH tail domain sites most probably plays a role in the regulation of neurofilament mediated axonal transport (Pierozan and Pessoa-Pureur, 2018), and increases the resistance of these subunits against protease activity (Khalil et al., 2018). The N-terminal of the NfL subunit also contains important phosphorylation sites, as the addition of phosphate molecules on these sites regulates the equilibrium of assembly and disassembly of NfM and NfH subunits. The enzymes catalyzing the phosphorylation of NfL head domain sites are c-AMP dependent protein kinase (PKA), protein kinase C (PKC), and Ca^{2+}/calmodulin-dependent protein kinase II (PKCaMII) (Pierozan and Pessoa-Pureur, 2018).

Under normal conditions, the phosphorylation-dephosphorylation processes of NFs are in equilibrium, however, an imbalance of these mechanisms can be detected under neurodegenerative conditions. While the NFs with normal phosphorylation are located in the distal parts of the axons, the hyperphosphorylated NFs can form phospho-neurofilament aggregates, which are usually found in the proximal part of axons and the cell body (Pierozan and Pessoa-Pureur, 2018). The accumulation of these aggregates in the cell body can have a cytotoxic effect, and the hyperphosphorylation can affect their interaction with other cytoskeletal components (Pierozan and Pessoa-Pureur, 2018). The misregulation of both kinase and phosphatase activity can lead to the inequilibrium of phosphorylation-dephosphorylation, which can be induced by stress, as well as a number of metabolites that can accumulate in the brain. Studies have shown that one such metabolite is QUIN. QUIN is one of the metabolites produced in the KP, that is the main metabolic pathway of TRP, whose end product is NAD^+. The enzymes of the KP are mostly located in glial cells in the brain, therefore QUIN is produced predominantly in microglia (Pierozan and Pessoa-Pureur, 2018).

Quinolinic acid is a neuroactive metabolite, that can upset the balance of cytoskeletal homeostasis in neurons by causing abnormal phosphorylation, leading to cell dysfunction and neurodegeneration. As an endogenous NMDA receptor agonist, QUIN activates the NMDA receptors, inducing Ca^{2+} influx into cells, activating phosphorylation enzymes, and consequently causing phosphorylation of cytoskeletal elements, including NFs (Pierozan et al., 2010). Pierozan et al. (2010, 2012) described, that intrastriatal QUIN administration in rat brain induced hyperphosphorylation of NFs in the short term, which effect was mediated by the influx of calcium via NMDA receptor channels under oxidative stress. The hyperphosphorylation of neurofilament was associated with protein kinase PKCaMII, PKA, and PKC activity (Pierozan et al., 2010; Chatterjee et al., 2019), and led to the destabilization of the neurofilament structure. The acutely injected QUIN altered neurofilament phosphorylation in a selective manner, progressing first from the striatum to the cerebral cortex, then to the hippocampus (Pierozan and Pessoa-Pureur, 2018). The activated enzymes catalyzing the phosphorylation differed in the different brain structures: while PKA and PKCaMII were responsible for hyperphosphorylation in the striatum and cortex, MAPKs (such as ERK1/2, JNK, and p38MAPK) were only activated in the hippocampus (Pierozan and Pessoa-Pureur, 2018). This suggests that MAPKs do not take part in the acute toxicity caused by QUIN, they do, however, have a role in the long-lasting effects. According to these findings, the accumulation neurofilament aggregates created by the phosphorylation of NfM and NfH

KSP repeats causes disturbances in the axonal transport, that leads to neural dysfunction and behavioral disturbances in the acute phase, followed by motor impairments in the long term, suggesting the role of the hippocampal involvement (Pierozan and Pessoa-Pureur, 2018).

A study made also by Pierozan et al. on acute striatal slices provides further insight into the cellular mechanisms of excitotoxicity caused by QUIN. In neuronal cells, QUIN activated metabotropic glutaminerg receptors (mGluR1 and mGluR5), caused Ca^{2+} influx through NMDA receptors, as well as initiating Ca^{2+} release from the ER. These events caused the downstream activation of the enzymes PKA, PKC, and PKCaMII, which phosphorylate the N-terminal sites of NfL (Pierozan and Pessoa-Pureur, 2018). The activation of the mGluR1 contributes to these actions: it is upstream from phospholipase C (PLC), which produces diacylglycerol (DAG) and inositol-3-phosphate (IP3). DAG contributes to the activation of PKC, while IP3 contributes to the release of Ca^{2+} from intracellular stores, leading to the phosphorylation of KSP repeats at the C-terminals of NfH and NfM subunits. The main enzyme catalyzing the phosphorylation of these sites, however, seems to be Cdk5, which is located downstream of mGluR5 (Pierozan and Pessoa-Pureur, 2018). The misregulation of these signaling pathways caused by QUIN therefore leads to the hyperphosphorylation of neurofilament subunits, which alters the neuronal homeostasis and the structure of the neuronal cytoskeleton, that can be detected by the changed neuron/neurite ratio and neurite outgrowth (Pierozan and Pessoa-Pureur, 2018).

The evidences shown above contribute to the suggestion, that NFs can have a significant role in the development of neurological disorders, as well as being excellent candidates for a biomarker of certain neurological conditions (Khalil et al., 2018). It is known that axonal damage releases NFs into the extracellular space and, consequently, to the CSF and blood, therefore an appropriately sensitive analytical method can be used to detect and quantify neurofilament concentration. It is relatively difficult, because NfL levels in the serum are relatively low, but in the recent years, the fourth generation single molecule array (Simoa) assay technologies presented the possibility to reliably quantify NfL levels in the serum, and detect even minor changes caused by aging or minor injuries (Khalil et al., 2020). There is good correlation between neurofilament levels in the serum and the extent of axonal damage; however, it is important to emphasize that even in healthy controls the blood level of NFs increases by an average of 2.2% per year, and metabolic alterations of neurofilament turnover can also affect the serum level (Khalil et al., 2018). Moreover, there are no standard reference values or intervals of NfL levels established yet, therefore the interpretation of the measured serum NfL levels is precarious at the time being. Elevated neurofilament levels have been detected in various diseases associated with axonal damage, including amyotrophic lateral sclerosis (ALS), AD, PD, stroke, HD, and MS (Khalil et al., 2018). This suggests that NFs are not specific to certain neurological diseases, therefore an elevated neurofilament level might indicate the need for thorough differential diagnosis.

Pathology of MS characterized by neurodegeneration and axonal injury, thereby NfL may be a putative biomarker for determination of neuronal damage. While the standard diagnostic tool for MS is currently MRI, it has its limitations: while it accurately shows lesions in the white matter, lesions in the gray matter are more difficult to detect; moreover, the traditional imaging cannot accurately determine the extent of neuro-axonal degradation, which is the most important factor in determining long-term functional disability (Khalil et al., 2018). In several studies, an elevated neurofilament level in CSF was observed in MS (Malmeström et al., 2003; Rejdak et al., 2008). Recently, an intensive study has been conducted to investigate to presence of NFs protein levels in CSF, as an increase NfL level has been observed in patients not only with RRMS (Lycke et al., 1998), but the NfL levels also seem to correlate well with PPMS (Pawlitzki et al., 2018). Based on immunoassay studies, the CSF levels of NfL correlated well with three aspects of MS: the degree of disability, the disease activity and the time passed from the last relapse in RRMS (Khalil et al., 2018). Recently, based on numerous studies, NfL is considered a promising and reliable prognostic factor for patients with MS, as it well reflects the degree of disease activity, the NfL levels correlate with clinically definitive MS transformation (Kuhle et al., 2015a), they correlate with atrophy of the brain and spinal cord, the relapse rate, or worsening of disability (Kuhle et al., 2016; Petzold et al., 2016; Barro et al., 2018). A long-term follow-up study has found a close correlation between serum NfL levels and MRI lesions and degree of atrophy measured 10 years later (Chitnis et al., 2018), while another found that patients with higher baseline serum NfL levels showed significantly more brain and spinal cord volumes over the 2 and 5 years follow-up (Khalil et al., 2018). Based on a study, patients with ongoing disease-modifying therapy (DMT) had significantly lower serum NfL levels, than untreated patients (Khalil et al., 2018), in line with this, Kuhle et al. (2015b) have determined that fingolimod therapy is associated with significantly lower blood NfL concentration . There is some evidence that KP metabolites can lead to neuro-axonal damage. A recently published study (Chatterjee et al., 2019) confirmed a positive association between KP metabolites, plasma NfL levels and amyloid-β concentration, possibly indicating that a high level of KP metabolites may be associated with NfL damage. Our own recently published study also provides support for this association. We have investigated the correlation and association between biomarkers of neurodegeneration (NfL) and kynurenine metabolites in the CSF of MS patients. Additionally, we have identified a strong positive correlation between NfL and QUIN levels (Rajda et al., 2020). Based on these results it can be concluded that QUIN destabilizes the neuronal cytoskeleton system via the phosphorylation of structural proteins which ultimately leads to neuro-axonal damage, contributing to neurodegeneration in MS. This evidence raises the exciting possibility that NFs could be a new target of neurotoxic QUIN; however, further studies are required to confirm this.

CONCLUSION

Several studies have demonstrated that the profile of KP changes in the course of MS progression and that changes in KP

metabolites can lead to neuro-axonal damage through multiple mechanisms. Additionally, NFs are good biomarkers of neuro-axonal damage and their levels closely correlate with the extent of damage. These results suggest on one hand that kynurenines are highly relevant to the process of neurodegeneration, and on the other hand that the metabolic profiling of the KP and neurofilament may be potentially useful in finding patients at risk of progression or worse disease outcome, and could be useful in devising individual therapeutic approaches in the future.

AUTHOR CONTRIBUTIONS

DP and HP contributed to the writing of the article. CR and LV revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Neurofilament Light Chain as Biomarker for Amyotrophic Lateral Sclerosis and Frontotemporal Dementia

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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are two related currently incurable neurodegenerative diseases. ALS is characterized by degeneration of upper and lower motor neurons causing relentless paralysis of voluntary muscles, whereas in FTD, progressive atrophy of the frontal and temporal lobes of the brain results in deterioration of cognitive functions, language, personality, and behavior. In contrast to Alzheimer's disease (AD), ALS and FTD still lack a specific neurochemical biomarker reflecting neuropathology *ex vivo*. However, in the past 10 years, considerable progress has been made in the characterization of neurofilament light chain (NFL) as cerebrospinal fluid (CSF) and blood biomarker for both diseases. NFL is a structural component of the axonal cytoskeleton and is released into the CSF as a consequence of axonal damage or degeneration, thus behaving in general as a relatively non-specific marker of neuroaxonal pathology. However, in ALS, the elevation of its CSF levels exceeds that observed in most other neurological diseases, making it useful for the discrimination from mimic conditions and potentially worthy of consideration for introduction into diagnostic criteria. Moreover, NFL correlates with disease progression rate and is negatively associated with survival, thus providing prognostic information. In FTD patients, CSF NFL is elevated compared with healthy individuals and, to a lesser extent, patients with other forms of dementia, but the latter difference is not sufficient to enable a satisfying diagnostic performance at individual patient level. However, also in FTD, CSF NFL correlates with several measures of disease severity. Due to technological progress, NFL can now be quantified also in peripheral blood, where it is present at much lower concentrations compared with CSF, thus allowing less invasive sampling, scalability, and longitudinal measurements. The latter has promoted innovative studies demonstrating longitudinal kinetics of NFL in presymptomatic individuals harboring gene mutations causing ALS and FTD. Especially in ALS, NFL levels are generally stable over time, which, together with their correlation with progression rate, makes NFL an ideal pharmacodynamic biomarker for therapeutic trials. In this review, we illustrate the significance of NFL as biomarker for ALS and FTD and discuss unsolved issues and potential for future developments.

Keywords: amyotrophic lateral sclerosis, frontotemporal dementia, cerebrospinal fluid, biomarkers, neurofilament light chain

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are two related neurodegenerative diseases. ALS is the most common motor neuron disease (MND) and affects both upper motor neurons (UMNs) located in the cerebral cortex and lower motor neurons (LMNs) located in the brainstem and spinal cord. Their degeneration results in progressive paralysis of voluntary muscles leading to death from respiratory failure after a median of 3–5 years from symptom onset (Masrori and Van Damme, 2020). The large majority of ALS cases occur sporadically, but 5–10% of ALS patients have a family history, usually with autosomal dominant inheritance, and approximately two thirds of them harbor mutations in one (or sometimes more) of > 20 genes, the most common being the $(G_4C_2)_n$ hexanucleotide repeat expansion (HRE) in the *C9orf72* gene and mutations in the genes *SOD1*, *TARDBP*, and *FUS* (Mejzini et al., 2019). The diagnosis of ALS is fundamentally clinical and is supported by investigations such as electromyography. There is no effective therapy for ALS, with the only two approved specific drugs, riluzole and edaravone, producing modest beneficial effects in terms of prolongation of survival and slowing of functional deterioration, respectively (Masrori and Van Damme, 2020).

FTD is the second most common form of dementia in people younger than 65 years and is classified into three main variants: the behavioral variant (bvFTD) and the two classic variants of primary progressive aphasia (PPA), i.e., the non-fluent/agrammatic variant (nfvPPA) and the semantic variant (svPPA) (Convery et al., 2019). The third PPA variant, the logopenic one (lvPPA), is usually considered separately because it is most commonly due to underlying AD pathology. While bvFTD is characterized by progressive changes in personality and behavior accompanied by a dysexecutive type of cognitive deterioration, the PPAs show selective deficits of speech production (nfvPPA) or of semantic knowledge (svPPA) resulting in progressive language disturbance and absent or only limited impairment of other cognitive domains. Also for FTD, there is no effective therapy, and therefore, the disease poses a heavy burden on often relatively young patients and caregivers (Convery et al., 2019). The neuropathological substrate of bvFTD, nfvPPA, and svPPA is frontotemporal lobar degeneration (FTLD), which is characterized by progressive atrophy of the frontal and temporal lobes of the brain and is in turn classified into three distinct pathological entities: FTLD-tau (comprising approximately 45% of cases), FTLD-TDP (50%), and the rare FTLD-FUS (5%), characterized by intracellular inclusions of pathologically modified forms of the proteins tau, TDP-43 and FUS, respectively (Mann and Snowden, 2017).

TDP-43 pathology is the fundamental element linking FTD and ALS: indeed, TDP-43 inclusions are the neuropathological substrate of virtually all sporadic and the large majority of genetic ALS cases (Neumann et al., 2006). Accordingly, up to 40–50% of ALS patients show at least subtle cognitive or behavioral alterations of the FTD spectrum upon specific neuropsychological investigation, with up to 10–15% fulfilling diagnostic criteria for FTD itself. The co-occurrence of the two diseases is, indeed, not uncommon, both at the individual level and within families, especially considering that the *C9orf72* HRE can cause not only ALS but also FTLD-TDP or both (Masrori and Van Damme, 2020). The other two main genes causing familial forms of FTD, which overall represent up to a third of all FTD cases, are *GRN* and *MAPT*, associated, respectively, with FTLD-TDP and FTLD-tau (Convery et al., 2019). ALS and FTD due to FTLD-TDP are now most commonly considered as two diseases belonging to the same neuropathological spectrum of TDP-43 proteinopathies (de Boer et al., 2020). A further, far less common, neuropathological link between ALS and FTD is represented by FUS pathology, occurring in rare instances of both diseases; however, notably, whereas cases of ALS with FUS pathology are associated with mutations in the corresponding gene *FUS*, the vast majority of FTLD-FUS cases appear to be sporadic (Mann and Snowden, 2017).

Both for ALS and for FTD, especially in the last years, considerable developments have taken place in the field of neurochemical biomarkers, following the example of the successfully established cerebrospinal fluid (CSF) biomarkers of AD (Blennow et al., 2010). Biomarkers are needed for several aims: to support clinicians in the diagnosis and especially in the differential diagnosis; to enable early diagnosis, thus allowing prompt initiation of disease-modifying treatments or early enrollment of patients in clinical trials; to stratify patients in the trials; to demonstrate target engagement by an experimental treatment; and to measure treatment effects as pharmacodynamic biomarkers. The most investigated biomarkers in ALS and FTD are the neurofilaments and especially the light chain (NFL) (Verde et al., 2019a; Swift et al., 2021). Neurofilaments are structural components of the axonal cytoskeleton and are released from the axon as a consequence of its damage or degeneration. This increases their concentration in the CSF and hence in the blood compared with physiological conditions and constitutes the foundation of their use as biomarkers for neurological diseases.

NEUROFILAMENTS: PHYSIOLOGY, BIOLOGICAL RATIONALE AS BIOMARKERS, AND MEASUREMENT

Neurofilaments are a class of intermediate filaments and are a major constituent of the cytoskeletal scaffold of the central (CNS) and peripheral nervous system (PNS) neurons (Yuan et al., 2017). They are most abundant in large myelinated axons; smaller amounts are also found in cell bodies, dendrites, and synapses (Gafson et al., 2020). Neurofilaments are heteropolymers composed of four different subunits: the light, middle, and heavy chains (NFL, NFM, and NFH, respectively), plus α-internexin in the CNS and peripherin in the PNS (Yuan et al., 2017). All neurofilament subunits comprise an N-terminal head domain, a highly conserved central rod domain, and a tail domain of variable length. In addition to this molecular structure, they undergo posttranslational modifications, including phosphorylation, O-linked glycosylation, nitration, and ubiquitination. The molecular weights of the different

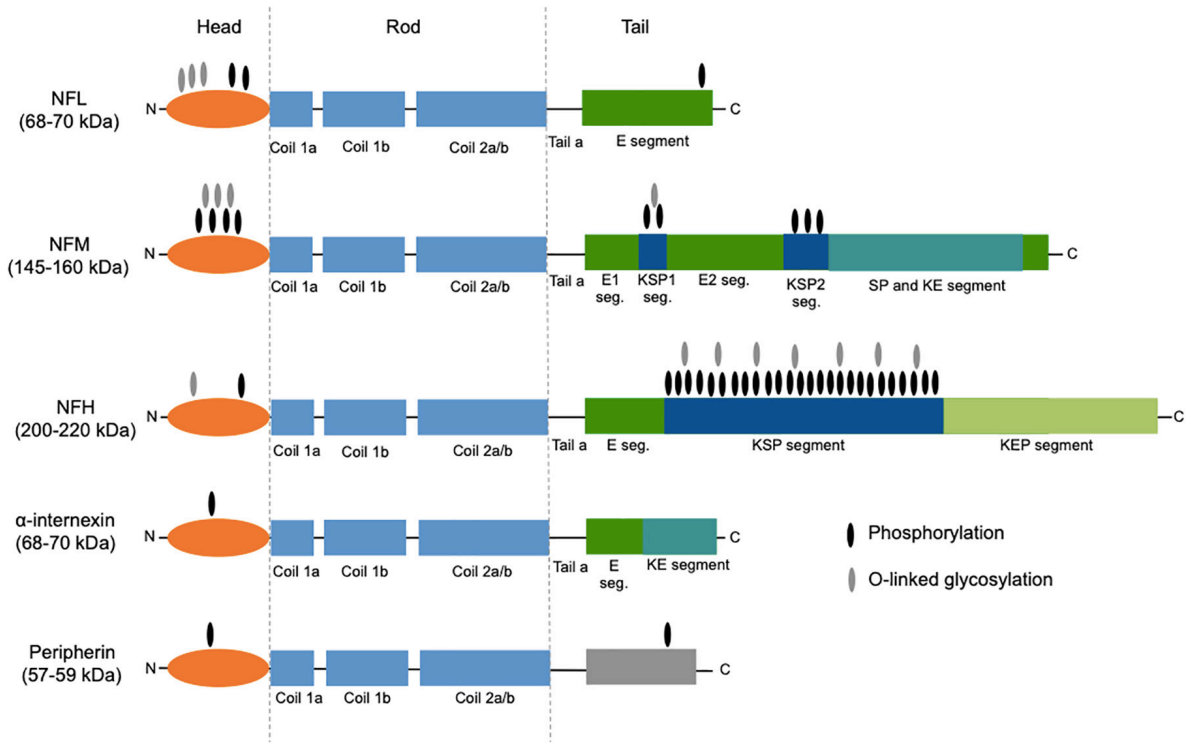

FIGURE 1 | Structure of neurofilament subunits. All neurofilament subunits share a common structure composed of a globular amino-terminal head domain, a central conserved α-helical rod domain (comprising several coiled coils), and a carboxy-terminal tail of variable length. NFM and NFH are characterized by long C-terminal tail domains rich in heavily phosphorylated serine-proline-lysine (KSP) repeats. The two main posttranslational modifications, namely, phosphorylation and O-linked glycosylation, are shown. The figure was created taking Figure 3 from Yuan et al. (2017) as model, with the authors' permission. NFL, neurofilament light chain; NFM, neurofilament middle chain; NFH, neurofilament heavy chain; E segment (seg.), glutamic acid-rich segment; KSP, lysine-serine-proline; SP, serine-proline; KE, lysine-glutamic acid; KEP, lysine-glutamic acid-proline.

subunits, predicted based on the genetic sequence, are as follows: 112.5, 102.5, 61.5, 55.4, and 53.7 kDa (NFH, NFM, NFL, alpha-internexin, and peripherin, respectively). Because of the abundance in glutamate residues and of posttranslational modifications, when measured by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), their molecular weights are slightly higher, namely, 200–220, 145–160, 68–70, 58–66, and 57–59 kDa, respectively (**Figure 1**). The backbone of neurofilaments is composed of NFL plus α-internexin or peripherin, all three having short tail domains, while NFM and NFH are placed more peripherally, with their long tails projecting radially (Yuan et al., 2017).

The basic assembly of neurofilaments is a paired coiled-coil dimer, which then associates in an antiparallel way with another dimer to form a non-polar tetramer. Eight tetramers associate circumferentially giving rise to a cylindrical structure called unit length filament (ULF). ULFs then undergo end-to-end annealing and radial compaction forming elongated structures with the typical 10-nm diameter of intermediate filaments. These structures have side arms constituted by the long tails of NFH and NFM, which are important for connecting neurofilaments among them as well as with other cytoskeletal components and cellular organelles, e.g., microtubules and mitochondria (**Figure 2**; Yuan et al., 2017). NFM and NFH contain the most abundant phosphorylation sites, and neurofilament phosphorylation inhibits proteolysis and is therefore important for the structural stability of the whole scaffold (Gafson et al., 2020). Neurofilaments are synthesized in the cell body and are then rapidly but intermittently transported distally along axons, resulting in a net slow anterograde movement, in accordance with the slow turnover of the whole neurofilament network of myelinated axons (Roy et al., 2000; Gafson et al., 2020). At the synaptic level, neurofilaments are more abundant in the postsynaptic compartment, and the synaptic neurofilament pool shows structural as well as biochemical differences from the axonal one (Yuan et al., 2003). The factors regulating neurofilament turnover are not precisely known; degradation involves the ubiquitin–proteasome system and probably also autophagy (Gafson et al., 2020). Neurofilaments have several biological functions: most notably, they are important for the stability of axons, especially of large myelinated ones, and for their radial growth, which determines their fast conduction properties (Barry et al., 2012). They also contribute to maintaining the stability of the mitochondria and the cytoskeletal content of microtubules (Bocquet et al., 2009; Gentil et al., 2015). At the synaptic level, they have a role in maintaining the structure and function of dendritic spines as well as a role in regulating glutamatergic

and dopaminergic neurotransmission: as an example, NFL interacts with the cytoplasmic C-terminal domain of the GluN1 subunit of the NMDA glutamate receptor (Yuan et al., 2015; Yuan et al., 2018).

Alterations of neurofilament structure and function could be involved in the pathogenesis of neurodegenerative diseases, with the largest body of evidence pointing toward a possible role in ALS. Neurofilament accumulations are observed in spinal motor neurons in ALS (Corbo and Hays, 1992), and alterations of the genes coding for NFH and peripherin were found in a small number of ALS patients (Figlewicz et al., 1994; Gros-Louis et al., 2004). A mutation of the NFL gene in a genetic mouse model of ALS causes degeneration of spinal motor neurons with accumulation of neurofilaments and atrophy of skeletal muscle (Lee et al., 1994), while genetic deletion of the NFL subunit in the *SOD1*-mutant ALS mouse model slowed disease onset and progression, at the same time reducing selectivity of the disease process toward motor neurons (Williamson et al., 1998). Noteworthy is also the finding that TDP-43 binds the mRNA of NFL and stabilizes it, preventing its degradation (Strong et al., 2007).

The role of neurofilaments as biomarkers is thought to be due to their release through the axonal plasma membrane as a consequence of axonal damage or degeneration (Khalil et al., 2018). Following leakage into the extracellular fluid (ISF) and hence into the CSF, neurofilaments penetrate into the blood, where they are usually present with a concentration gradient of about 1:40 relative to the CSF (Gaetani et al., 2019; **Figure 3**). This is the reason why elevated neurofilament levels in the CSF and in the blood have been described in a variety of neurological conditions characterized by neuroaxonal damage. This is true not only for neurodegenerative diseases but also for other pathophysiological processes, including multiple sclerosis (MS) (Disanto et al., 2017), HIV encephalopathy (Gisslen et al., 2016), and traumatic brain injury (Shahim et al., 2016). For the above reasons, neurofilaments are rather an unspecific marker of axonal damage/degeneration than a pathology-specific marker as, for example, Aβ or phosphorylated tau for AD (Gaetani et al., 2019). However, in the diagnostic field, they can be useful in discriminating between conditions characterized by a higher vs. a lower amount or rate of degeneration of large myelinated axons. This is the case of ALS, in which NFL elevations exceed those observed in most other neurological diseases (Bridel et al., 2019). Moreover, in the same wide range of neurological conditions, neurofilaments can have prognostic significance: as an example, in MS, baseline CSF and serum NFL levels predict longitudinal functional disability (Disanto et al., 2017).

Neurofilament levels in the CSF can be measured by means of traditional sandwich enzyme-linked immunosorbent assays (ELISAs) (Petzold et al., 2010). A major technological advance has been represented by the introduction of electrochemiluminescence (ECL) assays, enabling the measurement of neurofilaments also in the blood of patients with neurological diseases (Gaiottino et al., 2013). Finally, the introduction of the ultrasensitive single molecule array (Simoa) technology, based on simultaneous digital counting of single capture microbeads, has allowed precise quantification also of the low concentrations of neurofilaments in the blood (serum or plasma) of healthy individuals (Rissin et al., 2010; Gisslen et al., 2016). This has paved the way for an expansion of the field of biomarkers of neurodegenerative diseases from the CSF only toward peripheral blood, with considerable advantages in terms of reduced invasiveness, scalability, and opportunities of longitudinal evaluations. Although current important limitations to a large-scale application of neurofilament measurement with the Simoa technology are represented by its presently limited diffusion outside major research centers and relatively high costs, it can be envisioned that this technology as well as similar ultrasensitive ones will undergo increasing diffusion in the next few years. While in ALS the phosphorylated neurofilament heavy chain (pNFH) has been studied as CSF biomarker to a similar extent as NFL, in the field of FTD and other dementias, NFL has been more widely investigated than pNFH (Verde et al., 2019a; Swift et al., 2021). Moreover, pertaining to measurements in peripheral blood, a larger body of evidence has been produced for NFL, not least because of technical reasons regarding measurement, including the earlier availability of ultrasensitive assays for detecting this neurofilament subunit (Verde et al., 2019b).

NFL AS BIOMARKER FOR AMYOTROPHIC LATERAL SCLEROSIS

NFL as ALS Diagnostic Biomarker

ALS is characterized by a relatively rapid degeneration of motor neurons, and these cells, given their large myelinated axons of considerable length, contain a great amount of neurofilaments. These are the main reasons why ALS shows the most massive elevation of NFL concentrations in the CSF among the commonest neurodegenerative diseases (Olsson et al., 2019). In comparison with neurologically healthy controls, CSF NFL levels are more than sevenfold increased in ALS (Skillbäck et al., 2017; Bridel et al., 2019). Rosengren et al. (1996) were the first to show the potential of NFL as biomarker for ALS: in 1996, they developed a new ELISA assay for NFL and found that the protein was present at higher concentrations in the CSF of 12 ALS patients compared with 34 neurologically healthy individuals. Several years later, increased NFL levels in the CSF of ALS patients were reported also in comparison with other neurological, including neurodegenerative, diseases (Zetterberg et al., 2007; Tortelli et al., 2012).

One of the main investigations on NFL (and also on pNFH) as biomarker of ALS is the large German study by Steinacker et al. (2016) (**Table 1**). They measured NFL and pNFH levels in the CSF of 253 MND patients, 85 patients presenting with MND mimics (i.e., diseases clinically mimicking MND), and 117 patients with other neurological diseases. The levels of the two neurofilaments were strongly correlated, as confirmed in other cohorts (Rossi et al., 2018). Both neurofilaments showed higher levels in ALS compared with every other diagnostic category. For CSF NFL, this enabled discrimination of MND cases from MND mimics with an area under the ROC curve (AUC) of 0.866 and from all non-MND patients with an AUC of 0.851. In particular, at

TABLE 1 | Representative studies on NFL as diagnostic biomarker for ALS.

Study (authors and year)	ALS patients	Controls	Biological fluid	Type of assay	NFL levels in ALS patients (pg/ml; median, range)	NFL levels in controls (pg/ml; median, range)	Cutoff for discrimination between ALS and controls (pg/ml)	AUC (95% CI)	Sensitivity and specificity (95% CI)
Steinacker et al. (2016)	253 MND patients (222 ALS, 11 PLS, 20 familial/genetic ALS)	85 MND mimic patients, 28 AD patients, 26 patients with Parkinsonisms, 33 patients with polyneuropathies, 30 patients with facial palsy	CSF	ELISA	MND: 5,068 (100–38,350) ALS: 4,990 (100–38,350) PLS: 3,750 (100–26,650) Genetic/familial ALS: 6,452 (785–22,040)	MND mimics: 865 (168–10,000) AD: 1,510 (625–9,507) Parkinsonian syndromes: 1,455 (439–4,841) Polyneuropathies: 1,034 (277–24,330) Facial palsy: 585 (100–2,676)	2,200	MND vs. MND mimics: 0.866 (0.821–0.911) MND vs. all control groups: 0.851 (0.813–0.888)	MND vs. MND mimics: Se 77% (71–82%), Sp 88% (79–94%) MND vs. all control groups: Se 77% (71–82%), Sp 85% (79–90%)
Poesen et al. (2017)	220	50 ALS mimic patients 316 neurological disease controls	CSF	ELISA	9,427 (370–108,909)	ALS mimic patients: 1,407 (613–36,597) Neurological disease controls: 1,790 (262–53,677)	ALS vs. ALS mimics: 2,453 ALS vs. disease controls: 3,819	ALS vs. ALS mimics: 0.863 (0.808–0.908) ALS vs. disease controls: 0.809 (0.763–0.849)	ALS vs. ALS mimics: Se 85.4% (78.8–90.6%), Sp 78.0% (64.0–88.5%) ALS vs. disease controls: Se 78.8% (71.4–85.0%), Sp 72.7% (66.0–78.8%)
Feneberg et al. (2018)	48 ALS patients sampled ≤ 6 months after symptom onset ("early ALS") (CSF: 48; serum: 40) 128 ALS patients sampled > 6 months after symptom onset ("late ALS") (CSF: 128; serum: 112)	65 patients with ONDs (CSF: 65; serum: 48) 27 patients with MND mimics (CSF: 27; serum: 21)	CSF, serum	ELISA (CSF), Simoa (serum)	Early ALS, CSF: 6,802 (1,053–25,650) Early ALS, serum: 255 (51–879) Late ALS, CSF: 5,266 (985–24,240) Late ALS, serum: 196 (24–4,235)	ONDs, CSF NFL: range 152–4,874 ONDs, serum NFL: range 9–427 MND mimics, CSF NFL: range 219–3,390 MND mimics, serum NFL: range 15–95	CSF NFL, early ALS vs. ONDs: 2,300 CSF NFL, early ALS vs. MND mimics: 2,183 Serum NFL, early ALS vs. ONDs: 128 Serum NFL, early ALS vs. MND mimics: 97 CSF NFL, late ALS vs. ONDs: 2,146 CSF NFL, late ALS vs. MND mimics: 2,089 Serum NFL, late ALS vs. ONDs: 116 Serum NFL, late ALS vs. MND mimics: 95	CSF NFL, early ALS vs. ONDs: 0.95 (0.91–0.99) CSF NFL, early ALS vs. MND mimics: 0.94 (0.94–1) Serum NFL, early ALS vs. ONDs: 0.92 (0.85–0.99) Serum NFL, early ALS vs. MND mimics: 0.99 (0.97–1) CSF NFL, late ALS vs. ONDs: 0.93 (0.9–0.96) CSF NFL, late ALS vs. MND mimics: 0.96 (0.93–0.99) Serum NFL, late ALS vs. ONDs: 0.9 (0.83–0.97) Serum NFL, late ALS vs. MND mimics: 0.97 (0.94–1)	CSF NFL, early ALS vs. ONDs: Se 94% (83–99%), Sp 86% (75–93%) CSF NFL, early ALS vs. MND mimics: Se 89% (71–98%), Sp 94% (83–99%) Serum NFL, early ALS vs. ONDs: Se 88% (73–96%), Sp 92% (80–94%) Serum NFL, early ALS vs. MND mimics: Se 100% (84–100%), Sp 90% (76–97%) CSF NFL, late ALS vs. ONDs: Se 89% (82–93%), Sp 84% (73–92%) CSF NFL, late ALS vs. MND mimics: Se 89% (71–98%), Sp 89% (81–93%) Serum NFL, late ALS vs. ONDs: Se 79% (70–86%), Sp 92% (80–98%) Serum NFL, late ALS vs. MND mimics: Se 100% (84–100%), Sp 84% (76–90%)

(Continued)

TABLE 1 | Continued

Study (authors and year)	ALS patients	Controls	Biological fluid	Type of assay	NFL levels in ALS patients (pg/ml; median, range)	NFL levels in controls (pg/ml; median, range)	Cutoff for discrimination between ALS and controls (pg/ml)	AUC (95% CI)	Sensitivity and specificity (95% CI)
Gille et al. (2019)	149	19 ALS mimic patients 82 disease controls (48 GBS, 20 CIDP, 14 HSP)	Serum	ECL	179 (0.3–1,141)	ALS mimics: 29 (6–1,053) Disease controls: GBS: 123 (22–9,045); CIDP: 101 (29–2,863); HSP: 37 (8–639)	ALS vs. ALS mimics: 93 ALS vs. (GBS + CIDP): 139 ALS vs. HSP: 55	ALS vs. ALS mimics: 0.85 (0.79–0.90) ALS vs. (GBS + CIDP): 0.58 (0.51–0.64) ALS vs. HSP: 0.84 (0.78–0.90)	ALS vs. ALS mimics: Se 79.2% (71.8–85.4%), Sp 84.2% (60.4–96.6%) ALS vs. (GBS + CIDP): Sp 63.2% (50.7–74.6%) ALS vs. HSP: Se 89.3% (83.1–93.7%), Sp 78.6% (49.2–95.3%)
Verde et al. (2019b)	124	50 patients without neurodegenerative diseases (non-neurodegenerative controls), 44 patients with conditions in the differential diagnosis of ALS (disease controls), 20 FTD patients, 20 AD patients, 19 PD patients, 6 CJD patients	Serum	Simoa	125 (14.6–908)	Non-neurodegenerative controls: 16.2 (5.4–79.9) Disease controls: 27.3 (0.7–210) FTD: bvFTD: 46.5 (19.4–103); nfvPPA: 49.6 (28.2–124); svPPA: 53.9 (45.3–62.5); PPA, unspecified: 76.3 (51.6–101) AD: 38.6 (21.6–240) PD: 27.5 (7.7–81.5) CJD: 162.5 (121–288)	ALS vs. non-neurodegenerative controls: 49 ALS vs. disease controls: 62 ALS vs. all non-ALS categories: 62	ALS vs. non-neurodegenerative controls: 0.971 (0.950–0.991) ALS vs. disease controls: 0.873 (0.810–0.935) ALS vs. all non-ALS categories: 0.887 (0.849–0.926)	ALS vs. non-neurodegenerative controls: Se 89.5% (82.7–94.3%), Sp 92% (80.8–97.8%) ALS vs. disease controls: Se 85.5% (78.0–91.2%), Sp 77.3% (62.2–88.5%) ALS vs. all non-ALS categories: Se 85.5% (78.0–91.2%), Sp 81.8% (74.9–87.4%)

AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AUC, area under the ROC (receiver operating characteristic) curve; CJD, Creutzfeldt–Jakob disease; CI, confidence interval; CIDP, chronic inflammatory demyelinating polyneuropathy; CSF, cerebrospinal fluid; ECL, electrochemiluminescence; ELISA, enzyme-linked immunosorbent assay; FTD, frontotemporal dementia; GBS, Guillain–Barré syndrome; HSP, hereditary spastic paraparesis; MND, motor neuron disease; NFL, neurofilament light chain; nfvPPA, non-fluent variant of primary progressive aphasia; ONDs, other neurological diseases; PD, Parkinson's disease; PLS, primary lateral sclerosis; PPA, primary progressive aphasia; Se, sensitivity; Simoa, single molecule array; Sp, specificity; svPPA, semantic variant of primary progressive aphasia.

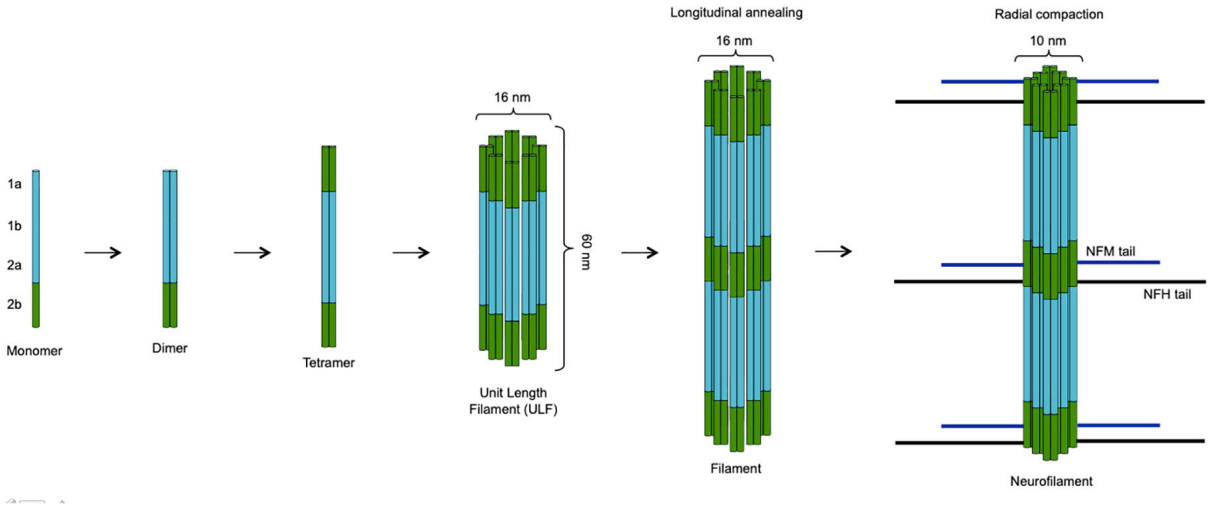

FIGURE 2 | Assembly of neurofilaments. Two neurofilament subunits combine to form a paired coiled-coil dimer, which then undergoes antiparallel association with another dimer giving rise to a non-polar tetramer. The circumferential association of eight tetramers produces a cylindrical unit length filament (ULF) with a diameter of 16 nm. ULFs undergo end-to-end annealing forming elongated filaments, whose radial compaction results in reduction of the diameter to the typical 10-nm size of intermediate filaments. While the backbone of the neurofilament scaffold is constituted by NFL plus α-internexin or peripherin (in the CNS and in the PNS, respectively), NFM and NFH molecules associate more peripherally with their long tails projecting outside and interacting with other cytoskeletal components (microtubules) and organelles (mitochondria). The figure was created taking Figure 4 from Yuan et al. (2017) as model, with the authors' permission.

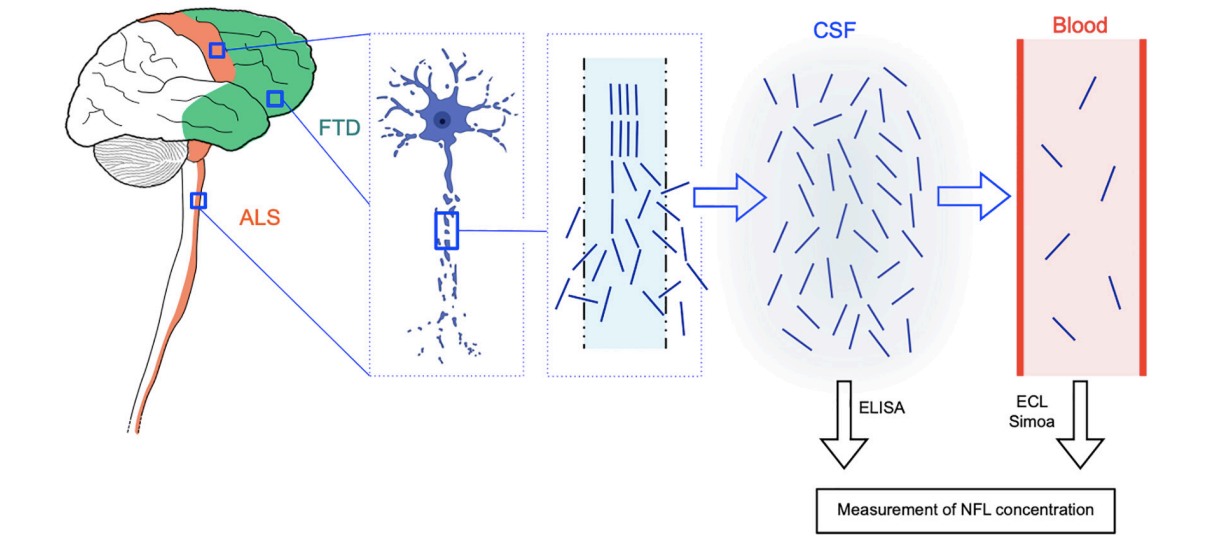

FIGURE 3 | Rationale of NFL as CSF and blood biomarker for ALS and FTD. ALS is characterized by degeneration of upper motor neurons of the motor cortex and of lower motor neurons of the brainstem and spinal cord, while in FTD, the disease process affects neurons of the frontal and temporal cortices. In both diseases, neuroaxonal degeneration causes the release of neurofilament (and in particular NFL) molecules from the axon to the interstitial fluid and hence to the CSF and finally to the blood, where they are present at much lower concentrations (approximate CSF:blood ratio for NFL: 40:1). NFL levels in the CSF can be quantified by means of conventional ELISA assays, while more sensitive techniques are needed for the measurement of the lower blood (serum or plasma) levels, i.e., electrochemiluminescence (ECL) and the ultrasensitive single molecule array (Simoa) technology. The image of the degenerating neuron was taken from BioRender.com (2021).

a cutoff of 2,200 pg/ml, CSF NFL distinguished between MND and MND mimics with 77% sensitivity and 88% specificity. The diagnostic performance of pNFH was similar (Steinacker et al., 2016). Poesen et al. (2017) conducted an analogous study on CSF neurofilaments in a comparably large cohort. Notably, they found that CSF NFL was less specific than pNFH in discriminating between ALS and neurological disease controls, because patients with FTD and some of the patients with inflammatory radiculoneuropathies [i.e., chronic inflammatory demyelinating polyneuropathy (CIDP) and Guillain–Barré syndrome (GBS)]

showed NFL elevations in the range of ALS patients. Also in the differentiation between ALS and ALS mimics, NFL performed slightly but significantly less well than pNFH, with an AUC of 0.863 corresponding to a sensitivity of 78.2% and a specificity of 63% (Poesen et al., 2017). Other studies on CSF NFL reported AUCs as high as 0.922 for the discrimination between ALS and ALS mimics (Abu-Rumeileh et al., 2020).

Feneberg et al. (2018) investigated the diagnostic performance of CSF NFL for ALS patients who are evaluated early in the disease course, i.e., within 6 months from symptom onset: also these patients had higher CSF NFL levels than the other categories, including MND mimics, resulting in good discrimination of ALS from MND mimics with AUC 0.94, sensitivity 89%, and specificity 94%. This supports a role of NFL measurement as an aid to the early diagnosis of ALS, which is fundamental for timely initiation of disease-modifying treatments and for enrollment of patients in clinical trials.

A meta-analysis published in 2019 and examining 11 studies conducted on a total of 1,239 patients with ALS and 806 patients with ALS mimic diseases confirmed the significant difference in CSF NFL levels between the two conditions. This corresponded to a ratio of means of 3.35 in NFL levels between ALS and mimics (Forgrave et al., 2019).

Gaiottino et al. (2013) were the first to measure NFL concentrations in the blood, namely, in the serum, of ALS patients using an ECL assay. ALS patients had higher NFL levels compared with healthy controls and patients with neurological complaints but no evidence of CNS pathology; serum NFL enabled discrimination between ALS and healthy controls with a sensitivity of 91.3% and a specificity of 91%. Importantly, CSF and serum levels of NFL were strongly correlated in ALS (r = 0.70), with a mean ratio of concentrations between CSF and serum of 57.8 (Gaiottino et al., 2013). The high correlation between CSF and serum levels of NFL has been subsequently confirmed by several other investigations (Steinacker et al., 2017a; Benatar et al., 2018). The same ECL assay was used in another study to demonstrate increased NFL levels also in the plasma of ALS patients compared with healthy controls, resulting in an AUC of 0.869 (Lu et al., 2015).

Gille et al. (2019) measured, by means of ECL as well, serum NFL in a cohort of patients with ALS (n = 149) and patients with ALS mimics and other neurological diseases. Given serum NFL levels on average sixfold higher in ALS compared with ALS mimics, the biomarker enabled differentiation between the two conditions with an AUC of 0.85 (best cutoff: 93 pg/ml). Notably, ALS had significantly higher serum NFL levels compared with the UMN diseases primary lateral sclerosis (PLS) and hereditary spastic paraparesis (HSP), while the differences between ALS and the LMN disease progressive muscular atrophy (PMA), CIDP, and GBS did not reach statistical significance. The performance in discriminating between ALS and PLS was good with an AUC of 0.89, corresponding to a sensitivity of 80.5% and a specificity of 90.9% (Gille et al., 2019).

We and others conducted a similar study investigating serum NFL in ALS patients (n = 124), patients with other neurodegenerative diseases, patients with conditions in the differential diagnosis of ALS, and neurological patients without neurodegenerative diseases. Serum NFL levels, measured with the Simoa technology, were significantly higher in ALS compared with each other category with the notable exception of Creutzfeldt–Jakob disease (CJD). In the discrimination between ALS and conditions in its differential diagnosis, serum NFL showed an AUC of 0.873 (best cutoff: 62 pg/ml) (Verde et al., 2019b). Importantly, similar to what has been described for CSF NFL, also serum NFL was shown to be already elevated in ALS patients presenting within 6 months from symptom onset, enabling excellent discrimination from MND mimics with AUC of 0.99 (Feneberg et al., 2018).

The already mentioned meta-analysis of 2019 examined four studies on serum NFL comprising 458 ALS cases and 181 cases of ALS mimics and confirmed significantly higher levels in the former category, with a ratio of means of 8.15 (Forgrave et al., 2019).

Relationship With Demographic and Clinical Characteristics

Nearly all investigations on CSF NFL in ALS agree that the levels of the biomarker do not differ between male and female patients (Gaiani et al., 2017; Skillbäck et al., 2017; Rossi et al., 2018). However, some studies reported higher blood NFL concentrations in female ALS patients: in one cohort, the finding could be explained by females suffering from more advanced disease (and being on average older than male patients), but in general, it warrants further investigation (Lu et al., 2015; Benatar et al., 2020; De Schaepdryver et al., 2020). Most studies did not find an association between CSF and serum NFL levels and age of ALS patients, whereas in healthy controls and in other neurological diseases, the biomarker is correlated with age (Zetterberg et al., 2007; Rossi et al., 2018; Verde et al., 2019b). This is probably due to the massive elevation of NFL concentrations resulting from axonal degeneration in ALS, which largely exceeds the mildly increased rate of axonal loss occurring in normal aging or the moderately increased rate characterizing slower disease processes such as those observed in other neurodegenerative diseases. A meta-analysis of 2019 confirmed an increase of 3.3% per year of age in CSF NFL levels in healthy controls but not in ALS (Bridel et al., 2019). However, some studies reported a weak correlation between CSF or serum NFL levels and age also in ALS (Gaiottino et al., 2013; Benatar et al., 2020). Although a recent study suggested an association between higher serum NFL levels and bulbar onset in ALS (Benatar et al., 2020), according to most other investigations, CSF and blood concentrations of NFL are not influenced by site of onset (Poesen et al., 2017; Rossi et al., 2018; Verde et al., 2019b).

The relationship of NFL with the differential involvement of UMNs vs. LMNs and with the anatomical extent of disease represents a more complex issue. The early investigation of Rosengren et al. (1996) already reported higher CSF NFL levels in ALS patients showing signs of UMN in comparison with those with LMN signs only. Another study confirmed higher concentrations in patients with typical ALS, ALS with predominant UMN signs (UMN-ALS), and progressive bulbar palsy (PBP) compared with the LMN-predominant variants flail

arm and flail leg syndromes and the LMN-only variant PMA (Gaiani et al., 2017). In partial agreement with this, both CSF and serum NFL levels were reported to be higher in ALS patients with clinical UMN and clinical/electromyographic LMN signs in two or three body regions compared with patients with UMN and LMN signs in only one region (Poesen et al., 2017; Gille et al., 2019). Gille et al. (2019) showed that, at least for serum NFL, this effect seemed to depend on UMN involvement, with a significant difference between patients with UMN signs in one vs. three regions and no difference between patients with LMN signs in one vs. two or three regions. Other studies reported a correlation of CSF or serum NFL levels with clinical scores of UMN burden, such as the Penn score, in parallel with a negative correlation with fractional anisotropy (FA) and a positive correlation with radial diffusivity (RD) of the corticospinal tracts (CSTs) in diffusion tensor imaging (DTI) studies, reflecting CST, and therefore UMN, degeneration (Menke et al., 2015; Schreiber et al., 2018). However, not all investigations confirm the association of NFL levels with DTI measures of CST integrity (Steinacker et al., 2016). Contrary to the abovementioned findings, another study on CSF NFL in ALS reported no association with anatomical extent of clinical UMN involvement, a borderline association with UMN + LMN involvement, and an association with the number of regions with electromyographic evidence of LMN degeneration; however, no correlation was found between CSF NFL levels and an EMG-based denervation score (Abu-Rumeileh et al., 2020). In general, the associations between NFL levels and anatomo-clinical features of ALS described above were reported by single or limited numbers of investigations and are therefore controversial; it is likely that the inconsistent results between the different studies are due not only to inherent variability between the relative patient cohorts but also to differences in the methods of measurement of the variables involved.

Pertaining to anatomical extent of motor degeneration, neither CSF nor serum NFL levels differ among patients belonging to the different diagnostic categories of the El Escorial criteria (Feneberg et al., 2018), and serum NFL levels are not influenced by neuroimaging-based disease stages as defined by DTI measures of degeneration of cerebral white matter tracts reflecting the neuropathological staging of ALS (Brettschneider et al., 2013; Verde et al., 2019b). No significant or consistent associations were found between CSF or blood (mostly serum) NFL levels and other clinical data in ALS, including measures of muscle strength based on the scoring system of the Medical Research Council (MRC) (Tortelli et al., 2012), cognitive performance as reflected by a global cognitive *z* score or by the score on the Edinburgh Cognitive and Behavioral ALS Screen (ECAS) (Steinacker et al., 2017a; Illán-Gala et al., 2018), or the albumin quotient in basic biochemical analysis of the CSF, reflecting the function of the blood–CSF barrier (Tortelli et al., 2012; Steinacker et al., 2016). A study reported a weak negative correlation between CSF NFL and forced vital capacity (FVC) at pulmonary function testing, reflecting the strength of respiratory muscles (Poesen et al., 2017). Notably, serum and plasma NFL levels do not differ between patients already taking riluzole and still untreated patients (Lu et al., 2015; Verde et al., 2019b).

NFL as Prognostic Biomarker

Although some investigations reported a weak correlation of CSF NFL with the score on the revised ALS Functional Rating Scale (ALSFRS-R), reflecting the degree of functional impairment (Tortelli et al., 2012; Steinacker et al., 2016; Schreiber et al., 2018), most studies do not confirm this finding (Illán-Gala et al., 2018; Verde et al., 2019b; Benatar et al., 2020). On the contrary, in the majority of investigated cohorts, including the recent large study by De Schaepdryver et al. (2020) on 383 ALS patients, CSF or serum NFL levels correlate moderately with the disease progression rate as expressed by the number of points lost on the ALSFRS-R score from disease onset divided by the disease duration from onset to sampling expressed in months (Gaiani et al., 2017; Illán-Gala et al., 2018; Verde et al., 2019b; Abu-Rumeileh et al., 2020; De Schaepdryver et al., 2020; Thouvenot et al., 2020; **Table 2**). In the cohort of Poesen et al. (2017), patients with fast and intermediate progression rates showed significantly higher CSF NFL levels compared with patients with slow disease progression, and the biomarker enabled discrimination between patients with fast and those with slow progression with an AUC of 0.814. The same authors confirmed the findings also for serum NFL, observing significantly higher levels in patients with fast progression compared with those with intermediate or slow progression: this resulted in good discrimination between patients with rapid and those with slow progression with AUC of 0.87 (Gille et al., 2019). The moderate correlation between NFL levels and disease progression rate was confirmed also for plasma and, importantly, when considering the final progression rate, i.e., that measured based on the ALSFRS-R score at the last follow-up visit, which better reflects the entire progression and has stronger prognostic significance due to its longitudinal nature (Lu et al., 2015).

In parallel, the large majority of studies agree that CSF or blood (mostly serum but also plasma) NFL levels have a moderate negative correlation with disease duration at sampling (Lu et al., 2015; Steinacker et al., 2016; Gaiani et al., 2017; Gille et al., 2019; Verde et al., 2019b; Abu-Rumeileh et al., 2020). This is most probably not due to a change in NFL levels along the natural history of the disease, but rather reflects the fact that patients with a more rapid disease course come earlier to medical attention and are thus investigated with a shorter delay from symptom onset, as demonstrated by the association between rapid disease progression rates and short disease durations at sampling observed in the same cohorts (Gille et al., 2019; Verde et al., 2019b). The consistent correlation with disease progression rate, as compared with the inconsistent associations with anatomical burden of disease, represents the most important feature of NFL as CSF or blood biomarker for ALS apart from its diagnostic potential: indeed, it indicates that NFL levels reflect the rate of degeneration of the motor system (determining the release of NFL from the axons of diseased motor neurons) rather than its spatial extent and can therefore be considered as an index of the biological aggressiveness of the disease (Verde et al., 2019b). This fundamental characteristic of NFL in ALS represents the basis for its role as a prognostic biomarker for the disease. This also explains why PLS has lower NFL levels than ALS, given that,

TABLE 2 | Representative studies on the association of NFL with progression rate and survival in ALS.

Study (authors and year)	ALS patients	Biological fluid	Type of assay	DPR (median, range)	Correlation between NFL and DPR (*r*, 95% CI)	Association between NFL and survival (HR, 95% CI)
Lu et al. (2015)	London cohort (plasma): 103 Oxford cohort (serum/CSF): serum: 64; paired CSF: 38	Plasma, serum, CSF	ECL	n.p.	London cohort, correlation between plasma NFL and DPR at baseline: $r = 0.47$ Oxford cohort, correlation between serum NFL and DPR at baseline: $r = 0.51$ London cohort, correlation between plasma NFL and DPR at last visit: $r = 0.48$ Oxford cohort, correlation between serum NFL and DPR at last visit: $r = 0.53$	London cohort (plasma NFL): mid tertile vs. low tertile: HR = 1.91 (0.86–4.23) London cohort (plasma NFL): high tertile vs. low tertile: HR = 3.78 (1.68–8.50) Oxford cohort (serum NFL): mid tertile vs. low tertile: HR = 2.68 (0.87–8.27) Oxford cohort (serum NFL): high tertile vs. low tertile: HR = 6.05 (1.68–21.87) Oxford cohort (CSF NFL): mid tertile vs. low tertile: HR = 3.64 (0.77–17.25) Oxford cohort (CSF NFL): high tertile vs. low tertile: HR = 31.82 (3.75–269.71)
Steinacker et al. (2016)	253 MND patients (222 ALS, 11 PLS, 20 familial/genetic ALS)	CSF	ELISA	n.p.	$r = 0.3264$ (0.2023-0.4402)	Significant differences between survival curves of patients with CSF NFL $\leq$ median, between median and 75th percentile, and $\geq$ 75th percentile
Steinacker et al. (2017a)	125	Serum	ECL	0.48 (0.26–0.75)	$r = 0.291$ (0.1113–0.4515)	Comparison between survival curves of the three tertiles: chi-square = 11.54
Gille et al. (2019)	149	Serum	ECL	0.672 (0.058–5.00)	$r = 0.51$ AUC to discriminate between fast and slow progressors (i.e., low and high tertiles of DPR): 0.87 (95% CI, 0.76-0.94) at a cutoff of 159 pg/ml	Mid vs. low NFL tertile: HR = 4.47 (1.08–18.63) High vs. low NFL tertile: HR = 5.34 (1.39–20.56)
Verde et al. (2019b)	124	Serum	Simoa	0.375 (0–6)	$r = 0.3359$ (0.1404-0.5062)	NFL > median vs. NFL $\leq$ median: HR = 2.392 (1.236–4.63)
Thouvenot et al. (2020)	207	Serum	Simoa	n.p.	$r = 0.571$	NFL $\geq$ median vs. NFL < median: HR = 4.7 (3.0–7.4)
Abu-Rumeileh et al. (2020)	80	CSF	ELISA	IQR: 0.24–1.15	$r = 0.391$	High vs. low NFL tertile: HR = 3.943 (1.097–14.167)
De Schaepdryver et al. (2020)	383	Serum	ECL	0.67 (0.03–10.13)	$r = 0.519$ (0.437-0.592)	NFL $\geq$ median vs. NFL < median: HR 2.21 (1.51–3.24)

ALS, amyotrophic lateral sclerosis; AUC, area under the ROC (receiver operating characteristic) curve; CI, confidence interval; CSF, cerebrospinal fluid; DPR, disease progression rate; ECL, electrochemiluminescence; ELISA, enzyme-linked immunosorbent assay; HR, hazard ratio; IQR, interquartile range; MND, motor neuron disease; NFL, neurofilament light chain; n.p., not provided; PLS, primary lateral sclerosis; Simoa, single molecule array.

although selectively involving UMNs, it generally has a much slower progression (Gille et al., 2019). A potential drawback of the relationship between NFL and disease progression rate is the possibly lower accuracy of NFL in diagnosing slowly progressive ALS forms, which could especially apply to LMN-predominant cases; however, this issue requires further investigations on large and properly selected patient cohorts.

In agreement with its association with disease progression rate, NFL is also associated with survival according to nearly all studies conducted on CSF (Steinacker et al., 2016; Gaiani et al., 2017; Skillbäck et al., 2017; Illán-Gala et al., 2018; Rossi et al., 2018; Abu-Rumeileh et al., 2020), serum (Steinacker et al., 2017a; Gille et al., 2019; Verde et al., 2019b; De Schaepdryver et al., 2020; Thouvenot et al., 2020), and plasma (Lu et al., 2015). Regarding CSF NFL, Abu-Rumeileh et al. (2020) reported a hazard ratio (HR) of 3.943 for patients with NFL concentrations in the highest tertile compared with those with concentrations in the lowest tertile. As for serum NFL, in the large cohort of De Schaepdryver et al. (2020) of 383 ALS patients, those with NFL levels above the median had a HR of 2.21 compared with those with levels below the median. In their elegant work, Thouvenot et al. (2020) demonstrated that in a multivariate analysis of survival on a cohort of 198 patients, only baseline serum NFL, site of onset, and weight loss were independent predictors of survival, with 0.74% increase in the risk of death for every 1 pg/ml increase in baseline serum NFL concentration.

Other prognostic parameters have been associated with NFL by single investigations. Tortelli et al. (2015) reported a moderate negative correlation between CSF NFL levels and the time to generalization, i.e., the time from onset of symptoms in the bulbar region to involvement of spinal regions in patients with bulbar onset or vice versa for patients with spinal onset: patients with NFL levels above the median had a 7.9-fold increased risk of generalization compared with those with lower NFL

levels, corresponding to a shortening of 2.8 months of the time to generalization for every 1,000 pg/ml increase of CSF NFL concentration. Finally, baseline CSF NFL was shown to predict longitudinal functional deterioration in ALS patients as assessed by both the ALSFRS-R and the related Milano-Torino Staging (MiToS) system (Gaiani et al., 2017).

Longitudinal Kinetics of NFL

A particularly relevant issue is represented by the longitudinal evolution of NFL concentrations over the disease course. In this regard, data on CSF are limited because of the need for repeated lumbar punctures, which have been performed only in subcohorts of patients. The largest investigation is that of Skillbäck et al. (2017), who found increased NFL levels at follow-up CSF examinations in 67% of the 69 MND patients undergoing a repeat lumbar puncture. On the contrary, a decrease of NFL at follow-up was reported for the 11 patients of the large MND cohort of Steinacker et al. (2016) undergoing a second CSF sampling. However, in a slightly larger subcohort of ALS patients, Poesen et al. (2017) found increased follow-up CSF NFL levels in a subset of intermediate and fast progressors, while levels were stable in the remaining patients, including those with slow progression. Finally, Lu et al. (2015) reported a slight increase in both slow and fast progressors, with stable levels in intermediate progressors. The same authors investigated longitudinal kinetics of NFL also in the blood, whereby the less invasive sampling procedure enables the study of larger cohorts and therefore more solid conclusions. While in the cohort with plasma samples NFL levels did not change significantly longitudinally, in the serum cohort (overlapping with the CSF cohort mentioned above), a slight increase of NFL was observed in fast progressors, with stable levels in slow and intermediate progressors (Lu et al., 2015). Other studies, including that of Steinacker et al. (2017a) on 125 ALS patients undergoing at least a second blood sampling, show mostly stable longitudinal NFL levels (Steinacker et al., 2017a; Gille et al., 2019; Verde et al., 2019b).

NFL in Genetic Forms of ALS

NFL in the CSF and serum of patients with genetic forms of ALS has been investigated by few studies in the past. In 2007, Zetterberg et al. (2007) reported lower CSF NFL levels in patients with both familial and apparently sporadic ALS harboring mutations in the *SOD1* gene, although NFL did not differ in general between familial and sporadic ALS cases of the cohort. While other studies did not find significant differences in CSF or serum NFL levels between patients with mutations in *SOD1*, *TARDBP*, or *FUS* or the HRE of *C9orf72* and sporadic cases (Weydt et al., 2016; Verde et al., 2019b), a recent larger study reported higher levels in patients with the *C9orf72* HRE (Benatar et al., 2020). Weydt et al. (2016) investigated CSF and serum NFL (as well as CSF pNFH) in a cohort of ALS patients carrying disease-causing mutations, related presymptomatic mutation carriers, and healthy non-carriers belonging to the same families. With limitations due to the cross-sectional nature of their study, they found that NFL (and pNFH) levels in presymptomatic mutation carriers did not differ from those in non-carriers, while elevated levels were consistently observed in symptomatic mutation carriers, starting shortly after symptom onset.

In their fundamental work, Benatar et al. (2018) conducted a longitudinal investigation of CSF and serum NFL levels in the large North-American *Pre-fALS* cohort of familial ALS cases and related presymptomatic ALS gene mutation carriers, thus overcoming the limitations of the abovementioned cross-sectional study. Baseline serum NFL levels were higher in patients compared with those in both healthy controls and presymptomatic carriers (n = 84; 52 with *SOD1* mutations, 27 with the *C9orf72* HRE, and 5 with mutations in *TARDBP*, *FUS*, and *VCP*), with lack of significant difference between healthy controls and presymptomatic carriers. Whereas in both patients and controls serum NFL levels were stable over time, an average increase of 2.41 pg/ml per 10-year increase in age was observed in presymptomatic carriers. In the additional group of so-called converters (n = 10), i.e., carriers converting from presymptomatic to symptomatic disease during the study period, elevated serum NFL levels (i.e., levels above the highest value observed in controls) were found as early as 11.6 months before symptomatic conversion and continued to increase for at least 6 months after conversion. Similar patterns were observed for CSF NFL (Benatar et al., 2018). Notably, in a subsequent expansion of their study, the same investigators observed elevated CSF and serum levels of NFL in converters as far back as 6–12 months prior to phenoconversion in *SOD1* mutation carriers, as far back as 2 years in the single converter with a *FUS* mutation, and as far back as 3.5 years in the single motor converter (i.e., developing ALS) with a *C9orf72* HRE (Benatar et al., 2019).

NFL as Pharmacodynamic Biomarker

Given its correlation with disease progression rate and its generally stable longitudinal blood levels, NFL has gained much attention as a candidate pharmacodynamic biomarker for ALS, i.e., a biomarker able to reflect target engagement by a hypothetical experimental treatment and possibly also to quantify the beneficial effect thereof. Although in general the stability of a clinical parameter over time is not a fundamental prerequisite for its validity as a marker of effectiveness of an experimental drug as demonstrated by the usefulness of normally increasing or decreasing clinical scores as outcome measures, the longitudinal stability of NFL over the natural disease course of ALS makes it easier to attribute possible changes in its levels observed during an experimental trial to an effect of the treatment itself. Moreover, it can be envisioned that in the future, when hopefully multiple effective and personalized therapies will be available for ALS patients, longitudinal changes in the levels of NFL in single patients could confirm biological response to treatment or, on the contrary, indicate the need to modify the therapeutic regimen, as is partially the case for MS (Gafson et al., 2020).

In their elegant study, Benatar et al. (2020) analyzed the theoretical performance of serum NFL as pharmacodynamic biomarker in a cohort of 220 ALS patients. In contrast to baseline pNFH, baseline serum NFL both predicts survival and improves prediction of longitudinal ALSFRS-R slope relative to

the information provided by the initial ALSFRS-R slope only: these two features qualify serum NFL as a true prognostic biomarker for ALS. The biomarker shows a longitudinal increase of 0.011 log units per month (with 95% confidence interval including 0) and correlates with age increasing by 1.3% per 1-year increase in age. Importantly, thanks to its ability to predict longitudinal ALSFRS-R slope, adding baseline serum NFL to the available information at the beginning of an ALS drug trial would enable an 8.2% reduction of the cohort size necessary to detect a significant treatment effect. According to the authors, including serum NFL (but, again, not pNFH) as a pharmacodynamic biomarker would allow a much larger reduction of the sample size: indeed, in order to detect—with 90% power and a two-tailed *t*-test with 0.05 significance level—a clinically meaningful lowering in longitudinal serum NFL concentrations (i.e., corresponding to lowering from the level of fast progressors to that of slow progressors) as a sign of treatment effect, it would be necessary to enroll 64 patients, whereas 1,054 or 470 patients would be required to detect a 20 or 30% reduction in the ALSFRS-R slope, respectively (Benatar et al., 2020). It should be pointed out, however, that this comparison does not seem to be completely balanced, as in this study fast progressors and slow progressors had ALSFRS-slopes of > 1 and < 0.5 points/month, respectively: this means that reducing the slope from the level of fast progressors to that of slow progressors would imply a $> 50\%$ reduction thereof.

A practical demonstration of the concepts theorized by Benatar et al. (2018) comes from the recent study of Dorst et al. (2020), who retrospectively analyzed serum NFL levels in ALS patients who had been enrolled in the LIPCAL-ALS trial of high-caloric fatty diet (HCFD). This trial had had an overall lack of effect on survival, but *post hoc* analysis had demonstrated a beneficial effect on patients with fast disease progression (Ludolph et al., 2020). Indeed, analysis of serum NFL levels demonstrated that while in the placebo group of the trial serum NFL levels had increased longitudinally, in HCFD-treated patients, serum NFL had decreased, and this difference was attributable to an effect in patients with fast progression, i.e., those benefiting from treatment in terms of survival. Moreover, within the subgroup of patients with baseline high serum NFL levels, survival was prolonged in HCFD-treated patients compared with those receiving placebo, and also in this subgroup, a corresponding longitudinal decrease of serum NFL levels was observed in the former compared with the latter (Dorst et al., 2020). Finally, NFL measurement was included in the historic phase 1–2 trials of the anti-SOD1 antisense oligonucleotide tofersen in patients with ALS due to *SOD1* mutations, whose results were published in 2020. The highest dose of the drug administered intrathecally over a period of 12 weeks produced a significant decrease in CSF SOD1 concentrations; importantly, this was accompanied by a decrease of both CSF and plasma concentrations of NFL (and pNFH), representing one of the first examples of the use of neurofilaments as pharmacodynamic biomarkers for an ALS treatment trial (Miller et al., 2020).

NFL AS BIOMARKER FOR FRONTOTEMPORAL DEMENTIA

NFL as FTD Diagnostic Biomarker

The first reports of NFL elevations in the CSF of patients with FTD are those of Rosengren et al. (1999) and Sjögren et al. (2000). In particular, Rosengren et al. (1999) demonstrated higher CSF NFL levels in patients with FTD, AD, and vascular dementia (VaD) in comparison with healthy individuals and hypothesized that raised NFL levels were the consequence of degeneration of the brain white matter in those diseases (**Table 3**). More than 10 years later, Scherling et al. (2014) measured NFL in the CSF of 79 patients with the three forms of FTD [bvFTD, nfvPPA (there called progressive non-fluent aphasia), and svPPA (there called semantic dementia)] and found increased levels compared with both controls and patients with AD. The difference between FTD and AD remained significant also if the comparison was limited to the 44 FTD patients with increased level of confidence of FTLD pathology (due to the presence of a FTLD-causing genetic mutation, autopsy neuropathological evidence, or negative result of amyloid PET) vs. the 14 AD patients with increased level of confidence of amyloid pathology (positive amyloid PET or neuropathological evidence). Notably, a recent study on neuropathologically confirmed cases showed that higher CSF NFL levels are still observed in FTLD in comparison with AD even after excluding cases of FTLD-ALS from the analysis (Cousins et al., 2020).

In their large cohort of FTD patients [$n = 361$, actually including also patients with corticobasal syndrome (CBS) and progressive supranuclear palsy (PSP), which belong to the spectrum of FTLD syndromes], Meeter et al. (2018b) demonstrated that each clinical form of FTD had higher CSF NFL levels compared with neurologically healthy controls, with the highest levels found in FTD-MND. This resulted in an AUC of 0.87 for discriminating between FTD patients and controls (corresponding to a sensitivity of 79% and a specificity of 89% at a cutoff of 1,613 pg/ml). The only exception was represented by lvPPA, a finding which is not surprising considering that in most cases this phenotype is due to underlying AD pathology. Accordingly, both nfvPPA and svPPA show higher CSF levels of NFL compared with lvPPA, enabling discrimination between the former two entities and lvPPA with an AUC of 0.8744 in the study of Steinacker et al. (2017b). In a very large multicenter cohort of svPPA patients ($n = 162$), CSF NFL had an excellent diagnostic performance in discriminating between patients and neurologically healthy controls (AUC 0.98, sensitivity 93%, specificity 98%) (Meeter et al., 2019).

CSF NFL is higher also in FTD cases compared with cases of AD with early onset (≤ 65 years), with one study reporting an AUC of 0.80 for the discrimination between the two, which is clinically meaningful considering the higher prevalence of FTD in presenile dementia (de Jong et al., 2007). In a cohort in which the diagnostic certainty was increased based on genetic, neuropathological, or CSF AD biomarker data or, in the case of FTLD, on the co-occurrence of ALS, given the differences in CSF NFL levels between patients with FTLD

TABLE 3 | Representative studies of NFL as biomarker for FTD.

Study (authors and year)	Patients	Biological fluid	Type of assay	Main findings
Rosengren et al. (1999)	5 patients with FTD, 39 HCs	CSF	ELISA	CSF NFL levels are increased in ALS compared with HCs.
Scherling et al. (2014)	79 FTD patients (45 bvFTD, 18 nfvPPA, 16 svPPA), 8 presymptomatic carriers of FTD-causing gene mutations, 22 PSP patients, 50 AD patients, 6 PD patients, 17 CBS patients, 47 HCs	CSF	ELISA	CSF NFL levels are higher in all FTD subgroups compared with HCs, AD patients, presymptomatic carriers of FTD mutations, and PD patients. CSF NFL in all FTD subgroups correlates moderately with CDR-SB (r = 0.359). CSF NFL has moderate negative correlations with MMSE score (r = -0.549) and with the performance in several neuropsychological tests (mostly of frontal-executive functions). In FTD and bvFTD, CSF NFL levels correlate negatively with gray matter volume of frontal, temporal, parietal, occipital, and cingulate cortices and, to a lesser extent, with volume of associated white matter.
Steinacker et al. (2017b)	99 PPA patients (40 nfvPPA, 38 svPPA, 21 lvPPA), 35 HCs	Serum, CSF	ECL (serum), ELISA (CSF)	Serum NFL levels are higher in each PPA variant compared to HCs. Both nfvPPA and svPPA have higher levels compared with lvPPA. Similar findings for CSF NFL. Performance in discriminating between PPA and HCs: sensitivity 95%, specificity 70%. Performance in discriminating between nfvPPA + svPPA vs. lvPPA: sensitivity 81%, specificity 67%. Longitudinal serum NFL (subcohort of 37 PPA patients): increase in nfvPPA and svPPA, no significant change in lvPPA. In the whole PPA cohort, longitudinal increase of serum NFL correlates with longitudinal atrophy progression in left frontal lobe. In patients with nfvPPA and svPPA, longitudinal increase of serum NFL correlates with longitudinal atrophy progression in right middle frontal gyrus. Longitudinal serum NFL change in PPA correlates moderately with longitudinal change in CDR-SB and, in nfvPPA and svPPA, also with longitudinal change in CDR-FTD-SB.
Meeter et al. (2018b)	361 FTD patients (179 bvFTD, 17 FTD-MND, 36 svPPA, 19 nfvPPA, 4 lvPPA, 42 CBS, 64 PSP), 45 HCs. Definite pathology known in 68 patients (49 FTLD-TDP, 18 FTLD-tau, 1 FTLD-FUS)	CSF	ELISA	All clinical entities except for lvPPA have higher CSF NFL levels compared with HCs, with the strongest elevation in FTD-MND. CSF NFL is higher in FTD-MND compared with bvFTD. Performance in discriminating between FTD patients and controls: AUC 0.87, sensitivity 79%, specificity 89%. CSF NFL levels do not differ significantly between pathology-proven FTLD-tau and FTLD-TDP, but when clinically suspected cases of the two types are added, FTLD-TDP has higher CSF NFL levels compared with FTLD-tau. CSF NFL correlates moderately with CDR-SB and weakly with FAB and (negatively) with MMSE. FTD patients with *GRN* mutations have higher CSF NFL levels than those with *C9orf72*, *MAPT*, or no mutations. CSF NFL levels are negatively associated with survival in FTD and bvFTD.
Steinacker et al. (2018)	74 bvFTD patients, 26 AD patients, 17 MCI patients, 15 HCs	Serum, CSF	Simoa (serum), ELISA (CSF)	In bvFTD, serum and CSF NFL correlate strongly (r = 0.706). CSF NFL levels are higher in bvFTD compared with AD and MCI. Serum NFL is higher in bvFTD compared with AD, MCI, and HCs. Diagnostic performance of serum NFL for bvFTD: vs. AD: AUC 0.6762; vs. MCI: AUC 0.9094; vs. HCs: AUC 0.8514. In bvFTD, serum NFL correlates moderately with CDR-SB (r = 0.4402) and CDR-FTD-SB (r = 0.5297) and negatively with MMSE (r = -0.3242). In bvFTD, serum NFL has a moderate negative correlation with volumes of the frontal lobe (r = -0.5857), striatum (r = -0.5244), right amygdala (r = -0.4951), and frontal lobe white matter (r = -0.5382). In bvFTD, serum NFL increases at follow-up (subcohort of 64 patients).

(Continued)

TABLE 3 | Continued

Study (authors and year)	Patients	Biological fluid	Type of assay	Main findings
van der Ende et al. (2019)	59 FTD patients with *MAPT* (*n* = 10), *GRN* (*n* = 25), or *C9orf72* (*n* = 24) mutations, 149 presymptomatic mutation carriers (24 *MAPT*, 79 *GRN*, 46 *C9orf72*), 127 non-carrier relatives	Serum	Simoa	Serum NFL is higher in symptomatic mutation carriers compared with presymptomatic carriers (AUC for discrimination: 0.93) and non-carriers (AUC: 0.95). Symptomatic *GRN* mutation carriers have higher serum NFL levels compared with symptomatic *MAPT* and *C9orf72* mutation carriers. Serum NFL strongly correlates with age in the whole cohort (*r* = 0.770) and in non-carriers (with an estimated increase of 1.2% per year). Serum NFL levels do not differ between presymptomatic carriers and non-carriers in general, but do from the age of 48 years onward. Longitudinal serum NFL levels are stable in non-carriers but increase in presymptomatic carriers (which is due to *C9orf72* mutation carriers). The rate of serum NFL increase is higher in converters than in non-converters. Among presymptomatic carriers, baseline serum NFL discriminates between converters (*n* = 9; 6 *GRN*, 2 *MAPT*, 1 *C9orf72*) and non-converters (AUC: 0.93). Among symptomatic carriers, longitudinal serum NFL levels are stable in *MAPT* and *C9orf72* mutation carriers but increase in *GRN* mutation carriers. Across all groups, the rate of serum NFL change is associated with the change in volume of the frontal lobe, insula, cingulate gyrus, hippocampus, putamen, whole brain, temporal lobe, amygdala, and cerebellum and with change in MMSE over time.
Katisko et al. (2020)	91 FTD patients (66 bvFTD, 16 nfvPPA, 4 svPPA, 5 FTD-MND), 34 patients with primary psychiatric disorders (psychoses and/or mood disorders)	Serum	Simoa	Serum NFL discriminates between FTD and primary psychiatric disorders with AUC 0.850 (sensitivity 80%, specificity 85%) and between bvFTD and primary psychiatric disorders with AUC 0.830 (sensitivity 79%, specificity 85%).
Benussi et al. (2020)	291 patients with FTLD syndromes (134 bvFTD, 48 nfvPPA, 27 svPPA, 51 CBS, 31 PSP), 63 AD, 63 HCs	Serum	Simoa	Serum NFL levels are higher in bvFTD, nfvPPA, and svPPA compared with HCs. Serum NFL discriminates between FTLD syndromes and HCs with AUC 0.862 (sensitivity 71.5%, specificity 92.1%). Serum NFL levels are higher in nfvPPA compared with svPPA. Serum NFL levels in FTLD syndromes are higher in patients with *GRN* or *MAPT* pathogenic mutations (*n* = 30 and *n* = 3, respectively) than in those without mutations. Serum NFL levels weakly correlate with several measures of functional impairment, cognitive function, and behavioral disturbance. Serum NFL levels correlate negatively with thickness of left dorsolateral prefrontal cortex. Serum NFL levels correlate with SICI and LICI (reflecting postsynaptic inhibition at the level of cortical interneurons). Higher serum NFL levels are associated with shorter survival.
Illán-Gala et al. (2021)	167 patients with FTLD syndromes (43 bvFTD, 28 nfvPPA, 18 svPPA, 36 PSP, 32 CBS, 10 ALS-FTD), of whom 70 pathology-proven (50 FTLD-tau, 18 FTLD-TDP, 2 FTLD-FUS), 43 AD patients, 55 HCs	Plasma, CSF	Simoa (plasma), ELISA (CSF)	Plasma and CSF NFL correlate strongly in FTLD syndromes (*r* = 0.82). All FTLD syndromes have higher plasma NFL levels compared with AD, with the highest levels observed in ALS-FTD. Plasma NFL discriminates very well between FTLD and HCs (AUC 0.97) but less well between FTLD and AD (AUC 0.75). Plasma NFL levels are higher in FTLD-TDP compared with FTLD-tau. In FTLD syndromes, plasma NFL has strong negative correlations with cortical thickness in frontal regions. Baseline plasma NFL is associated with faster annual worsening of CDR-FTD-SB. Plasma NFL increases over time in FTLD. Higher plasma NFL is associated with shorter survival.
Cousins et al. (2020)	27 FTLD patients, 67 AD patients (both autopsy-confirmed)	CSF	ELISA	CSF NFL is higher in FTLD compared with AD. Replacing CSF total tau with CSF NFL in the neurochemical AT(N) framework increases the accuracy of the scheme at diagnosing FTLD as suspected non-Alzheimer pathophysiology (SNAP), with sensitivity increasing from 44 to 93% and specificity remaining high at 94%.

Relevant data with reference to NFL in FTD are reported.

AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; bvFTD, behavioral variant of frontotemporal dementia; AUC, area under the ROC (receiver operating characteristic) curve; CBS, corticobasal syndrome; CDR-FTD-SB, Clinical Dementia Rating Scale for Frontotemporal Dementia—Sum of Boxes; CDR-SB, Clinical Dementia Rating Scale—Sum of Boxes; FAB, Frontal Assessment Battery; FTD, frontotemporal dementia; FTD-MND, frontotemporal dementia—motor neuron disease; FTLD, frontotemporal lobar degeneration; FTLD-FUS, frontotemporal lobar degeneration with FUS pathology; FTLD-tau, frontotemporal lobar degeneration with tau pathology; FTLD-TDP, frontotemporal lobar degeneration with TDP-43 pathology; HCs, healthy controls; lvPPA, logopenic variant of primary progressive aphasia; LICI, long-interval intracortical inhibition; MCI, mild cognitive impairment; nfvPPA, non-fluent variant of primary progressive aphasia; PD, Parkinson's disease; PPA, primary progressive aphasia; PSP, progressive supranuclear palsy; SICI, short-interval intracortical inhibition; svPPA, semantic variant of primary progressive aphasia.

(showing the highest levels), patients with AD (intermediate levels), and cognitively normal controls (lowest levels), CSF NFL was able to correctly assign 85.2% of patients to each of the three categories (Alcolea et al., 2017). CSF NFL is higher in FTD compared also with dementia with Lewy bodies (DLB), VaD, mixed dementia (i.e., dementia with both Alzheimer and vascular pathology), Parkinson's disease dementia (PDD), and other forms (Skillbäck et al., 2014). The difference from DLB was also demonstrated in a cohort of pathologically confirmed cases (Olsson et al., 2019). Conversely, CSF NFL is lower in FTD than in CJD (Antonell et al., 2020). Olsson et al. (2019) showed that adding CSF NFL to a neurochemical diagnostic algorithm based on the three classical CSF biomarkers $A\beta_{1-42}$, total tau, and phosphorylated tau increased diagnostic accuracy for the discrimination between FTD and healthy controls from 63 to 81%. The increased CSF levels of NFL in FTD have been confirmed by meta-analyses examining studies conducted on > 1,800 FTD patients, with ratios of means of 3.41 between FTD and cognitively healthy controls, 2.08 between FTD and AD, 2.50 between FTD and DLB, and 1.56 between FTD and VaD (Bridel et al., 2019; Forgrave et al., 2019). In their recent work on a cohort of neuropathologically confirmed FTLD and AD cases (n = 27 and n = 67, respectively), Cousin et al. (2020) examined the result of including CSF NFL instead of CSF total tau in the neurochemical AD AT(N) framework (based on CSF levels of $A\beta_{1-42}$, total tau, and phosphorylated tau) in order to correctly classify FTLD cases as SNAP (suspected non-Alzheimer pathophysiology). Importantly, the replacement of total tau with NFL improved the diagnostic classification, with sensitivity rising from 44 to 93% and specificity remaining high at 94%. This elegant work might pave the way for the introduction of CSF NFL in the AT(N) framework in the near future, thus increasing correct pathological attribution of cases of cognitive impairment, with relevant consequences for diagnosis and inclusion in clinical trials.

In the last 5 years, NFL has been increasingly studied in peripheral blood in FTD. Given the strong correlation between serum and CSF levels of NFL, Wilke et al. (2016) demonstrated that serum NFL was higher in FTD patients compared with that in neurologically healthy controls and that the diagnostic performance of the blood biomarker for the discrimination between the two conditions was similar to that of CSF NFL (AUCs, 0.81 for serum and 0.88 for CSF, respectively). Indeed, the recent study of Benussi et al. (2020) on a large cohort of FTD patients (n = 291, actually including also patients with CBS and PSP) reported an AUC as high as 0.862 for serum NFL in distinguishing FTD patients from healthy controls. The sensitivity and specificity for discriminating between FTD and healthy controls are as high as 84 and 96%, respectively, and serum NFL is higher in all three clinical forms of FTD, i.e., bvFTD, svPPA, and nfvPPA, compared with controls (Rohrer et al., 2016). CSF and serum NFL levels strongly correlate with each other also when limiting the analysis to bvFTD cases (Steinacker et al., 2018). According to the work of Steinacker et al. (2018) on 74 bvFTD cases, serum NFL enables the discrimination of this condition from neurologically healthy controls, patients with MCI, and patients with AD with sensitivities of 91, 74, and 74%, respectively, and specificities of 79, 74, and 58%, respectively. The same group measured serum NFL in 99 patients with primary progressive aphasia (PPA) and showed it to be higher in each of the three categories of PPA (nfvPPA, svPPA, and lvPPA), enabling discrimination between patients with PPA and neurologically healthy controls with 95% sensitivity and 70% specificity (Steinacker et al., 2017b). Similar to what was observed for CSF NFL, serum NFL was able to distinguish nfvPPA and svPPA from lvPPA (Steinacker et al., 2017b), while, in another cohort, nfvPPA was reported to have higher serum NFL levels than svPPA (Benussi et al., 2020). The already mentioned meta-analysis of Forgrave et al. (2019) conducted in 2019 reported a ratio of means of serum NFL between FTD patients and cognitively normal controls of 2.65.

In their recently published study, Illán-Gala et al. (2021) measured plasma NFL in a large cohort of FTD patients (n = 167, including CBS and PSP), of whom 70 had neuropathological confirmation. While not correlating with plasma tau, plasma NFL strongly correlated with CSF NFL. Plasma NFL was higher in clinically diagnosed FTD syndromes than in clinically diagnosed AD and in neurologically healthy controls and had an excellent performance in discriminating between FTD syndromes and controls (AUC 0.97), but was less good in discriminating between FTD syndromes and clinically diagnosed AD (AUC 0.75). Notably, in the subcohort of neuropathologically confirmed FTD cases, plasma NFL was higher both in FTLD-TDP and in FTLD-tau compared with AD cases with neuropathological or PET evidence of amyloid pathology.

A special issue regarding NFL as diagnostic biomarker for FTD is represented by the differentiation from primary psychiatric disorders (PPDs), which can present with similar behavioral features and be accompanied by cognitive impairment. Importantly, patients with bvFTD were shown to have higher CSF NFL levels compared with those suffering from PPDs, enabling discrimination between the two categories with AUC as high as 0.93 (Vijverberg et al., 2017). Similar results were obtained by Katisko et al. (2020) for serum NFL measured in a cohort of 91 FTD patients and 34 patients with PPDs. A study investigating the diagnostic performance of serum NFL in discriminating between bvFTD and specific psychiatric diseases reported AUCs of 0.89 for depression, 0.94 for bipolar disorder, and 0.90 for schizophrenia (Al Shweiki et al., 2019). Given these promising findings, a consensus paper of the recently established Neuropsychiatric International Consortium for Frontotemporal Dementia (NIC-FTD) highlights the potential role of CSF and serum NFL for distinguishing bvFTD from psychiatric disorders (Ducharme et al., 2020).

Relationship With Underlying Neuropathology and Presence of ALS

Higher CSF NFL levels have been reported in cases of probable or definite FTLD with TDP-43 pathology (FTLD-TDP) compared with cases of FTLD with tau pathology (FTLD-tau), whereby definite cases are pathologically proven or carry a pathology-causing mutation (*GRN* or *C9orf72* for FTLD-TDP, *MAPT* for FTLD-tau) while probable cases are clinically defined (with

FTD-MND and svPPA pointing to TDP-43 pathology and PSP and CBS pointing to tau pathology). CSF NFL may have an AUC as high as 0.861 for the discrimination between definite and probable cases of the two pathological categories, corresponding to 80% sensitivity and 81% specificity (Abu-Rumeileh et al., 2018). Probable or definite FTLD-TDP has been reported to have higher CSF NFL levels than FTLD-tau not only when accompanied by ALS but also in the absence of the motor phenotype (Pijnenburg et al., 2015; Abu-Rumeileh et al., 2018). Other studies, however, did not confirm in genetically or neuropathologically defined cases the difference which is observed when including phenotypically defined ones (Goossens et al., 2018; Meeter et al., 2018b). On the contrary, the recent investigation of Illán-Gala et al. (2021) demonstrated higher plasma NFL levels in pathology-proven FTLD-TDP cases compared with pathological FTLD-tau cases, a difference which held true also after excluding cases with ALS-FTD from the FTLD-TDP neuropathological cohort. In agreement with the tendency of FTLD-TDP toward higher NFL levels is the demonstration, in the large neuropathological investigation of Olsson et al. (2019), of a correlation between CSF NFL and TDP-43 load in 13 of 17 brain regions in the whole neuropathological cohort (n = 120) encompassing several neurodegenerative diseases.

Patients with ALS have higher median NFL levels both in CSF and in serum compared with those with FTD (Wilke et al., 2016; Gaiani et al., 2017; Skillbäck et al., 2017; Verde et al., 2019b). ALS-FTD is the form of FTD with the highest NFL levels both in CSF and in plasma (Meeter et al., 2018b; Illán-Gala et al., 2021). Accordingly, in the large FTD cohort of Meeter et al. (2018b) (total FTD cohort, n = 361; ALS-FTD subcohort, n = 17), ALS-FTD had significantly higher CSF NFL levels compared with bvFTD. An investigation of the Sant Pau Initiative on Neurodegeneration (SPIN) shows that the biomarker could enable diagnosis of ALS within a cohort of FTD patients with AUC of 0.705 (Delaby et al., 2020). The same study showed a gradient of CSF NFL concentrations with the highest levels in ALS, intermediate levels in ALS-FTD, and lowest levels in FTD; however, the only statistically significant differences were those between ALS and FTD and between ALS and ALS-FTD, whereas ALS-FTD did not significantly differ from FTD alone (Delaby et al., 2020). However, not all studies have found a significant difference in CSF NFL levels between ALS and ALS-FTD (Steinacker et al., 2016; Illán-Gala et al., 2018).

In studies differentiating FTD cases according to definite (pathologically proven or carrying a gene mutation) or probable (inferred from phenotype) pathological subtype, there is no agreement as to whether FTLD-TDP cases with ALS have higher CSF NFL levels than FTLD-TDP cases without ALS (Pijnenburg et al., 2015; Abu-Rumeileh et al., 2018). In the investigation of Olsson et al. (2019), although in the clinical cohort ALS patients had higher CSF NFL levels than FTD patients, among pathologically confirmed cases, ALS showed only a trend toward higher levels compared with FTD, without reaching statistical significance (Olsson et al., 2019). Notably, among patients with FTD with the *C9orf72* HRE, those with ALS-FTD were shown to have higher CSF NFL levels compared with those with FTD alone (Meeter et al., 2018a).

Relationship With Clinical Features and Longitudinal Kinetics

In a cohort of autopsy-confirmed FTLD cases, no relationship was found between sex and CSF NFL levels (Cousins et al., 2020). However, a very recent study on a large cohort comprising patients with FTD and asymptomatic individuals with a family history of FTD (n = 277) found higher plasma NFL levels in women even after correction for disease severity, age, and clinical phenotype (Rojas et al., 2021). While CSF NFL levels correlate with age in neurologically healthy controls and in patients with AD, no such correlation is observed in FTD patients (Skillbäck et al., 2014; Goossens et al., 2018), a finding confirmed by the meta-analysis of Bridel et al. (2019). The same lack of correlation with age in FTD was found in the recent investigation of Illán-Gala et al. (2021) on plasma NFL. Pertaining to FTD clinical subtypes, no correlation between serum NFL and age was found in bvFTD by Steinacker et al. (2018), whereas the same group observed a weak correlation between serum NFL and age at onset in all forms of PPA together as well as in nfvPPA and svPPA considered individually, while the correlation was stronger for lvPPA, which, again, is not surprising considering that AD pathology most commonly underlies this clinical variant (Steinacker et al., 2017b). Although Meeter et al. (2016, 2018b) reported a weak association of CSF NFL with disease duration at sampling in their large FTD cohort, the finding has not been replicated in studies investigating serum NFL in bvFTD and PPAs and CSF or serum NFL in genetic forms of FTD (Meeter et al., 2016, 2018b; Steinacker et al., 2017b, 2018; van der Ende et al., 2019).

NFL concentrations in the CSF and blood in FTD patients are associated with cognitive features. Although not according to all studies, CSF NFL concentrations negatively correlate with the score in the Mini Mental State Examination (MMSE) (Sjögren et al., 2000; Scherling et al., 2014; Skillbäck et al., 2014); in the study of Scherling et al. (2014), this is true also when considering only the cases with increased level of certainty of FTLD pathology (due to autopsy evidence, presence of gene mutations, or negative result of amyloid PET scan). CSF NFL also correlates with the score of the Clinical Dementia Rating Scale—Sum of Boxes (CDR-SB) in FTD as a whole and in the three subclasses bvFTD, nfvPPA, and svPPA, as well as, in bvFTD, with the score of the modified version of the CDR for FTD (CDR-FTD-SB) (Scherling et al., 2014; Ljubenkov et al., 2018). CSF NFL is weakly associated with the score of the Frontal Assessment Battery (FAB) in FTD and correlates with the scores of several neuropsychological tests, especially regarding frontal-executive functions, both in FTD and in the subclasses bvFTD and nfvPPA (Scherling et al., 2014; Meeter et al., 2018b). In svPPA, the biomarker shows a weak negative correlation with the score of the Boston Naming Test (Meeter et al., 2019). CSF NFL has also relationships with longitudinal cognitive data: CSF NFL at baseline correlates with worsening of the MMSE score

at follow-up evaluation in FTD and with worsening of CDR-FTD-SB score and other neuropsychological parameters both in bvFTD and in nfvPPA (Ljubenkov et al., 2018; Olsson et al., 2019). For blood NFL, similar associations were reported. In the large cohort of Benussi et al. (2020), serum NFL correlated with functional impairment, dementia severity as measured with the CDR-FTD-SB, performance in several cognitive tests (especially regarding frontal-executive functions), and behavioral alterations. Also, in the subclass bvFTD serum, NFL correlates with the CDR-FTD-SB, as well as with the traditional CDR-SB (Steinacker et al., 2018). In svPPA, plasma NFL correlates with neuropsychological measures of semantic impairment (Heller et al., 2020a). Regarding longitudinal associations, plasma NFL at baseline correlates with worsening of CDR-FTD-SB score both in FTD as a whole and in the subclasses bvFTD, svPPA, and ALS-FTD (Illán-Gala et al., 2021).

Most investigations agree that CSF NFL is associated with survival, with Meeter et al. (2018b) reporting a HR for tertiles of CSF NFL concentrations of 1.7. The association is true also when limiting the analysis to bvFTD patients or to FTD patients with definite or probable FTLD-TDP pathology based on neuropathology, genetics, or phenotype (Pijnenburg et al., 2015; Skillbäck et al., 2017). Importantly, NFL is associated with survival in FTD also when measured in serum or in plasma (Benussi et al., 2020; Illán-Gala et al., 2021).

Although most studies on longitudinal NFL levels in FTD have been conducted on blood because of the less invasive nature of blood sampling compared with CSF sampling, two investigations reported longitudinal data regarding CSF NFL: whereas in the 27 patients with a follow-up lumbar puncture in the cohort of Ljubenkov et al. (2018) no consistent longitudinal trend of NFL could be recognized, 11 of the 14 FTD patients with a longitudinal CSF sample studied by Skillbäck et al. (2017) showed higher levels of NFL compared with the first sample (Skillbäck et al., 2017; Ljubenkov et al., 2018). Longitudinal studies on blood NFL enable investigation of larger cohorts. The group of Steinacker et al. (2017b, 2018) reported an increase in serum NFL at follow-up 1 year after baseline sampling in bvFTD as well as in nfvPPA and svPPA, while such an increase was not observed in lvPPA. In nfvPPA and svPPA, longitudinal change in serum NFL correlates with longitudinal worsening of the CDR-FTD-SB score (Steinacker et al., 2017b). An increase at follow-up was also observed for plasma NFL in FTD in the recent study of Illán-Gala et al. (2021).

NFL in Genetic Forms of FTD

NFL has been investigated in patients with genetic FTD, i.e., those carrying mutations in the three main genes *MAPT*, *GRN*, and *C9orf72*. Most studies on CSF NFL report higher levels in patients with *GRN* mutations compared with patients without known gene mutations, patients with *MAPT* mutations or the *C9orf72* HRE, or patients with FTLD-tau as neuropathologically or genetically defined (Meeter et al., 2016, 2018b; Goossens et al., 2018).

Results of studies on blood NFL are less clear-cut, with some investigations not reporting significant differences in serum NFL between bvFTD cases with and without gene mutations or between FTD patients with mutations in different genes (Rohrer et al., 2016; Steinacker et al., 2018). Moreover, higher serum or plasma NFL levels have been reported in FTD patients with the *C9orf72* HRE compared with FTD patients without mutations, who, in turn, do not differ from patients with *GRN* or *MAPT* mutations (Cajanus et al., 2020; Illán-Gala et al., 2021). Higher plasma NFL levels have also been reported in FTD patients with the *C9orf72* HRE compared with patients with *MAPT* mutations (Heller et al., 2020b). Notwithstanding this, also regarding blood NFL, several investigations reported higher serum or plasma levels in patients with *GRN* mutations as compared with patients without mutations in known genes, patients with *MAPT* mutations, or patients with the *C9orf72* HRE (including patients with ALS-FTD) (Meeter et al., 2016; van der Ende et al., 2019; Benussi et al., 2020; Heller et al., 2020b; Rojas et al., 2021).

The relationship between NFL and cognitive impairment has also been investigated in cohorts of genetic FTD. In patients with mutations in the three main genes *MAPT*, *GRN*, and *C9orf72*, both CSF and serum NFL correlated with the score of the CDR-SB (Meeter et al., 2016). In a cohort of patients with the *C9orf72* HRE, CSF NFL correlated both with CDR-SB score and, negatively, with the MMSE score (Meeter et al., 2018a). Also in cohorts of FTD patients carrying mutations in the three genes, both CSF and serum NFL were associated with survival (Meeter et al., 2016, 2018a; Cajanus et al., 2020). Pertaining to longitudinal kinetics, in FTD patients carrying mutations in the three genes as a whole, serum NFL does not show a consistent change over time, but when analyzing patients with the three genetic forms separately, an increase is observed in *GRN* mutation carriers but not in the other two groups (van der Ende et al., 2019).

Several important studies conducted by centers collaborating in the Genetic FTD Initiative (GENFI) have investigated NFL in presymptomatic carriers of FTD-causing gene mutations. Meeter et al. (2016) measured NFL in the CSF of 86 FTD patients with mutations in *MAPT* and *GRN* and the *C9orf72* HRE, 40 presymptomatic carriers of the same mutations, and 48 neurologically healthy controls. Median NFL levels were more than eight times higher in patients than in presymptomatic carriers and controls but did not significantly differ between presymptomatic carriers and controls nor between presymptomatic carriers with mutations in the three different genes. Diagnostic performance of CSF NFL in discriminating between patients and presymptomatic carriers was excellent, with AUC 0.97 and sensitivity and specificity of 84 and 100%, respectively. On the contrary, performance for the discrimination between presymptomatic carriers and controls was poor (AUC 0.65). Notably, CSF NFL correlated with age in presymptomatic carriers, and two presymptomatic *GRN* mutation carriers converting to symptomatic FTD during the study period showed a three to fourfold increase in CSF NFL levels during follow-up. Results obtained in the smaller and partially overlapping cohort in which NFL was measured in serum were similar, in agreement with the strong correlation observed between CSF and serum NFL levels (r = 0.87) (Meeter et al., 2016). Similar results for CSF NFL were obtained for carriers of the *C9orf72* HRE (Meeter et al., 2018a). Two investigations on the Danish FTD-3 family

with the rare genetic form of FTD due to mutation of the gene *CHMP2B* demonstrated higher levels of CSF and serum NFL not only in symptomatic carriers of the mutation ($n = 12$ in the larger and more recent study) compared with both presymptomatic carriers ($n = 10$) and healthy non-carriers ($n = 16$), but also in presymptomatic carriers compared with non-carriers (Rostgaard et al., 2018; Toft et al., 2021).

In their fundamental study, van der Ende et al. (2019) from the GENFI analyzed serum NFL in a large cohort ($n = 140$) of presymptomatic carriers of *MAPT* and *GRN* mutations and the *C9orf72* HRE, 59 symptomatic carriers with FTD, and 127 neurologically healthy non-carriers belonging to the same families. Serum NFL at baseline was higher in symptomatic carriers compared with both non-carriers and presymptomatic carriers, a finding which was confirmed also when separating participants according to mutated genes. Serum NFL had a good diagnostic performance in distinguishing symptomatic from asymptomatic carriers, with AUC 0.93, corresponding to 86% sensitivity and 87% specificity. Although in general serum NFL at baseline did not significantly differ between presymptomatic carriers and non-carriers, when including age in the analysis, a significant difference was observed, with presymptomatic carriers showing higher serum NFL levels after the age of 48 years. Importantly, serum NFL at baseline was higher in the nine presymptomatic carriers who converted to symptomatic disease during the follow-up period of the study in comparison with non-converters, enabling good discrimination at baseline between converters and non-converters, with AUC 0.93 and corresponding sensitivity and specificity of 100 and 84%, respectively. As expected, in the follow-up period, an increase in serum NFL was observed in converters. An increase in serum NFL was observed also in the whole group of non-converting presymptomatic carriers, but separate analyses conducted according to gene mutations showed that this increase was attributable to cases carrying the *C9orf72* HRE and not to cases with *MAPT* or *GRN* mutations. Not surprisingly, the rate of serum NFL increase during follow-up was higher in converters compared with non-converting presymptomatic carriers (van der Ende et al., 2019). A very recent study of the GENFI including presymptomatic and symptomatic *GRN* mutation carriers investigating the temporal cascade of multimodal biomarkers by means of discriminative event-based modeling (DEBM) demonstrated that, both in bvFTD and in nfvPPA, serum NFL is—together with language—the first biomarker to become abnormal in this genetic form of FTD (Panman et al., 2021).

Heller et al. (2020b) investigated plasma NFL in a large cohort comprising 196 presymptomatic and 90 symptomatic carriers of *GRN* and *MAPT* mutations and the *C9orf72* HRE as well as 183 neurologically healthy non-carriers belonging to the same families. As expected, for each of the three genes, symptomatic carriers had higher plasma NFL levels than presymptomatic carriers. Additionally, plasma NFL was higher in presymptomatic carriers of the *C9orf72* HRE compared with non-carriers, whereas no statistically significant difference was observed between presymptomatic carriers of *MAPT* or *GRN* mutations and non-carriers (Heller et al., 2020b). A very recent longitudinal study on plasma NFL examined both a cohort from the GENFI ($n = 297$) and one from the similar North-American network LEFFTDS/ARTFL ($n = 277$), including patients with mild cognitive impairment (MCI) or mild behavioral impairment (MBI) as defined by a score of 0.5 in the CDR-FTD: in the original cohort (from the LEFFTDS/ARTFL), baseline plasma NFL was higher in asymptomatic patients converting to MCI/MBI or to dementia in the next 2 years than in non-converters, and in the validation cohort (from the GENFI), asymptomatic participants or mildly symptomatic ones (MCI/MBI) had higher plasma NFL at baseline compared with corresponding non-converting individuals. However, baseline plasma NFL discriminated only poorly between asymptomatic and MCI/MBI participants (AUCs: 0.676 in the original cohort and 0.641 in the validation cohort) (Rojas et al., 2021).

Relationship With Neuroimaging

Several studies have investigated the relationship between CSF or blood NFL and magnetic resonance imaging (MRI) data, in most cases finding correlations between NFL and brain atrophy, particularly in frontal and temporal lobes. In the cohort of Scherling et al. (2014), CSF NFL in FTD and in bvFTD correlated negatively with gray matter volume of frontal, temporal, parietal, occipital, and cingulate cortices and, to a lesser extent, with the volume of the white matter associated with most of these regions. In FTD, serum NFL has been reported to correlate inversely with thickness of the prefrontal, temporal, and parietal cortices (Benussi et al., 2020). In their work on bvFTD, Steinacker et al. (2018) demonstrated negative correlations between serum NFL and volumes of frontal lobe, striatum, right amygdala, and frontal lobe white matter. The negative correlation with cortical thickness of frontal regions in FTD was recently confirmed also for plasma NFL (Illán-Gala et al., 2021).

Other studies investigated the relationship between baseline NFL levels and longitudinal changes in brain MRI in FTD or bvFTD, finding an association of CSF NFL with faster frontotemporal volume loss and faster decline in frontotemporal FA in bvFTD and correlations of serum NFL with atrophy rates of the frontal lobe as well as of the thalamus, caudate, putamen, pallidus, and overall subcortical white matter (Rohrer et al., 2016; Ljubenkov et al., 2018; Cajanus et al., 2020). Interestingly, in their large FTD cohort, Benussi et al. (2020) also reported a correlation of serum NFL with the neurophysiological measures, obtained with transcranial magnetic stimulation (TMS), short-interval intracortical inhibition (SICI), and long-interval intracortical inhibition (LICI), reflecting short-lasting postsynaptic inhibition mediated by GABA A and B receptors, respectively, at the level of cortical interneurons.

In svPPA, CSF NFL was reported to negatively correlate with gray matter volume of the parahippocampal gyrus of the dominant atrophic side, while for plasma NFL, a negative correlation was observed with gray matter volume of the combined temporal lobes (Meeter et al., 2019; Heller et al., 2020a). In nfvPPA, baseline CSF NFL was shown to be associated with faster frontotemporal volume loss and faster decline in frontotemporal FA (Ljubenkov et al., 2018). Pertaining to longitudinal NFL data, Steinacker et al. (2017b) reported

correlations of increases in serum NFL at follow-up with atrophy rate of the left frontal lobe in patients with PPA and with atrophy rate of the right middle frontal gyrus in the subcohort of patients with nfvPPA and svPPA.

In the study of Meeter et al. (2016) from the GENFI on patients with FTD carrying mutations in the three main genes *MAPT*, *GRN*, and *C9orf72*, NFL correlated negatively with volumes of the whole brain, frontal cortex, and insular cortex. In presymptomatic carriers, NFL correlated with volumes of the whole brain and frontal, temporal, and parietal cortices. In mutation carriers with follow-up MRI data, baseline CSF NFL correlated with atrophy rate of whole brain and of frontal, temporal, parietal, insular, and cingulate cortices. In the large investigation of van der Ende et al. (2019) on serum NFL in carriers of FTD gene mutations, negative correlations were observed at baseline between NFL and mostly frontotemporal brain volumes; moreover, in the whole cohort, the rate of change of serum NFL over time was associated with the longitudinal change in the volume of the whole brain and in the volumes of the frontal lobe, insula, cingulate gyrus, hippocampus, putamen, temporal lobe, amygdala, and cerebellum.

CONCLUSION AND PERSPECTIVES

A large body of evidence demonstrates that NFL is increased in the CSF and, consequently, in the blood, in both ALS and FTD in comparison with normal conditions (Scherling et al., 2014; Steinacker et al., 2016; Gille et al., 2019; Illán-Gala et al., 2021). In the case of ALS, the diagnostic potential of CSF and blood NFL is not much lower than that of the established CSF biomarkers for Alzheimer's disease (Blennow et al., 2010; Steinacker et al., 2016; Verde et al., 2019b). Therefore, it seems reasonable to consider the introduction of NFL in diagnostic criteria for ALS as a supportive element, with measurement in blood substituting for that in CSF when a lumbar puncture cannot be performed. Blood NFL measurement could also be implemented in the future as a large-scale screening test on individuals complaining of early neuromuscular symptoms (e.g., fasciculations), in order to identify those with "abnormal" values deserving prioritization for specialized evaluation (Verde et al., 2019b). However, the putative added value of NFL for the diagnosis of ALS should be evaluated by means of prospective studies including assessment of benefits for patients and the healthcare system. Measurement of NFL could be most beneficial in individuals at genetic risk for ALS—and for FTD—enabling optimization of the timing of prophylactic/therapeutic interventions aimed at opposing the disease process still in its subclinical phase (Benatar et al., 2018; van der Ende et al., 2019). At least as promising as its diagnostic performance is the potential of NFL as prognostic biomarker in ALS, resulting from its correlation with the disease progression rate (Verde et al., 2019b; Abu-Rumeileh et al., 2020), its longitudinal stability (Steinacker et al., 2017a; Verde et al., 2019b), and its measurability on peripheral blood. For these reasons, NFL deserves inclusion in future treatment trials of ALS and possibly inclusion into multiparameter prognostic models.

Regarding FTD, NFL shows promising diagnostic potential for the differentiation from primary psychiatric disorders (Katisko et al., 2020); on the contrary, NFL alone does not provide enough accuracy for the differential diagnosis between FTD and other dementias on an individual patient basis (Illán-Gala et al., 2021). However, it would be informative to evaluate—possibly in prospective studies—the added value of multiparameter models including CSF or blood NFL for early diagnosis of FTD, in a similar manner to what has been shown for the incorporation of CSF NFL into the neurochemical AT(N) scheme in order to more correctly identify FTLD as SNAP (Cousins et al., 2020). The same applies for the *in vivo* differentiation between the two main neuropathologic forms of FTD (i.e., FTLD-tau and FTLD-TDP), because this would impact stratification of patients in proteinopathy-oriented drug trials and, in the future, choice of the appropriate disease-specific treatment. Moreover, as its levels correlate with several measures of disease severity in FTD, NFL deserves consideration for inclusion as pharmacodynamic biomarker in therapeutic trials for FTD, which are in general at an earlier phase of their history compared with those for ALS (Scherling et al., 2014; Boxer et al., 2020; Illán-Gala et al., 2021).

Finally, several further issues regarding NFL biology and kinetics and its role as biomarker remain incompletely solved and warrant further investigation. Examples thereof include the following:

1) It is reasonable to assume that the cause of the elevation of NFL levels in the CSF and, hence, in the blood is leakage through a damaged axonal membrane. However, it cannot be excluded that other mechanisms of emission are involved, including active secretion or exosomes (Gafson et al., 2020). Moreover, damage to the axonal membrane *per se* could not most properly explain the release of neurofilaments: rather, more proximal mechanisms could contribute, e.g., imbalances in neurofilament transport or turnover or loss of integrity of the axonal cytoskeletal scaffold. The assumption itself that neurofilaments are markers of axonal pathology could also not be totally correct, as increasing evidence points to a role of neurofilaments in synapses (Yuan et al., 2015). Finally, elevation of neurofilament levels in neurodegenerative conditions could reflect not only cell damage but also more complex pathophysiological events, as suggested by the putative etiologic role of neurofilament gene mutations in rare cases of ALS as well as by the biological impact of experimental manipulation of neurofilament genes in ALS animal models (Williamson et al., 1998).
2) Although it is generally stated that neurofilaments are released from neurons into the CNS ISF and from there pass to the CSF and hence to the blood, the actual route followed by neurofilament molecules is not completely known. A relevant role could be played by ISF drainage along intramural perivascular (mostly periarterial but also perivenous) spaces and/or by lymphatic and glymphatic routes (Albargothy et al., 2018). The relative contribution of these mechanisms could also change in different CNS diseases (Gafson et al., 2020).

3) Even the kinetics of neurofilaments in healthy conditions in the human body are not completely known. A deeper knowledge of neurofilament metabolism and turnover, e.g., by means of SILK (stable isotope labeling kinetics) studies, would be essential for complete elucidation of the potential of NFL as biomarker (Sato et al., 2018; Gafson et al., 2020).
4) It is possible that neurofilaments undergo different biochemical modifications in different pathological conditions and in different disease stages (Gafson et al., 2020). Such modifications cannot be detected by current quantitative measurement techniques but deserve investigation both for mechanistic understanding and for exploration of diagnostic–prognostic potential.
5) The controversial issue of the relationship between NFL elevation and the extent of UMN vs. LMN degeneration in ALS should be clarified. This will require investigations using homogeneous methods, longitudinal observations, and large cohorts. An aid could also be offered by the development of assays specific for neurofilament forms reflecting CNS vs. PNS pathology, e.g., targeting α-internexin or peripherin, but also of hypothetical assays capable of recognizing biochemical differences which could exist between NFL forms released by UMNs and LMNs (Sato et al., 2018; Gafson et al., 2020).
6) The notion that NFL measurement is useful in the differential diagnosis of ALS is derived from studies on cohorts of ALS mimics which are admittedly quite large but are heterogeneous, including several forms of UMN, LMN, or related diseases (Verde et al., 2019b). To fully elucidate the usefulness of the biomarker in this context, it would be highly informative to compare ALS patients with large and homogeneous cohorts of single categories of mimic diseases. In these investigations, the ALS category itself should be stratified in different disease forms, thus analyzing also most problematic differential diagnostic issues such as the distinction between slowly progressive LMN-predominant ALS forms and neuromuscular diseases exclusively involving LMNs, or between slowly progressive UMN-ALS and PLS.
7) The reason why NFL is more elevated in FTD compared with most other forms of dementia is not yet fully understood (Skillbäck et al., 2014). On one hand, this could simply reflect a more rapid neurodegenerative process as opposed, for example, to what happens in Alzheimer's disease. On the other hand, a deeper mechanism could be represented by subclinical motor neuron degeneration occurring in a subset of FTD patients harboring TDP-43 pathology (Brettschneider et al., 2014), as suggested by the higher levels of NFL often observed in FTLD-TDP compared with FTLD-tau (Illán-Gala et al., 2021) and by the correlation existing between CSF NFL and the burden of TDP-43 pathology (Olsson et al., 2019).

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Neurofilament Proteins as Biomarkers to Monitor Neurological Diseases and the Efficacy of Therapies

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Biomarkers of neurodegeneration and neuronal injury have the potential to improve diagnostic accuracy, disease monitoring, prognosis, and measure treatment efficacy. Neurofilament proteins (NfPs) are well suited as biomarkers in these contexts because they are major neuron-specific components that maintain structural integrity and are sensitive to neurodegeneration and neuronal injury across a wide range of neurologic diseases. Low levels of NfPs are constantly released from neurons into the extracellular space and ultimately reach the cerebrospinal fluid (CSF) and blood under physiological conditions throughout normal brain development, maturation, and aging. NfP levels in CSF and blood rise above normal in response to neuronal injury and neurodegeneration independently of cause. NfPs in CSF measured by lumbar puncture are about 40-fold more concentrated than in blood in healthy individuals. New ultra-sensitive methods now allow minimally invasive measurement of these low levels of NfPs in serum or plasma to track disease onset and progression in neurological disorders or nervous system injury and assess responses to therapeutic interventions. Any of the five Nf subunits – neurofilament light chain (NfL), neurofilament medium chain (NfM), neurofilament heavy chain (NfH), alpha-internexin (INA) and peripherin (PRPH) may be altered in a given neuropathological condition. In familial and sporadic Alzheimer's disease (AD), plasma NfL levels may rise as early as 22 years before clinical onset in familial AD and 10 years before sporadic AD. The major determinants of elevated levels of NfPs and degradation fragments in CSF and blood are the magnitude of damaged or degenerating axons of fiber tracks, the affected axon caliber sizes and the rate of release of NfP and fragments at different stages of a given neurological disease or condition directly or indirectly affecting central nervous system (CNS) and/or peripheral nervous system (PNS). NfPs are rapidly emerging as transformative blood biomarkers in neurology providing novel

insights into a wide range of neurological diseases and advancing clinical trials. Here we summarize the current understanding of intracellular NfP physiology, pathophysiology and extracellular kinetics of NfPs in biofluids and review the value and limitations of NfPs and degradation fragments as biomarkers of neurodegeneration and neuronal injury.

Keywords: neurofilament, NFL, pNfH, biomarker, CSF, blood, neurodegeneration, neuronal injury

INTRODUCTION

It is widely accepted that the pathophysiology underlying many neurodegenerative disorders, such as Alzheimer's disease (AD), originates many years prior to clinical symptoms. AD evolves through three stages – an early, preclinical stage with no detectable symptoms; a middle stage of mild cognitive impairment; and a late stage marked by symptoms of dementia. The lack of success in identifying treatments that cure AD or alter its progression has been attributed in part to the implementation of candidate treatments at a disease stage that is too advanced to blunt the disease triggering mechanism(s) or halt early progression before momentum builds to irreversible levels. There is a growing need for reliable non-invasive blood-based biomarkers for AD that can facilitate diagnosis, predict disease progression, and provide evidence of disease modification.

Neurofilament proteins (NfPs) appeared in the last few years as the most promising blood biomarkers of neuroaxonal integrity or damage. Nfs are classified as a type IV class of intermediate filaments (IFs) specific to neurons (Yuan et al., 2017). They are protein polymers measuring 10 nm in diameter and many micrometers in length. Together with microtubules (25 nm) and microfilaments (7 nm), they form the neuronal cytoskeleton. Much interest in the field has been recently focused on the detection of NfPs and degradation fragments released from neurons into blood as surrogate markers of neuronal damage in neuropathic states. The rationale for NfPs and fragments as biomarkers of neuronal damage is that they are not only responsive to neuronal injury but are also prominent components of abnormal intraneuronal aggregates in varied neurodegenerative diseases, including AD, dementia with Lewy bodies (DLB), Parkinson's disease (PD), frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS), Charcot-Marie-Tooth disease (CMT), multiple sclerosis (MS), giant axonal neuropathy (GAN) and toxic neuropathies. Although amyloid-beta and tau proteins are widely regarded as useful diagnostic biomarkers of AD, tau proteins increase only in specific neurodegenerative diseases such as AD and unaltered in other neurological diseases that are clearly neurodegenerative, such as tau-negative FTD caused by granulin or C9orf72 mutations (Foiani et al., 2018) where, by contrast, CSF and serum neurofilament light chain (NfL) fragment levels are more than 8 times higher in patients than in pre-symptomatic carriers or healthy controls (Meeter et al., 2016). Furthermore, in Huntington disease (HD), CSF NfL fragment levels correlate more strongly with disease progression than do CSF tau levels (Niemela et al., 2017). Moreover, studies using a stable isotope labeling method to investigate tau metabolism demonstrate that the production rate of tau positively correlates with the amount of amyloid plaques, suggesting that increased tau levels in AD could be due to elevated transcription, synthesis or secretion from neurons in response to amyloid-beta pathology rather than reflect actual neurodegeneration (Sato et al., 2018). Thus, as general neuronal integrity markers, NfPs and their fragments may be more sensitive to neurodegeneration than is tau.

In individuals with inherited forms of AD, levels of NfL fragments in blood may be altered 22 years before symptoms begin (Quiroz et al., 2020). NfL responds more sensitively to subclinical cognitive decline than amyloid-beta or tau (Bos et al., 2019; Kern et al., 2019; Merluzzi et al., 2019). Moreover, mean NfL fragment plasma levels increased 3.4 times faster in subjects who developed AD compared to those who remained dementia-free in a trajectory analysis of 4444 non-demented participants in the Rotterdam study at baseline and up to 14 years follow-up. In this review, we summarize the current understanding of NfPs and fragments as biomarkers in neurodegeneration and neurological injuries and draw attention to important unanswered questions.

PROPERTIES OF NEUROFILAMENTS RELEVANT TO THEIR USE AS BIOMARKERS

The Physiological Basis of Neurofilament Proteins as Biomarkers of Neuronal Structural Integrity

For a blood-based biomarker to reflect the structural integrity of neurons in human brains, it has to be a structural constituent of the neuron, impacted by the neuropathological process, and easily detectable in blood. The composition of intermediate filament subunits in neurons varies depending on the nerve cell type and stage of development (**Figure 1**). At the earliest stage of embryonic development, neural stem cells express nestin (NES), a type VI intermediate filament protein that is down-regulated after differentiation and replaced by cell type-specific intermediate filament proteins (Lendahl et al., 1990). Vimentin (VIM), a type III intermediate filament protein of mesenchymal cells, is also transiently co-expressed with nestin in precursor nerve cells (Yabe et al., 2003). VIM is gradually replaced by peripherin (PRPH), alpha-internexin (INA), neurofilament medium chain (NfM), and NfL during embryonic development. Neurofilament heavy chain (NfH) chain expression is low in developing neurons and increases postnatally (Shaw and Weber, 1982; Pachter and Liem, 1984).

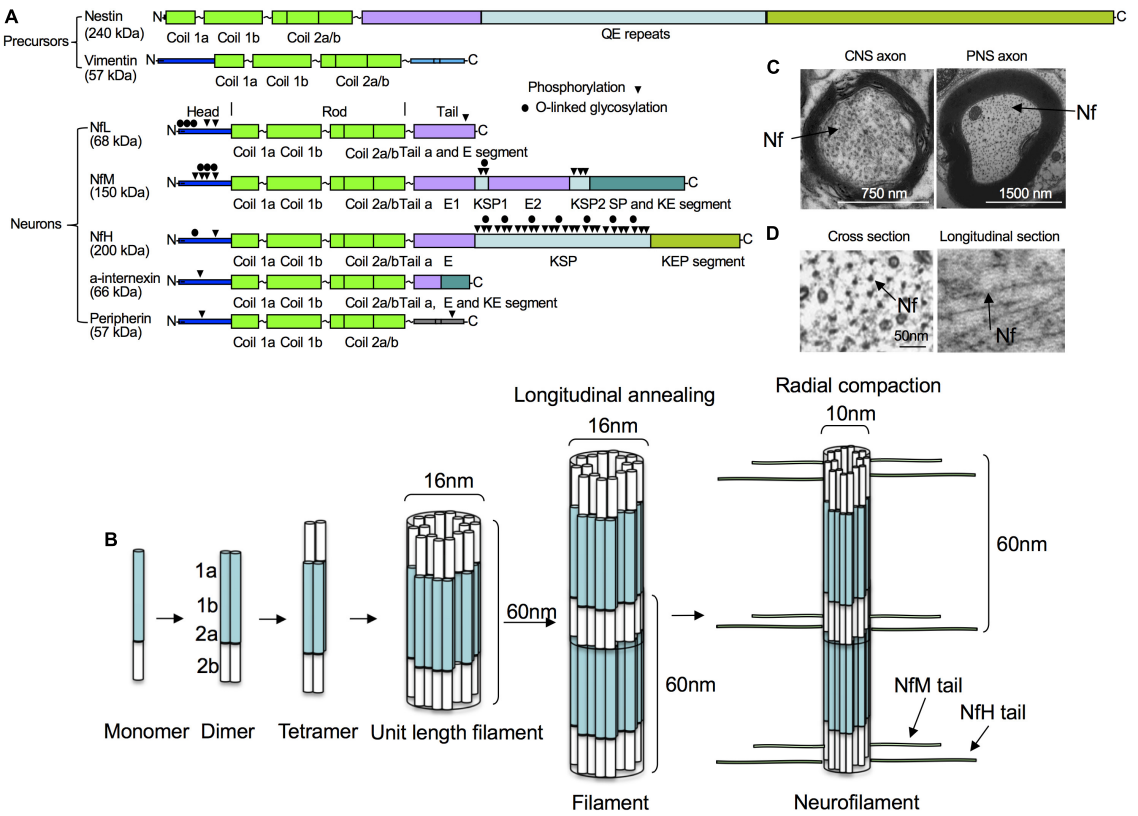

FIGURE 1 | Structure, assembly and cytoarchitecture of Nfs. **(A)** Domain structure of Nfs in precursor and mature neurons. Precursor neurons contain nestin and vimentin while mature neurons have NfPs consisting of NfL, NfM, NfH, INA, and/or PRPH. All Nf subunits include a conserved alpha-helical rod domain, amino-terminal globular head regions and carboxy-terminal tail domains. Phosphorylation and O-linked glycosylation sites are shown. **(B)** Nf assembly. Nf monomers form coiled-coil heterodimers, then tetramers and unit-length filaments and gradual end-to-end annealing of which results in filament elongation to form mature Nfs with a diameter of about 10 nm after radial compaction. **(C)** Moderate number of Nfs in corpus callosum axons vs. large number of Nfs in sciatic axons in mice. **(D)** Ultrastructural representations of Nfs from mouse optic nerves in cross and longitudinal sections.

Mature mammalian neurons usually express five different NfPs: NfL, NfM and NfH chains, as well as INA and PRPH. In mature neurons in the CNS, Nfs are generally composed of NfL, NfM, NfH, and INA (Yuan et al., 2006), whereas, in the peripheral nervous system, they mainly consist of NfL, NfM, NfH and PRPH (Yuan et al., 2012). Like all IF proteins, NfPs all share a common alpha-helical rod domain that assembles to form a filament backbone, flanked by variable amino and carboxy-terminal domains that regulate polymer assembly and interactions. NF heteropolymer assembly starts with the formation of NfP dimers and antiparallel aggregation of these dimers leads to formation of tetramers which are thought to be the basic subunit of NFs during assembly (Mucke et al., 2018) and usually consist of NfL and one or more of the other Nf proteins. NfPs of mature neurons *in vivo* are mainly stable polymers and the pool of soluble NfP is small.

Neurofilament proteins are mainly synthesized in the cell body and transported as hetero-oligomeric assemblies and short filaments into axons and dendrites (Pachter and Liem, 1984; Yuan et al., 2003, 2009; Yan and Brown, 2005) to establish a highly stable regionally specialized NF network (Nixon and Logvinenko, 1986; Nixon et al., 1994; Sanchez et al., 1996). Nf mRNAs are also transported out of cell bodies into dendrites, spines, and axons and localized NfP synthesis in these cytoplasmic extensions is used to spatially and temporally regulate their protein content in these subcellular domains (Alami et al., 2014). NfPs can be proteolyzed by calpains, the proteasome, and autophagy into many smaller degradation products (Yuan et al., 2017).

The Neuropathological Basis for Neurofilament Proteins as Biomarkers

Biochemical, genetic, and animal model evidence implicates NfPs as a pathogenic culprit playing primary or secondary roles in nervous system diseases. NfPs are involved in the pathophysiological processes underlying many states of neurological injury and neurodegeneration, reflecting changes in structural integrity and abnormal accumulation or maldistribution of NfPs (Hamberger et al., 2003).

Animal Studies

Proper levels of NfPs are important for the normal functions of nervous systems in animals. Absence of NfL from neurons reduces axon diameters and causes sensorimotor and cognitive impairments in quails (Yamasaki et al., 1991) and mice (Zhu et al., 1997; Yuan et al., 2018). Single deletion of NfM, NfH or PRPH

in mice can lead to age-related atrophy of motor axons (Elder et al., 1999), decrease in conduction velocity (Kriz et al., 2000) and reduced numbers of unmyelinated sensory axons (Lariviere et al., 2002), respectively. Deletion of INA in the absence of NfL (Yuan et al., 2003) or both NfL and NfH results in reduced transport of NfM into axons (Yuan et al., 2015b). Overexpression of NfL, NfM, NfH or PRPH in animals can produce neuropathology of motor neuron diseases (Cote et al., 1993; Xu et al., 1993; Beaulieu et al., 1999; Gama Sosa et al., 2003) while overexpression of INA leads to motor coordination deficits (Ching et al., 1999). In addition to the importance of NfP levels, expression of an NfL mutation in mice which causes human disease (Zuchner et al., 2004; Filali et al., 2011; Liu et al., 2011; Shen et al., 2011; Pisciotta et al., 2015) also leads to motor neuropathology (Lee et al., 1994) and phenotype of CMT (Filali et al., 2011) probably due to disruption of Nf assembly (Perez-Olle et al., 2002; Tradewell et al., 2009) and transport (Brownlees et al., 2002), and abnormal Nf accumulation (Zhai et al., 2007).

Human Studies

Clinical studies demonstrate presence, normal structure and assembled network of NfPs are critical for human health. NfL loss of function mutations in cases of human neuropathy which cause markedly lowered NfL protein levels reduce axon diameters and cause sensorimotor and cognitive impairments in humans (Yum et al., 2009; Sainio et al., 2018). NfL and NfH mutations can cause Nf accumulation in CMT type 2E/1F/CMTDIG (Lerat et al., 2019) and CMT2CC (Ikenberg et al., 2019). In AD, NfPs are integral components of neurofibrillary tangles (Rudrabhatla et al., 2011; **Figure 2**) and NfH and NfM are 4–8-fold more phosphorylated than normal (Rudrabhatla et al., 2010). In PD, Lewy bodies contain NfPs (Goldman et al., 1983) and a cage-like Nf structure encapsulates Lewy bodies (Moors et al., 2019). In Nf inclusion disease, a form of FTD, prominent aggregations of NfPs, especially INA, are the neuropathologic hallmark of the condition (Cairns et al., 2004). Abnormal NfP accumulations are also a hallmark pathologic feature of ALS (Cleveland and Rothstein, 2001). In MS, increased expression of phosphorylated NfH (pNfH) is observed in spinal motor neuron perikarya (Muller-Wielsch et al., 2017) and Nfs accumulate excessively in axons in GAN (Bomont et al., 2000).

Neurofilament Proteins Released From Neurons Gain Access to Blood Under Physiological and Pathological Conditions

Recent Technology Breakthroughs for the Reliable Detection of Neurofilament Proteins in the Peripheral Circulation

Low levels of NfPs are constantly released from neurons into CSF and blood under physiological conditions and rise above normal in pathological states.

Rosengren et al. (1996) first tested NfPs as possible biomarkers using enzyme-linked immunosorbent assay (ELISA) with polyclonal rabbit antisera specific against the individual NfPs and showed that CSF NfL levels were increased in patients with ALS and AD compared to controls. However, the sensitivity of ELISA and the later developed electrochemiluminescence (ECL) immunoassay does not allow small, disease-related changes to be reliably detected in peripheral circulation. In 2010, single-molecule enzyme-linked immunosorbent assay (Simoa) was initially described (Rissin et al., 2010) which later enabled reliable quantification of NfL in serum or plasma samples (Gisslen et al., 2016) using NfL-specific monoclonal antibodies (mAb47:3) (Norgren et al., 2002). More recently, Meso Scale Discovery, immunomagnetic reduction technologies and the Ella platform based on microfluidic channels have also been developed to detect low NfP levels in blood (Liu H.C. et al., 2020; Lombardi et al., 2020; Gauthier et al., 2021).

Neurofilament Proteins in Exosomes

The fact that plasma NfL levels are enriched in neuron-derived exosomes compared to total exosomes isolated from blood in healthy individuals (Sun et al., 2017) suggests the NfPs are released from neurons at least in the form of exosomes (**Figure 3**). Moreover, plasma neuron-derived exosomes contain about 74-fold more NfL than plasma astrocyte-derived exosomes, which have only negligible amounts (Winston et al., 2019). NfP-containing exosomes or NfPs or degradation fragments released into the extracellular space may be eliminated from the CNS along intramural peri-arterial drainage pathway (Engelhardt et al., 2017).

Neurofilament Protein Forms in Peripheral Circulation

Identity of the NfL forms in plasma exosomes is still unclear but a 22 kDa NfL degradation fragment has been revealed with an anti-NfL antibody and shown to be increased in ALS patients (Lombardi et al., 2020). Also identified are a 30 kDa fragment of NfL in Nf-containing aggregates from human blood (Adiutori et al., 2018) and a 10 kDa fragment of NfL in mouse CSF. Since no full length NfL has been ever reported in CSF or blood (Brureau et al., 2017), the detected Simoa signal is, therefore, NfL immunoreactivity or NfL breakdown product. By contrast, full length (200 kDa) or oligomeric NfH were predominant in CSF and blood (Petzold et al., 2003; Shaw et al., 2005; Lewis et al., 2008). Recent studies also suggest full length (150 kDa) or trimeric NfM (450 kDa) in blood (Haggmark et al., 2014). A comprehensive list of widely used capture and detection antibodies to NfPs in ELISA is shown in **Table 1**.

Neurofilament Light Chain Levels in Normal Individuals

Intracellular NfPs have long half-lives ranging from 55 days in axons (Nixon and Logvinenko, 1986; Yuan et al., 2015a) to 64–72 days at synapses (Heo et al., 2018), indicating their slow turnover rates inside neuronal compartments. Upon release into the extracellular space, serum or plasma NfL levels in healthy individuals are about 2.5% of the levels in CSF and correlate highly with the 40-fold higher NfL concentrations in CSF with typical R values ranging from 0.6 to 0.7 (Disanto et al., 2017; Pereira et al., 2017; Khalil et al., 2020; Alagaratnam et al., 2021), suggesting that most of the NfL signal in blood is CNS-derived and could be used as a proxy measure for CSF NfL

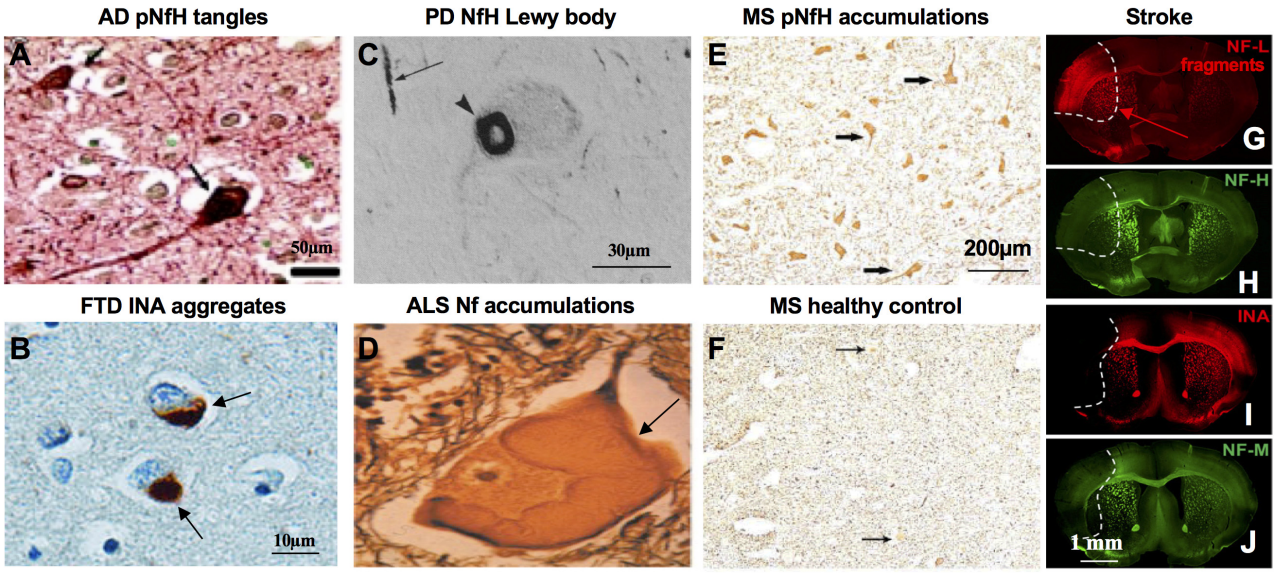

FIGURE 2 | Pathological basis of NfPs as biomarkers in neurologic diseases and neuronal injury. **(A)** NFTs in AD brain are stained with pNfH with mouse monoclonal phospho-NfH antibody RT97 under the condition it does not cross-react with phosphor-tau (adapted from Rudrabhatla et al., 2010). **(B)** Cytoplasmic inclusions in NIFID brain, a type of FTD, are stained with antibody to alpha-internexin (adapted from Cairns et al., 2004). **(C)** Cytoplasmic Lewy bodies in PD brain are stained with antibody to NfH (adapted from Goldman et al., 1983). **(D)** Masses of Nf swelling in ALS spinal cord are stained with Silver (adapted from Cleveland and Rothstein, 2001). **(E)** Anterior horn cell perikarya in MS spinal cord are prominently stained with antibody to pNfH (SMI31) whereas healthy controls remain almost non-reactive **(F)** (adapted from Muller-Wielsch et al., 2017). Ischemia-affected areas in mouse brain 24 h after experimental stroke induction are demarcated by an increase of NfL degradation fragments immunoreactivity **(G)**, while the immunosignals for NfH **(H)**, alpha-internexin (INA) **(I)**, and NfM **(J)** are decreased (adapted from Mages et al., 2018).

levels (Gisslen et al., 2016). The NfL levels in blood are most often tested in serum and less frequently in EDTA-plasma with serum levels slightly higher than in plasma (Hviid et al., 2020b). Either specimen type is acceptable, however, when used in either research or clinical setting a single specimen should be selected for use. Plasma NfL levels measured in the morning may be more than 10% higher than those measured in the evening, suggesting that synaptic remodeling during sleep might alter NfL kinetics (Benedict et al., 2020; Thebault et al., 2021). CSF NfL levels in healthy females are about 20% lower than levels in healthy males (Bridel et al., 2019) although the reverse was true in an ALS cohort (Thouvenot et al., 2020). Concentrations of CSF and serum NfL increase with age in healthy controls (Yilmaz et al., 2017) with an increase in adult control serum NfL levels of 2.2% per year of age (Disanto et al., 2017; Barro et al., 2018). These increases accompany hippocampal atrophy in cognitively healthy older adults, which has suggested possible AD-independent, age-expected hippocampal decline (Idland et al., 2017). However, younger children have higher serum NfL levels than older children reaching the lowest level between the age of 10 and 15 years, then increasing in a linear fashion until the age of 60 years and accelerating non-linearly afterward (Evers et al., 2020; Khalil et al., 2020; Reinert et al., 2020). There are various proposed bases for serum NfL elevation in aging, including subclinical senescence with greater neuronal apoptosis (Khalil et al., 2020) and increased disruption of blood-brain barrier (Sweeney et al., 2018). Levels of serum NfL may also be affected by race, systolic blood pressure, decreased renal function, glycemic control measured by hemoglobin A1C (Korley et al., 2019) and pregnancy (Cuello et al., 2019). The multiplicity of influences on these levels prompts caution in controlling stringently for confounding variables in clinical studies.

Contribution of Neurofilament Proteins or Fragments From Different Neuronal Compartments

Besides calpains, the proteasome and autophagy (Smerjac et al., 2018), other non-specific proteases, including cathepsin D (Nixon and Marotta, 1984) and caspases 6 and 8 (Shabanzadeh et al., 2015) can also trigger Nf turnover and generate Nf peptides. Nf assembly confers significant proteolytic resistance to Nf subunits: deletion of three Nf subunits leads to degradation of the fourth subunit (Yuan et al., 2015b). Phosphorylation also protects Nfs against proteolysis (Goldstein et al., 1987; Pant, 1988; Rao et al., 2012). NfPs or their degradation fragments are released into biofluids following any damage to nervous system. Therefore, they are neither able to determine brain region specific alterations nor differentiate disease specific pathophysiological process.

Mechanisms for Neurofilament Protein and Fragment Release From Neurons

The exact mechanisms governing NfP release into biofluid are not fully understood. Release of NfPs or fragments from neurons may be a direct passive consequence of the loss of membrane integrity or may follow the known pathways for active secretion of other neuronal peptides and proteins. Intracellular endosomal organelles known as multivesicular bodies may play

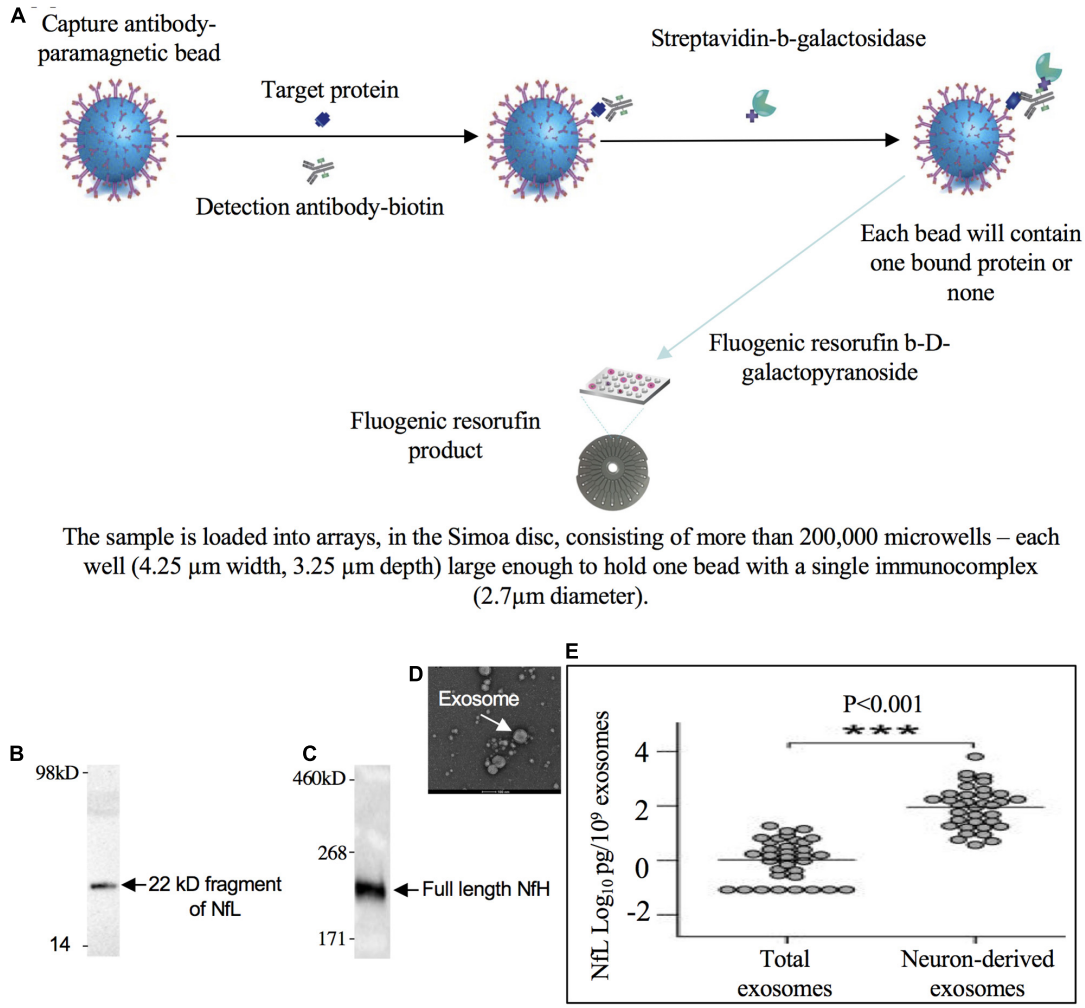

FIGURE 3 | 22 kD fragment of NfL and full length NfH in blood. **(A)** Low levels of NfPs in blood can be detected with single molecule array technology (Simoa/digital ELISA). A 22 kDa degradation fragment of NfL (**B**, adapted from Lombardi et al., 2020) and full length NfH (adapted from Adiutori et al., 2018) were detected in blood **(C)**. **(D)** Isolated exosomes from blood (adapted from Zhang et al., 2020). **(E)** NfL signal is enriched in neuron-derived exosomes compared to total or astrocyte-derived exosomes in blood (adapted from Sun et al., 2017). *** Indicates highly significant.

critical roles in the release of peptides (Von Bartheld and Altick, 2011). This may happen though "back-fusion" events and budding from the plasma membrane to generate micro-vesicles (Kleijmeer et al., 2001) or through release of smaller endosomally derived exosomes (Lachenal et al., 2011). Levels of NfL signals in isolated neuron-derived exosomes accounting for a small percentage of total NfL concentration in plasma suggest that active secretion is at least one of the mechanisms for NfP release from neurons. After release from neurons, some NfPs and fragments can be degraded and cleared by varied extracellular proteinases and microglia and these processes may even further generate the fragments from a larger form. Pathways for degradation could be differentially critical in the context of healthy, injured or chronically damaged neurons. Expression of NfP genes is not elevated in ALS (Wong et al., 2000) and neither NfP gene (Robinson et al., 1994) nor protein expression (Ashton et al., 2019) is elevated in AD, suggesting that the increased NfP signal in biofluids is not due to a compensatory overproduction.

Major Determinants of Neurofilament Protein and Fragment Levels in Cerebrospinal Fluid and Blood

Studies have linked NfP levels in blood to changes in white matter (Moore et al., 2018; Spotorno et al., 2020; Maggi et al., 2021), gray matter (Jakimovski et al., 2019a; Kang et al., 2020), or both (Johnson et al., 2018), yielding a confusing picture of what variables dictate the highly variable levels found in different disorders. Some likely determinants of blood/CSF levels, however, include the composition of the diseased or injured area (relative abundance of large caliber axons that have high Nf-content) and size of the damaged region. NfL and NfH content in spinal cord is several fold higher than in corpus callosum (Yuan et al., 2012) and at least 10-fold higher than in cortex (Shaw et al., 2005). Accordingly, a spinal cord injury released

TABLE 1 | NfP and fragment measurement as biomarkers.

	Capture antibodies	Detection antibodies	References
ELISA for measuring Nf subunits as biomarkers			
NfL	Chicken polyclonal anti-NfL	Rabbit polyclonal anti-NfL	Rosengren et al., 1996
	NfL mAb 47:3, core domain (aa92-396)	NfL mAb 2:1	Norgren et al., 2003
	Polyclonal antibody R61d to NfL, NfM and NfH	NfL mAb NR4	Hu et al., 2002
	NfL 21 mAb, core domain (aa93-396)	NfL 23 mAb	Gaetani et al., 2018
NfH	SMI31 mAb anti-NfH and NfM	Anti-NfH NA211	Hoglund et al., 2012
	SMI34 mAb anti-NfH	Rabbit polyclonal anti-NfH	Zucchi et al., 2018
	SMI35 mAb anti-NfH	Chicken polyclonal anti-NfH	Petzold et al., 2003
	Chicken polyclonal anti-NfH	Rabbit polyclonal anti-NfH	Shaw et al., 2005
	AH1 mAb anti-NfH	NAP4 mAb anti-NfH	Boylan et al., 2009
	Polyclonal antibody R61d to NfL, NfM and NfH	SMI31, SMI32, SMI33 and SMI34	Hu et al., 2002
	pNfH mAb 9C9	NfH polyclonal antibody	Koel-Simmelink et al., 2014
NfM	Polyclonal antibody R61d to NfL, NfM and NfH	SMI31, SMI32 and SMI33	Hu et al., 2002
	Monoclonal anti-NfM	Monoclonal anti-NfM	Martinez-Morillo et al., 2015
	Polyclonal anti-NfM	Polyclonal anti-NfM	Zucchi et al., 2018
PRPH	Rabbit polyclonal anti-PRPH	Chicken polyclonal anti-PRPH	Finderlater, 2010
	Unknown	Unknown	Sabbatini et al., 2021
Proteomics for measuring NF subunits as biomarkers			
NfM			Haggmark et al., 2014; Martinez-Morillo et al., 2014; Remnestal et al., 2020
INA			Martinez-Morillo et al., 2014
PRPH			Liang et al., 2019

about 12-fold more NfH into blood than a brain injury of comparable size (Shaw et al., 2005). Demyelinating damage to CNS axons associated with clinical or MRI (magnetic resonance imaging) disease activity in MS can cause a spike of more than 20-fold in the levels of serum NfL which may be lowered with effective treatment (Akgun et al., 2019). Only about 20% of the NfL fragment present in blood comes from neuron-derived exosomes (Altick et al., 2009; Winston et al., 2019; Guedes et al., 2020) so the extent of loss of membrane integrity affecting NF-rich axons, or even to a lesser extent synapses, is likely the major determinant of NfP and fragment levels in CSF and blood. In a limited region of involvement as in the substantia nigra pars compacta in PD, NfL fragment level increases in CSF and serum are modest. By contrast, in FTD/ALS, widespread degeneration of large caliber Nf-rich axonal fibers in the spinal cord and brain results in one of the highest elevations of NfP and fragment blood levels among neurodegenerative diseases. More studies are warranted to determine the relative gray and white matter contributions to NfP and fragment levels in biofluids at different stage of a specific disease.

Mechanisms of Neurofilament Protein or Peptide Trafficking Between Brain and Blood

Since the main source of serum NfPs is the CNS, it is not fully clear how NfPs traffic between parenchymal, CSF and blood compartments. NfPs or their degradation fragments could also follow the apparent general pathways by which molecules such as amyloid-beta peptides pass from the interstitial fluid (ISF) of the brain into CSF and blood. Soluble metabolites or peptides from cells in most organ are absorbed directly into the blood or drain via lymphatic vessels to regional lymph nodes (Engelhardt et al., 2017). Soluble tracers such as serum albumin injected into ISF of the brain drain to cervical lymph nodes along the walls of cerebral arteries (Szentistvanyi et al., 1984) through intramural peri-arterial drainage pathway (IPAD) (Albargothy et al., 2018) including initially along basement membranes that surround capillaries and then along the basement membranes between smooth muscle cells in the tunica media of intracerebral and leptomeningeal arteries (Carare et al., 2008). About 85% of a tracer injected into the cerebral hemispheres passes to cervical lymph nodes via IPAD (Szentistvanyi et al., 1984) while only 10–15% passes into the CSF (Szentistvanyi et al., 1984; McIntee et al., 2016). Future studies need to measure the proportion of NfPs and degradation fragments released from neurons that reaches the CSF. Drainage of CSF into lymphatic vessels of the nasal mucosa via the cribriform plate appears to be a major lymphatic drainage pathway (Kida et al., 1993; De Leon et al., 2017) and may also include dural lymphatics (Aspelund et al., 2015). The glial-lymphatic or glymphatic pathway is recently identified in rodent brain, which sub-serves the flow of CSF into the brain along perivascular spaces and then into the brain interstitium facilitated by aquoporin-4 water channels (Rasmussen et al., 2018). This pathway then directs flow toward the venous perivascular and perineuronal spaces, ultimately clearing solutes from neuropil into meningeal and cervical lymphatic drainage vessels.

Dynamics of Extracellular Neurofilament Proteins and Fragments

In acute neurological diseases with a known timepoint for neuronal or axonal damage such as traumatic brain injury (TBI)

(Bergman et al., 2016; Shahim et al., 2017), stroke (Gattringer et al., 2017; Tiedt et al., 2018) and MS (Rosso et al., 2020), CSF and serum NfP signals increased over a few days and remained elevated over many months. It may be difficult to investigate the dynamics of CSF and serum NfPs in chronic neurodegenerative diseases such as AD and PD without treatments that can cure them. Because it is suggested that serum NfL fragments may be cleared by the kidneys, renal function ought to be considered when interpreting serum NfL levels (Korley et al., 2019; Van Der Plas et al., 2021).

Neurofilament Proteins as Biomarkers in Animal Models

Neurofilament Proteins as Biomarkers in Animal Models of Neurological Diseases

Increased levels of plasma NfL have been observed in mouse models of PD A53T- alpha-synuclein, tauopathy P301S-Tau and AD APP/PS1 (amyloid precursor protein/presenilin 1) (Bacioglu et al., 2016). Increases in NfL in CSF and blood coincide with the onset and progression of the corresponding proteopathic lesions in brain. Experimental induction of alpha-synuclein lesions increase blood NfL levels, while blocking the development of amyloid-beta lesions attenuates NfL increases (Bacioglu et al., 2016). Prolonged expression in mice of p25 (the calpain-mediated truncated product of p35, the regulatory subunit of Cdk5 – cyclin-dependent-like kinase 5) causes severe synaptic and neuronal loss and brain atrophy which are accompanied by cognitive deficits (Fischer et al., 2005). In these inducible CamKII-TetOp25 transgenic mouse models of neurodegeneration, serum NfL levels increase after induction of neurodegeneration by switching on p25 transgene expression via removal of doxycycline but do not increase further if induction is stopped by switching off p25 expression. Increased levels of serum NfL correlate with induced neuronal damage in the cortex and hippocampus of CamKII-TetOp25 mice, indicating that NfL levels mirror the ongoing neurodegeneration and neuronal loss and may be used as a dynamic biomarker of neurodegeneration (Brureau et al., 2017). In HD R6/2 mice, increased levels of NfL in CSF and serum is associated with neurodegeneration and disease severity (Soylu-Kucharz et al., 2017). In 304Q knock-in spinocerebellar ataxia type 3 (SCA3) mouse model, serum NfL and pNfH are elevated at the pre-symptomatic stage of 6 months of age and correlate with ataxin 3 aggregation and Purkinje cell loss in the brain (Wilke et al., 2020). Increased CSF pNFH levels were also observed in horses with equine neuroaxonal dystrophy/degenerative myeloencephalopathy (Edwards et al., 2021). Plasma pNfH levels also closely reflect later stages of disease progression and therapeutic response in the SOD1 (superoxide dismutase 1) G93A mouse model of ALS (Lu et al., 2012). Recently, serum NfL concentration in sheep with prion disease was more than 15 times higher than that found in control samples (Zetterberg et al., 2019). More recently, plasma NfL levels were also reported to reflect disease severity in mice inoculated with prions and fell significantly in antisense oligonucleotide-treated mice compared to the immediate pre-dose timepoint, suggesting a reversal of pathology driving the 53% increase in survival time (Minikel et al., 2020).

Neurofilament Proteins as Biomarkers in Animal Models of Neurological Injuries

Following experimental spinal cord injury (SCI) in adult rats, serum pNfH showed an initial peak of expression at 16 h and a second peak at 3 days while no serum pNfH is detectable in sham control animals (Shaw et al., 2005). The maximum level of pNfH in these SCI experiments was 250 ng/ml pNfH in the 3–5 day post-injury period following injury. Serum pNfH showed a similar trajectory in TBI in adult rats but the average peak level of expression of serum pNfH was only about 20 ng/ml, much lower than that seen in the SCI model (Shaw et al., 2005). Recent studies found serum NfL levels were substantially elevated at all acute and subacute time-points after a single mild TBI (mTBI), peaked at 1-day, and remained elevated 14-days post-injury (O"Brien et al., 2021). Increased serum NfL levels were also mTBI dose-dependent and correlated with the degree of sensorimotor impairment (O"Brien et al., 2021). In more recent studies using an experimental rat model of blast-induced TBI, pNfH levels increased at 24 hr, returned to normal levels at 1 month, but increased again at 6 months and 1 year post-blast exposure (Arun et al., 2021). Moreover, the changes in CSF pNfH correlate with pNfH levels in brain regions and with neurobehavioral function in the rats (Arun et al., 2021).

Neurofilament Proteins as Biomarkers in Neurological Diseases

Cerebrospinal fluid or serum NfL and pNfH have been widely studied as biomarkers in a number of neurological diseases (**Table 2**) or conditions directly or indirectly affecting central and peripheral nervous systems (**Table 3**). NfPs are not only elevated in neurological diseases but may also track disease progression. Different subunits might reflect different neurodegenerative processes. In addition to commonly used NfL and pNfH, some studies also found potential values of other Nf subunits, i.e., NfM (Hu et al., 2002; Haggmark et al., 2014; Martinez-Morillo et al., 2014; Zucchi et al., 2018; Remnestal et al., 2020), INA (Martinez-Morillo et al., 2014) and PRPH (Finderlater, 2010; Liang et al., 2019; Sabbatini et al., 2021) as biomarkers in CSF or serum in neurological diseases or injuries.

Multiple Sclerosis

Patients with MS have up to 60% axonal loss at all spinal levels involving all fibers regardless of their diameter (Tallantyre et al., 2009; Petrova et al., 2018). The concentrations of CSF and serum NfPs represent the degree of axonal loss and therefore, could be a biomarker of MS disease activity. Accordingly, CSF NfL levels in relapsing MS were 3-fold higher than in healthy controls (951.8 vs. 284.4 pg/ml) and associated with relapse and cortical lesions (Damasceno et al., 2019). Serum NfL levels was first reported to be increased in early relapsing MS and correlated with MRI measures of disease severity using an electro-chemiluminescence assay (Kuhle et al., 2016). This finding of serum NfL as a biomarker of MS disease activity was later substantiated with higher sensitivity Simoa digital immunoassay

TABLE 2 | NfPs and fragments as biomarkers in neurodegeneration and neuronal injuries.

Neurological diseases and injuries	Association of NF subunit level with								Association of NF subunit level with			References
	NfL		pNfH (smi35)		NfH (smi34)		NfH (smi31 and others)					
	B	C	B	C	B	C	B	C	Disease activity	Prognosis	Treatment response	
Multiple sclerosis and clinically isolated syndrome	+	+	+	+	+	+	+	+	yes	yes	yes	Linker et al., 2009; Disanto et al., 2016, 2017; Herrera et al., 2019; Calabresi et al., 2020; Saraste et al., 2021
Alzheimer's disease	+	+		+			+	+	yes	yes	yes	Rosengren et al., 1996; Hu et al., 2002; Kuhle et al., 2010; Hoglund et al., 2012; Zetterberg et al., 2016; Mattsson et al., 2017; Gaetani et al., 2018; Benedet et al., 2020
Adult Down syndrome	+								yes	yes		Fortea et al., 2018; Strydom et al., 2018; Shinomoto et al., 2019; Delaby et al., 2020; Carmona-Iragui et al., 2021; Petersen et al., 2021
Mild cognitive impairment	+	+							yes	yes		Zhou et al., 2017; Mayeli et al., 2019; Osborn et al., 2019
Vascular dementia		+				+		+	yes	yes		Hu et al., 2002; Skillback et al., 2014
Mixed dementia		+							yes	yes		Skillback et al., 2014
Frontal temporal dementia		+							yes	yes		Skillback et al., 2014; Remnestal et al., 2020
Dementia with Lewy body				+								De Jong et al., 2007
HIV-associated dementia	+								yes	yes	yes	Gisslen et al., 2016
Stroke	+	+		+					yes	yes		Norgren et al., 2003; Petzold et al., 2003; Kuhle et al., 2010; Martinez-Morillo et al., 2014; Tiedt et al., 2018; Garland et al., 2021; Peng et al., 2021; Wang Z. et al., 2021
Traumatic brain injury	+								yes	yes		Shahim et al., 2016; Liang et al., 2019; Yang et al., 2019
Sport-related concussion	+											Shahim et al., 2017; McDonald et al., 2021

(Continued)

TABLE 2 | (Continued)

Neurological diseases and injuries	Association of NF subunit level with								Association of NF subunit level with			References
	NfL		pNfH (smi35)		NfH (smi34)		NfH (smi31 and others)					
	B	C	B	C	B	C	B	C	Disease activity	Prognosis	Treatment response	
Spinal cord injury	+						+		yes	yes		Shaw et al., 2005; Kuhle et al., 2015
Amyotrophic lateral sclerosis	+	+	+	+			+	+	yes	yes		Rosengren et al., 1996; Kuhle et al., 2010; Haggmark et al., 2014; Rossi et al., 2018; Benatar et al., 2019
Parkinson's disease	+	+							yes	yes		Lin et al., 2018, 2019; Backstrom et al., 2020; Ye et al., 2021
Huntington disease	+								yes	yes		Byrne et al., 2017; Rodrigues et al., 2020
Bipolar disorder		+										Jakobsson et al., 2014
Autism spectrum disorder	+											He et al., 2020
Neuronal ceroid lipofuscinosis type 2 and 3	+										yes	Ru et al., 2019; Dang Do et al., 2020
Spinal muscular atrophy	+								yes	yes	yes	Olsson et al., 2019
Cortico-basal degeneration	+											Hansson et al., 2017
Multiple system atrophy	+											Hansson et al., 2017
Progressive supranuclear palsy	+											Hansson et al., 2017
Spinocerebellar ataxia	+								yes	yes		Li et al., 2019; Coarelli et al., 2021
Friedreich ataxia	+								Yes			Clay et al., 2020
Epilepsy								+				Rejdak et al., 2012
Charcot-Marie-Tooth disease	+								yes			Sandelius et al., 2018; Millere et al., 2021
Hereditary transthyretin amyloidosis	+								yes	yes	yes	Kapoor et al., 2019; Ticau et al., 2019
Guillain-Barre syndrome	+			+								Kuhle et al., 2010; Mariotto et al., 2018
Chronic inflammatory demyelinating polyneuropathy	+								Yes			Hayashi et al., 2021
Neuromyelitis optica				+								Miyazawa et al., 2007; Liu et al., 2021
Creutzfeldt-Jacob disease (prion disease)	+								yes	yes	yes	Steinacker et al., 2016; Minikel et al., 2020; Thompson et al., 2021
Canine cognitive dysfunction syndrome	+								yes			Vikartovska et al., 2020

B, blood; C, CSF.

(Hansson et al., 2017; Novakova et al., 2017; Kuhle et al., 2019; Szilasiova et al., 2021). Increased levels of serum NfL are also associated with MS brain T2 lesion load (Disanto et al., 2017). A recent study showed that serum NfL levels were associated with T1, T2 and gadolinium-enhancing lesion volumes at baseline and higher serum levels of NfL at baseline were associated with greater atrophy of the whole brain, gray matter and deep gray matter nuclei in the long term (Jakimovski et al., 2019a). Serum NfL may also detect MS disease activity that escapes detection in routine MRI (Akgun et al., 2019). The levels of serum NfL increased 6 years before clinical MS onset, indicating MS may have a prodromal phase lasting several years and that neuronal damage occurs already during this phase (Bjornevik et al., 2019). After clinical onset, a 1-point Expanded Disability Status Scale (EDSS) increase corresponds to an serum NfL increase of about 14% (Disanto et al., 2017).

Serum NfL concentrations have been used to assess disease progression in MS. Clinically isolated syndrome (CIS) is one of the MS disease courses and refers to a first episode of neurological symptoms that last at least 24 h and is caused by inflammation or demyelination of in the CNS. Elevated NfL levels in both pediatric and adult patients with CIS have been reported to be associated with a shorter time to clinically definite MS diagnosis independent of other prognostic factors (Van Der Vuurst De Vries et al., 2019). In patients with confirmed relapsing or progressive MS, baseline serum NfL can predict short-term outcomes including clinical and cognitive performance (Disanto et al., 2017; Jakimovski et al., 2019b; Filippi et al., 2020). Serum NfL levels sampled within the first 5 years of MS symptom onset was shown to independently predict long-term worsening EDSS score and risk of developing progressive MS in patients followed longitudinally for 15–26 years (Thebault et al., 2020a). Notably, patients with serum NfL levels less than 7.62 pg/ml were 7.1 times less likely to develop progressive MS (Thebault et al., 2020a).

Serum/plasma or CSF NfPs have potential utility for assessing treatment efficacy in single patients and beginning at an earlier stage in the disease course. Treatment with any disease-modifying therapy in MS has been reported to be associated with significantly lower serum NfL levels compared to untreated individuals (Disanto et al., 2017; Harris et al., 2021), proving that CSF or serum/plasma NfL is a therapeutic response biomarker in MS that may be related to consequent prevention of ongoing neuronal damage.

Fingolimod significantly reduced plasma NfL levels after 6 months and until the end of the studies (24 months) (Kuhle et al., 2019). Similarly, CSF NfL and NfH^{SMI35} levels were significantly lowered after 12 months of natalizumab treatment. A 4 fold greater reduction of NfL than of NfH^{SMI35} suggests differential sensitivity to therapeutic changes using different subunits as the biomarker (Kuhle et al., 2013) although NfH^{SMI35} antibodies detect NfH phosphorylation rather than the protein itself and may reflect different aspects of a given disease. Caution should be taken when MS patient are at risk for other treatment-induced neurological complications that can cause serum NfL levels to rise, such as natalizumab-induced progressive multifocal leukoencephalopathy (Dalla Costa et al., 2019) and ablative hemopoietic stem cell transplantation (Thebault et al., 2020b).

Amyotrophic Lateral Sclerosis

Mutation carriers with ALS symptoms have higher NfPs than those without ALS symptoms (CSF NfL 37-fold, 7388 vs. 195.7 pg/ml) (Weydt et al., 2016), suggesting that elevated NfP levels are linked to disease progression and the symptomatic disease phase (Benatar et al., 2019; Gille et al., 2019). Moreover, elevated serum NfL levels were observed as far back as 1 to 3.5 years before symptom onset depending on different gene mutations (SOD1, 12 months; FUS, 2 years and C9orf72, 3.5 years) (Benatar et al., 2018, 2019). CSF NfL levels also correlate with the extent of upper motor neuron and lower motor neuron involvement in ALS (Poesen et al., 2017). The time to generalization in ALS is an early clinical parameter of disease progression and CSF NfL concentrations have been shown to predict the conversion from bulbar/spinal to generalized ALS (Tortelli et al., 2015). Levels of NfL and pNfH also correlate with survival length in ALS (Brettschneider et al., 2006; Zetterberg et al., 2007; Lu et al., 2015). Higher serum NfL at diagnosis is also one of several factors that predict time of death in ALS (Thouvenot et al., 2020). In a recent clinical trial, levels of pNfH and NfL in plasma and CSF were largely unchanged in placebo-treated patients due to superoxide dismutase 1 (SOD1) mutations and decreased in patients treated with tofersen administered intrathecally over a period of 12 weeks, an antisense oligonucleotide that mediates the degradation of SOD1 messenger RNA to reduce SOD1 protein synthesis (Miller et al., 2020). Moreover, CSF SOD1 concentration decreased in these tofersen-treated patients with evidence of a slowing in the disease in the total scores on the ALS functional rating scale and the handheld dynamometry megascore.

Alzheimer's Disease

Plasma NfL is significantly higher in patients with MCI (mild cognitive impairment) (42.8 pg/ml) and patients with AD (51.0 pg/ml) compared with healthy controls (34.7 pg/ml) (Mattsson et al., 2017). This finding was further confirmed by other studies (Zhou et al., 2017; Lewczuk et al., 2018). Moreover, higher NfL levels were associated with cognitive decline in non-dementia older adults (He et al., 2021). Interestingly, elevated plasma NfL is associated with the presence of amyloid-beta plaques in pre-symptomatic individuals whereas NfL levels is associated with the load of tau in symptomatic patients (Benedet et al., 2020). Plasma NfL is also associated with AD progression independent of amyloid-beta (Moscoso et al., 2021a). Plasma NfL levels also correlate with Braak staging and longitudinal increases in plasma NfL are observed in all Braak groupings (Ashton et al., 2019). In addition, normal plasma NfL level (20.24 pg/ml) is also linked with resistance to PS1 familial AD in apolipoprotein E3 (APOE3) Christchurch mutation (Arboleda-Velasquez et al., 2019). The role of NfL as a potential biomarker for AD has been extensively reviewed and recent meta-analysis regarding its association with AD can be found elsewhere (Olsson et al., 2016; Khalil et al., 2018; Bridel et al., 2019; Jin et al., 2019). Recent studies further demonstrated plasma NfL levels or together with cognitive testing as predictors of fast progression (Santangelo et al., 2021) and future declines in cognition and function in AD (Li et al., 2021).

TABLE 3 | NfPs and fragments as biomarkers in conditions affecting nervous system.

Conditions	Association of NF subunit level with						Association of NF subunit level with			References
	NfL	pNfH (smi35)			NfH (smi31 and others)					
	B	C	B	C	B	C	Disease activity	Prognosis	Treatment response	
Acute bacterial meningitis	+									Gronhoj et al., 2021
Anesthesia and surgery	+									Evered et al., 2018
Anorexia nervosa	+									Nilsson et al., 2019
Autoimmune encephalitis		+		+					yes	Kortvelyessy et al., 2018; Fominykh et al., 2019; Piepgras et al., 2021
Brain metastasis and glioma	+						yes	yes		Hepner et al., 2019
Cardiac arrest	+						yes	yes		Moseby-Knappe et al., 2019
Cerebral small vessel disease	+						yes	yes		Egle et al., 2021; Qu et al., 2021
Chemotherapy-induced cognitive impairment					+		yes			Natori et al., 2015
Chorea-acanthocytosis	+						yes			Peikert et al., 2020
Diabetic neuropathy					+					Qiao et al., 2015
Hypoxic-ischemic encephalopathy						+	yes			Douglas-Escobar et al., 2010
Idiopathic normal pressure hydrocephalus		+								Jeppsson et al., 2013
Intrapartum asphyxia	+						yes			Toorell et al., 2018
Mcleod syndrome	+									Peikert et al., 2020
Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes	+						yes			Zheng et al., 2021
Mitochondrial encephalopathy		+					yes	yes		Sofou et al., 2019
MOG-Abs-associated disorders				+						Sara et al., 2021
Neurosarcoidosis	+	+								Byg et al., 2021
Peri/intraventricular hemorrhage	+	+					yes	yes		Goeral et al., 2021
Postoperative delirium	+									Casey et al., 2019
Preeclampsia	+						yes	yes		Evers et al., 2018
Preterm infants	+									Depoorter et al., 2018
Sepsis-associated encephalopathy	+						yes	yes		Ehler et al., 2019
Severe COVID-19	+									Sutter et al., 2021
Thoracolumbar intervertebral disk herniation					+		yes	yes		Nishida et al., 2014
Wilson's disease	+						yes			Shribman et al., 2021
X-linked adrenoleukodystrophy	+						yes		yes	Weinhofer et al., 2021

B, blood; C, CSF.

Hyperphosphorylation of tau neurofibrillary tangles is one of the hallmarks in AD. CSF ptau181 levels were first found to be increased significantly in patients with AD compared to healthy controls over two decades ago (Vanmechelen et al., 2000). This finding was subsequently verified by others (Lewczuk et al., 2004; Fagan et al., 2011; Tang et al., 2014) and later also was confirmed with the measurement of serum ptau181 (Shekhar et al., 2016). Recent studies demonstrated that blood ptau181 can predict cortical brain atrophy (Llibre-Guerra et al., 2019; Tissot et al., 2021), tau and amyloid-beta pathology (Lantero Rodriguez et al., 2020; Clark et al., 2021; Moscoso et al., 2021b), differentiate AD from other neurodegenerative diseases (Mielke et al., 2018; Thijssen et al., 2020; Grothe et al., 2021) and identify AD across the clinical continuum (Janelidze et al., 2020a; Karikari et al., 2020b, 2021). In familial AD, plasma ptau181 levels may rise as early as 16 years before clinical onset (O'connor et al., 2020). In addition to ptau181, some studies demonstrated ptau217 (Janelidze et al., 2020b; Karikari et al., 2020a; Palmqvist et al., 2020) and ptau231 are also useful biomarkers for AD (Kohnken et al., 2000; Suarez-Calvet et al., 2020; Ashton et al., 2021b). The combined use of these AD-specific biomarkers ptau181, ptau217, ptau231 with NfL as a disease-non-specific biomarker of neuronal integrity could improve prediction and monitoring of disease progression in AD (Moscoso et al., 2021a).

Frontotemporal Dementia

Serum NfL levels in patients with FTD were about 4-fold higher than in healthy controls (77.9 vs. 19.6 pg/ml) and the elevations correlate with disease severity (Rohrer et al., 2016). Moreover, increased serum NfL levels were observed 1 to 2 years before the clinical onset of symptoms (Van Der Ende et al., 2019), indicating pathophysiology of the disease in the preclinical phase.

Dementia With Lewy Bodies

Plasma NfL levels in patients with DLB were about 2-fold higher than in healthy controls (55.3 vs. 25.7 pg/ml) and the elevations correlate with disease severity and plasma NfL is the best predictor of cognitive decline compared to age, sex and baseline severity variables over a follow-up of 2 years (Pilotto et al., 2021).

Peripheral Neuropathy

Neurofilament proteins are most abundant in peripheral large-caliber myelinated axons such as sciatic nerves (Hoffman et al., 1987). Plasma NfL levels were about 2-fold higher in patients with inherited peripheral neuropathy CMT than in healthy controls (13.2 vs. 5.2 pg/ml) and correlated with disease severity (Sandelius et al., 2018; Millere et al., 2021). Serum NfL was also significantly elevated in acquired peripheral neuropathy and their levels correlated not only with disease severity and outcome (Mariotto et al., 2018) but also declined with remission (Bischof et al., 2018). These studies suggest that NfL might be a promising biomarker for disease activity monitoring of peripheral neuropathy.

Parkinson's Disease

Plasma NfL levels were about 1.6-fold higher in patients with advanced Hoehn-Yahr stage and patients with PD dementia than in healthy controls (17.6 vs. 10.6 pg/ml) and correlated with disease severity (Lin et al., 2019). Higher baseline plasma levels of NfL were also associated with greater motor and cognitive decline after a follow-up period of 3 years in patients with PD, suggesting value of NfL as a predictive biomarker of disease severity and progression in this disease (Lin et al., 2019; Ma et al., 2021; Zhu et al., 2021). A recent study also suggests that higher serum NfL levels were also associated with dopamine transporter concentration (Ye et al., 2021).

Huntington Disease

Plasma NfL levels were about 3-fold higher in patients with HD than in healthy controls (3.63 vs. 2.68 log pg/ml) and also significantly higher in manifest HD than premanifest HD (Byrne et al., 2017, 2018). Increased CSF and plasma NfL appeared in young adult carriers of HD gene mutation approximately 24 years before the clinical onset of symptoms (Scahill et al., 2020). Each CAG (cytosine, adenine and guanine trinucleotide repeat) increase is associated with higher, more steeply rising NfL levels (Byrne et al., 2017).

Stroke

Cerebrospinal fluid NfL was first reported to correlate with outcome after aneurysmal subarachnoid hemorrhage (Nylen et al., 2006) followed by the observations of increased CSF pNfH levels in acute ischemic stroke (8-fold at week 3 after stoke, 2.96 vs. 0.35 ng/ml in controls) (Singh et al., 2011). The findings were replicated with the measurements of serum NfL levels that patients with recent subcortical infarcts had higher NfL baseline levels compared to healthy controls (Gattringer et al., 2017; Pujol-Calderon et al., 2019; Peters et al., 2020). Elevated plasma NfL was also associated with poor functional outcome and mortality rate after spontaneous subarachnoid hemorrhage (Hviid et al., 2020a). The elevated NfL levels continued at the 3-month follow-up and seemed to return to normal at 15-month after stroke, indicating that levels of NfL could be a tool for monitoring infarct extent (Tiedt et al., 2018), predicting cognitive function (Peng et al., 2021; Wang J.H. et al., 2021) and mortality in patients with stroke (Gendron et al., 2020). Serum NfL levels also correlate with disease severity, disease progression and 17-year survival in patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) caused by mutations in the NOTCH3 gene (Gravesteijn et al., 2019). In addition to NfL and pNfH, CSF and serum NfM levels were also elevated in patients with stroke (Martinez-Morillo et al., 2015).

Traumatic Brain Injury and Spinal Cord Injury

One month after neurosurgical trauma, there was a distinct peak in CSF (6-fold increase, 2460 vs. 409 ng/ml at baseline) and plasma NfL concentration, which peaked at 1-month post-surgery, returning to baseline after 6 to 9 months (Bergman et al., 2016). Boxers who received severe head impact (>15 hits to the head or experienced grogginess during or after bout) had elevated plasma NfL at 7–10 days after a bout compared to boxers who received mild head impact (<15 head hits) (Shahim et al., 2017). In TBI, both CSF and serum NfL levels were elevated over the first 1–2 weeks compared to healthy controls (Al Nimer et al., 2015; Shahim et al., 2017; Hossain et al., 2019),

decreased over 5 years and correlated with measures of functional outcome (Shahim et al., 2020). Similar to TBI, CSF and serum NfL concentrations are also increased in SCI patients compared to healthy controls (Guez et al., 2003), correlated with motor outcome 3–12 months after trauma and minocycline treatment showed decreased NfL levels in a subgroup of injured patients (Kuhle et al., 2015). Care must be taken when TBI patients are over 60 years old or having pre-existing neurological conditions (Iverson et al., 2019). In addition to NfL, serum pNfH was also increased in TBI (Shibahashi et al., 2016) and SCI patients (Hayakawa et al., 2012; Singh et al., 2017) and appears to be a predictive biomarker for outcome.

Spinal Muscular Atrophy

Spinal Muscular Atrophy (MSA) is a rare neuromuscular disorder due to a mutation of survival of motor neuron 1 gene that results in the loss of motor neurons and progressive muscle wasting. Baseline levels of CSF NfL (31-fold, 4598 vs. 148 pg/ml) and tau (2.3-fold, 939 vs. 404 pg/ml) were significantly higher in children with SMA than in controls (Olsson et al., 2019). Treatment with nusinersen, a drug that increases the level of SMN protein in the CNS normalized NfL and tau levels which correlated with degree of motor improvement in children with SMA (Olsson et al., 2019). Plasma pNFH levels were also observed to correlate with disease activity and treatment response in infants with MSA treated with nusinersen (Darras et al., 2019).

Spinocerebellar Ataxia Type 3

Spinocerebellar ataxia type 3 is a condition characterized by progressive problems with movement due to mutations in the ataxin 3 gene. Plasma NfL levels were about 4-fold higher in patients with SCA3 than in healthy controls (34.8 vs. 8.6 pg/ml) and correlate with disease severity, disease progression and CAG repeat length of ataxin 3 gene mutation (Li et al., 2019; Peng et al., 2020; Wilke et al., 2020). Increased serum NfL appeared in mutation carriers 7.5 years before the clinical onset of symptoms (Wilke et al., 2020).

Human Immunodeficiency Virus Infection

Human immunodeficiency virus (HIV) invades brain and leads to the CNS injury, most severely manifesting as HIV-associated dementia with high morbidity and mortality (Price and Brew, 1988). CSF and plasma NfL levels were elevated in HIV infection, especially in HIV-associated dementia (44-fold increase for CSF NfL, 16185 vs. 363 nmol/L in HIV-negative controls), and is markedly reduced after antiretroviral treatment-induced viral suppression (Abdulle et al., 2007; Jessen Krut et al., 2014; Gisslen et al., 2016). Plasma NfL is also negatively associated with neuropsychological performance in HIV-infected individuals and their levels decline with initiation of antiretroviral therapy (Anderson et al., 2018).

Prion Diseases

Prion diseases are a family of rare progressive neurodegenerative disorders that affect both humans and animals. CSF and blood NfL levels are significantly higher (about 4-fold increase) in both sporadic and genetic prion disease compared to healthy controls (Steinacker et al., 2016; Thompson et al., 2018; Kanata et al., 2019; Zerr et al., 2021). Increased plasma NfL appeared in adult carriers of prion gene mutation as early as 2 years before the clinical onset of symptoms (Thompson et al., 2021).

Hereditary Transthyretin-Mediated Amyloidosis

Hereditary transthyretin-mediated amyloidosis is a condition with adult onset caused by mutation of transthyretin and characterized by extracellular deposition of amyloid and destruction of the somatic and autonomic PNS. Plasma NfL levels in patients with hereditary transthyretin-mediated (hATTR) amyloidosis with polyneuropathy were 4-fold higher than in healthy controls (69.4 vs. 16.3 pg/ml) (Ticau et al., 2019). Levels of NfL at 18 months increased in placebo-treated patients (99.5 pg/ml) and decreased in patients treated with patisiran (48.8 pg/ml), a gene-silencing drug that interferes with the production of an abnormal form of transthyretin (Ticau et al., 2019). The levels of 66 proteins in blood were significant changed following patisiran treatment relative to placebo, with change in NfL being the most significant (Ticau et al., 2019). Moreover, at 18 months, improvement in mNIS + 7 (a robust and clinically meaningful measure of neuropathy progression) compared to baseline in patisiran-treated patients significantly correlated with a reduction of plasma NfL levels.

Late Infantile Neuronal Ceroid Lipofuscinosis Type 2

Ceroid lipofuscinosis type 2 (CLN2) disease is an inherited disorder that primarily affects the nervous system. Before treatment in CLN2 patients, plasma NfL levels were 48-fold higher than in healthy controls (153.2 vs. 3.21 pg/ml) and in CLN2 disease, subjects receiving replacement therapy with cerliponase alfa, plasma NfL levels decreased by 50% each year over 3 years of treatment (Ru et al., 2019). Cerliponase alfa-treated patients demonstrated fewer declines in motor and language function than that in historical controls (Schulz et al., 2018). The fold change of CSF NfL compared with healthy controls has been shown varied extensively between individual conditions, with the smallest effect sizes observed in subjective cognitive decline and PD, and the largest effect sizes observed in cardiac arrest, HIV-associated dementia, FTD/ALS, ALS and HD (Rosen et al., 2004; Bridel et al., 2019). The pre-treatment plasma NfL levels observed in CLN2 disease patients is at the high end of neurological disease levels – similar to that seen in ALS, FTD/ALS, HIV-associated dementia and higher than many other neurodegenerative diseases. Even within ALS group, the CSF NfL levels in patients with lower motor neuron signs (346 pg/ml) only had 2.6-fold increase compared with healthy controls (138 pg/ml) while 17.6-fold increase was observed in those with signs of upper motor neuron disease (2435 pg/ml) (Rosengren et al., 1996). Therefore, the fold change in CSF and serum NfL levels could be due to damage to different neuronal compartments in different nervous system regions.

Brain Cancer

Neurofilament light chain levels in serum are sensitive to any neuronal damage. As CNS tumors grow bigger and bigger, they could affect function and integrity of neighboring neurons and/or may cause increased intracranial pressure that compromises

neuronal function. Accordingly, levels of serum NfL in patients with CNS tumors with progressive disease were 33-fold higher than in healthy controls (239.3 vs. 7.2 pg/ml) and vary closely with tumor activity (Hepner et al., 2019). Similarly, neurons could be damaged by the infiltration of the brain metastasis in the brain parenchyma, brain compression caused by metastasis, vascular disturbance and toxic products diffusing from tumor cells (Zhang and Olsson, 1997). In fact, serum NfL levels in patients with metastatic solid tumors with known brain metastasis were 19-fold higher than in healthy controls (142.3 vs. 7.2 pg/ml) (Hepner et al., 2019). This finding was later confirmed and expanded that an increase in serum NfL could be detected 3 months before brain metastasis diagnosis and a high level of NfL at time of brain metastasis correlated with an inferior survival (Winther-Larsen et al., 2020; Lin et al., 2021). These studies imply serum NFL is a potential clinical biomarker for both CNS tumors and metastatic solid tumors with brain metastasis.

Cardiac Arrest

Neurons in the brain can be damaged due to prolonged oxygen and sugar deprivation within 3 min of the heart stopping. CSF NfL levels were first reported to be increased in adult patients with cardiac arrest (52-fold, 11,381 vs. 217 pg/ml in healthy controls) and highly predictive of poor outcome (Rosen et al., 2004). This finding was later confirmed (Rosen et al., 2014) and also with plasma (Wihersaari et al., 2021) or serum NfL levels (Rana et al., 2013; Disanto et al., 2019). Recently, similar findings were also reported in pediatric patients with cardiac arrest (Kirschen et al., 2020). Cardiac arrest over 3 min can lead to not only hypoxic-ischemic brain damage but also reperfusion injury, the restoration of blood flow after resuscitation placing oxidative stress on the brain as pooled toxins flood already-damaged tissues (Sekhon et al., 2017). Future studies in large dedicated cardiac arrest cohorts with serial longitudinal measurements of serum NfL and parallel analyses to assess changes caused by hypoxia, ischemia and reperfusion in brain are warranted.

Delirium

Serum NfL levels in delirium in hip fracture patients were 1.7-fold higher than in controls (94 vs. 54 pg/ml) (Halaas et al., 2018) and plasma NfL was associated with delirium severity (Fong et al., 2020) independent of changes in inflammation (Casey et al., 2019). In addition to elevated NfL, higher serum pNfH levels also correlated with more severe postoperative delirium (Inoue et al., 2017; Mietani et al., 2019). These results suggests NfPs can be sensitive markers of neuronal injury associated with delirium.

The Value of Neurofilament Proteins in Differential Diagnosis Is Limited

Although NfPs are not disease-specific, they may have limited utility in differential diagnosis in some cases. Some neurodegenerative diseases share part of their symptomatology and neuropathology, making it difficult to differentiate between them. The differentiation between multiple system atrophy (MSA) and PD is difficult, particularly in early disease stages. Increased CSF NfL may offer clinically relevant, high accuracy discrimination between MSA and PD (Herbert et al., 2015) and also between PD and other atypical parkinsonian disorders including progressive supranuclear palsy and corticobasal degeneration (Constantinescu et al., 2010; Ashton et al., 2021a). The overlap of FTD and ALS has been well documented in FTD patients with co-morbid motor neuron degeneration and in ALS patients with frontotemporal dysfunction (Lomen-Hoerth, 2011). CSF NfL levels are higher in ALS than in FTD (Skillback et al., 2017) and also significantly higher in patients with FTD-ALS than in patients with FTD without ALS (Pijnenburg et al., 2015). CSF pNfH has also been shown to be a better biomarker than CSF NfL in differentiating ALS from other diseases mimicking ALS symptomatology (Poesen et al., 2017). Early symptoms of patients with FTD typically do not include memory impairment but instead often manifest changes in their behavior, personality and social interaction, which are often confused with symptoms occurring in psychiatric disorders. About 50% behavioral variant FTD patients received a prior diagnosis of a psychiatric disorder in a large retrospective study (Woolley et al., 2011). Patients with FTD have significantly higher serum NfL levels than patients with psychiatric disorders (Al Shweiki et al., 2019; Katisko et al., 2020), suggesting NfL as a promising tool to help differentially diagnose FTD and psychiatric disorders.

CURRENT RESEARCH GAPS AND POTENTIAL DEVELOPMENT OF NEUROFILAMENTS AS BIOMARKERS

Blood-Brain and Blood-Cerebrospinal Fluid Barriers

The effects of blood-brain barrier (BBB) and blood-CSF barrier (BCB) on serum NfP levels are not fully understood. Aging and neurodegenerative disease can cause increased disruption of the BBB (Sweeney et al., 2018) which could contribute to the elevated levels of serum NfP signals observed in these conditions. Recent evidence suggests that serum NfL level does not correlate with opening of the blood brain barrier after cranial irradiation (Kalm et al., 2017). Consistent with this finding, higher CSF/serum-albumin ratios were observed in FTD-3 patients, but this did not affect the significant associations among serum NfL levels and pre-symptomatic, symptomatic CHMP2B (charged multivesicular body protein 2B) mutation carriers and healthy family controls (Toft et al., 2021).

The Exact Form of Extracellular Neurofilament Proteins and Degradation Fragments

Because full length NfL proteins have never been detected in CSF and blood, it seems likely that most or all of the NfL detected in the CSF or serum are peptides generated from partial degradation of NfL in neurons or after their release. The identity and form(s) of NfPs detected by the commonly used NfL antibodies is not fully clarified. Recent studies suggest that a 22 kDa degradation fragment could be the detected plasma signal of NfL since it is also increased in ALS patients (Lombardi et al., 2020). The peptide species of INA and PRPH in CSF and plasma are not known. If a

Nf subunit such as INA is fully and rapidly degraded into amino acids upon release from neuronal compartments into blood, then no signals of Simoa assay can be measured and no value of utility as a biomarker. Determination of the form of detected NfP immunosignals (full length or degradation fragments) in blood will not only impact their utility as blood biomarkers but also help to better understanding the pathophysiological process in a given neurological diseases.

The Relationship Among Different Neurofilament Subunits

Neurofilament proteins are not identical and each has a distinct structure and could potentially have differential diagnostic value as biomarkers. The relationships among NfPs are complex and interrelated. When NfL is absent in mice, NfH levels decline most, followed by the decreased levels of NfM and PRPH (Yuan et al., 2012) and the levels of INA is only marginally declined (Yuan et al., 2003). When NfM is absent in mice, NfL levels decline most, followed by the lowered levels of NfH and INA (Yuan et al., 2006). NfL and NfM are co-regulated in mammalian brain and only marginally affected by the deletion of NfH, INA or both (Yuan et al., 2006). The first ELISA for NfPs was developed (Rosengren et al., 1996) prior to the recognition of INA and PRPH as additional Nf subunits (Yuan et al., 2006, Yuan et al., 2012). NfL is the most intensively studied subunit as a biomarker followed by phosphorylated NfH, especially after introduction of a highly sensitive digital assay (Gisslen et al., 2016).

Despite less attention being paid to NfM, INA and PRPH as biomarkers in neurological diseases, their potential utility is considerable. In addition to the well-established increase of NfL during aging, a highly significant increase in the levels in CSF of both phosphorylated and non-phosphorylated NfM and NfH are also seen in aged individuals as compared with young controls (Hu et al., 2002). NfPs are an integral part of neurofibrillary tangles in AD brain (Rudrabhatla et al., 2011) and C-terminal phosphorylation sites of both NfM and NfH are 4- to 8-fold more abundant in AD compared with control brain (Rudrabhatla et al., 2010). Levels of specific phosphorylation sites on NfM and NfH in blood could potentially be used as a biomarker to discriminate AD from normal brain aging and other neurological conditions.

Alpha-internexin is enriched in CNS and its prominent aggregation in Nf inclusion disease (Cairns et al., 2004) could qualify INA as a CNS-selective biomarker. However, the intact form of INA is difficult to detect in laboratory practice due to its instability. A possible solution could be to test for blood levels of its more stable degradation products. INA was identified by proteomics as a novel biomarker in the CSF of patients with hemorrhagic stroke (Martinez-Morillo et al., 2014). In contrast to INA, PRPH is enriched in PNS (Yuan et al., 2012) and therefore could potentially be developed as a PNS-specific biomarker. Moreover, PRPH is also sensitive to diffuse axonal injury (Liang et al., 2019) and its aggregate-inducing isoform Per 28 is upregulated in ALS and is associated with disease pathology (Xiao et al., 2008). A recent report suggests high serum levels of PRPH might be a general biomarker of axon disorders of lower motor neurons (Sabbatini et al., 2021). Future studies should therefore aim to develop assays of appropriate specificity for each of the NfP subunits or degradation fragments to explore the complementary information they may contribute to NfP pathobiology and use as biomarkers.

Stable Isotope Labeling Kinetics Coupled With Mass Spectrometry

The levels of NfP and peptide in CSF and blood depend on the rates of synthesis of NfPs or mechanism and rates of NfP peptide release. A recently developed stable isotope labeling method coupled with mass spectrometry may be useful to define the kinetics of NfP turnover in healthy individuals, with aging and in patients with neurological conditions associated with elevated NfP signals in CSF and blood. Special attention should be paid to the extremely slow turnover of NfPs incorporated into the filamentous lattice in axons (Nixon and Logvinenko, 1986; Yuan et al., 2015a). This method uses hours-long infusions of ^{13}C and ^{15}N stable isotopes before measuring the labeled proteins in CSF, blood or brain tissue samples (Bateman et al., 2006; Paterson et al., 2019). The incorporation of newly synthesized labeled proteins gradually increases until a steady state is reached. Following stop of infusions, the proportion of the labeled amino acid in the target protein gradually declines as a result of protein clearance or degradation. Alterations in the isotopic enrichment of the target proteins allow the calculation of protein synthesis and clearance rates from the ratio of labeled to non-labeled protein. This method was used to measure the kinetics of tau isoforms and fragments in human CNS (Sato et al., 2018). The elevated CNS tau levels in AD patients was initially interpreted as resulting from passive release of this protein by degenerative neurons. However, results from stable isotope labeling kinetics (SILK) studies suggest that the bulk of tau in human CSF is released by an active process that is stimulated by neuronal exposure to aggregated amyloid-beta. On the one hand, the concentration of NfPs in CSF or serum measured at a given time represents a static biomarker whose equilibrium could be affected by various factors. On the other hand, NfP-SILK can provide dynamic measure of production and clearance of newly synthesized NfPs that might provide a more detailed understanding of the mechanisms underlying these alterations in NfP levels.

Confounding Factors

Since there are significant variations of measured blood NfL levels among different methods and labs, standardization of blood NfL measurement globally is needed. Care must be taken when interpreting results obtained in different studies. Community-based large populations of healthy individuals are required to generate normative data for reference intervals. As discussed earlier, there are numerous demographic, life style, and comorbidity factors that potentially influence NfP levels in biological samples. With the increasing use of blood assays, variables such as exercise (Joisten et al., 2021), blood volume, body mass index need to be considered (Manouchehrinia et al., 2020; Perino et al., 2021). Trace amounts of NfPs relative to those in neurons have been reported

in erythrocytes (Granger and Lazarides, 1983; Terasawa et al., 2006), T lymphocytes (Murphy et al., 1993), podocytes (Wang et al., 2018), and oocytes (Takahashi and Ishizuka, 2012), which could be confounds in certain disease conditions. Because blood NfL alteration is associated with aging, future studies are also needed to establish the age-adjusted normal values of serum NfL levels across all age groups. The recent establishment of reference intervals of serum NfL in 342 Scandinavian reference subjects from 18 to 87 years of age is a step in the right direction (Hviid et al., 2020b). Comparative studies of two or more neurological disorders will be valuable to clarify the relative magnitude of change and its disease significance using the same methodologies. Sporadic AD patients are often older individuals associated with higher prevalence of cardiovascular conditions that is also associated with CNS ischemic damage and subsequent release of NfPs into blood (Gattringer et al., 2017). Co-existing peripheral neuropathy with CNS diseases may also weaken the correlation between CSF and serum NfP signals. Longitudinal measurements should also be encouraged to minimize intra- and inter-individual variation due to transient confounding variables and emerging co-morbidities (Khalil et al., 2020; Liu S. et al., 2020).

CONCLUSION

The development of minimally invasive ultrasensitive assays of NfPs released from neurons into in blood has increased the potential use of NfPs as biomarkers especially for repeated measurements during longitudinal studies such as in MS. The degree of elevation of NfPs in serum could easily differentiate behavioral FTD from primary psychiatric disorders where significant clinical overlaps of these two conditions exist and the sensitivity and specificity of structural and functional imaging methods remain imperfect. Monitoring the kinetics of NfPs in blood can increase our ability to assess disease activity, neuronal injury, and neurodegeneration in real time and to measure treatment effectiveness. Much interest has been focused on the detection of blood NfPs by high-sensitivity assays as a surrogate marker of neuronal structural damage and degeneration. However, the majority of these reports are cross-sectional, more longitudinal data are required to better elucidate the place of NfPs in the clinical settings. Due to their lack of specificity for a given disease, NfPs will most likely be of limited value as a diagnostic tool except when levels drastically differ between two conditions with similar clinical presentations. No single test or value of NfPs can currently be used to rule in or exclude the diagnosis of a specific disease. Nevertheless, NfPs can potentially be used to monitor disease progression and the effects of therapeutic intervention in combination with clinical judgment in almost any neuronal injury and neurological diseases. Serum NfPs are relatively easily measured. Treatment-induced decrease in blood NfPs levels as a complement to the more lengthy process of measuring clinical outcomes may, in the future, be more important in the validation and regulatory approval of new drugs for neurological conditions.

AUTHOR CONTRIBUTIONS

AY and RN wrote the manuscript and have approved it for publication. Both authors contributed to the article and approved the submitted version.

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frontiers in Neuroscience

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Neurofilament Levels Are Reflecting the Loss of Presynaptic Dopamine Receptors in Movement Disorders

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Aims: Neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH) are biomarkers for neuroaxonal damage. We assessed whether NfL and other biomarker levels in the CSF are correlated to the loss of presynaptic dopamine transporters in neurons as detected with dopamine transporter SPECT (DaTscan).

Methods: We retrospectively identified 47 patients (17 Alzheimer's dementia, 10 idiopathic Parkinson's disease, 7 Lewy body dementia, 13 progressive supranuclear palsy or corticobasal degeneration) who received a DaTscan and a lumbar puncture. DaTscan imaging was performed according to current guidelines, and z-scores indicating the decrease in uptake were software based calculated for the nucleus caudatus and putamen. The CSF biomarkers progranulin, total-tau, alpha-synuclein, NfL, and pNfH were correlated with the z-scores.

Results: DaTscan results in AD patients did not correlate with any biomarker. Subsuming every movement disorder with nigrostriatal neurodegeneration resulted in a strong correlation between putamen/nucleus caudatus and NfL (nucleus caudatus right $p < 0.01$, putamen right $p < 0.05$, left $p < 0.05$) and between pNfH and putamen (right $p < 0.05$; left $p < 0.042$). Subdividing in disease cohorts did not reveal significant correlations. Progranulin, alpha-synuclein, and total-tau did not correlate with DaTscan results.

Conclusion: We show a strong correlation of NfL and pNfH with pathological changes in presynaptic dopamine transporter density in the putamen concomitant to nigrostriatal degeneration. This correlation might explain the reported correlation of impaired motor functions in PD and NfL as seen before, despite the pathological heterogeneity of these diseases.

Keywords: neurofilament light chain, movement disorders, DaTscan, Parkinson's Disease, CSF, alpha-synuclein, progranulin, neurofilament heavy chain

INTRODUCTION

Currently the diagnosis of a movement disorder (MD) is based on clinical symptoms (Balestrino and Schapira, 2020). It is supported by imaging of the presynaptic dopamine transporter density with semi-quantitative determination in single photon emission computed tomography (SPECT) (Bajaj et al., 2013). Movement disorders are histopathologically heterogeneous entities with the idiopathic Parkinson's disease (PD) being the most common form. It involves the degeneration of dopaminergic neurons in the pars compacta of the substantia nigra, which inhibits the motor-inhibiting part of the striatum resulting in a nigro-striatal degeneration (Balestrino and Schapira, 2020). Also, the putamen is affected early in the disease course (Kordower et al., 2013). There are a number of other parkinsonian-like neurodegenerative diseases associated with nigrostriatal degeneration called atypical parkinsonism or parkinsonian plus syndromes including diseases such as multiple system atrophy (MSA), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD). Dementia with Lewy bodies (LBD) is also associated with PD but, in contrast, starts with mnestic syndromes and fads into PD with the mnestic syndromes being predominant throughout the disease (Cummings et al., 2011). There are numerous biomarkers established mirroring general, neuronal, or neuroaxonal neurodegeneration, but so far, no biomarker is known that specifically detects dopaminergic neurodegeneration nor proteinopathies caused by aggregated alpha-synuclein protein (Mollenhauer and Trenkwalder, 2009; Fayyad et al., 2019).

Dopaminergic neurodegeneration in the striatum can be visualized by dopamine transporter imaging. Nigrostriatal degeneration, in general, is associated with a decreased striatal presynaptic dopamine transporter density, which can be detected with SPECT using (123I) Ioflupane [iodine-123-fluoropropyl (FP)-carbo-methoxy-3 β-(4-iodophenyltropane) (CIT) FP-CIT as contrast agent (DaTscan) (Bajaj et al., 2013)]. DaTscan is an established *in vivo* imaging to diagnose suspected or unclear parkinsonian syndromes (Bajaj et al., 2013). Reduced density of dopamine transporters in the striatum is a suitable marker for nigrostriatal degeneration in PD or DLB. The binding pattern of the radioactive substance 123I correlates with the loss of presynaptic dopamine transporter detecting the radioactivity with SPECT indirectly mirroring the dopaminergic neurodegeneration in these neurons (Cummings et al., 2011). The neuronal fiber disintegrity in the striatum is already present in early stages of the disease (Bernheimer et al., 1973; Niznik et al., 1991; Duncan et al., 2013; Fazio et al., 2018), and therefore, DaTscan should be an early marker of neurodegeneration in these patients.

Alpha-synuclein (α-Syn) plays a major role in the pathogenesis of synucleinopathies, which include PD, LBD, and MSA. The presence of α-Syn in an aggregated form is a pathological hallmark in these diseases and may be responsible for neurodegeneration (Waxman and Giasson, 2008). In recent years, numerous biomarkers have been studied to support the clinical diagnosis of PD, LBD, and other diseases in cerebrospinal fluid (CSF) and serum (Mollenhauer and Trenkwalder, 2009; Parnetti et al., 2013; Van Dijk et al., 2013a,b; Hall et al., 2016; Majbour et al., 2016; Farotti et al., 2017). For alpha-synucleinopathies with movement disorders, some biomarkers indicating neurodegeneration, in general, show a reduced or increased level in patients with neurodegenerative movement disorders but lack a high specificity (Mollenhauer and Trenkwalder, 2009), although it has been shown that the combination of several biomarkers in CSF and serum can increase sensitivity and specificity for alpha-synucleinopathies (Parnetti et al., 2014; Majbour et al., 2016; Oosterveld et al., 2020).

Neurofilament light chain (NfL) is one of the four subunits of neurofilament proteins (Petzold, 2005; Oosterveld et al., 2020). This cytoskeletal protein is exclusively expressed in neurons and located particularly abundant in axons (Bridel et al., 2019). Neurofilaments in body fluids such as CSF are considered to be markers of neuronal and axonal injury (Bacioglu et al., 2016). Significantly elevated concentrations of NfL in CSF have been described in some neurological conditions, but no association with direct dopaminergic neurodegeneration has been described so far. However, the magnitude of increase shows a high degree of variability in clinically similar conditions. Parkinson's disease must be differentiated from atypical parkinsonian syndromes as frontotemporal dementia from Alzheimer's disease (AD) (Bridel et al., 2019). Human post-mortem brain studies have shown that NfL may be involved in Lewy body formation (Chu et al., 2012; Kordower et al., 2013; Moors et al., 2018). Another subtype of the neurofilament proteins is neurofilament heavy chain that can be abnormally phosphorylated (pNfH) in neurological disease. As a marker for axonal damage, pNfH gained attention as a diagnostic marker for neurological diseases such as amyotrophic lateral sclerosis (Gaiottino et al., 2013).

Progranulin (PGRN) is a protein with numerous functions in the brain involving lysosomal and microglial pathways (Kao et al., 2017). The involvement of PGRN in the PD pathomechanisms has been discussed lately (Tayebi et al., 2020). To our knowledge, there are no studies about CSF PGRN levels in patients with movement disorders. We added this precursor protein for granulin as a possible biomarker for neurodegeneration to the CSF biomarkers. Furthermore, we included total-tau (T-tau), which is associated with AD, PSP, and CBD, as a neuronal biomarker (Wang et al., 2013; Goedert et al., 2017).

As mentioned above, recent studies demonstrated that the diagnostic value may increase by combining biomarkers reflecting different pathological mechanisms in PD, such as axonal degeneration and α-Syn aggregation. For instance, CSF and serum NfL levels in combination with CSF α-Syn species achieve a high discriminative potential (Oosterveld et al., 2020). We aimed to assess whether these five CSF biomarkers could be linked to nigrostriatal degeneration as seen in the DaTscan in order to increase the significance of biomarkers indicating neurodegeneration in the dopaminergic system.

TABLE 1 | Epidemiology data on patients.

Characteristic[a]	AD (n = 17/X = 9)[e]	MD (n = 30/X = 13)[e]	PD (n = 10/X = 5)[e]	LBD (n = 7/X = 2)[e]	PSP + CBD (n = 13/X = 6)[e]
Age (years)	70.2 ± 9.1	69.5 ± 6.9	71.6 ± 6.4	72.1 ± 7.6	66.5 ± 6.2
Gender					
No. (%) male	12 (70.6)	16 (53.3)	6 (60)	2 (28.6)	8 (61.5)
No. (%) female	5 (29.4)	14 (46.7)	4 (40)	5 (71.4)	5 (38.5)
NfL, pg/ml	1,859 ± 468.6[d]	2,041.77 ± 749.16[c]	1,301 ± 144.3[f]	2,107 ± 289.9[b]	2,637.3 ± 710[f,d]
pNfH, ng/ml	0.41 ± 0.18	0.50 ± 0.55	0.51 ± 0.39	0.45 ± 0.17	0.61 ± 0.76
PGRN, pg/ml	0.89 ± 0.18	0.87 ± 0.25	0.86 ± 0.22	0.81 ± 0.24	0.91 ± 0.29
T-tau, pg/ml	513.4 ± 240.8	301.6 ± 240.3[f]	247.8 ± 168.3[f]	319.00 ± 152.9[f]	333.6 ± 320.8
α-Syn, pg/ml	2012.9 ± 449.1	2,035.6 ± 1,043.9	1,885.2 ± 626.6	2,587.2 ± 1339.2	1,854.2 ± 1,100.3
Z Putamen right	1.79 ± 1.65	3.79 ± 1.45[h]	3.37 ± 1.56[f]	3.59 ± 1.12[f]	4.20 ± 1.59[h]
Z Nucleus caudatus right	2.04 ± 1.74	3.50 ± 1.38[g]	2.75 ± 1.43	3.51 ± 0.88[f]	4.01 ± 1.40[g]
Z Putamen left	1.75 ± 1.68	3.69 ± 1.78[h]	3.20 ± 1.86	3.02 ± 1.69	4.40 ± 1.65[h]
Z Nucleus caudatus left	1.99 ± 1.34	3.20 ± 1.64[f]	2.67 ± 1.60	2.93 ± 1.49	3.73 ± 1.71[g]

Legends AD, Alzheimer's disease; MD, movement disorders; PD, Parkinson's disease; LBD, Lewy body dementia; PSP + CBD, progressive supranuclear palsy + corticobasal degeneration; NfL, neurofilament light chain; pNfH, phosphorylated neurofilament heavy chain; PGRN, progranulin; T-tau, Total-tau; α-Syn, alpha-synuclein.

[a] *Data given as mean ± standard deviation.*

[b] *Compared with control group, $p < 0.05$.*

[c] *Compared with control group, $p < 0.01$.*

[d] *Compared with control group, $p < 0.001$.*

[e] *n, group sample size; X, number of NfL measurements.*

[f] *Compared with AD group, $p < 0.05$.*

[g] *Compared with AD group, $p < 0.01$.*

[h] *Compared with AD group, $p < 0.001$.*

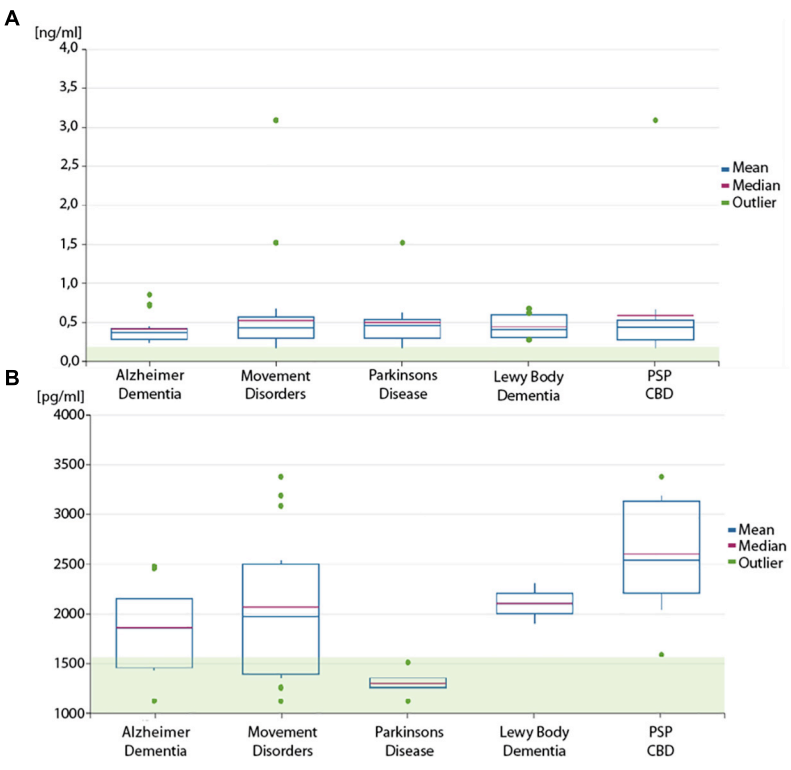

FIGURE 1 | Box plot of neurofilament levels. Boxplots of **(A)** phosphorylated neurofilament heavy chain (pNfH) levels and **(B)** neurofilament light chain (NfL) in the cerebrospinal fluid (CSF) of the disease cohorts and subgroups. The transparent green box is indicating the normal range (mean + 1 standard deviation).

MATERIALS AND METHODS

This retrospective study is part of the Magdeburg Dementia Cohort Study approved by the Ethical Committee at the University Hospital Magdeburg, Germany (Approval Number 22/19). The study population included a cohort of 47 patients who received a lumbar puncture and presynaptic dopamine transporter imaging using SPECT. Every patient was diagnosed at the Department of Neurology, University Hospital Magdeburg, Germany between May 2012 and August 2017 according to the German clinical consensus criteria as proposed by the German Neurological Society for PD. We included patients with a nigrostriatal neurodegeneration (n = 30) suffering from PD (n = 10), LBD (n = 7), PSP (n = 12), and CBD (n = 1). Lewy body dementia, PSP, and CBD were diagnosed according to the international criteria at that time (Litvan et al., 1996; McKeith et al., 2005; Gilman et al., 2008; Armstrong et al., 2013) because of the small number of patients with PSP and CBD and because of the similar tau-mediated pathogenesis, we summarized these two clinical entities into one subcohort. Furthermore, a disease control with AD patients (n = 17) was used as a reference. In these 17 AD patients, DaTscan were performed as part of the clinical diagnostics due to unclear movement disorders and/or adjacent mnestic symptoms to differentiate, e.g., AD from LBD. Diagnosis of AD was made due to clinical course, neuropsychological data, and CSF biomarkers showing at least a pathologically affected amyloid metabolism (Jack et al., 2018). We used an age-matched control group (n = 13, mean age 64.1 ± 9.68; 52.4% female, 47.6% male) from Magdeburg, Germany, to compare the NfL levels with patients suffering from other neurological diseases without deviations in CSF. We used controls with non-neurological patients from the CSF laboratory at the department of Neurology, Magdeburg for PGRN (mean age = 69.87 ±, mean level = 0.77 ± 0.13 ng/ml). The NfL and PGRN control cohorts are published elsewhere (Körtvélyessy et al., 2015; Körtvelyessy et al., 2018). We designed a new non-immunological and non-neurodegenerative control cohorts for alpha-synuclein (mean age = 60.29 ± 8.8 years, mean level = 2,218.31 ± 888.19 pg/ml). For total-tau and pNfH levels, cutoffs according to the recommendation of the manufacturers were used (De Schaepdryver et al., 2018).

The dopamine transporter imaging was performed at the Department of Nuclear Medicine of the University Hospital Magdeburg (Otto-von Guericke University, Magdeburg Germany). SPECT imaging was performed 3 h after intravenous injection of 123-I-FP-CIT [180 MBq (± 2 Mbq); GE Medical]. An E.CAM (Siemens) with a fan-beam collimator (128 × 128 matrix; SPECT; 60 steps; 40 s/steps) was used. The relative uptake in the striatum was semi-quantified by using the three-dimensional automated functional brain analysis software BRASS^{TM} (Hermes BRASS software, Hermes Medical Solutions, Sweden). These semi-quantitative results are expressed in z-scores, which indicate the decrease in uptake of Ioflupane compared with a normal collective. The z-scores were calculated separately for the nucleus caudatus and putamen of each side.

The lumbar puncture was performed at the Department of Neurology at the University Hospital Magdeburg, Germany. The biomarker NfL (measured with an ELISA from Umandiagnostics, Sweden), PGRN (measured with an ELISA from Mediagnost, Germany), and T-tau (measured with an ELISA from Fujirebio, Belgium) were all measured at the Department of Neurology, Magdeburg, Germany, with PGRN and T-tau measured prospectively and NfL retrospectively. Due to the retrospective design of this study, NfL levels could not be measured in every patient. Alpha-synuclein and pNfH were measured in a batch in the EUROIMMUN laboratory in Lübeck, Germany.

Biomarker levels with log10 transformations were used to fit them to standard distribution.

Every statistical analysis was performed using Jasp 0.14 (University of Amsterdam, 2020). We correlated the CSF biomarker levels of PGRN, T-tau, α-Syn, pNfH, and NfL with z-scores obtained from the DaTscan for each brain region and performed a Pearson's correlation once for all subgroups and once for combined groups. We used paired t-test to compare biomarker levels within cohorts and controls. Box plots were made with StatMacPlus V7.3.3.0 (AnalystSoft, United States).

TABLE 2 | t-test of cerebrospinal fluid (CSF) biomarkers compared with non-neurodegenerative controls.

α -Synuclein		*P*	Mean difference	Standard deviation
	AD	0.384	205.464	233.355
	MD	0.487	182.753	261.231
	PD	0.287	333.152	307.744
	LBD	0.389	−368.918	422.246
	PSP + CBD	0.272	364.116	326.813
Progranulin				
	AD	0.038	−0.123	0.057
	MD	0.086	−0.100	0.057
	PD	0.265	−0.089	0.076
	LBD	0.580	−0.044	0.079
	PSP + CBD	0.130	−0.138	0.086
NfL				
	AD	0.004	−644.692	197.929
	MD	0.004	−827.462	252.907
	PD	0.545	−86.692	140.042
	LBD	0.019	−892.692	332.683
	PSP + CBD	< 0.001	−1423.026	265.794

Significant results are marked in red.

RESULTS

Epidemiology

Forty-seven patients received a dopamine transporter imaging *via* DaTscan and a spinal tap fulfilling the inclusion criteria. The mean age of all included patients was 69.7 (± 7.7) years, and 19 (40.4%) patients were female, 28 (59.6%) were male. We divided the patients into two cohorts (AD and MD) and subcohorts according to clinical characteristics. The highest mean age was observed in the subcohort LBD, the lowest in PSP and CBD.

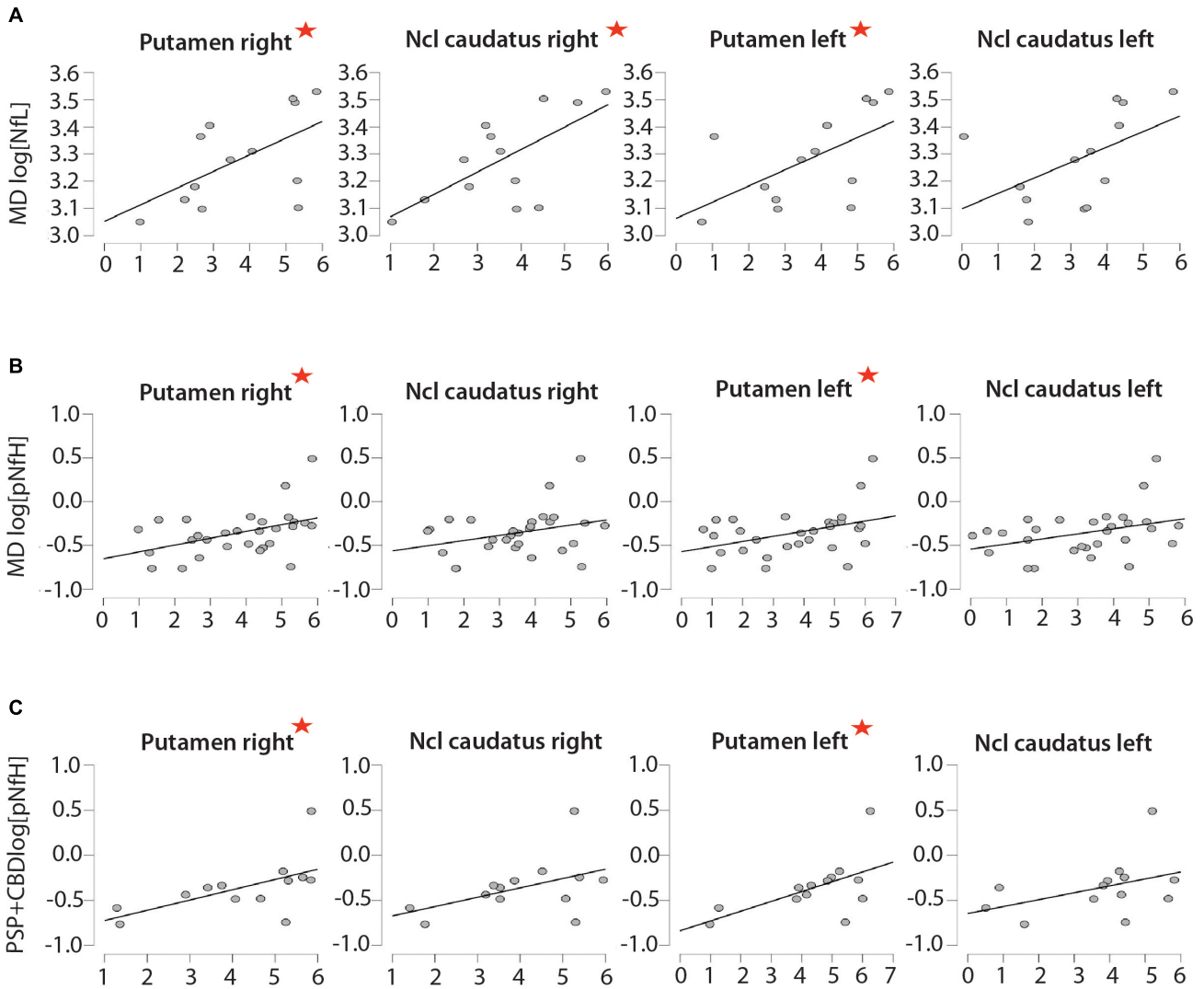

FIGURE 2 | Correlations between neurofilament proteins and dopamine transporter single photon emission computed tomography (DaTscan). Correlations between CSF levels of **(A)** NfL and **(B,C)** pNfH and the results of DaTscan (Z-scores indicating the decrease in uptake compared with a normal collective) in patients with **(A,B)** movement disorders and **(C)** progressive supranuclear palsy + corticobasal degeneration (PSP + CBD). The red asterisk is indicating the significant difference. The *x*-axis shows the Z-scores of the DaTScan and on the *y*-axis the log (biomarker level).

The mean age of all subcohorts did not differ significantly. Demographic characteristics for the cohorts and subgroups are presented in **Table 1**.

Dopamine Transporter Single Photon Emission Computed Tomography Are More Pathological in Movement Disorder Patients

Based on the software used and individual assessments at the division of nuclear medicine, 34 of 47 DaTscans were identified as pathological. In the AD cohort, 7 of 17 DaTscans revealed signs of pathological deviations, and in the MD cohort, 27 of 30 pathological results on the DaTscans have been seen. In patients with PD, 8 out of 10 scans were pathological, in patients with LBD, 7 out of 7 scans, and 11 out of 12 scans in patients with PSP and CBD. Only considering the quantitative results as z-scores, the lowest deviation from normal could be observed in patients with AD, the highest in patients with PSP and CBD (see **Table 1**).

Neurofilament Light Chain Correlates Positively With Single Photon Emission Computed Tomography Pathology

When comparing NfL levels from MD and AD patients to the control group (mean 1,214.31 ± 448.1 pg/ml), we could find a significant increase in patients with AD (mean 1,859 ± 468.6 pg/ml; $p < 0.001$), MD (mean 2,041.77 ± 749.16 pg/ml; $p < 0.01$), LBD (mean 2,107 ± 289.9 pg/ml, $p < 0.05$), and PSP and CBD

(mean 2,637.3 ± 710 pg/ml, $p < 0.001$) but not in patients with PD (mean 1,301 ± 144.3 pg/ml) (see **Tables 1, 2** and **Figure 1**). In a second step, we analyzed the correlations between NfL levels and DaTscan results. No correlation between z-scores and CSF NfL levels could be found in the Alzheimer's cohort (see **Table 3** and **Figure 2**). Subsuming all movement disorders with nigrostriatal degeneration, positive correlations between NfL levels and nearly every striatal regions were found except the left nucleus caudatus showing a trend ($r = 0.520$, $p = 0.069$), (putamen right, $r = 0.565$, $p < 0.05$; nucleus caudatus right, $r = 0.663$, $p < 0.05$, and putamen left, $r = 0.583$, $p < 0.05$). No significant correlations between z-scores and CSF biomarkers could be observed for each of the MD subgroups.

Phosphorylated Neurofilament Heavy Chain Also Correlates Positively With Dopamine Transporter Single Photon Emission Computed Tomography Pathology

Every patient in the AD and MD cohort had pathologically high pNfH levels according to the cutoff levels as provided by the manufacturer. The lowest levels of pNfH could be observed in patients with AD (0.41 ± 0.18 ng/ml), followed by patients with LBD (0.45 ± 0.17 ng/ml) and PD (0.51 ± 0.39 ng/ml) (see **Table 1** and **Figure 1**). The highest levels could be observed in patients with PSP and CBD (0.61 ± 0.76 ng/ml). The pNfH levels are moderately elevated in the MD cohort (0.50 ± 0.55 ng/ml).

There is no correlation between CSF pNfH levels and z-scores of each brain region in patients with AD (see **Table 4** and **Figure 2**). In the combined group of nigrostriatal movement disorders, positive correlations are seen in between pNfH levels in CSF and putamen right ($r = 0.383$, $p < 0.05$) and putamen left ($r = 0.384$, $p < 0.05$). Also, the CSF levels and DaTscan pathology correlated in the PSP and CBD cohort (putamen right $r = 0.575$, $p < 0.05$ and putamen left $r = 0.571$, $p < 0.05$).

Alpha-Synuclein, Progranulin, and Total-Tau Show no Significant Correlation in Dopamine Transporter Single Photon Emission Computed Tomography and Cerebrospinal Fluid Levels

Comparing α-Syn levels of every subcohort with normal controls (mean level = 2,218.31 ± 888.19 pg/ml) revealed no significant deviation at all (see **Table 4**). The lowest levels could be observed in patients with PSP + CBD (1,854.2 ± 1,100.27 pg/ml). AD patients (2,012.85 ± 449.11 pg/ml) as well as patients with alpha-synucleinopathies had no significant deterioration of α-Syn levels (PD 1,885.16 ± 626.59 pg/ml; LBD 2,587.23 ± 1,339.17 pg/ml) (see **Table 1**). None of the subcohorts revealed a correlation between DaTscan and α-Syn CSF levels.

TABLE 3 | Pearson's correlation neurofilament light chain (NfL).

Pearson's correlation	AD	MD	PD	LBD	PSP + CBD
Putamen right					
Pearson's r	0.058	0.565[a]	0.186	0.130	0.247
p-value	0.882	0.044	0.765	0.780	0.637
Nucleus caudatus right					
Pearson's r	0.037	0.663[a]	0.214	0.187	0.684
p-value	0.952	0.013	0.730	0.688	0.134
Putamen left					
Pearson's r	−0.183	0.583[a]	0.302	−0.163	0.620
p-value	0.637	0.037	0.621	0.727	0.189
Nucleus caudatus left					
Pearson's r	−0.041	0.520	−0.317	−0.524	0.688
p-value	0.917	0.069	0.603	0.228	0.131

AD, Alzheimer's disease; MD, movement disorders; PD, Parkinson's disease; LBD, Lewy body dementia; PSP + CBD, progressive supranuclear palsy + corticobasal degeneration. Significant results are written in red.
[a]$p < 0.05$.
[b]$p < 0.01$.
[c]$p < 0.001$.

TABLE 4 | Pearson's correlation phosphorylated neurofilament heavy chain (pNfH).

Pearson's correlation	AD	MD	PD	LBD	PSP + CBD
Putamen right					
Pearson's r	$6.473e^{-10}$	0.438[a]	0.520	−0.319	0.575[a]
p-value	0.998	0.017	0.151	0.485	0.040
Nucleus caudatus right					
Pearson's r	−0.140	0.308	0.299	−0.137	0.464
p-value	0.592	0.104	0.434	0.770	0.110
Putamen left					
Pearson's r	0.021	0.397[a]	0.438	−0.034	0.571[a]
p-value	0.973	0.033	0.238	0.943	0.042
Nucleus caudatus left					
Pearson's r	−0.085	0.362	0.403	0.251	0.417
p-value	0.746	0.054	0.282	0.587	0.156

AD, Alzheimer's disease; MD, movement disorders; PD, Parkinson's disease; LBD, Lewy body dementia; PSP + CBD, progressive supranuclear palsy + corticobasal degeneration. Significant results are written in red.
[a]$p < 0.05$.
[b]$p < 0.01$.
[c]$p < 0.001$.

Progranulin levels in CSF were similar throughout the cohorts (AD 0.89 ± 0.18 pg/ml; MD 0.87 ± 0.25 pg/ml), no significant differences could be observed throughout the subgroups. In addition, we could not identify any significant correlations between CSF levels and dopamine transporter imaging.

Total tau is pathologically elevated in patients with AD (513.35 ± 240.76 pg/ml) with a cutoff at 450 (pg/ml) as given by the manufacturer. The second highest level can be found in patients with PSP and CBD (333.616 ± 320.77 pg/ml). There are no significant differences in T-tau in between each cohort or subgroup (see **Table 2**). T-tau did also not correlate with DaTscan z-scores.

DISCUSSION

We observed a strong correlation of CSF neurofilament levels with a pathological functional integrity of presynaptic dopamine neurons in patients with nigrostriatal degeneration across the different pathomechanisms. We report significant correlations for NfL for most striatal regions on both hemispheres. Another positive correlation could be observed between CSF pNfH levels and the left and right putamen in movement disorders.

A positive correlation of early neuronal damage in the nucleus caudatus and higher NfL levels in CSF have already been described but not for the putamen (Bäckström et al., 2020). One major role of the putamen is supporting the execution of the intended motorical plans (Vicente et al., 2012). The putamen has been shown as one of the first important brain locations to be affected in MD patients by a decreased dopaminergic metabolism (Kordower et al., 2013). Our results showing the predominance of the correlation with z-scores of the putamen are well in line with the pathophysiology since all of our patients received their spinal tap and DaTscan at the beginning of the disease. Earlier studies in PD showed that denervation of dopaminergic neurons in the putamen precedes denervation in the nucleus caudatus (Brooks and Piccini, 2006; Jakobson et al., 2013). Also, NfL has been shown as an early predictor of motor impairment in PD (Bäckström et al., 2020). Taken together, these two factors may explain the significant correlation as found here, reflecting the assumed pathophysiology and clinical course of diseases within nigrostriatal neurodegeneration. Also, both biomarkers, DaTscan and neurofilaments, reflect the underlying pathogenetic neurodegeneration. In general, the lowest NfL levels could be found in patients with PD and AD, the highest levels could be observed in patients with PSP and CBD. Pathologically high CSF NfL levels were only found in the PSP and CBD group. These elevated levels are in agreement with other studies (Hall et al., 2012; Magdalinou et al., 2015; Olsson et al., 2019). CSF levels for pNfH are also elevated in all groups in a similar manner. Thus, pNfH has a similar correlation also indicating direct or indirect involvement of neurofilaments in the loss of the presynaptic dopamine transporters. On the contrary, NfL CSF levels are not elevated in PD in general (Bridel et al., 2019; Bäckström et al., 2020). Neurofilament light chain is a well-known marker for axonal–neuronal degeneration with, on the one hand, low specificity for the underlying pathomechanisms except for Tar-DNA-binding protein with 43 kDa induced neurodegeneration such as in ALS (Weydt et al., 2016; Goossens et al., 2018; Körtvelyessy et al., 2018; Bridel et al., 2019). On the other hand, NfL levels do correlate with the speed of neurodegeneration as seen in ALS (Steinacker et al., 2016; Feneberg et al., 2018) and, e.g., Creutzfeld–Jacob disease (Palermo et al., 2020) meaning that high NfL levels reflect a fast disease progression. It is intriguing to speculate whether NfL is more specific for a loss of trajectories in between the striatal regions than just a neuronal degeneration. More studies are recommended to further investigate this hypothesis.

Progranulin has been of some interest in PD and related disorders because of its role in lysosomalen degeneration and in microglial activity (Tayebi et al., 2020). Progranulin levels were similar across every cohort and subgroup and did not differ from an age-related control cohort. Thus, PGRN levels do reflect the PGRN metabolism as it has been shown for patients with frontotemporal dementia with and without *GRN* mutations (Körtvélyessy et al., 2015; Wilke et al., 2017; Goossens et al., 2018; Körtvelyessy et al., 2018). In analogy, we could not see any change in the PGRN metabolism as mirrored in the CSF PGRN concentration in the entire movement disorder cohort.

Again, alpha-synuclein in CSF measured with our ELISA has not proven its biomarker properties as it has been several times before and is reviewed elsewhere (Mollenhauer and Trenkwalder, 2009; Fayyad et al., 2019).

Total-tau is a well-known marker for general neurodegeneration announced as one of the key biomarkers in AD (Jack et al., 2018). Here, this biomarker did not mirror the nigrostriatal neurodegeneration going on in our MD patients. This probably emphasizes the putative involvement of neurofilament and not general neuronal degeneration in the loss of presynaptic dopaminergic neurodegeneration.

This analysis has a number of limitations that should be acknowledged. One limitation is the small sample size of the groups, which leads to a higher risk of false-positive statistical test results, which could not be cross-checked in an independent cohort. Due to the fact that this study was done at the Department of Neurology and not at a movement disorder outpatient clinic, cohort and subgroup distributions are different with PSP + CBS being as frequent as PD, which is not a normal PD/PSP + CBD ratio for a movement disorder outpatient clinic or any neurological outpatient clinic.

CONCLUSION

Neurofilament light chain and pNfH concentrations in the CSF are probably reflecting the specific loss of presynaptic dopamine transporter loss in the putamen only in patients with nigrostriatal neurodegeneration and concomitant movement disorders. We could also think of the DaTscan and NfL, pNfH levels reflecting two sides of the pathomechanisms.

We encourage further studies to correlate dopaminergic or amyloid imaging with fluid biomarkers such as YKL-40 (Baldacci et al., 2019) or neurogranin (Mazzucchi et al., 2020) to elucidate the systemic effect of neurodegeneration.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethikkommission der Universitätsklinik

Magdeburg. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

PK, EDi, PG, and EDü: study concept and design. PK and EDi: writing the first draft and statistics. PK, EDi, IG, and SV: clinical data acquisition. All authors: writing and revising. All authors contributed to the article and approved the submitted version.

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Serum Neurofilament Light Chain: A Marker of Nervous System Damage in Myopathies

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Purpose: Neurofilament light chain in serum (sNfL) has been suggested as a biomarker for the assessment of neuroaxonal damage. Since NfL are not expressed in muscle, elevated sNfL in patients with primary myopathies suggest additional nervous system involvement. To verify this hypothesis, we measured sNfL in a series of patients with myopathies.

Methods: sNfL were determined in 62 patients with molecular proven primary myopathies in whom some nervous system involvement may be predicted: myotonic dystrophy type I and II (DM I, II) and mitochondrial disease. In addition, sNfL were measured in 8 patients with facioscapulohumeral muscular dystrophy (FSHD) and in a disease control group caused by genetic defects exclusively expressed in muscle.

Results: sNfL values were significantly elevated in the DM I, the DM II and the mitochondrial group, with FSHD patients showing the lowest sNfL elevations. sNfL levels in the disease control group were not different from the healthy controls. A significant correlation between repeat length and sNfL levels was found in the DM I patients, but not in the DM II patients. Mitochondrial patients with encephalopathy showed significantly higher sNfL concentrations compared to patients with only muscular symptoms.

Conclusion: sNfL levels are elevated in myopathies with, based on the underlying molecular defect or clinical features, established nervous system involvement, i.e., myotonic dystrophies and mitochondrial disorders. sNfL were also raised in FSHD, where involvement of the nervous system is not usually clinically apparent. Thus, sNfL concentrations may serve as a biomarker for additional neuronal damage in primary myopathies.

Keywords: myopathy, serum neurofilament, nervous system involvement, mitochondriopathies, myotonic dystrophies (DM1 and DM2), facio scapulo humeral dystrophy

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INTRODUCTION

Primary myopathies are a diverse group of genetic and acquired conditions, which may present with a plethora of clinical symptoms, and it is becoming increasingly evident that many hereditary primary myopathies can be ascribed to underlying multisystem disorders. MRI studies have uncovered central nervous system (CNS) involvement, with evidence of cerebral atrophy, gray and

white matter lesions and ventricular enlargement in a number of muscle diseases (Angelini and Pinzan, 2019). These diseases include certain muscular dystrophies (e.g., Duchenne), the dystrophic myotonias and mitochondrial myopathies.

The myotonic disorders are classified into dystrophic (myotonic dystrophy Type I/DM1 and myotonic dystrophy Type II/DM 2/PROMM) and non-dystrophic myotonias. The underlying genetic defect in the myotonic dystrophies affects transcription and translation of multiple cellular genes, thus also causing extramuscular pathology, with cardiac, endocrinological and CNS-symptoms.

The mitochondrial diseases represent the archetype of multisystem disorders because of the ubiquitous occurrence of mitochondria, but they also display a high degree of heterogeneity due to the involvement of either the nuclear or mitochondrial genome and, in the case of mitochondrial DNA mutations, frequent disparity in the level of mutations between the different tissues. Not surprisingly, because of their high energy requirements, both the peripheral and the central nervous system are frequently involved in mitochondrial diseases. Our cohort included patients with proven defects of nuclear genes (*POLG*, encoding the mitochondrial polymerase gamma and *DNAJC30*, encoding DnaJ heat shock protein family (Hsp40) member C30) and of mtDNA encoded mitochondrial genes (*MT-ATP6*, encoding ATPase 6, *MT-TL1*, encoding tRNA Leucine 1, *MT-TK* encoding tRNA Lys, *MT-T1* encoding tRNA Ile) and patients with a single, large scale deletion in mtDNA.

Clinically evident CNS involvement is rare in facioscapulohumeral muscular dystrophy (FSHD), but detailed neurophysiological testing has shown subclinical involvement in a number of cases (Stübgen, 2007).

Until now, the assessment of CNS involvement in myopathies has mainly been based upon clinical parameters such as neuropsychological testing or cerebral imaging by MRI. Therefore, there is a need for reliable and easily accessible biomarkers for the detection of relevant nervous system damage, possibly even at a pre-symptomatic stage.

Neurofilaments constitute an important part of the neuronal cytoskeleton and are relevant for axonal growth and transport. Elevated levels of serum neurofilament light chain have been shown to specifically reflect neuroaxonal damage in a variety of neurodegenerative conditions such as amyotrophic lateral sclerosis (Steinacker et al., 2016), Alzheimer's dementia (Mattsson et al., 2017) and multiple sclerosis (Kuhle et al., 2017). Plasma or serum NfL have also been measured in peripheral nervous system disorders, including Charcot-Marie-Tooth disease (Sandelius et al., 2018), the acquired polyneuropathies (Mariotto et al., 2018; Van Lieverloo et al., 2019) and Guillain-Barré syndrome (Martín-Aguilar et al., 2021). In all cases, increased NfL levels were associated with greater disease activity and severity, thus monitoring of serum NfL levels may also be used to assess disease progression.

The aim of this study was to ascertain whether, using sNfL as a non-invasive, easily accessible biomarker, nervous system involvement can be demonstrated in patients with primary myopathies. We chose three multisystem disorders, DM1, DM2/PROMM, and mitochondrial disease, with frequent nervous system involvement and one myopathy without overt nervous system involvement (FSHD) to test this hypothesis. In addition, we measured sNfL concentrations in a group of disease controls, consisting of monogenic primary myopathies caused by pathogenic mutations in genes which are expressed in muscle tissue only. These comprise structural muscle proteins, channel proteins of the muscle membrane and proteins related to muscle energy metabolism.

METHODS

Clinical data and blood samples were prospectively collected in the Neuromuscular Outpatient Clinic of the University Hospital Dresden, Germany. Molecular genetic confirmation of the diagnosis was available for all patients. Patients with known acquired diseases or injuries of the CNS were excluded from the study. The NfL study was approved by the local Ethics committee (ID: EK394102018), conforms with World Medical Association Declaration of Helsinki and the patients or their authorized representatives consented to the use and storage of the biosamples and publication of their data.

All serum samples were frozen immediately and stored at −20°C. sNfL concentrations were determined using the single-molecule array (SIMOA) analysis as previously described (Akgün et al., 2019; Sutter et al., 2020). The mean intra-assay coefficient of variation of duplicates and the mean inter-assay coefficient of variation was < 10%. The control group comprised 485 samples from healthy volunteers, which were collected in the University Hospital Basel as previously described (Sutter et al., 2020).

To account for the known positive correlation of NfL with age, age-corrected sNfL percentile values have been derived based on healthy controls using a Generalized Additive Model for Location, Scale and Shape (GAMLSS) as described previously (Sutter et al., 2020). The percentile value was then calculated for each data point. Unadjusted group differences in sNfL levels were visualized using boxplots and were assessed using the Wilcoxon rank sum test. To additionally account for the minor age differences between groups, a multivariable linear regression model with log (sNfL) as the dependent variable was built with group and age as predictors. Estimates were back-transformed (exponentiated) and therefore represent multiplicative effects on the geometric mean of sNfL. Correlation of sNfL with age and repeat length within a patient group were assessed using Pearson correlation coefficients. Differences between different groups of mitochondrial patients were assessed using the unpaired *t*-test (GraphPad Prism). All remaining analyses were done with the statistical Software R (version 4.1.0). We used the STARD reporting guidelines (Bossuyt et al., 2015).

RESULTS

Between December 2019 and September 2021, 62 patients with either DM1, DM 2/PROMM, mitochondrial disease or FSHD and 13 patients for the disease control group were recruited for the study. Clinical characteristics and demographics of the patient cohorts are shown in **Table 1**.

TABLE 1 | Clinical characteristics and demographics of the patient cohorts.

A.

Patient group	sNfL [pg/ml]	Age/Gender	Co-morbidity/Neurological medication	Repeat expansion/Diagnosis (Mutation)	Tissue expression
DM I					
1	**6**	32, f	Lamotrigine (myotonia)	150	
2	**11**	37, f	—-	n.a.	
3	**24.1**	52, m	—-	500	
4	**8.5**	39, m	Dissociative seizures/lamotrigine (myotonia)	250	
5	**11.5**	57, f	—-	220	
6	**11.2**	29, f	—-	400	
7	**55.8**	67, f	—-	220	
8	**10.7**	31, m	Lamotrigine (myotonia)	300	
9	**9.06**	55, f	—–	200	
DM II					
1	**11.3**	53, f	—-	2,000	
2	**9.8**	49, f	—-	n.a.	
3	**15.5**	71, f	—-	5000	
4	**21.4**	70, f	—-	n.a.	
5	**7.5**	33, f	—-	n.a.	
6	**11.5**	58, f	—-	7,000	
7	**8.7**	51, f	Depression/citalopram	4,000	
8	**15.6**	60, f	—-	n.a.	
9	**12.6**	53, f	Fibromyalgia	n.a.	
10	**12.8**	55, f	—-	n.a.	
11	**9.8**	38, m	—-	7,000	
12	**26.4**	64, m	—-	7,000	
13	**8.8**	51, m	Chronic pain/duloxetine, mirtazapine, pregabalin	4,500	
14	**4.7**	25, f	—-	3,500	
15	**3.6**	19, f	Migraine/—	3,000	
16	**12.1**	41, f	—-	4,000	
17	**9.3**	50, m	—-	6,500	
18	**4.2**	33, m	—-	1,500	
19	**8.0**	44, m	Epilepsy/valproate	n.a.	
20	**6.1**	41, f	Restless legs syndrome	4,000	
21	**4.6**	33, m	Essential tremor/venlafaxine	n.a.	
22	**17.8**	57, f	Restless legs syndrome, depression/levodopa	4,000	
FSHD					
1	**10.9**	65, m	—-		
2	**13.7**	62, m	—-		
3	**11.3**	48, f	—-		
4	**4.8**	37, m	—-		
5	**9.0**	56, m	—-		
6	**10.3**	59, m	—-		
7	**16.7**	49, f	—-		
8	**6.9**	19, m	—-		
Disease controls					
1	**11.6**	41, f	Depression/duloxetin; lamotrigine (myotonia)	Myotonia congenita Thomsen	Muscle only
2	**5.9**	29, f	Migraine	McArdle's disease	Muscle only
3	**6.2**	17, f	Lamotrigine (myotonia)	Myotonia congenita Becker	Muscle only
4	**8.3**	62, m	Restless legs syndrome	RYR1: het. (p.V2280I)	Muscle only

(Continued)

TABLE 1 | (Continued)

Patient group	sNfL [pg/ml]	Age/Gender	Co-morbidity/Neurological medication	Repeat expansion/Diagnosis (Mutation)	Tissue expression
5	**5.9**	36, m	—	Bethlem myopathy/COL6A3: het. (p.Gly2068fs)	Muscle only
6	**12.4**	42, m	—-	Myotonia congenita Becker	Muscle only
7	**4.8**	32, f	—-	Ocular myositis	Muscle only
8	**8.2**	60, m	—-	LGMDR1/CAPN3: het. (p.Cys442Tyr, c.1746-20C > G)	Muscle only
9	**7.3**	53, f	Gabapentin, amitriptyline (myotonia)	Myotonia congenita Becker	Muscle only
10	**11.4**	64, f	—-	MYH7: het. (p.Met1429del)	Muscle only
11	**6.6**	43, m	—-	MYH7: het. (p.Met1429del)	Muscle only
12	**9.1**	42, m	Depression/mirtazapine	Pompe	Muscle only
13	**6.5**	21, m	—-	CPT2: (p.Arg231Trp; p.Leu178_Ile186delinsPhe)	Muscle only

B.

Mitochondrial myopathy	sNfL [pg/ml]	Age/Gender	Non-myopathic symptoms	Diagnosis/Mutation	Tissue expression/Heteroplasmy level
1	**10.7**	53, m	—-	CPEO/mtDNA—single deletion	Muscle
2	**11.3**	54, f	—-	CPEO/mtDNA- single deletion	Muscle, urine, (not blood)
3	**8.7**	61, f	—-	CPEO/mtDNA—single deletion	Muscle
4	**10.1**	47, f	—-	CPEO/mtDNA—single deletion (6.3 kB)	Muscle, urine, (not blood), (70% in muscle)
5	**14.5**	68, m	—-	CPEO/mtDNA—single deletion (4.5 kB)	Muscle, urine, not blood
6	**30.9**	62, m	Ataxia, cataract, visual impairment, SNHL	CPEO-plus/mtDNA—single deletion (4.5 kB)	Muscle, urine, (not blood)(60% in muscle)
7	**23.4**	20, f	Short stature	CPEO- plus/mtDNA—single deletion (7.8kB)	Muscle, urine, blood
8	**12.2**	42, f	Dysarthria	CPEO-plus/mtDNA—single deletion	Muscle, urine, (not blood)
9	**25.9**	43, m	SNHL, encephalomyopathy, short stature, cognitive impairment, ataxia	CPEO-plus/mtDNA—single deletion	Muscle, urine, blood
10	**17.4**	41, m	Optic atrophy (10 year-history)	LHON/DNAJC30: hom. (p.Y51C)	Nuclear encoded
11	**13.7**	59, m	Polyneuropathy	POLG: het. (p.R627Q; Q1236H in cis)	Nuclear encoded
12	**6.3**	34, f	—-(daughter of patient 11)	POLG: het. (p.R627Q; Q1236H in cis)	Nuclear encoded
13	**18.2**	63, f	SANDO, Parkinson, SNHL	CPEO-plus/mtDNA—multiple deletions; POLG: het., domin. (p.F961S)	Nuclear encoded
14	**12.7**	32, f	Short stature, polyneuropathy, dysarthria	MT-ATP6: m.9185T > C (p.L220P)	Blood (100%)
15	**33.8**	48, m	Ataxia, dysarthria, epilepsy, optic atrophy, cognitive impairment, polyneuropathy	MT-ATP6: m.9198delC (p.D224Efs*)	Urine, blood, fibroblasts (100%)
16	**30.8**	59, m	Myoclonus, epilepsy, ataxia, SNHL	MT-TK: m.8344A > G in tRNA-Lys	Blood (79%)
17	**11.9**	55, m	Ataxia, myoclonus, epilepsy, headache	MT-TK: m.8344A > G in tRNA-Lys	Urine (88%)
18	**1250**	48, f	Cerebral atrophy, stupor, stroke-like episodes, MRI: necrotizing encephalomyelopathy, onset age 16 years	Leigh/MT-T1: m.4290T > C in tRNA-Ile	Blood, muscle, fibroblasts (100%)

(Continued)

TABLE 1 | (Continued)

Mitochondrial myopathy	sNfL [pg/ml]	Age/Gender	Non-myopathic symptoms	Diagnosis/Mutation	Tissue expression/Heteroplasmy level
19	**66.3**	34, f	Dementia, neuropathy, ataxia, epilepsy, SNHL/anticonvulsants	MT-TL1: m.3243A > G in tRNA-Leu1	Urine (71%), blood (37%)
20	**70.4**	62, f	Stroke-like episode, only SNHL until age 62	MT-TL1: m.3243A > G in tRNA-Leu1	Urine (49%), blood (18%)
21	**14.5**	30, f	Mild SNHL, migraine	MT-TL1: m.3243A > G in tRNA-Leu1	Urine (49%), blood (25%)
22	**26.5**	57, f	SNHL, depression/citalopram. Mother of P21	MT-TL1: m.3243A > G in tRNA-Leu1	Urine (57%), blood (15%)
23	**33.2**	31, f	Migraine, rhabdomyolysis	MT-TL1: m.3243A > T in tRNA-Leu1	Urine, blood (low), muscle (80%)

DM I, myotonic dystrophy type 1; DM II, myotonic dystrophy type 2; FSHD, facioscapulohumeral muscular dystrophy; sNfL, serum neurofilament light chain; RYR1, Ryanodine receptor 1; LGMDR1, Limb-girdle muscular dystrophy, recessive Type 1; MYH7, Myosin heavy chain 7; CPT2, Carnitine palmitoyltransferase 2.
CPEO, chronic progressive ophthalmoplegia; mtDNA, mitochondrial DNA; SNHL, sensorineural hearing loss; LHON, Lebers hereditary optic neuropathy; POLG, Polymerase gamma; MELAS, Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes. SANDO, sensory ataxia, neuropathy, dysarthria and ophthalmoplegia.

Concomitant neurological diseases (depression, dissociative seizures, fibromyalgia syndrome, migraine, chronic/neuropathic pain, restless legs syndrome, essential tremor) in some patients with DM I/DM II and the medication taken by the patients are not, according to current knowledge, associated with structural neuronal damage and would therefore not affect sNfL levels. Several patients in the mitochondrial group showed, in addition to the myopathy, clinically apparent CNS-symptoms (**Table 1**). In these patients with mitochondrial myopathy, the CNS-symptoms are a feature of the primary mitochondrial disorder and therefore clinically confirm neuronal damage underlying the sNfL elevation.

All disease groups showed significantly higher median serum NfL levels than the healthy controls, whilst the smallest elevation of sNfL concentrations was found in the FSHD group (**Figure 1**). After adjustment for age, serum NfL was on average 2.1 times higher (CI: 1.6;2.7, p-value: < 0.0001) in DM I, compared to levels in healthy controls, 1.4 times (CI: 1.2;1.7, p-value: < 0.0001) in DM 2/PROMM, 1.4 times (CI: 1.1;1.9, p-value: 0.0227) in FSHD and 3.2 times (CI: 2.7;3.8, p-value: < 0.0001) in the Mito-patient cohort.

Compared to the controls, serum NfL levels were above the 90th percentile in 6/9 (66%) patients with DM I and in 9/22 (41%) patients with PROMM (**Figure 2**). In the FSHD group, only 3/8 (38%) patients were above the 90th percentile. The mitochondrial patients had the highest NfL levels of all groups, and 16/23 (70%) patients had values above the 90th percentile (**Figure 2**).

Within each group, only the DM I and DM 2/PROMM patients showed a significant correlation between age and sNfL levels ($r = 0.69$, $p = 0.04$ and $r = 0.83$, $p < 0.0001$, respectively) (**Supplementary Figures 1A,B**), whilst no significant correlation was seen within the other groups (**Supplementary Figures 1C–E**).

No significant correlation was found between sNFL levels and repeat length in the DM2/PROMM patients ($r = 0.5$; $p = 0.07$), but there was a significant correlation in the DM I patients ($r = 0.86$; $p = 0.014$). P7 was omitted from the regression analysis as an outlier value, because the clinical symptoms (gait ataxia out of proportion to the myopathy), noted at the last examination when the sample was obtained, suggested additional neuronal pathology.

The mitochondrial cohort is a more heterogeneous group than the other groups, in terms of both clinical symptoms and underlying genetic cause. Within the mitochondrial group, those patients with only clinically apparent muscle involvement had lower sNFL levels than patients with additional non-muscle symptoms. Thus, patients with a single deletion in mtDNA and manifesting CPEO only, had significantly lower sNFL levels than patients with CPEO + and a single or multiple deletions (due to a mutation in *POLG*) in mtDNA in muscle (**Table 1** and **Supplementary Figure 2**; 11.3 ± 2 vs. 24.6 ± 5.3; mean $\pm$ SD; $p < 0.05$). The greatest range in sNFL levels occurred in the group of patients with a mutation in mtDNA, where the levels ranged from 11.9 (patient 17 with a heteroplasmic m.8344A > G mutation in tRNA lysine) to 1,250 pg/ml (patient 18, with a homoplasmic m.4290T > C mutation in tRNA Ile) (**Table 1** and **Supplementary Figure 2**). Four patients from the mitochondrial group harbored a heteroplasmic m.3243A > G mutation in tRNA leucine 1 (the so-called MELAS mutation, **Table 1**). Although this group is small, the sNFL levels in these 4 patients showed no correlation with the mutation load in either blood or urine (Pearson correlation test, $p = 0.7$ and 0.6, respectively). Interestingly, the two patients with less severe neuronal involvement (P11 and P14 with polyneuropathy only) had similar sNFL levels 13.7 and 12.7 pg/ml), despite the difference in the underlying genetic cause. P11 harbors a heterozygous mutation in the nuclear gene *POLG*, whilst P14 harbors a m.9185T > C in the mitochondrial gene *MT-ATP6* which encodes a subunit of complex V.

DISCUSSION

This study demonstrates that sNfL levels can be used as a sensitive biomarker of ongoing neuronal damage in primary myopathies. As proof of concept, sNFL levels were significantly

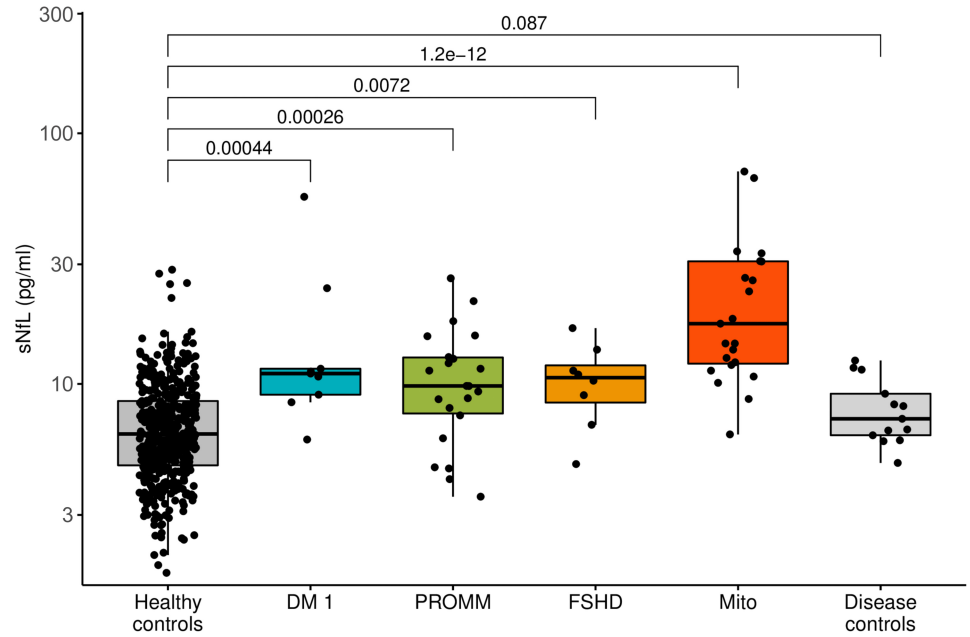

FIGURE 1 | Boxplot diagram of serum neurofilament light chain (sNfL) concentrations, comparing the disease groups against healthy control samples. Boxes represent median and interquartile range (IQR) and whiskers the extreme value within 1.5 × IQR above and below the median. The *p*-values listed were determined using a Wilcoxon rank-sum test.

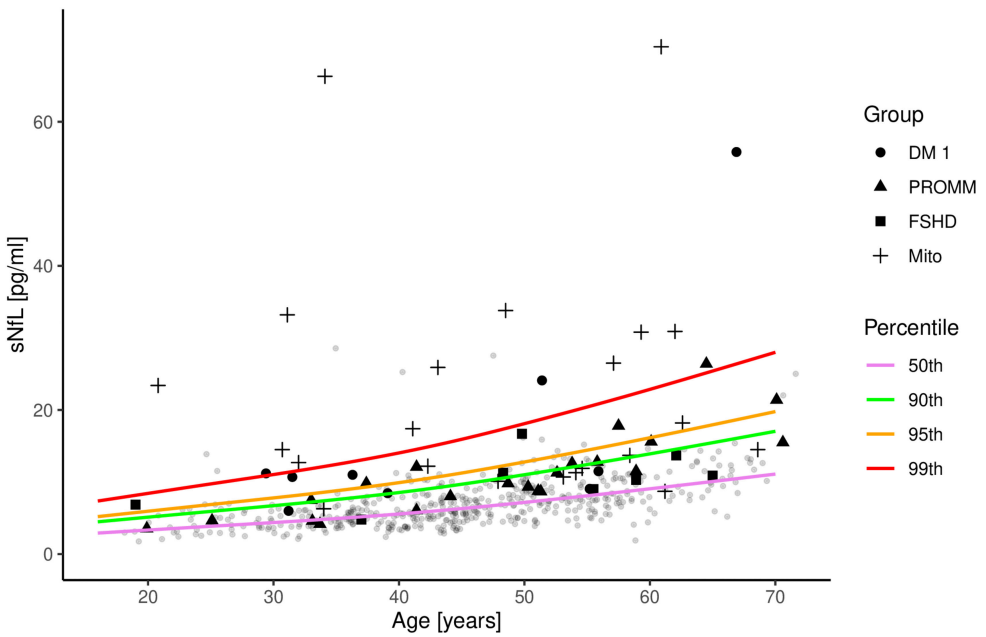

FIGURE 2 | Serum neurofilament light chain (sNfL) concentrations in the four disease groups are shown in comparison to 485 control samples. The distribution of sNfL is shown as a function of age and expressed as percentile curves (coloured lines), based on the control samples (gray circles). Patient 18 from the mitochondrial group is not included in this figure as the sNfL value is outside the range of the axes.

elevated in patients suffering from myopathies in which CNS-manifestations have been established, either as a clinically apparent symptom of the underlying genetic defect or by auxiliary clinical investigations (MRI, neurophysiology). Thus, we found sNfL to be high in patients with DM1, PROMM and mitochondrial disease, but also—even though to a lesser degree—in patients with FSHD. Our concept of sNfL as a biomarker for nervous system involvement in primary myopathies is further

validated by the lack of sNfL elevations in patients from the disease control group (myopathies due to mutations in genes exclusively expressed in muscle tissue).

The severity of neuronal damage appeared to be loosely correlated with the degree of sNfL elevation: disease groups with clinically common and more severe CNS-symptoms (DM1, mitochondrial myopathy) showed on average higher median sNfL levels than disorders in which signs of CNS-involvement are mild and less common (PROMM/FSHD).

Within the Mito-group, patients with a single deletion in mtDNA and only CPEO had on average, a significantly lower sNfL level than patients with CPEO plus (e.g., P6 with ataxia and P9 with sensorineural hearing loss and cognitive impairment). In addition, the highest sNfL levels were observed in patients with the most severe involvement, even when the underlying mutation resides in the same mtDNA gene. Thus, in the two patients with a homoplasmic mutation in the mtDNA gene *MT-ATP6* (P14 and P15), the highest sNFL levels were detected in P15, with ataxia, dysarthria, epilepsy, optic atrophy, cognitive impairment and polyneuropathy as compared to P14 with polyneuropathy. The highest sNfL levels were observed in patients who experienced symptoms such as epilepsy, ataxia or stroke-like episodes (**Table 1**: P15, P16, P18, P19, P20). Patient 18 (described in Limongelli et al., 2004) had the highest sNFL levels of all of the mitochondrial patients (1,250 pg/ml), and she has the most severe neurological involvement of the group with Leigh disease with onset at the age of 16 years and necrotizing encephalopathy of basal ganglia and brain stem.

One of the greatest problems associated with reaching a prognosis in individuals who harbor a mutation in mtDNA is that these mutations are often heteroplasmic, i.e., both mutant and wild type mtDNA are present in an affected individual, and the mutation load may vary from tissue to tissue. The mutation level in urinary epithelial cells can be a useful predictor of the mutation load in muscle (Whittaker et al., 2009), but the extent to which the level in this tissue mirrors that in nervous tissue is unknown. Three of our four patients with the m.3243A > G mutation (tRNA leucine 1) harbor similar mutation loads in urinary epithelial cells, but show different degrees of neuronal involvement which suggests that the sNFL levels may be a better indicator of neuronal involvement than the degree of heteroplasmy in urinary epithelial cells. Thus, regular measurement of sNFL levels may provide an important prognostic tool for treatment of these patients. Indeed, higher sNfL concentrations in patients with MELAS-syndrome during acute stroke-like episodes than in the attack-free interval suggest the applicability of sNfL for the assessment of neuronal damage in mitochondrial diseases (Zheng et al., 2021). Also in our cohort, P20 had only manifested sensorineural hearing loss until the age of 61, when she developed stroke-like episodes with a very high sNFL level of 70.4 pg/ml.

Interestingly, only patients with DM I and DM2/PROMM showed a significant correlation of sNfL with age (**Supplementary Figures 1A,B**) within their cohort. This suggests that additional neuronal damage, above that which occurs with normal aging, accumulates in the myotonic dystrophies. Thus, our observations could support the concept of myotonic dystrophies as progeroid disorders with premature aging, as suggested previously (Meinke et al., 2018).

Regarding repeat length, sNfL correlated well to repeat length for the DM I patients, which is supported by previous observations of a negative correlation between brain volume on neuroimaging and CTG repeat length (van der Plas et al., 2019). However, one patient (P7) presented sNfL greatly above the range of all other DM I patients; this patient, as mentioned above, also exhibited clinical symptoms which indicated additional neuronal involvement separate from DM I.

In contrast, no correlation could be demonstrated between sNfL and repeat length in the DM2/PROMM patients. This is not surprising, however, considering the lack of a correlation between repeat length and the severity of the myopathy in these patients (Schoser, 2006).

Interestingly, even patients with FSHD demonstrated elevated sNfL compared to age matched controls, although CNS-involvement is not generally regarded to be clinically relevant in FSHD. However, neurophysiological (Stübgen, 2007) and cognitive (Sistiaga et al., 2009) assessment revealed alterations of CNS function in up to 70% of patients with FSHD.

Owing to the rarity of the myopathies examined in this study, the size of the disease groups was small, but despite this drawback, the estimated differences in sNfL levels were very high. Other confounding variables affecting NfL concentrations, such as a subclinical age-dependent vascular encephalopathy (Thebault et al., 2020), were accounted for by comparison with NfL values in a large group of healthy controls of a similar age range. Thus, NfL elevations due to an age-dependent encephalopathy in the patient groups would be matched in the age-adjusted controls. Other concomitant CNS-disease which might influence NfL levels, was excluded on clinical grounds.

We propose from this pilot study that sNfL could be used as simple and non-invasive biomarker to detect and monitor neuronal damage in myopathies, even in those without clinically evident CNS-involvement. sNfL might also be helpful to assess treatment effects in view of upcoming new gene therapies for myopathies with nervous system involvement, such as the dystrophinopathies, the myotonic dystrophies or mitochondrial disorders. Larger longitudinal studies comparing sNfL levels with imaging or neuropsychological course parameters, are required to elucidate the value of sNfL as an outcome parameter in clinical practice.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Local Ethics committee of the Technische Universität Dresden (ID: EK394102018). The

patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS, JS, and SJ designed the concept of the study. AS, JS, JK, PB, SJ, EW, TZ, and KA contributed to data acquisition and analysis. KA and TZ developed ethics protocol. AS, JS, SJ, and PB drafted the manuscript and figures. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Exploring Neurofilament Light Chain and Exosomes in the Genetic Forms of Frontotemporal Dementia

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Differential diagnosis of neurological disorders and their subtype classification are challenging without specific biomarkers. Genetic forms of these disorders, typified by an autosomal dominant family history, could offer a window to identify potential biomarkers by exploring the presymptomatic stages of the disease. Frontotemporal dementia (FTD) is the second cause of dementia with an age of onset < 65, and its most common mutations are in *GRN*, *C9orf72*, and *MAPT* genes. Several studies have demonstrated that the main proteins involved in FTD pathogenesis can be secreted in exosomes, a specific subtype of extracellular vesicles able to transfer biomolecules between cells avoiding cell-to-cell contact. Neurofilament light chain (NfL) levels in central nervous system have been advocated as biomarkers of axonal injury. NfL concentrations have been found increased in FTD and have been related to disease severity and prognosis. Little information on the relationship between NfL and exosomes in FTD has been collected, deriving mainly from traumatic brain injury. Current review deals with this matter in the attempt to provide an updated discussion of the role of NfL and exosomes as biomarkers of genetic forms of FTD.

Keywords: neurofilament, NfL, exosomes, neurodegeneration, genetic frontotemporal dementia, presymptomatic carriers

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INTRODUCTION

Early diagnosis of dementia is challenging, that's why there is an impelling need for specific biomarkers. Frontotemporal dementia (FTD) encompasses a heterogenous group of neurodegenerative disorders with a wide range of clinical, genetic, and neuropathological features (Bang et al., 2015). About one-third of FTD patients have an autosomal dominant family history (Rohrer et al., 2009), typified by mutations in three genes: granulin (*GRN*; Baker et al., 2006; Cruts et al., 2006), chromosome 9 open reading frame 72 (*C9orf72*) (DeJesus-Hernandez et al., 2011; Renton et al., 2011) and microtubule-associated protein tau (*MAPT*; Hutton et al., 1998). It has been demonstrated that several proteins involved in FTD pathogenesis can be secreted by cells in association with exosomes (Ghidoni et al., 2011; Benussi et al., 2016). Furthermore, mutations in *GRN* strongly reduce the number of released exosomes also altering their composition

(Benussi et al., 2016). Exosomes are a specific subtype of extracellular vesicles (EVs) of 30–150 nm, originating in the endosomal/multivesicular body system and widely distributed in body fluids, including blood. Exosomes can carry a wide variety of DNA, RNA, proteins and lipids, allowing communication between cells avoiding cell-to-cell contact (Raposo and Stoorvogel, 2013; Rajendran et al., 2014). They have been reported as "Trojan horses" of toxic proteins (Ghidoni et al., 2008a), that's why they may serve as novel biomarkers in neurodegenerative diseases (Rajendran et al., 2014; Longobardi et al., 2021). Exosomes have a lipid bilayer membrane and can cross the blood brain barrier bidirectionally, thus reflecting and tracking neuropathological changes (Chen et al., 2013; Lai et al., 2014). In this context, a number of studies have shown the potential of peripheral blood EVs enriched for neuronal origin (nEVs) to identify biomarkers in several neurological disorders (Mustapic et al., 2017). Moreover, encouraging studies have been published that illustrate how certain biomarkers of AD carried within circulating nEVs, can identify individuals with age-related cognitive decline at an early pre-clinical stage, when symptoms are milder than mild cognitive impairment (Eren et al., 2020). In the same line, significantly lower levels than controls of several excitatory synaptic proteins have been found in plasma nEVs in AD patients (Goetzl et al., 2018). In several neurological diseases, levels of neurofilament light chain (NfL) released from the Central Nervous System (CNS) have been demonstrated to be altered, mainly in the cerebrospinal fluid (CSF; Bridel et al., 2019) but also in serum (Mariotto et al., 2020). Concentrations of NfL, biomarker of axonal damage, are increased in serum of FTD patients and might be related to disease severity and prognosis (Meeter et al., 2016; Rohrer et al., 2016; van der Ende et al., 2019; Benussi et al., 2020). In the present review, the potential role of NfL and exosomes as promising biomarkers for FTD diagnosis are briefly explained in the context of the FTD forms typified by autosomal dominant mutations that allow investigations in the early or even in the presymptomatic stages of the disease.

GENETIC BASES OF FRONTOTEMPORAL DEMENTIA

Frontotemporal dementia is an early-onset form of dementia, with a mean age of symptoms presentation before the age of 65 (Ratnavalli et al., 2002; Knopman et al., 2004). This early dementia is highly hereditary: 30–40% of FTD patients have a positive family history, (Rohrer et al., 2009; Wood et al., 2013; Fostinelli et al., 2018). In FTD families, null mutations in *GRN* leads to the production of a non-functional or no progranulin protein at all (Baker et al., 2006; Cruts et al., 2006; Ghidoni et al., 2008b; Finch et al., 2009; Sleegers et al., 2009). Mutations in *MAPT*, encoding for tau protein, typify FTD patients with tau-positive brain inclusions (Hutton et al., 1998; Poorkaj et al., 1998). Furthermore, an intronic expansion of a hexanucleotide repeat in *C9orf72* has been found in some families with an autosomal dominant inheritance form of FTD (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Most forms of FTD, encompassing both genetic and sporadic FTD, are characterized by cell inclusion bodies composed of tau or transactive response DNA-binding protein of 43 kDa (TDP-43) (Greaves and Rohrer, 2019). TDP-43 cytoplasmatic inclusion can be found in the CNS of patient with FTD and/or amyotrophic lateral sclerosis (ALS) and could explain neuropathological overlap between these neurodegenerative diseases (Elman et al., 2008).

THE ROLE OF NEUROFILAMENT LIGHT CHAIN IN THE GENETIC FORMS OF FRONTOTEMPORAL DEMENTIA

Neurofilaments are a family of neuronal cytoplasmic proteins divided into three subunits: heavy (NfH), medium (NfM) and light (NfL) chain. They are expressed primarily in neuronal axons where they provide structural support and stabilization of myelinated axons and interact with many proteins and organelles, including mitochondria (Petzold, 2005). NfL is the most abundant and soluble Nf subunit and can be released into blood and CSF in diverse neurological diseases reflecting neuroaxonal injury (Petzold, 2005; Lu et al., 2015; Mattsson et al., 2017; Khalil et al., 2018; Steinacker et al., 2018; Verde et al., 2019). NF gene mutations can cause multiple familial neurodegenerative disorders typified by NF aggregation and transport failure leading to further NF accumulations, including 14 NF-L gene mutations known to cause type 2E and 1F forms of Charcot–Marie–Tooth disease (Yuan et al., 2017). Recently NfL alterations have been also associated with FTD. NfL detection can provide some utility as a biomarker to differentiate specific FTD subtypes and, to support differential diagnosis of FTD from psychiatric disorders. To this regard, it has been recently reported an increase of serum NfL (sNfL) levels in behavioral-FTD but not in psychiatric disorders (Al Shweiki et al., 2019). Furthermore, low CSF level of NfL (cNfL) have been found in presymptomatic carriers of genetic FTD in contrast to high concentration in the symptomatic ones (Scherling et al., 2014; Meeter et al., 2016): in FTD *GRN*, *MAPT* or *C9orf72* mutation carriers, cNfL levels have reached a 8-fold higher increase in the affected patients than in presymptomatic carriers. On this basis, a role as a biomarker of disease severity and for prediction of the conversion to full dementia has been proposed for cNfL (Scherling et al., 2014; Meeter et al., 2016). Furthermore, it has been shown that sNfL levels were strongly associated with cNfL concentrations in the affected patients (Scherling et al., 2014; Meeter et al., 2016), and that NfL levels associated with disease severity, brain atrophy and patient survival (Meeter et al., 2016). In line with this evidence, van der Ende et al., 2019 showed normal sNfL levels in presymptomatic FTD carriers of *GRN*, *MAPT* or *C9orf72* mutations and a significant increase of sNfL concentrations after conversion to full dementia. The authors also described higher concentrations of sNfL in presymptomatic converters few years before the disease onset, pointing out to a potential role of sNfL as a prognostic biomarker of genetic FTD (van der Ende et al., 2019).

TABLE 1 | Literature evidences of NfL alterations in genetic forms of FTD.

Mutated gene	Source	Presymptomatic mutation carriers	Symptomatic mutation carriers	References
GRN, MAPT, C9orf72	CSF	Levels of CSF NfL similar to CTRL	↑ CSF NfL vs. CTRL	Scherling et al., 2014
GRN, MAPT, C9orf72	CSF	Levels of CSF NfL similar to CTRL	↑ CSF NfL vs. CTRL and presymptomatic carriers (↑ CSF NfL in *GRN* patients compared to *MAPT* and *C9orf72* ones)	Meeter et al., 2016
CHMP2B	CSF	↑ CSF NfL vs. CTRL	↑ CSF NfL vs CTRL and presymptomatic carriers	Toft et al., 2020
GRN, MAPT, C9orf72	CSF	Levels of CSF NfL similar to CTRL	↑ CSF NfL vs CTRL and presymptomatic carriers	van der Ende et al., 2021
GRN, MAPT, C9orf72	SERUM	Levels of serum NfL similar to CTRL	↑ serum NfL vs CTRL and presymptomatic carriers (↑ serum NfL in *GRN* patients compared to *MAPT*)	Meeter et al., 2016
GRN, MAPT, C9orf72	SERUM	Levels of serum NfL similar to CTRL (↑ serum NfL in converters *vs* non-converter)	↑ serum NfL vs CTRL and presymptomatic carriers (↑ serum NfL in *GRN* patients compared to *MAPT* and *C9orf72*)	van der Ende et al., 2019
CHMP2B	SERUM	↑ serum NfL vs CTRL	↑ serum NfL vs CTRL and presymptomatic carriers	Toft et al., 2020
GRN, MAPT, C9orf72	SERUM	Levels of serum NfL similar to CTRL (↑ serum NfL in converters *vs* non-converter)	↑ serum NfL vs CTRL and presymptomatic carriers	Wilke et al., 2021
GRN, MAPT, C9orf72	SERUM	Levels of serum NfL similar to CTRL	↑ serum NfL vs. CTRL and presymptomatic carriers	van der Ende et al., 2021

THE ROLE OF EXOSOMES IN THE GENETIC FORM OF FRONTOTEMPORAL DEMENTIA

Exosomes are biologically active entities, facilitating the intercellular communication and the transfer of biomolecules from one cell to another without direct cell-to-cell contact (Raposo and Stoorvogel, 2013; Rajendran et al., 2014). Alteration in intercellular communication in FTD patients with *GRN* mutation have been previously reported (Benussi et al., 2016). The study (Benussi et al., 2016) showed not only that progranulin was secreted in association with exosomes but also that levels of exosomal progranulin released by fibroblasts as well as the whole release of exosomes were reduced in mutations carrier patients. In brain, Wren et al., 2015, showed a significant alteration in intracellular vesicles trafficking with an accumulation of endosomes and exosomes and a reduction of lysosomes in FTD patients carrying N279K mutation in *MAPT*. These patients also showed an increase of exosomal proteins in frontal and temporal cortex (Wren et al., 2015). Moreover, it has been shown that both full length TDP-43 and TDP-43 C-terminal fragments were enriched in exosomes isolated from CSF in ALS-FTD patients. On this basis, approaches tackling the transmission of exosomes containing pathological TDP-43 could be a promising therapeutic strategy to halt or delay FTD-ALS progression (Ding et al., 2015). Based on these studies, exosomes and their cargo appear attractive biomarkers that could achieve a high diagnostic efficiency.

TABLE 2 | Literature evidences of exosomes alterations in genetic forms of FTD.

Mutated gene	Source	Mutation carrier patients	References
GRN	Human primary fibroblasts	↓ Exosomes *vs* CTRL	Benussi et al., 2016
GRN	Brain, Plasma	↑ Exosomes *vs* CTRL (only the symptomatic carriers)	Arrant et al., 2020
MAPT	iPSC-derived neural stem cells	↑ Exosomes vs. CTRL	Wren et al., 2015

EVIDENCE ON THE ATTRACTIVE ROLE OF NEUROFILAMENT LIGHT CHAIN IN EXOSOMES

The interaction of NfL and exosomes in FTD has been preliminary explored in subjects with traumatic brain injury (TBI). A recent study focused on veterans evidenced that repetitive events of TBI were associated with elevated exosomal and plasma NfL: the years from the first TBI were associated with both plasma and exosomal NfL levels. However, the years since the last TBI positively correlated only with exosomal NfL (Guedes et al., 2020). Similarly, in the study from Peltz et al., 2020 on TBI, NfL in CNS-enriched exosomes isolated from plasma were associated with cognitive impairment, suggesting the utility of exosomal NfL as biomarker of cognitive loss. Conversely, the analysis of plasmatic NfL didn't show any positive results (Peltz et al., 2020). Alongside a longitudinal study explored exosomal sNfL in patients with moderate-to-severe TBI in association with the free-circulating counterpart (Mondello et al., 2020). The authors found that sNfL levels were higher than their exosomal counterpart and that they positively correlated each other, likely part of a common disease process but pertaining to different pathways. Furthermore, exosomes enriched in sNfL were significantly higher in patients with diffuse TBI rather than in patients with focal lesions, supporting their potential utility in the prediction of neuronal damage (Mondello et al., 2020). In the same line, a study on HIV patients complaining neuropsychological impairment (Sun et al., 2017) showed that neuron-derived exosomes isolated from plasma had increased levels of NfL compared to exosomes from neuropsychologically

normal subjects highlighting their usefulness in tracking the worsening of cognitive impairment.

CONCLUSION

Even though not exhaustive, the present overview summarizes the most relevant evidence collected on the potential role of NfL and exosomes in the genetic form of FTD (**Tables 1**, **2**). The latter can provide information on the presymptomatic stage of the disease, offering a good chance to identify early or prognostic biomarkers and the opportunity to deliver preventive therapeutic strategies in this ideal time to obtain the greatest possibility of success.

Knowledge on the matter discussed is still at the beginning, and further investigation is needed to dissect the potential of this promising field of research and reveal whether the potential that emerged in the TBI study could also apply to genetic FTD. Exosomes represent an important subtype of EVs for the release and transfer of biomolecules among cells, without cells-to-cells contact. The study of the EV content, such as NfL in exosomes, from different tissues and fluids may provide information about the source of origin, reflecting the pathological changes. Moreover, it may predict the course of the disease and the prognosis for the patients, as well as establish a more reliable diagnosis. Since the first EVs description, ultracentrifugation has been the "gold standard" for EVs isolation. Nowadays, additional methodologies have been proposed for a more rapid and efficient EV isolation, such as several commercial kits, based on size exclusion.

Further prospective studies are greatly needed specifically to clarify the performance of exosomal biomarkers in genetic FTD diagnosis and prognosis.

AUTHOR CONTRIBUTIONS

RZ, CS, LB, RS, and RG gave their substantial contribution to conception and design of the manuscript and drafting the manuscript, revising it critically for important intellectual content. All authors have approved the manuscript in its present form for publication and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Plasma Neurofilament Light Chain Levels Are Elevated in Children and Young Adults With Wolfram Syndrome

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Wolfram syndrome is a rare disease caused by pathogenic variants in the *WFS1* gene with progressive neurodegeneration. As an easily accessible biomarker of progression of neurodegeneration has not yet been found, accurate tracking of the neurodegenerative process over time requires assessment by costly and time-consuming clinical measures and brain magnetic resonance imaging (MRI). A blood-based measure of neurodegeneration, neurofilament light chain (NfL), is relatively inexpensive and can be repeatedly measured at remote sites, standardized, and measured in individuals with MRI contraindications. To determine whether NfL levels may be of use in disease monitoring and reflect disease activity in Wolfram syndrome, plasma NfL levels were compared between children and young adults with Wolfram syndrome (n = 38) and controls composed of their siblings and parents (n = 35) and related to clinical severity and selected brain region volumes within the Wolfram group. NfL levels were higher in the Wolfram group [median (interquartile range) NfL = 11.3 (7.8–13.9) pg/mL] relative to controls [5.6 (4.5–7.4) pg/mL]. Within the Wolfram group, higher NfL levels related to worse visual acuity, color vision and smell identification, smaller brainstem and thalamic volumes, and faster annual rate of decrease in thalamic volume over time. Our findings suggest that plasma NfL levels can be a powerful tool to non-invasively assess underlying neurodegenerative processes in children, adolescents and young adults with Wolfram syndrome.

Keywords: Wolfram syndrome, neurofilament light chain, neurodegeneration, axonal injury, thalamus, *WFS1* gene

INTRODUCTION

Wolfram syndrome is an ultrarare genetic disorder, with features including childhood-onset insulin dependent diabetes mellitus, optic nerve atrophy, sensorineural hearing loss, and diabetes insipidus (Wolfram, 1938; Barrett et al., 1995; Minton et al., 2003). Affecting approximately 1/500,000 worldwide, Wolfram syndrome is a devastating disease, with reported shortened lifespan due to health complications (Barrett et al., 1995; Minton et al., 2003). Wolfram syndrome is caused by pathogenic variants in the *WFS1* gene, which encodes wolframin, a transmembrane ER glycoprotein involved in intracellular calcium homeostasis and regulation of unfolded protein response. In Wolfram syndrome, absent or reduced levels of wolframin disrupt normal ER functioning, leading to ER stress-induced apoptosis (Takeda et al., 2001; Ishihara et al., 2004; Fonseca et al., 2005; Riggs et al., 2005; Yamada et al., 2006; Akiyama et al., 2009; Fonseca et al., 2009; Fonseca et al., 2010).

Recent clinical and brain MRI data from our group's ongoing natural history study of Wolfram syndrome in children, adolescents and young adults describe an early neurophenotype of ophthalmologic deficits, impaired balance, smell identification, and hearing. Brain MRI analyses from our study and case studies of adults with Wolfram syndrome reveal reduced volumes in ventral pons, cerebellar white matter, thalamus, optic nerve, and total ICV (Rando et al., 1992; Saiz et al., 1995; Barrett et al., 1997; Hadidy et al., 2004; Pakdemirli et al., 2005; Yang et al., 2005; Ito et al., 2007; Mathis et al., 2007; Nickl-Jockschat et al., 2008; Waschbisch et al., 2011; Hershey et al., 2012; Pickett et al., 2012a,b; Karzon and Hullar, 2013; Marshall et al., 2013; Hoekel et al., 2014; Bischoff et al., 2015; Lugar et al., 2016; Zmyslowska et al., 2019; Samara et al., 2020), among other regions, even early in the disease progression (Hershey et al., 2012; Lugar et al., 2016, 2019). Over time and age in children, adolescents and young adults, white matter volume increases in controls but specific white matter volumes (brainstem and ventral pons) decrease in Wolfram syndrome (Lugar et al., 2019). In addition, gray matter subcortical thalamic and cerebellar cortex volumes remain stable in controls but decrease over time in Wolfram syndrome (Lugar et al., 2019). Microstructural integrity in major white matter tracts is lower in individuals with Wolfram syndrome relative to controls (Lugar et al., 2016) and declines over time in the brain's visual pathway accompanied by progressive deficits in visual acuity (Hoekel et al., 2018). These findings suggest that early neurodevelopmental deficits and neurodegenerative processes, accompanied by worsening clinical severity, occur in Wolfram syndrome.

Abbreviations: MRI, magnetic resonance imaging; NfL, neurofilament light chain; ER, endoplasmic reticulum; ICV, intracranial volume; CSF, cerebrospinal fluid; MS, multiple sclerosis; AD, Alzheimer's disease; WUSTL, Washington University in St. Louis; QC, quality control; WURS, Wolfram United Rating Scale; logMAR, Logarithm of the Minimum Angle of Resolution; UPSIT, University of Pennsylvania Smell Identification Test; MPRAGE, T1-weighted Magnetization-Prepared Rapid Gradient-Echo; eTIV, estimated total intracranial volume; ANCOVA, analysis of covariance; ANOVA, analysis of variance.

While MRI measures have proven instrumental in improving our understanding of the disease, they are costly, time-consuming, and require specialized on-site equipment and expertise. In contrast to MRI, a biofluid-based measure of neurodegeneration would be less invasive, easily standardized and repeatable, and able to be performed remotely and in individuals with contraindications for MRI. Thus, such a measure would be extremely useful for ongoing and future clinical trials designed to slow or halt neurologic progression in Wolfram syndrome.

One fluid biomarker protein, NfL, has shown excellent disease-monitoring potential in common neurodegenerative diseases. Neurofilaments are components of the microskeleton and are between microfilaments and microtubules in size (Gaetani et al., 2019). They maintain axonal caliber, facilitate the radial growth of axons, and ensure the structural integrity of neurons and their processes (Yuan et al., 2012, 2015, 2017; Yuan and Nixon, 2016; Gaetani et al., 2019). NfL is the most abundant component of axonal scaffolding and is released into CSF and blood during normal aging and following neuroaxonal injury in a range of neurological conditions, including inflammation, trauma, cerebrovascular disease and neurodegeneration (Petzold, 2005; Bacioglu et al., 2016; Zetterberg, 2016; Disanto et al., 2017; Bridel et al., 2019; Gaetani et al., 2019; Khalil et al., 2020). Although initially studied only in the CSF, recent technological improvements in sensitivity have made it possible to measure NfL in the blood. Serum and plasma NfL levels are highly correlated with CSF levels in disease states (Bacioglu et al., 2016; Disanto et al., 2017; Hansson et al., 2017; Piehl et al., 2018; Harp et al., 2019; Preische et al., 2019). Serum and plasma NfL, which are obtained through blood draws rather than more invasive and uncomfortable lumbar punctures necessary for CSF NfL, are measurable in healthy individuals, and appear to remain stable and at low levels from ∼6–18 years of age with a yearly estimated increase of 2.2% in adulthood (Disanto et al., 2017; Harp et al., 2019).

Neurofilament light chain presence in blood or CSF reflects neuroaxonal injury and relates to clinical severity and MRI measures in progressive neurological disease. NfL levels are not associated with a specific disease etiology but instead are sensitive to progressive neurodegeneration and may predict onset or progression across many diseases, such MS in adults and children, Alzheimer's disease (AD), Huntington disease, amyotrophic lateral sclerosis, and spinocerebellar ataxia (Disanto et al., 2017; Mattsson et al., 2017; Ashton et al., 2019; Bridel et al., 2019; Gaetani et al., 2019; Gordon, 2020; Reinert et al., 2020; Coarelli et al., 2021). In addition, elevated NfL predicts worse cognitive function and smaller brain volume in both AD and frontotemporal dementia (Rohrer et al., 2016; Mattsson et al., 2017), decreased cerebellar and pons volumes in spinocerebellar ataxia (Coarelli et al., 2021), decreased cerebellar gray matter volume in children with chronic kidney disease (van der Plas et al., 2021), reduced white matter integrity in dominantly inherited AD (Schultz et al., 2020), and decreased hippocampal volume, cortical thickness, white matter integrity, and worsening cognition in cognitively unimpaired older adults (Mielke et al., 2019). NfL levels are also useful as a biomarker for monitoring

therapeutic response. Decreasing NfL levels in pediatric and adult MS patients have been consistently shown following disease-modifying therapy (Disanto et al., 2017; Sejbaek et al., 2019; Hyun et al., 2020; Reinert et al., 2020). NfL has also demonstrated response to treatment in other disease including slowed rates of change in clinical trials of anti-amyloid therapies in dominantly inherited AD (Salloway et al., 2021).

Given this background, it is reasonable to hypothesize that NfL levels may be elevated in Wolfram syndrome, and that this measure could be useful for disease monitoring. The primary aim of this study was to compare plasma NfL levels between children, adolescents, and young adults with Wolfram syndrome and controls consisting of their parents and siblings. Second, in a subset of individuals with Wolfram syndrome, plasma NfL levels at ∼1.8 years after baseline were measured. We hypothesized that baseline and follow-up NfL levels would be elevated in individuals with Wolfram syndrome relative to controls independent of age and that higher NfL levels would relate to worse clinical severity and smaller regional brain volumes in individuals with Wolfram syndrome.

MATERIALS AND METHODS

Participants

Participants with genetically confirmed Wolfram syndrome diagnosis were recruited *via* self or physician referral to attend the annual Wolfram syndrome Research Clinic at Washington University in St. Louis (WUSTL), MO, United States. Participants with Wolfram syndrome and their unaffected parents or siblings attended the clinic between 2010 and 2017. The study protocol was approved by the Human Research and Protection Office at WUSTL and carried out in accordance with the Declaration of Helsinki. Participants <18 years of age gave informed assent, and their parents or legal guardians gave written informed consent. Participants ≥18 years gave written informed consent.

Plasma Sample Collection

Participants fasted overnight, and blood was collected into EDTA vacutainer tubes on ice and spun down at 1300 $g \times 10$ min. Plasma was aliquoted (100 μL) and frozen at −80°C. In total, 65 blood samples were obtained from the Wolfram group in 2014, 2016, and 2017 and 35 from the control group in 2014. Within Wolfram participants, 27 had plasma NfL measures for two consecutive time points, designated hereafter as time points 1 and 2.

Neurofilament Light Chain Measurements

Plasma NfL levels were assayed in duplicate per manufacturer instructions using the commercially available NfL immunoassay kit (Quanterix NfL Advantage Kit™, Quanterix Corp., United States) on the automated ultrasensitive Simoa® HD-X Analyzer (Quanterix Corp., United States) platform. Samples were diluted 1:2 prior to loading on to the HD-X to reduce the volume of plasma needed for the assay. QC parameters were described previously (Hendricks et al., 2019). The assay required four kits in total. Effects of year that the samples were collected and of separate kits on NfL levels were assessed. No individual sample had CV > 25% in duplicate assays.

Clinical Disease Severity Measures

Wolfram United Rating Scale

The WURS (Nguyen et al., 2012; Bischoff et al., 2015) was administered by a neurologist. The WURS instrument was developed to assess overall disease severity of Wolfram syndrome sequelae (e.g., vision, hearing, motor, urological, neurological, psychological, and mood problems) and validated in a subset of the participants currently described (Nguyen et al., 2012). The maximum score for the subscale used here to indicate clinical severity, the Physical Activity subscale, is 136, with higher scores indicating greater severity (Nguyen et al., 2012).

Visual Acuity

Using Snellen optotypes, best-corrected visual acuity was recorded and transformed into Logarithm of the Minimum Angle of Resolution scaled values, with higher values indicative of worse visual acuity, for each participant with Wolfram syndrome as described in Hoekel et al. (2014). Normal visual acuity is 20/20 (logMAR = 0, no loss of visual acuity). Color vision was assessed using Hardy-Rand-Rittler as described in Hoekel et al. (2014). The normal color vision score (number correct) is 51–52. In a study of a subset (n = 18) of the participants currently described, mean (range) vision acuity was 20/60 (20/2000–20/20) and color vision score was 13.2 (0–51), with 89 and 94% of individuals with Wolfram syndrome having subnormal visual acuity and deficits in color vision, respectively (Hoekel et al., 2014).

Smell Identification

Smell identification was assessed with the University of Pennsylvania Smell Identification Test (Doty et al., 1984) as described in Alfaro et al. (2020). Briefly, participants were asked to scratch and sniff stimuli with microencapsulated odorants and indicate which of four response alternatives best matched the perceived odor. Higher scores indicate more accurate smell identification. Relative to age-matched healthy controls and individuals with Type 1 diabetes, a sample (n = 40) including most of the individuals with Wolfram syndrome in the current study had less accurate smell identification (Alfaro et al., 2020).

Regional Brain Volumes

Regional brain volumes in each participant with Wolfram syndrome were obtained from MRI scans as described in Lugar et al. (2019). Briefly, individuals with Wolfram syndrome underwent MRI scans on a Siemens 3T Tim Trio at the Center for Clinical Imaging Research at Washington University. The analyses described here include data obtained from T1-weighted Magnetization-Prepared Rapid Gradient-Echo (MPRAGE) sequences. Regional brain volumes were determined using Freesurfer 5.3 (Fischl et al., 2002), averaged between left and right hemispheres and corrected for total ICV by dividing regional brain volume by estimated total intracranial volume (Buckner et al., 2004) and scaling the quotient by 1,400,000 mm^3,

an approximately average eTIV. *A priori* regions of interest were selected for analyses based on previous findings of decreased volume over time in a Wolfram patient study sample (n = 29), including most of the individuals with Wolfram syndrome described in the current study, compared to controls including ventral pons, brainstem, cerebellar cortex, and thalamus (Lugar et al., 2019).

Statistical Analyses

Raw plasma NfL levels were log10-transformed to normalize distributions, which is a standard way of analyzing NfL levels (Mattsson et al., 2017; Zeitlberger et al., 2018; Mielke et al., 2019; Preische et al., 2019; Reinert et al., 2020; Goeral et al., 2021). These and other relevant variables were compared between individuals with Wolfram syndrome and the control group with Student's between-subjects t-tests and between time points 1 and 2 in the Wolfram group with Student's within-subjects t-tests. Gender and ethnicity distributions were compared between control and Wolfram groups with Mann–Whitney U tests. A one-way ANCOVA was used to determine whether plasma NfL levels differed between control and Wolfram groups when age was controlled. Correlation of plasma NfL levels with age was evaluated within the Wolfram group and within controls using separate Pearson's r analyses. Effects of kit number and plasma sample collection year on plasma NfL levels were assessed with one-way ANOVA. For the group comparisons and correlations with age, alpha was set to $p < 0.05$, as these were primary *a priori* hypotheses. Additional analyses were considered exploratory in the interest of generating testable hypotheses in future studies and so were not corrected for multiple comparisons.

While plasma was collected annually for up to two consecutive time points (time points 1 and 2) in individuals with Wolfram syndrome, clinical and MRI measures were obtained annually for up to 7 years depending on when the participant started attending the clinic. In exploratory analyses within the Wolfram group, we performed Pearson's r or Spearman's ρ correlations between log10 plasma NfL levels and disease severity and MRI variables obtained during the corresponding clinic year. Annual percent change in volume was calculated for brain regions in which volume related to NfL levels at both time points (thalamus). Specifically, for each participant, average annual percent change in thalamic volume was calculated with the following formula:

$$\frac{\left(\frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2}\right)}{\bar{y}} \times 100$$

where slope is $\frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2}$, x is age at MRI visit, $\bar{x}$ is mean age across MRI visits, y is thalamic volume at MRI visit and $\bar{y}$ is mean thalamic volume across MRI visits. The mean thalamic volume across MRI visits was used to normalize slopes so that variability in thalamic volume over the study period, rather than just at baseline, could be removed from the slope calculation for each participant. Number of MRI visits varied from 2 to 7 in individuals with Wolfram syndrome depending on how many annual clinics were attended.

RESULTS

Participants

Descriptive statistics for the control and Wolfram groups are shown in **Table 1**. Within controls, all had a single time point at which NfL was measured; 28 were parents and 7 were siblings of individuals with Wolfram syndrome. Within the Wolfram group, 38 participants had plasma NfL data from time point 1 and 27 of these individuals also had plasma NfL data from time point 2. Scaled parent education levels were derived from the Barratt Simplified Measure of Social Status (Barratt, 2006) and were averaged when data from both parents were available. For parents, their own scaled education level was used. One individual with Wolfram syndrome had raw plasma NfL levels at both time points 1 (37.8 pg/mL) and 2 (34.2 pg/mL) >3 SD above the respective means, but were < 3SD above log10 means at time points 1 (log10 plasma NfL = 1.58) and 2 (1.53) within the Wolfram group and when controls were included in the calculation. Therefore, this participant's data points were included in data analyses. Due to many parents (n = 28, 80%) in the control group, the Wolfram sample was younger ($t_{71} = -8.5$, $p < 0.001$). Wolfram and control groups did not differ in gender or ethnicity distributions ($p \geq 0.63$) or in scaled parent education level ($p = 0.33$).

Clinical Disease Severity Variables

Individuals with Wolfram syndrome had worse visual acuity and smaller ventral pons, brainstem, cerebellar cortex and thalamic volumes at time point 2 relative to time point 1 (**Table 2**) as in previous publications that included a subset of these participants (Hoekel et al., 2014; Lugar et al., 2019). Mean (SD) annual decrease in thalamic volume over all MRI time points was −66.1 (80.9) mm^3 and thalamic-volume corrected mean (SD) annual decrease in thalamic volume was −1.0% (1.3).

Quality Control for Neurofilament Light Chain Measures

The average (SD) CV across 72 replicate samples on separate assay plates run by two separate technicians was 5.7% (4.8). For one individual with Wolfram syndrome, a replicate was not analyzed due to a processing error. Given the consistently low CV (i.e., high reproducibility), the single value for this participant was included in analyses. Within 73 time point 1 and 27 time point 2 samples, neither year of sample collection (1-way ANOVA, $p \geq 0.36$) nor kit number (1-way ANOVA, $p \geq 0.27$) affected NfL plasma concentrations.

Plasma Neurofilament Light Chain Level Comparisons Between Control and Wolfram Groups

Raw and log10 plasma NfL levels on average were higher in individuals with Wolfram syndrome at time point 1 [Raw: $t_{(1,71)} = 4.2$, $p < 0.001$, Cohen's $d = 1.0$; Log10: $t_{(1,71)} = 5.0$, $p < 0.001$, Cohen's $d = 1.2$] and time point 2 [Raw: $t_{(1,60)} = 3.9$, $p < 0.001$, Cohen's $d = 1.0$; Log10: $t_{(1,60)} = 4.5$, $p < 0.001$,

Cohen's $d = 1.1$] compared to controls (**Table 1** and **Figures 1A–C**), including when the raw plasma NfL outlier was excluded from these analyses (both time points $p < 0.001$ relative to controls). Raw and log10 plasma NfL levels were higher in individuals with Wolfram syndrome relative to both control group subsets (parents: both time points $p < 0.001$; siblings: both time points $p \leq 0.02$) including when the Wolfram group outlier was excluded ($p \leq 0.01$). Neither raw nor log10 plasma NfL levels were different between time points within the Wolfram group ($p \geq 0.66$) (**Table 1** and **Figures 2A,B**).

Plasma Neurofilament Light Chain Levels and Age

Both raw and log10 NfL levels were higher in the Wolfram group compared to controls when age was covaried [time point 1: $F_{(1,70)} = 26.7$, $p < 0.001$, Cohen's $d = 1.23$; time point 2: $F_{(1,59)} = 24.2$, $p < 0.001$, Cohen's $d = 1.28$], indicating that disease status, not age, drives the difference in NfL levels between groups. Age did not relate to raw or log10 plasma NfL levels within individuals with Wolfram syndrome (**Table 3**) or within controls ($r_{35} \leq 0.31$, $p \geq 0.07$). Results were similar when the raw plasma NfL outlier data point was excluded from the Wolfram syndrome group ($r_{37} \leq 0.09$, $p \geq 0.60$).

Plasma Neurofilament Light Chain Levels, Clinical Severity and Neurodegeneration in Individuals With Wolfram Syndrome

Correlation statistics for the relationship between log10 plasma NfL levels at time points 1 and 2 with measures of clinical severity are shown in **Table 3**. Briefly, higher log10 plasma NfL levels at time point 1 related to worse visual acuity and color vision at time point 1, worse visual acuity, less accurate smell identification and smaller brainstem and thalamic volumes at time point 2, and faster annual rate of decrease in thalamic volume [mean (SD) number of annual MRIs = 4.5 (1.6) per participant] (**Figures 3A–F**). Higher log10 plasma NfL levels at time point 2 related to worse visual acuity and color vision, less accurate smell identification, and smaller thalamic volume at time point 2 (**Figures 4A–D**).

TABLE 1 | Control and Wolfram group demographics and plasma neurofilament light chain levels.

	All controls	Parents	Siblings	Wolfram	Wolfram with two time points	
N (number of participants with NfL samples)	35	28	7	38	27[a]	
Female/male	22/13	20/8	2/5	22/16	18/9	
Race (ethnicity)	35 W (12 H)	28 W (10 H)	7 W (2 H)	38 W (11 H)	27 W (9 H)	
Mean scaled parental education ± SD	15.5 ± 4.5[b]	15.3 ± 4.6[b]	16.3 ± 4.2	14.5 ± 4.2[c]	14.9 ± 4.8[c]	
					TP 1[d]	TP 2
Median age (years, IQR)	41.0 (35.1–47.9)	44.8 (39.6–49.5)	12.5 (10.3–14.1)	14.4 (5.1–29.7)	14.0 (10.8–20.1)	16.0 (12.8–22.1)
Median plasma NfL (pg/mL, IQR)	5.6 (4.5–7.4)	5.9 (5.0–7.4)	4.5 (2.8–6.4)	11.3 (7.8–13.9)	10.6 (7.2–14.5)	10.7 (8.4–13.8)
Median log10 plasma NfL (IQR)	0.7 (0.7–0.9)	0.8 (0.7–0.9)	0.7 (0.5–0.8)	1.1 (0.9–1.1)	1.0 (0.9–1.2)	1.0 (0.9–1.1)

NfL, neurofilament light chain; W, White; H, Hispanic; IQR, interquartile range; TP, time point.
[a] *Of 38 individuals in the Wolfram group, 27 also had data from a second time point.*
[b] *Data missing from two participants.*
[c] *Data missing from four participants.*
[d] *TP 1 and TP 2 columns include data for 27 out of 38 individuals in Wolfram group who had NfL data from both time points.*

TABLE 2 | Clinical severity measures in individuals with Wolfram syndrome.

	TP 1	*N* with 1 TP	TP 1[a]	TP 2	*N* with 2 TPs
Disease duration (years)	4.5 ± 4.4	37	3.9 ± 4.1	5.6 ± 4.0	26
Plasma glucose (mg/dL)	187.8 ± 68.4	36	187.7 ± 72.5	181.6 ± 84.4	26
HbA1c	7.5 ± 1.5	36	7.5 ± 1.7	7.3 ± 1.1	26
WURS Physical Activity subscale score	5.0 ± 5.7	34	4.3 ± 3.2	4.5 ± 3.7	24
UPSIT total score (number correct)	24.9 ± 7.5	37	24.9 ± 7.8	24.0 ± 7.3	27
Visual acuity (logMAR)	0.56 ± 0.45	34	0.50 ± 0.34	0.56 ± 0.39*	27
Color vision (number correct)	9.2 ± 9.3	30	10.7 ± 9.4	10.2 ± 10	20
Ventral pons volume (mm^3)	6280 ± 1160	27	6133 ± 1190	6030 ± 1249*	20
Brainstem volume (mm^3)	15079 ± 1590	18	15079 ± 1590	14879 ± 1649**	18
Cerebellar cortex volume (mm^3)	46109 ± 4440	26	45573 ± 4393	44712 ± 4420**	19
Thalamus volume (mm^3)	6530 ± 478	26	6546 ± 381	6429 ± 425**	19
Follow-up duration (years, range)	NA	NA	NA	1.8 (0.99–2.0)	27

Mean (SD) shown except where noted.
TP, time point; NA, not applicable; WURS, Wolfram United Rating Scale; UPSIT, University of Pennsylvania Smell Identification Test.
***, *p < 0.01, 0.05 relative to time point 1.*
[a] *Column includes individuals with Wolfram syndrome who had data from both time points.*

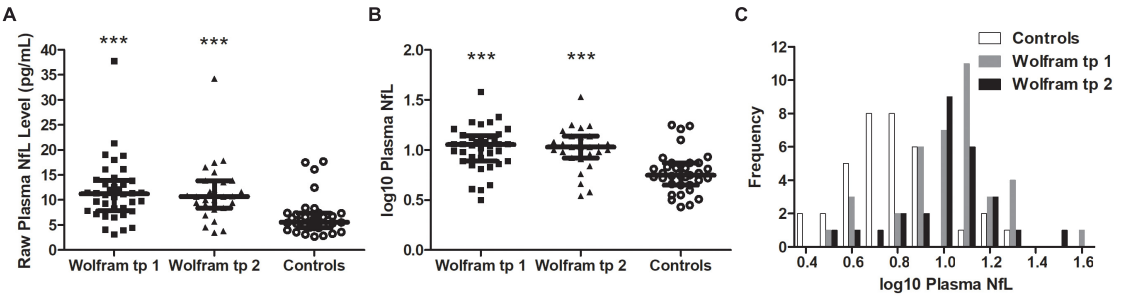

FIGURE 1 | Raw **(A)** and log10 **(B)** plasma NfL levels were higher in the Wolfram group at time points 1 and 2 relative to controls. Results were similar when outlier data were excluded. Median and IQR shown. **(C)** Frequency distribution of log10 plasma NfL levels for control and Wolfram groups at time points 1 and 2. NfL, neurofilament light; tp, time point. ***$p < 0.001$ relative to controls.

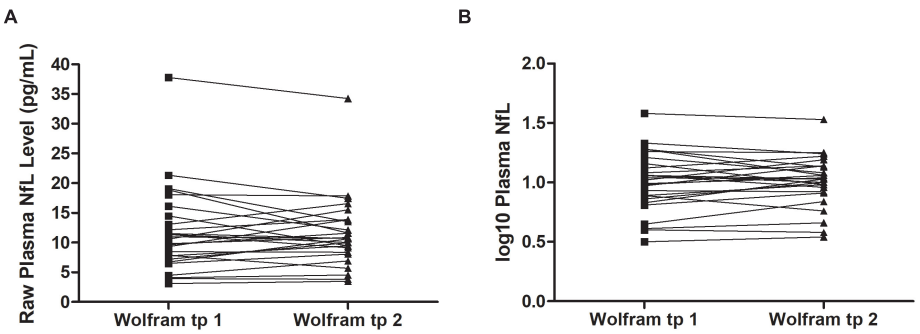

FIGURE 2 | Raw **(A)** and log10 **(B)** plasma NfL levels were similar at time points 1 and 2 within individuals in the Wolfram group who had NfL measures at both time points 1.8 years apart on average ($n = 27$). NfL, neurofilament light; tp, time point.

TABLE 3 | Correlations between log10 plasma neurofilament light levels with age and measures of clinical severity at time points 1 and 2 in individuals with Wolfram syndrome.

	NfL TP 1 vs. Clinical TP 1	***N***	**NfL TP 1 vs. Clinical TP 2**	***N***	**NfL TP 2 vs. Clinical TP 2**	***N***
Age	$r = 0.21$, $p = 0.21$	38	$r = 0.23$, $p = 0.25$	27	$r = 0.30$, $p = 0.13$	27
WURS Physical Activity subscore total	$\rho = -0.14$, $p = 0.40$	34	$\rho = -0.07$, $p = 0.72$	26	$\rho = 0.10$, $p = 0.63$	26
UPSIT (number correct)	$r = -0.26$, $p = 0.12$	37	$r = -0.38$, $\mathbf{p = 0.05}$*	27	$r = -0.46$, $\mathbf{p = 0.02}$*	27
Visual acuity (logMAR)	$r = 0.34$, $\mathbf{p = 0.05}$*	34	$r = 0.40$, $\mathbf{p = 0.04}$*	27	$r = -0.46$, $\mathbf{p = 0.02}$*	27
Color vision (number correct)	$r = -0.40$, $\mathbf{p = 0.03}$*	30	$r = -0.42$, $p = 0.07$	20	$r = -0.59$, $\mathbf{p = 0.01}$**	20
Ventral pons volume	$r = -0.24$, $p = 0.23$	27	$r = -0.34$, $p = 0.14$	21	$r = -0.35$, $p = 0.12$	21
Brainstem volume	$r = -0.48$, $\mathbf{p = 0.04}$*	18	$r = -0.50$, $\mathbf{p = 0.04}$*	18	$r = -0.43$, $p = 0.08$	18
Cerebellar cortex volume	$r = 0.12$, $p = 0.55$	26	$r = 0.21$, $p = 0.37$	20	$r = 0.25$, $p = 0.29$	20
Thalamic volume	$r = -0.36$, $p = 0.07$	26	$r = -0.60$, $\mathbf{p = 0.01}$**	20	$r = -0.57$, $\mathbf{p = 0.01}$**	20
Average annual rate of change in thalamic volume	NA		$r = -0.52$, $\mathbf{p = 0.01}$**	24	NA	

*NfL, neurofilament light chain; TP, time point; WURS, Wolfram Unified Rating Scale; UPSIT, University of Pennsylvania Smell Identification Test; NA, not applicable. *, **$p \leq 0.05, 0.01$. Bold p-values indicate statistical significance at $\alpha = 0.05$. N = number of participants with data points included in the analysis in the preceding column.*

DISCUSSION

Similar to other neurological diseases in which neuroaxonal injury is a core feature (Khalil et al., 2018; Gaetani et al., 2019), we found that NfL levels are higher in individuals with Wolfram syndrome compared to controls and related to measures of greater clinical severity and neurodegeneration. Between two time points ∼1.8 years apart, plasma NfL levels did not differ, indicating that any change in plasma NfL levels is not detectable over this brief interval in Wolfram syndrome. Our findings demonstrate that NfL levels are sensitive to clinical presentation and brain health in Wolfram syndrome, indicating that this blood-based marker may have prognostic value and is a promising biomarker to monitor response in future theraputic trials.

For NfL to be of use in detecting clinically relevant severity or rate of neuroaxonal injury, NfL levels must be different in individuals with disease relative to controls. Our primary finding is that plasma NfL levels discriminate between individuals with Wolfram syndrome and controls composed of parents

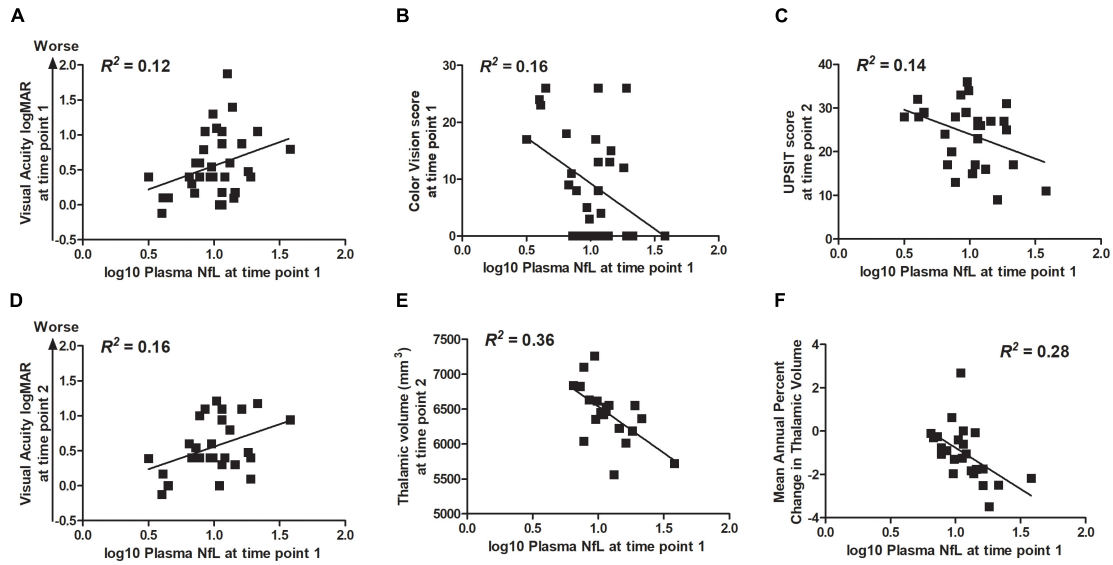

FIGURE 3 | Within the Wolfram group, higher log10 plasma NfL levels at time point 1 related to **(A,B)** worse visual acuity and color vision at time point 1; **(C–E)** worse visual acuity and color vision (data not shown), less accurate smell identification, and smaller thalamic volume at time point 2; and **(F)** faster rate of annual decrease in thalamic volume.

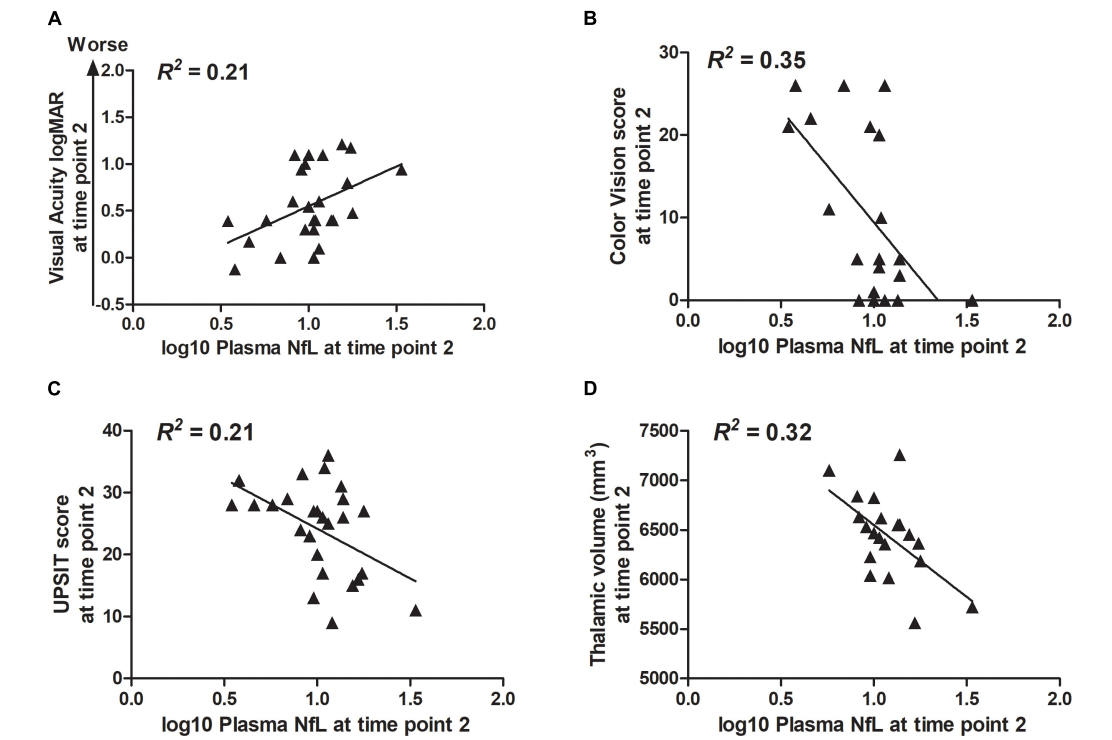

FIGURE 4 | Within the Wolfram group, higher log10 plasma NfL levels at time point 2 related to **(A–D)** worse visual acuity and color vision, less accurate smell identification, and smaller thalamic volume at time point 2.

and siblings of individuals in the Wolfram group. In addition, unlike in controls, these levels were higher relative to the normal reference range [median (IQR) plasma NfL = 5.9 (4.3–7.9) pg/mL] established by a previous study of NfL levels in healthy donors aged 24–64 years using the same assay on the SIMOA platform (Harp et al., 2019). The observed plasma NfL levels in individuals with Wolfram syndrome overlap with similar-aged, untreated children with pediatric MS (Reinert et al., 2020)

and appear similar to those in people with asymptomatic spinocerebellar ataxia type 3 [median (IQR) NfL = 12.2 (10.2–13.9) pg/mL] (Peng et al., 2020). The latter disease, like Wolfram syndrome, is caused by a genetic mutation with variable expressivity, rate of progression, age of onset, and phenotype, and is accompanied by cerebellar and brainstem atrophy (Brooker et al., 2021). The plasma NfL levels observed here in Wolfram syndrome also overlap with those obtained from older (mean age = 57.4 years) individuals with type 1 diabetes [mean (SD) plasma NfL = 13.3 (6.7) pg/mL]. It is possible that between-group factors other than central axonal injury such as peripheral nerve damage, impaired renal function and/or vascular neuropathy may be responsible for elevated plasma NfL levels in individuals with Wolfram syndrome (Bischof et al., 2018; Khalil et al., 2018; Sandelius et al., 2018; Akamine et al., 2020; Frempong et al., 2021). Future studies using CSF NfL samples and MR diffusion studies of tissue microstructural integrity, known to be impaired in Wolfram syndrome (Lugar et al., 2016), will help to determine whether the elevated NfL is primarily due to central axonal injury or other aspects of Wolfram syndrome (e.g., diabetes, ER dysfunction) (Piehl et al., 2018; Akamine et al., 2020; van der Plas et al., 2021). Unfortunately, the current study was not powered to detect relationships between diffusion MRI-based tissue microstructural integrity and NfL levels since these measures were not always obtained during the same clinic year in individuals with Wolfram syndrome. Nonetheless, elevated plasma NfL levels in individuals with Wolfram syndrome cross-sectionally related to smaller brainstem and thalamic volumes and faster rate of decreasing thalamic volume over time, providing indirect evidence that NfL levels likely reflect the current degree or rate of central axonal injury.

To determine whether NfL levels are altered over time and/or predict future disease activity, longitudinal studies are required over greater than 2 years. In contrast, this interval was sufficient for detection of increased clinical severity and neurodegeneration. Interestingly, in sera from a small sample of teens and young adults with Wolfram syndrome, expression of multiple microRNAs are altered after 2 years follow-up and relate to simultaneous MRI indicators of neurodegeneration including reduced macular average thickness and brainstem volume (Zmyslowska et al., 2020). These preliminary observations suggest that serum microRNA expression, influenced by ER stress, may be a sensitive marker of short-term progression in neurodegeneration in Wolfram syndrome (Zmyslowska et al., 2020). In the current study, NfL levels at time point 1 did relate to worsening accuracy in smell identification and visual acuity and decreased thalamic and brainstem volumes at time point 2, indicating that NfL levels may predict future disease activity in Wolfram syndrome. Of note, our findings are similar in nature to those of a study of spinocerebellar ataxia, in which NfL levels did not change over 24 mos but predicted worsening in clinical severity and decreased cerebellar and pons volumes over this time period (Coarelli et al., 2021). To test whether NfL levels truly predict future disease activity in Wolfram syndrome, a prospective, longitudinal study with sufficient sample size, duration, and sampling frequency is required.

Neurofilament light chain levels fluctuate non-linearly over the lifespan in healthy individuals and, in normal aging, elevated NfL levels cross-sectionally and longitudinally relate to brain volume loss presumably due to increasing levels of neuroaxonal injury (Bridel et al., 2019; Khalil et al., 2020; Ashton et al., 2021). Risk for or presence of disease often alters the relationship between age and NfL levels (Bridel et al., 2019; Khalil et al., 2020; Ashton et al., 2021; van der Plas et al., 2021). The subset of siblings in our control group, composed of children and adolescents, had plasma NfL levels similar to those of the control parents in our study and to serum NfL levels in similarly aged healthy controls in a pediatric MS study (Reinert et al., 2020). The subset of parents in our control group (age range = 26.6–59.7 years) had plasma NfL levels in line with the established reference range for the Quanterix/SIMOA platform (Harp et al., 2019) but tended to skew lower than other published control plasma and serum NfL samples from similarly aged adults (Kuhle et al., 2015; Weydt et al., 2016; Weston et al., 2017; Benatar et al., 2018; Korley et al., 2018; Thompson et al., 2018; Zeitlberger et al., 2018; Clay et al., 2020; Coarelli et al., 2021), likely due to differences in assay methods, specimen type, and variability in age ranges were studied. While, we did not find evidence of a relationship between age and plasma NfL levels at either time point in the Wolfram group, we observed that, in controls, older age related to higher log10 plasma NfL levels (age range = 3.0–59.7 years), albeit at trend-level statistical significance, similar to previous studies that included similarly aged controls (Clay et al., 2020; Hayer et al., 2020). Given the small sample size and limited age range in the Wolfram group (5.1–30.7 years), it is difficult to know whether Wolfram syndrome alters the relationship between age and NfL levels. Future longitudinal studies including a wide age range of individuals with Wolfram syndrome will help determine at what age(s) NfL levels differ from those of controls and if and how they relate to age.

A strength of the current study is the direct comparison of NfL levels between controls and individuals with Wolfram syndrome using the same assays at the same study site. Relatives of individuals with Wolfram syndrome served as convenient case controls that guarded against environmental confounds. Future sampling of unrelated control groups using the same assays at the same study site may yield further insight into differences in NfL levels between individuals with Wolfram syndrome and the general population. There are several weaknesses in the currently described study. The study sample is small due to the rarity of Wolfram syndrome and people with Wolfram syndrome with severe physical and/or psychological impairment that restricted travel to participate are not represented. Finally, the relatively short time between baseline and follow-up blood draws (2 years) limited our ability to detect whether NfL levels change over time in individuals with Wolfram syndrome.

CONCLUSION

We show that plasma NfL levels are higher in individuals with Wolfram syndrome relative to parent and sibling controls and that higher NfL levels are related to worse clinical symptoms, smaller brainstem and thalamic volumes, and greater annual percent loss of thalamic volume. Serial NfL measures in Wolfram syndrome using prospective, large, and longitudinal studies are

needed to determine whether NfL levels change over time, are prognostic of future disease activity, and reflective of response to treatment in this disease. This study suggests that such an investigation is warranted and could improve future clinical trials for Wolfram syndrome by providing a potential easily obtained outcome measure of neurodegeneration (Khalil et al., 2018).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Research and Protection Office at Washington University in St. Louis, MO, United States, and carried out in accordance with the Declaration of Helsinki. Participants < 18 years of age gave informed assent, and their parents or legal guardians gave written informed consent. Participants ≥ 18 years gave written informed consent.

AUTHOR CONTRIBUTIONS

SE: formal analysis, writing–original draft preparation, writing–review and editing, and visualization. RB: writing–original draft preparation, formal analysis, and writing–review and editing. CS: validation, investigation, and writing–review and editing. HL: formal analysis, investigation, data curation, and writing–review and editing. BG: writing–review and editing. BM and FU: investigation and writing–review and editing. AF: methodology, resources, writing–review and editing, and supervision. TH: conceptualization, methodology, formal analysis, investigation, resources, writing–original draft preparation, writing–review and editing, supervision, project administration, and funding acquisition. All authors made substantial contributions to the conception or design of the work or the acquisition, analysis, or interpretation of data for the work; drafted the work or critically revised it for important intellectual content; and agreed to be accountable for all aspects of the work.

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Conflict of Interest: FU is a Founder and President of CURE4WOLFRAM, INC. and employed by it. FU is an inventor of three patents related to the treatment of Wolfram syndrome, Soluble MANF in Pancreatic Beta Cell Disorders (US 9,891,231) and Treatment for Wolfram Syndrome and Other ER Stress Disorders (US 10,441,574 and US 10,695,324).

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Renal Function and Body Mass Index Contribute to Serum Neurofilament Light Chain Levels in Elderly Patients With Atrial Fibrillation

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Objective: Serum neurofilament light chain (sNfL) is increasingly used as a neuroaxonal injury biomarker in the elderly. Besides age, little is known about how other physiological factors like renal function and body mass index (BMI) alter its levels. Here, we investigated the association of estimated glomerular filtration rate (eGFR) and BMI with sNfL in a large sample of elderly patients with atrial fibrillation (AF).

Methods: This is a cross-sectional analysis from the Swiss-AF Cohort (NCT02105844). We measured sNfL using an ultrasensitive single-molecule array assay. We calculated eGFR using the chronic kidney disease epidemiology collaboration (CKD-EPI) creatinine ($eGFR_{crea}$) and creatinine–cystatin C ($eGFR_{crea\text{-}cys}$) formulas, and BMI from weight and height measurements. We evaluated the role of eGFR and BMI as determinants of sNfL levels using multivariable linear regression and the adjusted R^2 (R^2_{adj}).

Results: Among 2,277 Swiss-AF participants (mean age 73.3 years), $eGFR_{crea}$ showed an inverse curvilinear association with sNfL after adjustment for age and cardiovascular comorbidities. BMI also showed an independent, inverse linear association with sNfL. The R^2_{adj} of models with age, $eGFR_{crea}$, and BMI alone was 0.26, 0.35, and 0.02,

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respectively. A model with age and $eGFR_{crea}$ combined explained 45% of the sNfL variance. Sensitivity analyses (i) further adjusting for vascular brain lesions (N = 1,402 participants with MRI) and (ii) using $eGFR_{crea\text{-}cys}$ yielded consistent results.

Interpretation: In an elderly AF cohort, both renal function and BMI were associated with sNfL, but only renal function explained a substantial proportion of the sNfL variance. This should be taken into account when using sNfL in elderly patients or patients with cardiovascular disease.

Keywords: neurofilament light, renal function, glomerular filtration rate, body mass index, elderly, atrial fibrillation

INTRODUCTION

Neurofilament light chain (NfL) is a cytoskeletal protein exclusive to neurons. Following neuroaxonal damage, it is released into the extracellular space, cerebrospinal fluid, and eventually peripheral blood. Over the past years, NfL has been established as the first blood-based biomarker reflecting disease activity and treatment response in traumatic brain injury and neurodegenerative diseases (Khalil et al., 2018; Barro et al., 2020; Gafson et al., 2020). Considering the increasing use of blood NfL as a biomarker for neurological diseases in clinical research and the perspective of its diagnostic and prognostic applications in individual clinical practice, a deeper understanding of its homeostasis (including distribution and clearance) in the blood compartment is needed to elucidate physiological factors that might affect its association with disease processes (Barro et al., 2020; Gafson et al., 2020). This is becoming increasingly important for NfL-based investigations of normal aging (Khalil et al., 2020), as well as cerebrovascular disease (Gattringer et al., 2017; Duering et al., 2018; Tiedt et al., 2018; Peters et al., 2020), atrial fibrillation (AF) (Polymeris et al., 2020), and dementia (Zhao et al., 2019), where accumulating age-related comorbidities might both interfere with the homeostasis of NfL and directly induce neuronal damage *per se* (Barro et al., 2020; Gafson et al., 2020).

While the association of NfL blood levels with age has been consistently demonstrated across a variety of patient populations and healthy controls (Khalil et al., 2018, 2020), their association with renal function and body mass index (BMI) was only recently reported in elderly diabetic patients and younger patients with multiple sclerosis, respectively (Korley et al., 2019; Akamine et al., 2020; Manouchehrinia et al., 2020). However, little is known on how these factors impact NfL concentrations relative to age, one another, and cardiovascular comorbidities and vascular brain lesions, which are increasingly prevalent in the elderly (Vermeer et al., 2007; Wardlaw et al., 2013). Such data are necessary for a systematic appraisal of the importance of these factors as potential confounders and the need to account for them in future use of NfL as a laboratory measure in elderly individuals.

With this in mind, we investigated the association of (i) estimated glomerular filtration rate (eGFR) and (ii) BMI with serum NfL (sNfL) concentrations in a large, well-characterized cohort of elderly AF patients accounting for age, cardiovascular comorbidities, as well as vascular brain lesions and brain volume on neuroimaging.

MATERIALS AND METHODS

Study Design, Patient Population, and Data Collection

This was a cross-sectional analysis using baseline data from the prospective observational Swiss-AF cohort study (NCT02105844), which was designed to investigate the relationship between AF, structural brain changes, and cognition. We selected the Swiss-AF cohort for this analysis due to the large sample size, the detailed clinical and neuroimaging characterization with a relatively high prevalence of cardiovascular comorbidities, and the availability of blood biomarker measurements. Swiss-AF enrolled 2,415 patients with AF between 2014 and 2017 across 14 centers in Switzerland. Included were patients aged 65 years or older, with an additional 15% of patients aged < 65 years. Patients with a recent ischemic stroke, transient ischemic attack (TIA) or other acute illness (< 4 weeks), and those unable to provide consent (e.g., patients with dementia) were excluded. The detailed methodology of Swiss-AF has been described previously (Conen et al., 2017, 2019; Polymeris et al., 2020). Baseline investigations included a standardized clinical assessment (sociodemographic parameters, comorbidities), weight and height measurements [from which BMI was calculated as (weight in kg)/(height in m)^2], blood sampling, and brain MRI.

Baseline blood samples were collected following standard operating procedures. After centrifugation, serum samples were aliquoted into cryotubes and stored at -80°C in a centralized biobank. The concentration of sNfL was measured in duplicate using a previously described ultrasensitive single-molecule array assay (lower limit of quantification 1.0 pg/ml) (Disanto et al., 2017; Polymeris et al., 2020). Creatinine and cystatin C were measured using commercially available assays (cobas c 311 and Elecsys; Roche Diagnostics, Mannheim, Germany). In order to calculate eGFR as a measure of renal function, we used the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) (i) creatinine equation ($eGFR_{crea}$) and (ii) the combined creatinine–cystatin C equation ($eGFR_{crea\text{-}cys}$) (Inker et al., 2012).

On baseline MRI, we assessed the presence and volume of small non-cortical infarcts (SNCIs), large non-cortical or cortical infarcts (LNCCIs), and white matter lesions (WMLs); the presence and count of microbleeds (MBs); and estimated the normalized brain volume (nBV) using SIENAX

(Smith et al., 2002), as described previously in detail (Conen et al., 2019; Polymeris et al., 2020).

In this study, we included all Swiss-AF patients with quantifiable sNfL measurement and available data on clinical variables, creatinine and cystatin C (**Supplementary Figure 1**). The Ethics Committee of Northwest and Central Switzerland approved Swiss-AF, including this study (PB_2016-00793). Written informed consent was obtained from all study participants according to the Declaration of Helsinki. This study was conducted in accordance with the STROBE Statement for cross-sectional studies (von Elm et al., 2007).

Statistical Analyses

Main Analysis

As the first step to investigate the association of eGFR and BMI with sNfL, we fitted a multivariable linear regression model with log-transformed sNfL as the dependent variable and eGFR and BMI as independent variables. The model was adjusted for the following variables, known to be associated with sNfL from our previous work in this cohort (Polymeris et al., 2020): age, history of hypertension, diabetes mellitus, stroke or TIA, peripheral artery disease, heart failure, as well as mean arterial pressure [calculated as (1/3 × systolic blood pressure) + (2/3 × diastolic blood pressure)], smoking status (non, past, and current smoker), and alcohol consumption (in standard drinks per day). Continuous variables were centered on their mean (or, in case of skewed data, median) values, as appropriate. Visual inspection suggested curvilinear associations of age and eGFR with sNfL. We chose the best way to model these variables by fitting different univariable models (linear only, quadratic, cubic, cubic without quadratic term) and selecting the one with the best fit based on the Akaike's information criterion (AIC). Considering the known strong association of age and diabetes with sNfL (Polymeris et al., 2020), we also included in the multivariable model the interactions GFR by age, BMI by age, and GFR by diabetes. We report the backtransformed model-based estimates, which represent multiplicative effects on the geometric mean of sNfL and are denoted by β_{mult} (so that a one-unit increase in the independent variable is associated with an average β_{mult}-fold change in sNfL), along with 95% confidence intervals (95% CI) and two-sided *p*-values.

In a second step, to investigate the relative contribution of eGFR, BMI, and age to the variance of sNfL concentrations, we fitted linear models with log-sNfL as the dependent variable and these factors as independent variables, alone and in combination with one another and with their interactions with age. We report the coefficient of determination (R^2) and the adjusted R^2 (R^2_{adj}; penalized for larger number of independent variables) as a measure of the proportion of the observed sNfL variance explained by each model.

Sensitivity Analyses

Considering patients who also had baseline brain MRI available (**Supplementary Figure 1**), we further adjusted the multivariable linear model from the main analysis for the following imaging measures: log-volume of WMLs, presence and log-volume of LNCCIs, presence and log-volume of SNCIs, presence and count of MBs, and nBV, which were previously reported to be associated with sNfL concentrations (Polymeris et al., 2020). We reduced the model to a smaller set of variables via stepwise backward elimination based on AIC. As this is known to inflate the type I error, we refrained from providing *p*-values in this analysis and evaluated the explanatory importance of independent variables for sNfL concentrations based on whether they were selected or eliminated from the reduced model. Finally, we refitted the model without eGFR and report the R^2 and R^2_{adj} for both models. We repeated all models using $eGFR_{crea\text{-}cys}$ as a further sensitivity analysis.

All analyses were performed with R version 4.0.3 (2020-10-10).

RESULTS

Main Analysis

A total of 2,277 Swiss-AF patients were available for the main analysis, after exclusion of 77 patients without sNfL measurement (no or insufficient blood sample), 16 with sNfL measurement below the limit of quantification, and 45 with other data missing (**Supplementary Figure 1**). The mean [standard deviation (SD)] age was 73.3 (8.5) years, mean (SD) $eGFR_{crea}$/$eGFR_{crea\text{-}cys}$ was 59.1 (18.3)/58.6 (20.0) ml/min/1.73 m^2, mean (SD) BMI was 27.6 (4.7) kg/m^2, and the median (interquartile range) sNfL was 42.0 (29.0 – 65.1) pg/ml. All patient characteristics are provided in **Table 1**.

In the multivariable model adjusted for all clinical variables (**Table 2**), $eGFR_{crea}$ showed a strong inverse curvilinear association with sNfL (**Figure 1A**). Modeling $eGFR_{crea}$ with a linear, quadratic, and cubic component was chosen based on AIC (**Supplementary Table 1**). BMI also showed a strong, inverse linear association with sNfL in the multivariable model (**Figure 2A**).

Furthermore, age (modeled with a linear and cubic component based on AIC; **Supplementary Table 1**) was strongly, positively associated with sNfL in the multivariable model. There was evidence for an interaction between $eGFR_{crea}$ and age on their association with sNfL ($p_{interaction} < 0.001$), indicating that, with older age, the negative association of $eGFR_{crea}$ with sNfL was steeper for lower values of $eGFR_{crea}$ (**Figure 1B**). There was also evidence for a weaker interaction between BMI and age on their association with sNfL ($p_{interaction} = 0.013$), indicating a slightly stronger negative association of BMI with sNfL with increasing age (**Figure 2B**). **Supplementary Table 2** presents the model-based estimates for the association of $eGFR_{crea}$ and BMI with sNfL in four age-quartile subgroups.

Further variables with a strong association with sNfL in the multivariable model were diabetes mellitus and history of stroke or TIA. There was no evidence for an interaction between $eGFR_{crea}$ and diabetes mellitus on their association with sNfL.

Upon examination of the R^2_{adj} of different models fitted with sNfL as the dependent variable, models containing age, $eGFR_{crea}$, and BMI alone explained 26%, 35%, and 2% of the variance in sNfL concentrations, respectively. Adding $eGFR_{crea}$ to the

TABLE 1 | Patient characteristics.

Clinical characteristics of 2,277 Swiss-AF patients (main analysis)	
Age, years, mean (SD)	73.3 (8.5)
Sex, female, N (%)	615 (27.0)
History of atrial fibrillation, N (%)	2,277 (100.0)
History of hypertension, N (%)	1,599 (70.2)
History of diabetes mellitus, N (%)	395 (17.3)
History of stroke or transient ischemic attack, N (%)	452 (19.9)
History of peripheral artery disease, N (%)	183 (8.0)
History of heart failure, N (%)	604 (26.5)
Smoking status, N (%)	
Non-smoker	999 (43.9)
Past smoker	1,111 (48.8)
Current smoker	167 (7.3)
Alcohol consumption, std. drinks/day, median (IQR)	0.5 (0.1–1.3)
Mean arterial pressure, mmHg, mean (SD)	92.6 (12.6)
Body mass index, kg/m^2, mean (SD)	27.6 (4.7)
$eGFR_{crea}$, ml/min/1.73 m^2, mean (SD)	59.1 (18.3)
$eGFR_{crea\text{-}cys}$, ml/min/1.73 m^2, mean (SD)	58.6 (20.0)
Serum neurofilament light chain, pg/ml, mean (SD)	42.0 (29.0–65.1)
MRI characteristics of 1,402 Swiss-AF patients (sensitivity analysis)	
Small non-cortical infarcts, N (%)	308 (22.0)
Volume (if present), mm^3, median (IQR)	62 (30–150)
Large non-cortical and cortical infarcts, N (%)	299 (21.3)
Volume (if present), mm^3, median (IQR)	1,350 (252–7,086)
White matter lesions, N (%)	1,390 (99.1)
Volume (if present), mm^3, median (IQR)	3,753 (1,368–9,353)
Microbleeds, N (%)	302 (21.5)
Count (if present), median (IQR)	1 (1–2)
Normalized brain volume, cm^3, mean (SD)	1,416 (94)

SD: standard deviation, IQR: interquartile range, $eGFR_{crea}$/$eGFR_{crea\text{-}cys}$: estimated glomerular filtration rate based on creatinine/creatinine–cystatin C.

age model conferred a substantial increase in the sNfL variance explained by the model (R^2_{adj} 0.45 vs. 0.26), while adding BMI to age increased the model's explanatory power only marginally (R^2_{adj} 0.27 vs. 0.26). The combined age, $eGFR_{crea}$, and BMI model explained 46% of the total sNfL variance. Adding the interaction terms $eGFR_{crea}$ and BMI by age to the models conferred no substantial increase in R^2_{adj} (**Table 3**).

Sensitivity Analysis Adjusting for MRI Variables

A total of 1,402 Swiss-AF patients were available for the MRI sensitivity analysis (**Supplementary Figure 1**). In the multivariable model including all variables from the main analysis, vascular brain lesions and nBV, both $eGFR_{crea}$ and BMI remained in the model after stepwise backward elimination, as did age, its interaction with $eGFR_{crea}$, diabetes mellitus, and history of stroke or TIA (**Supplementary Table 3**). The R^2_{adj} of this model was 52%, and dropped to 36% after excluding $eGFR_{crea}$.

Sensitivity Analysis Using $eGFR_{crea\text{-}cys}$

As for the main analysis using $eGFR_{crea}$, a total of 2,277 Swiss-AF participants were available for sensitivity analysis using $eGFR_{crea\text{-}cys}$. Consistent with the main analysis, in a multivariable model adjusting for all clinical variables (**Table 2**), $eGFR_{crea\text{-}cys}$ was strongly associated with sNfL, with a curvilinear relationship including a linear, quadratic, and cubic component (modeled as such based on AIC, **Supplementary Table 1**). BMI was also strongly associated with sNfL, as was age, diabetes mellitus, and history of stroke or TIA. For all associations, the coefficients were of similar magnitude as in the main analysis. Consistent with the main analysis, there was evidence for an interaction between $eGFR_{crea\text{-}cys}$ and age ($p_{interaction} < 0.001$). The interaction between BMI and age was even weaker than in the main analysis ($p_{interaction} = 0.057$). Examination of the R^2_{adj} of different models containing age, $eGFR_{crea\text{-}cys}$, and BMI either alone or in combination with one another revealed similar results with the main analysis, with $eGFR_{crea\text{-}cys}$ explaining a substantial proportion of the sNfL variance beyond that explained by age (**Table 3**).

A total of 1,402 Swiss-AF patients were available for the sensitivity analysis including MRI data and using $eGFR_{crea\text{-}cys}$. As in the main analysis, in the multivariable model including all clinical variables, vascular brain lesions, and nBV, both $eGFR_{crea\text{-}cys}$ and BMI remained in the model after backward variable elimination, as did age, its interaction with $eGFR_{crea\text{-}cys}$, diabetes mellitus, and history of stroke or TIA (**Supplementary Table 3**). The R^2_{adj} of this model was 52% with $eGFR_{crea\text{-}cys}$, dropping to 36% after excluding $eGFR_{crea\text{-}cys}$.

DISCUSSION

This cross-sectional study on the association of eGFR and BMI with sNfL concentrations in a large elderly cohort of AF patients showed that both eGFR (estimated using either creatinine or creatinine and cystatin C) and BMI were strongly associated with sNfL concentrations. This was true even after adjustment for other parameters known to contribute to sNfL concentrations, including age, clinical comorbidities, and MRI characteristics. Furthermore, eGFR, but not BMI, conferred a substantial increase in the explanatory power of models predicting sNfL

TABLE 2 | Multivariable models for the association of eGFR and BMI with sNfL.

Variables (*N* = 2,277)	Using $eGFR_{crea}$			Using $eGFR_{crea-cys}$		
	β_{mult}	95%-CI	*p*-value	β_{mult}	95%-CI	*p*-value
Age* (per decade)	1.293	[1.244, 1.344]	< 0.001	1.229	[1.182, 1.278]	< 0.001
[Age* (per decade)]^3	0.988	[0.977, 0.999]	0.032	0.989	[0.978, 1.000]	0.046
eGFR* (per 10 ml/min/1.73 m^2)	0.888	[0.869, 0.907]	< 0.001	0.869	[0.854, 0.886]	< 0.001
[eGFR* (per 10 ml/min/1.73 m^2)]^2	1.030	[1.023, 1.036]	< 0.001	1.029	[1.024, 1.033]	< 0.001
[eGFR* (per 10 ml/min/1.73 m^2)]^3	0.998	[0.996, 1.000]	0.017	0.998	[0.997, 1.000]	< 0.001
BMI* (per 5 kg/m^2)	0.898	[0.878, 0.919]	< 0.001	0.891	[0.871, 0.910]	< 0.001
History of hypertension	1.043	[0.996, 1.094]	0.076	1.031	[0.985, 1.079]	0.195
History of diabetes mellitus	1.203	[1.137, 1.274]	< 0.001	1.181	[1.117, 1.249]	< 0.001
History of stroke or TIA	1.127	[1.072, 1.185]	< 0.001	1.120	[1.067, 1.176]	< 0.001
History of peripheral artery disease	1.071	[0.993, 1.154]	0.075	1.046	[0.972, 1.125]	0.229
History of heart failure	1.063	[1.014, 1.115]	0.012	1.023	[0.976, 1.071]	0.344
Mean arterial pressure (per 1 mmHg)	0.998	[0.997, 1.000]	0.031	0.999	[0.998, 1.001]	0.277
Past smoker (ref: non-smoker)	0.970	[0.930, 1.011]	0.151	0.967	[0.928, 1.007]	0.109
Current smoker (ref: non-smoker)	0.963	[0.887, 1.044]	0.357	0.934	[0.863, 1.010]	0.088
Alcohol consumption (per 1 std. drink/d)	1.009	[0.988, 1.015]	0.795	1.004	[0.991, 1.017]	0.555
Interaction eGFR × age	1.032	[1.015, 1.050]	< 0.001	1.035	[1.020, 1.049]	< 0.001
Interaction BMI × age	0.971	[0.948, 0.994]	0.013	0.978	[0.956, 1.001]	0.057
Interaction eGFR × diabetes	0.993	[0.967, 1.020]	0.603	1.004	[0.980, 1.029]	0.738

eGFR: estimated glomerular filtration rate, BMI: body mass index, TIA: transient ischemic attack.
**Centered on its mean.*

TABLE 3 | Performance of different models including age, eGFR, and BMI to predict serum neurofilament light concentrations.

	Model (*N* = 2,277)								
	Age* alone	eGFR† alone	BMI alone	Age* and eGFR†	Age* and eGFR† incl. interaction eGFR × age	Age* and BMI	Age* and BMI incl. interaction BMI × age	Age*, eGFR†, and BMI	Age*, eGFR†, and BMI incl. interactions eGFR × age, BMI × age
Using $eGFR_{crea}$									
R^2	0.26	0.35	0.02	0.45	0.45	0.27	0.27	0.46	0.47
R^2_{adj}	0.26	0.35	0.02	0.45	0.45	0.27	0.27	0.46	0.46
AIC	3,927	3,629	4,568	3,284	3,269	3,911	3,910	3,231	3,208
Using $eGFR_{crea-cys}$									
R^2	0.26	0.42	0.02	0.48	0.48	0.27	0.27	0.50	0.50
R^2_{adj}	0.26	0.42	0.02	0.48	0.48	0.27	0.27	0.49	0.50
AIC	3,927	3,386	4,568	3,148	3,129	3,911	3,910	3,073	3,044

R^2: coefficient of determination, R^2_{adj}: adjusted R^2, AIC: Akaike's information criterion.
**Modeled with a linear and cubic component.*
†Modeled with a linear, quadratic, and cubic component.

concentrations, which was additional and independent to the contribution of age.

Our finding of a strong negative association of eGFR with sNfL concentrations confirms and refines previous observations (Korley et al., 2019; Akamine et al., 2020). An inverse association between eGFR and blood NfL concentrations was recently shown in smaller samples of elderly patients with diabetes and healthy controls, and the renal clearance of blood NfL was proposed as one potential explanation (Korley et al., 2019; Akamine et al., 2020). Here, we show that this association is independent of age, BMI, and pre-existing disease (diabetes, stroke history, and other cardiovascular comorbidities). The association between eGFR and sNfL was maintained independent of the method used for calculating eGFR, that is, based on creatinine alone or combined creatinine–cystatin C. Importantly, the association persisted even after adjustment for brain volume, as well as for the presence and burden of ischemic infarcts and small vessel disease markers on neuroimaging, which are known to be associated with sNfL concentrations (Gattringer et al., 2017; Duering et al., 2018; Tiedt et al., 2018; Polymeris et al., 2020), indicating that it is not mediated through structural brain pathology. These findings further support that, apart from NfL release from damaged neurons, renal clearance seems to be a predominant factor determining NfL levels. Combined investigations of NfL in cerebrospinal fluid (CSF), blood, and urine to confirm this are now under way in our laboratory. Additionally, we show here that the association of eGFR with sNfL is non-linear, with a steeper slope in lower eGFR values. Taken together with our finding that the association between sNfL and eGFR depends on age (which indicates that the impact of renal impairment on

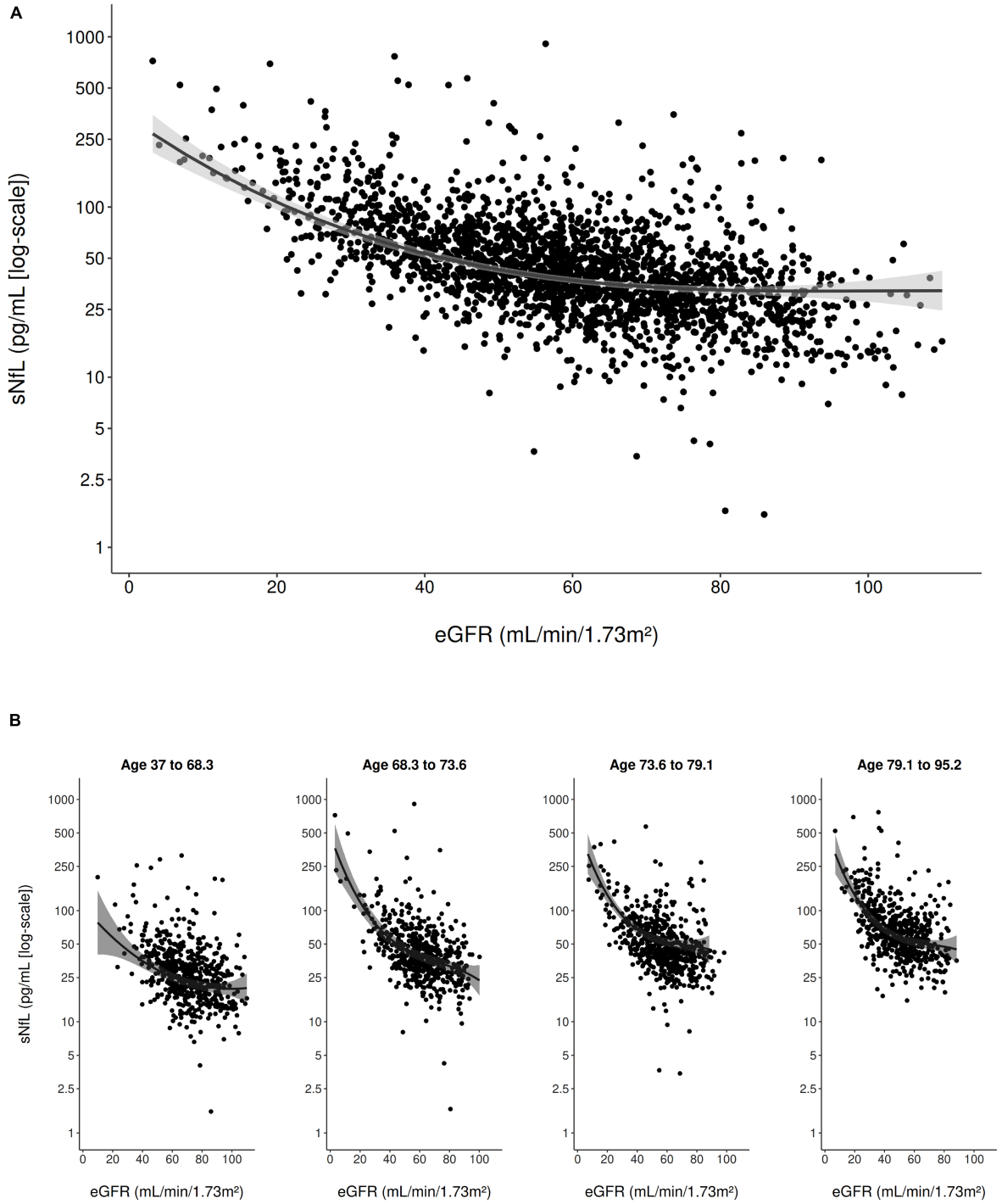

FIGURE 1 | Scatter plot of the association of $eGFR_{crea}$ with sNfL (using the log scale) in the entire study population **(A)** and stratified to age quartiles **(B)**. The solid line represents the predicted values from the main multivariable model and the gray shading represents the 95% confidence interval.

sNfL levels is even more pronounced in older than in younger patients), these data stress the importance of accounting for renal function when evaluating blood NfL concentrations in elderly populations, in whom chronic kidney disease is highly prevalent (Coresh et al., 2003).

We also found a strong inverse association between BMI and sNfL. This is in line with previous observations from large cohorts of young patients with multiple sclerosis and healthy controls, where a larger distribution volume and specifically a larger total blood volume was postulated to be a modifier

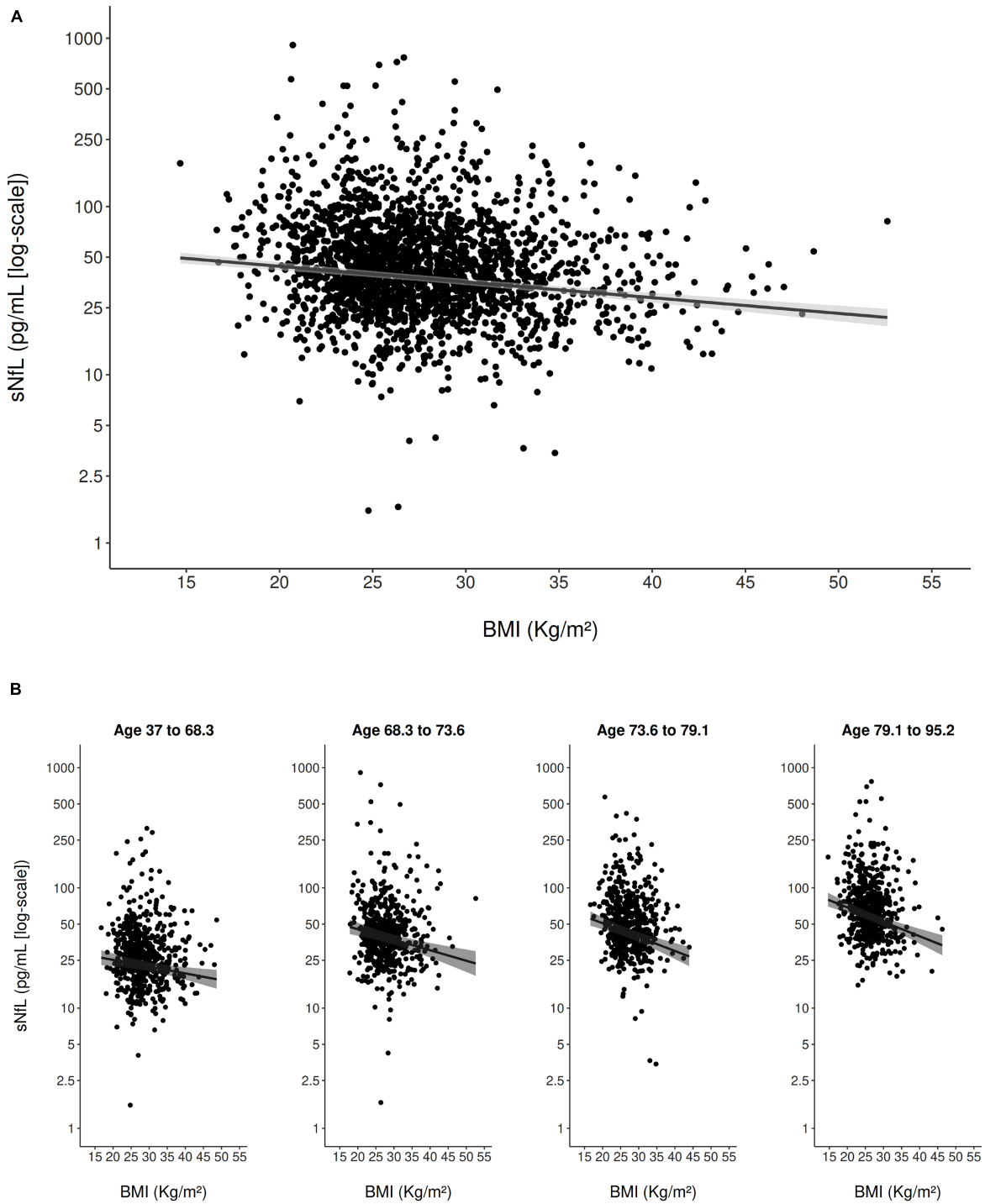

FIGURE 2 | Scatter plot of the association of BMI with sNfL (using the log scale) in the entire study population **(A)** and stratified to age quartiles **(B)**. The solid line represents the predicted values from the main multivariable model and the gray shading represents the 95% confidence interval.

of blood NfL levels (Manouchehrinia et al., 2020). Here, we expand on these findings by demonstrating that the association holds true also among elderly individuals, and is independent of age, eGFR, cardiovascular comorbidities, and the presence and burden of vascular brain lesions on neuroimaging. Furthermore, we confirmed the linearity of the association and found only a weak interaction with age. These findings strengthen the evidence for the validity of this relationship and for dilution as the underlying mechanism (Barro et al., 2020; Manouchehrinia et al., 2020). It seems therefore appropriate to account for

BMI when examining blood NfL concentrations across the entire age spectrum.

Our study provides a comprehensive assessment of the relative contribution of eGFR and BMI in determining NfL serum levels as physiological factors important in its homeostasis in the elderly. After adjustment for age and comorbidities, and regardless of the GFR estimation formula, both eGFR and BMI showed an independent, strong inverse association with sNfL levels, with effect sizes in a similar order of magnitude as age. However, only age and eGFR explained relevant proportions of the sNfL variance. Age alone explained about one-fourth, GFR alone explained approximately one-third, and their combination almost half of the variance of sNfL concentrations in this elderly cardiovascular cohort. Adding BMI did not substantially increase the explanatory power of the model. Taken together, these findings suggest that diagnostic and prognostic applications of sNfL in elderly populations should account not only for age, but also for renal function to increase their clinical meaningfulness, while the contribution of BMI seems to be less important.

Consistent with our findings, two very recent studies also demonstrated the importance of renal function as a contributor to sNfL levels (Koini et al., 2021; Ladang et al., 2022). While these studies featured smaller samples from normal aging cohorts, they further support the key conclusions of our study which examined a significantly larger sample of elderly patients with cardiovascular disease. Consequently, a large reference database for sNfL levels developed recently from data of younger individuals to optimize the use of sNfL for individual application in patients with multiple sclerosis excluded control persons with eGFR < 60 ml/min/1.73 m^2 (Benkert et al., 2022).

The strengths of this study include: (i) the large sample size of elderly patients with a detailed and standardized clinical and neuroimaging characterization, allowing for the exhaustive adjustment for multiple factors that are known to contribute to sNfL concentrations, indicating that the observed associations are not spurious but reflect true relationships; (ii) the estimation of GFR using two different approaches [the CKD-EPI formula using creatinine alone and the more accurate combined creatinine–cystatin C formula (Inker et al., 2012)] that yielded highly consistent results; and (iii) comprehensive statistical modeling investigating not only the association of eGFR and BMI with sNfL concentrations, but also their relative contribution to the variance of sNfL concentrations.

We acknowledge the following limitations: (i) The study's cross-sectional design, which allows only for the assessment of association but not causality thereof. (ii) Although our results persisted after adjustment for brain MRI characteristics, we were not able to adjust our analyses for diseases of the peripheral nervous system, which were not systematically collected in Swiss-AF but might contribute to sNfL concentrations (Khalil et al., 2018). (iii) As Swiss-AF included exclusively AF patients, we did not have a comparison group of elderly individuals without this arrhythmia. However, in light of recent studies showing consistent results in other patient populations, this limitation is unlikely to have influenced our key findings. (iv) As the Swiss-AF biosampling protocol did not include the acquisition of CSF or urine, this study was not able to examine whether the observed associations are exclusive to blood concentrations of NfL, and we may only speculate on their underlying mechanisms.

In conclusion, this study represents a comprehensive appraisal of how physiological factors including renal function and BMI are associated with and contribute to blood NfL concentrations in the elderly, thereby providing important insights into the homeostasis of this increasingly used biomarker. The role of renal function and BMI in the prediction of neurological outcomes with sNfL needs to be evaluated in prospective studies.

DATA AVAILABILITY STATEMENT

The Swiss-AF consent forms, as approved by the ethics committee, do not allow for the data to be made publicly available. Researchers may contact the authors for the potential submission of research proposals for future analyses or independent verification of our results.

ETHICS STATEMENT

Swiss-AF including this study was approved by Ethics Committee of Northwest and Central Switzerland (PB_2016-00793). The patients provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AP, LB, and JK conceived the study and drafted the manuscript, with additional support from FH, PB, and MC. FH performed the statistical analyses. All authors contributed to study design, data acquisition and analysis, and critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2022.819010/full#supplementary-material

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