Review

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Neurofilament light protein as a biomarker for spinal muscular atrophy: a review and reference ranges

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Abstract: Spinal muscular atrophy (SMA) is the leading genetic cause of infant mortality, characterized by progressive neuromuscular degeneration resulting from mutations in the survival motor neuron (*SMN1*) gene. The availability of disease-modifying therapies for SMA therapies highlights the pressing need for easily accessible and cost-effective blood biomarkers to monitor treatment response and for better disease management. Additionally, the wide implementation of newborn genetic screening programs in Western countries enables presymptomatic diagnosis of SMA and immediate treatment administration. However, the absence of monitoring and prognostic blood biomarkers for neurodegeneration in SMA hinders effective disease management. Neurofilament light protein (NfL) is a promising biomarker of neuroaxonal damage in SMA and reflects disease progression in children with SMA undergoing treatment. Recently, the European Medicines Agency issued a letter of support endorsing the potential utilization of NfL as a biomarker of pediatric neurological diseases, including SMA. Within this review, we comprehensively assess the potential applications of NfL as a monitoring biomarker for disease severity and treatment response in pediatric-onset SMA. We provide reference ranges for normal levels of serum based NfL in neurologically healthy children aged 0–18 years. These reference ranges enable accurate interpretation of NfL levels in children and can accelerate the implementation of NfL into clinical practice.

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Introduction

Spinal muscular atrophy (SMA) is a neuromuscular disorder characterized by the degeneration of alpha-motor neurons resulting in progressive proximal muscle weakness. SMA is among the most common pediatric genetic neurological diseases with a global live-birth incidence of ~1 in 5,000–10,000 [1, 2]. Despite its low incidence, SMA has the highest infant mortality rate among genetic neuromuscular disorders [1, 3]. SMA is caused by bi-allelic mutations in the survival motor neuron 1 (*SMN1*) gene, which results in impaired survival motor neuron (SMN) protein production [4]. The neighboring *SMN2* gene is paralogous to *SMN1* but a one nucleotide difference causes the exclusion of exon 7 in most *SMN2*-derived mRNAs (*SMN2*Δ7), which is translated into unstable SMN protein. A small portion of *SMN2*-mRNA transcripts includes exon 7 after splicing, leading to the generation of varying quantities of functional, full-length SMN. The main modifier of SMA severity is, therefore, the presence of a varying number of *SMN2* copies, as more *SMN2* copies result in higher levels of functional SMN protein, and consequently a milder disease course [5, 6]. SMA is traditionally categorized into four clinical subtypes based on the motor milestones achieved, with further distinctions based on the age at which symptoms first appear [3]. SMA type 1 manifests before 6 months of age and usually results in death before the child reaches 2 years. SMA type 2 is characterized by the ability to sit independently, while SMA type 3 allows individuals to stand and walk independently. Both these subtypes present a broader spectrum of symptoms including muscle weakness, increased fatigue, reduced lung function, and scoliosis, which can significantly impact their quality of life. On the other hand, SMA type 4 emerges in adulthood and allows for normal motor development and survival [7, 8].

Advancements in SMA research have now resulted in the approval of three SMN-augmenting therapies [9]. These therapies target upregulation of SMN protein in SMA patients either via enhanced correct splicing of *SMN2* (risdiplam; Evrysdi® [10] and nusinersen; Spinraza® [11]), or adeno-associated viral gene therapy (onasemnogene abeparvovec; Zolgensma® [12]). Despite the initial promise surrounding the approval and real-world use of these therapies for SMA, several uncertainties remain for patients, families, and clinicians [3]. Most importantly, although it is known that early start and better motor function before the start of treatment lead to better outcomes at the group level, it is currently not possible to provide patients with individual prognoses. Moreover, the long-term effects of SMA therapies remain to be unraveled to explain the sustained benefits or risks of treatment for SMA patients. Finally, the costs of lifetime SMA therapies and non-drug/health-related costs are a global burden [13]. Each of these examples highlights the need to develop precise, biological markers to better advise patients, families, and clinicians in the complex choices they need to make around the selection, start, and (dis-)continuation of therapies.

Because it is recognized that early treatment is the most effective, many Western countries are now including SMA in their newborn screening programs. This leads to a shift in how SMA is diagnosed: whereas initially clinical signs leading to a suspicion of SMA were confirmed by genetic testing for homozygous loss-of-function of *SMN1*, SMA is now diagnosed presymptomatically in most cases. This means that when treatment is started, clinicians and parents of children with SMA cannot be informed of the expected severity or treatment outcomes. This highlights an urgent need for better biomarkers for SMA. Currently, the only available prognostic factor for SMA is *SMN2* copy number. However, the correlation between *SMN2* copy number and disease severity is only about ~60 %, and therefore its use to inform decision-making in the clinic remains limited. Other biomarkers reflect SMA indirectly, for example, blood creatine kinase (CK) or creatinine levels, neurophysiological measurements (e.g., compound muscle action potential [CMAP]), magnetic resonance imaging (MRI), and muscle ultrasonography [14, 15]. However, these biomarkers are ineffective at monitoring disease progression over time and tracking neurodegeneration [16, 17]. Moreover, these currently used biomarkers provide insights into downstream processes of SMA such as muscle function but cannot monitor motor neuron degeneration directly. In this respect, neurofilament light protein (NfL) is a promising biomarker to add to the diagnostic and prognostic panel, as it directly reflects neuroaxonal damage leading to clinical dysfunction. Blood (plasma/serum)-based as well as cerebrospinal fluid (CSF)-based NfL levels are elevated in patients with SMA and reflect disease severity [18, 19]. Moreover, NfL levels rapidly decline upon administration of effective disease-modifying therapeutics in SMA [18–23]. Therefore, we hypothesize that NfL would be an accessible tool for monitoring treatment responses in SMA. We here review and discuss the recent data on the use of NfL as a monitoring or treatment response biomarker in SMA. First, we introduce the NfL protein and its metabolism. Next, we highlight its use as a biomarker in pediatric neurological diseases, especially SMA, based on recent studies. We then present normal physiological NfL levels in neurologically healthy children. Lastly, we address the challenges and steps to broadly adopt NfL as a biomarker to aid in SMA management.

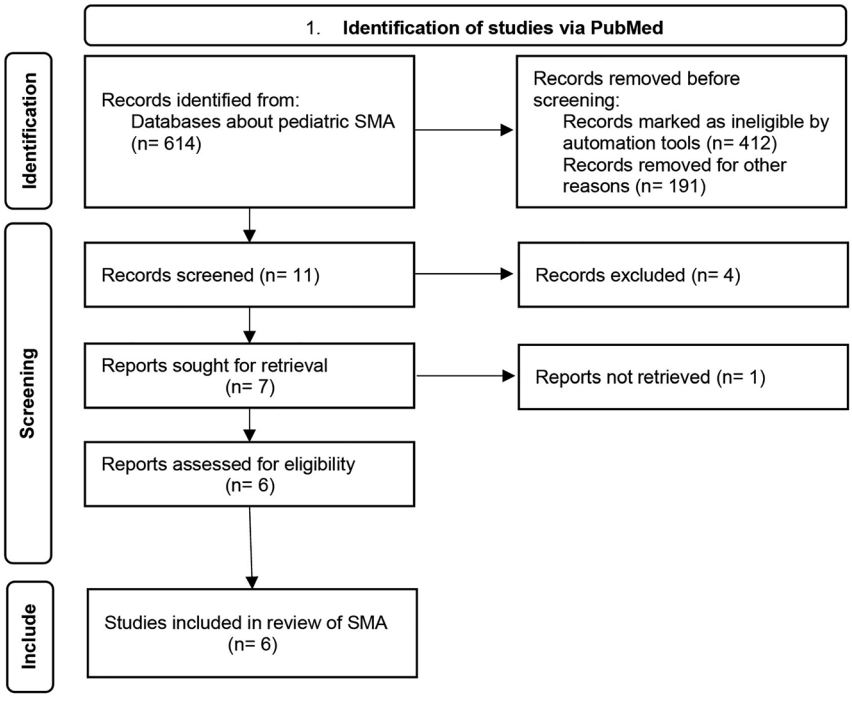
Methods

Data sources and record identification

To identify relevant studies, we performed an online search in PubMed until August 2023, applying the search terms ([neurofilament light AND pediatric spinal muscular atrophy] OR [neurofilament light AND pediatric sma] OR [nfl AND pediatric spinal muscular atrophy] OR [nfl AND pediatric sma] OR [nfl AND sma] [nf-l AND sma]). There was no date restriction. The search was restricted to articles published in English. We excluded reviews and studies conducted on animals. We included case-control and treatment-response studies. Data selection was in concordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [24]. We included six relevant studies of NfL in pediatric SMA. A flow diagram outlining the systematic review is shown in Figure 1A and the selection criteria in Figure 1B. Figures 1 and 3 were plotted using GraphPad Prism version 9.1.0 (221).

Statistical analysis

For Figures 2 and 4, and Table S1, we collected age, NfL levels, and subgroups of patients from the reviewed literature. We report the median/mean as presented by the authors of the studies.



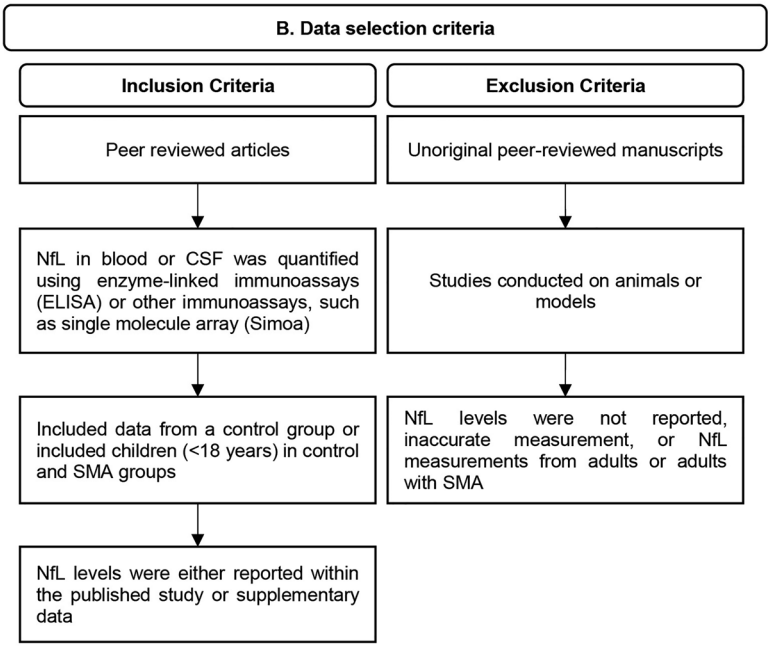


Figure 1: A. Flow diagram of the literature review. Records were searched on PubMed. B. Selection criteria for the reviewed data (inclusion and exclusion). From the literature reviewed, we extracted (1) bibliographic information (study title, year of publication, first author, study type), (2) clinical features of the sample (number of patients and controls, age, sub-groups, and treatments), (3) the used assay and (4) levels of the NfL.

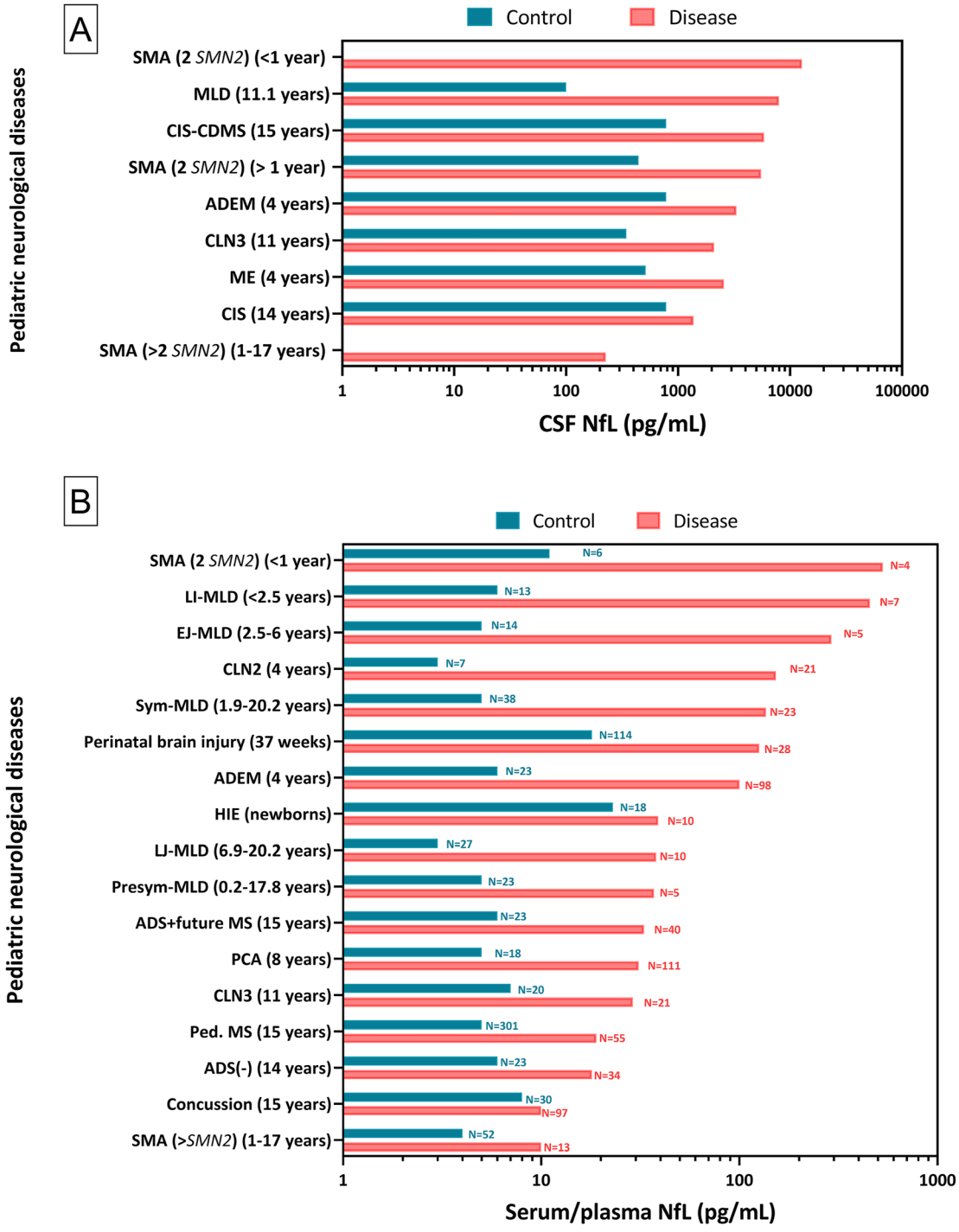


Figure 2: Levels of CSF-NfL and serum/plasma NfL levels in pediatric neurological diseases and controls as reported in the literature. A. Levels of CSF-NfL in pediatric neurological diseases and matched controls. B. Levels of plasma or serum NfL in pediatric neurological diseases and matched controls. Levels of NfL are presented in pg/mL on the X-axis and pediatric diseases are listed on the Y-axis. Column labels represent serum/plasma or CSF NfL levels and (n) within the tested cohorts. Age between brackets refers to the age of children for the related neurological diseases in each of the studies. The x-axes in A and B were log-transformed for better visualization of the NfL levels in control individuals. ME, mitochondrial encephalopathy [25]; LI-MLD, late infantile metachromatic leukodystrophy [26]; EJ-MLD, early juvenile metachromatic leukodystrophy [26]; CLN2, neuronal ceroid lipofuscinosis type 2 disease [27]; sym-MLD, of symptomatic MLD [26]; ADEM, acute disseminated encephalomyelitis [28]; HIE, perinatal hypoxic-ischemic encephalopathy [29]; LJ-MLD, late-juvenile MLD [26]; presym-MLD, presymptomatic MLD [26]; ADS + future MS, patients without acute disseminated encephalopathy with future multiple sclerosis [30]; PCA, pediatric cardiac arrest [31]; CLN3, neuronal ceroid lipofuscinosis type 3 [32]; MS, pediatric multiple sclerosis; ADS(−), Monophasic acute disseminated encephalopathy [30]. The selection criteria of the articles are listed in Supplementary Figure 1B. The NfL data was

For the reference serum NfL levels, we compiled the data from four different cohorts [19, 26, 35, 36]. Categorical parameters (i.e., cohorts and sex) were described by counts and percentages, and continuous parameters by the medians and interquartile ranges (IQR). Quantile regression was employed to determine the quantile percentiles of NfL levels [34, 37]. We calculated age-specific percentile ranges for the neurologically healthy children by simple quantile regression with age as the predictor over the age range (i.e., 0–18 years). Before performing the quantile regression and plotting the percentiles, we adjusted the data for age and sex to account for potential confounding effects. Age was treated as a continuous variable, and sex was included as a binary categorical variable. The NfL levels were divided based on the age of 9 years, differentiating individuals into two groups: young (<9 years) and old (>9 years). The rationale behind applying this age split at 9 years, was driven by its superior fit during the data analysis. We applied a segmented regression to model the association over the entire age range. Segmented regression was used to estimate the breakpoint for the relationship between the log2-transformed NfL levels and Age. The breakpoint was found to be approximately 8.72 pg/mL. This suggested that there is a significant change in the relationship between NfL levels and the age of the neurologically healthy children in our data around the age of 8.72 years. Before this age, the relationship might follow one trend, and after this age, it might follow another trend. We calculated 5th, 10th, 25th, 50th, 75th, 90th and 95th percentiles. The percentiles express the percentage of the general population aged 0–18 years, expected to have serum/plasma NfL levels lower than that given level of NfL. NfL levels were log-transformed to meet the assumptions of normality. We performed the Wilcoxon rank sum test with continuity correction to compare the ages between males and females. Statistical tests were two-tailed, and p-values <0.05 were considered statistically significant. R was used for statistical computing (v.4.0.3, with the packages 'tidyverse' [38], 'lme4' [39], 'rmcorr' [40], and 'ggplot2' [41]).

Literature review on the potential use of neurofilament light protein in pediatric SMA

Neurofilament light protein and its metabolism

NfL is a major cytoskeleton intermediate filament protein [42–44]. NfL belongs to the family of highly phosphorylated intermediate filaments, which additionally includes neurofilament heavy (NfH), neurofilament medium (NfM), perinephrin, and alpha-internexin proteins [42, 45]. Together with alpha-internexin, NfL forms the backbone of the neurofilament to which the NfM and NfH subunits attach. NfL is highly abundant in the cytoplasm of long myelinated neurons and is present in neurons in the central and peripheral nervous system (CNS and PNS) [44, 46]. The full function of NfL is not fully understood but is hypothesized to play a role in radial growth, enable rapid nerve conduction, provide structural support for axons, and regulate the axonal diameter [45]. NfL is primarily found as a full-length protein in brain tissue and appears in various fragments in the CSF [47]. The mechanisms by which NfL or its fragments move between the brain, CSF, and blood compartments remain unclear. It is also unknown whether NfL or its fragments differ between CNS and PNS sources. Several hypotheses regarding NfL release, turnover, and degradation have been proposed [48]. One possible mechanism of clearance from the extracellular space could be through retro-fusion events of multivesicular bodies from the axonal plasma membrane. Multivesicular bodies are present in axons and axon terminals of the CNS and PNS [48]. Such multivesicular bodies were reported to contain various accumulated protein aggregates in the case of neurodegenerative diseases and aging, including aggregated neurofilaments [45]. Additionally, proteolytic [45] or autophagy [49] pathways have been proposed as possible mechanisms of NfL release, turnover, and degradation.

Neurofilament light protein as a fluid biomarker

Pathological processes that cause acute axonal damage lead to the release of NfL into the CSF and peripheral blood (Figure 2). As a result, NfL in CSF and serum/plasma has proved to be a reliable biomarker of axonal injury, axonal loss, and neuronal death in numerous neurological diseases [42]. The correlation between CSF NfL and serum/plasma NfL levels was reported to be moderate-strong to strong and agreement between changes in CSF NfL and serum/plasma NfL levels in response to ongoing neuro-axonal injury was reported [19, 26, 50–53], implying that serum/plasma NfL could reliably replace CSF NfL measurements.

Many studies on NfL have been conducted in adult-onset neurological diseases, including but not limited to, multiple sclerosis [54, 55], spinal cord injury [51], amyotrophic lateral sclerosis [56], and Alzheimer's dementia [57]. Studies about the potential role of NfL in pediatric neurological diseases are relatively scarce compared to studies investigating the use of NfL in adult-onset diseases but have provided

reported as means ± SD in the following studies [25, 32, 33], geometric mean [30], or as medians in the remaining studies. NfL concentrations were plotted as reported in the earlier-mentioned studies. Some studies [26, 27, 29–32] utilized the NF-light Advantage Simoa kit™ (Quanterix), while others [23, 25, 28] used NF-light ELISA from UmanDiagnostics (Umeå, Sweden). However, before plotting NfL levels in the Figure, we converted the NfL levels generated using the UmanDiagnositcs ELISA kit into the Simoa kit according to the equation generated in a previous study by our group [34]. PCA is included for comparative purposes but is not considered a neurological disease. PCA data is presented to provide insights into a disease with neurological implications along the pediatric neurological diseases.

valuable insights into axonal and neuronal loss in pediatric SMA, pediatric multiple sclerosis (MS) [55], metachromatic leukodystrophy (MLD) [26], pediatric traumatic brain injury [58], brain damage in premature babies and neonates [59–61] and others. We summarized the results in Figure 2. The findings from the studies indicate that levels of NfL in CSF and serum/plasma are consistently higher in children suffering from neurological disorders when compared to typical levels of the control subjects included in the studies. Notably, the elevation in NfL levels was particularly pronounced in cases of SMA and MLD. Following these, neuronal ceroid lipofuscinosis (CLN) and pediatric MS also showed significant elevations, though not as extreme as in MLD and SMA. Accordingly, NfL is considered a cross-disease neuroaxonal biomarker, associated with disease state, and not considered a disease-specific biomarker [42, 43].

The integration of NfL findings in pediatric neurological disease research has led to a pivotal development, wherein the European Medicines Agency (EMA) has recently acknowledged the potential of NfL as a promising biomarker to quantify axonal damage and its several contexts of use, especially in pediatric neurological diseases. This recognition was officially conveyed through a letter of support issued by the EMA, endorsing the application of NfL as a monitoring tool for pediatric neurological diseases [62]. The most extensive data on NfL in pediatric neurological diseases has been documented for SMA. Studies indicate that NfL levels are notably elevated in children with SMA, particularly those with ≤2 *SMN2* copies, compared to those with other pediatric neurological conditions (as depicted in Figure 2 and Supplementary Table 1) [18–20, 23]. Since the largest body of evidence has been obtained in patients with SMA, this review further focuses on the potential use of serum/plasma or CSF NfL as a biomarker for different intended uses in SMA. Figure 3 presents a simplified illustration of the release of NfL into serum/plasma or CSF following neuroaxonal injury in children with SMA, compared to neurologically healthy children.

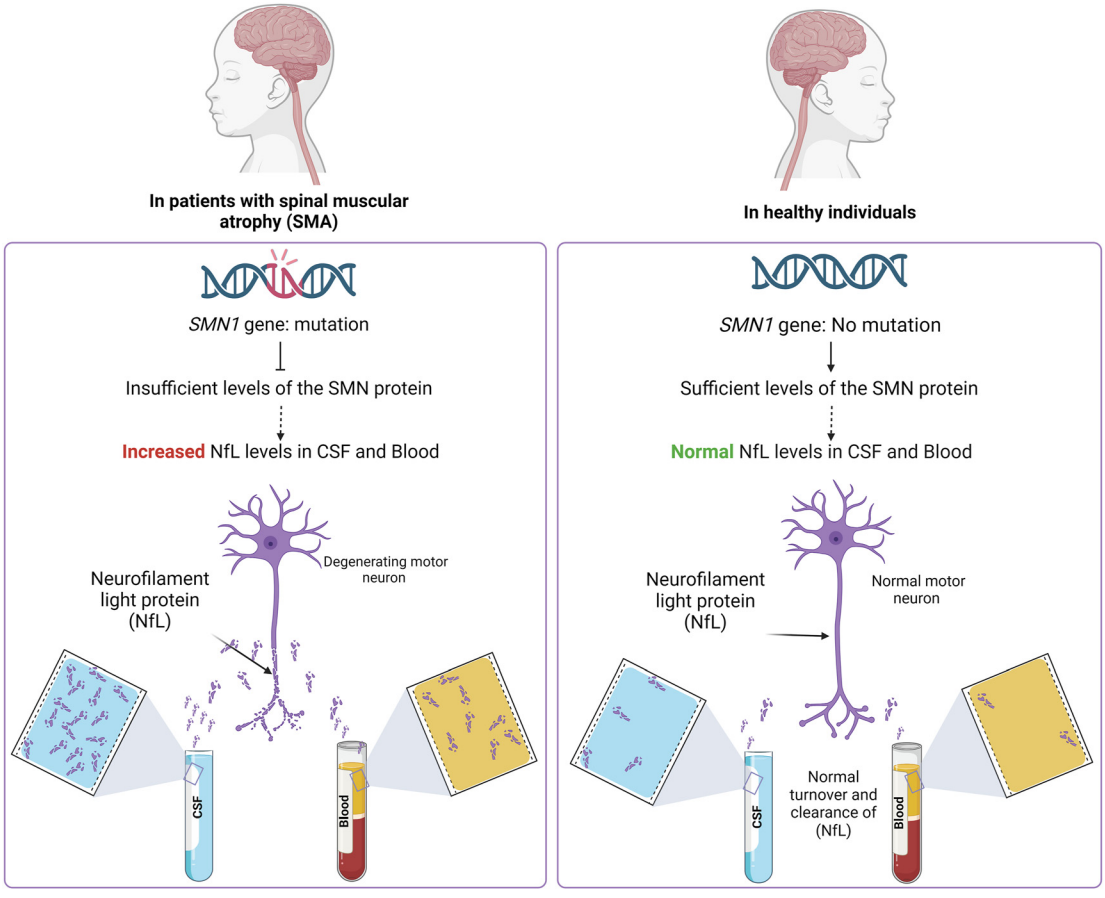


Figure 3: Neurofilament light (NfL) protein is released into body fluids upon neuroaxonal damage in patients with SMA. When an axon is injured neurofilament proteins, including NfL, are released into the extracellular space and cerebrospinal fluid (CSF) and, at relatively lower levels, into the blood. Created with Biorender.com.

Neurofilament light protein as a biomarker for disease severity in SMA

Serum/plasma and CSF NfL levels are the highest in SMA patients with 2 *SMN2* copy numbers, compared to those with >2 *SMN2* copy numbers [18, 19, 22] (Figure 2; Supplementary Table 1). In agreement, higher baseline NfL levels were shown to be associated with earlier age of disease onset [18]. This suggests that NfL level in SMA is dependent not only on disease severity but also on disease stage. Since *SMN2* copy number correlates with disease severity, NfL levels could predict disease severity in treatment-naive SMA patients, on a group level [18]. For example, compared to controls, serum NfL levels were 11- to 50-fold higher in SMA patients with ≤2 *SMN2* copies [18, 19], 3 to 9-fold higher in SMA patients with 3 *SMN2* copies, and 2 to 4-fold higher in SMA patients with 4 *SMN2* copies [18, 19]. So far, the most elevated serum NfL level in SMA was reported in a treatment-naive patient younger than 1 year carrying ≤2 *SMN2* copies, which reached 1,100 pg/mL, compared with ~20 pg/mL in the age-matched controls [19]. However, serum/plasma NfL measurements may overlap in patients with 2 and 3 *SMN2* copies, which requires CSF NfL measurements. Further, in the CSF of patients with ≤2 *SMN2* copies, NfL levels were around 30-fold higher, compared to controls [23].

The fact that NfL is a cross-disease biomarker, precludes its use as an SMA-specific diagnostic marker. However, given the correlation of elevated levels of NfL with *SMN2* copy number, NfL might be a suitable marker for disease severity, and as such indicative of the prognosis. In addition, in patients with a mild disease course (e.g., infants with >4 *SMN2* copies) in whom treatment will not be initiated directly after receiving new-born genetic test results, increasing NfL levels can aid in a timely start of therapeutic interventions, which could lead to better long-term health outcomes. However, due to the expeditious change in rules between countries and reimbursement rules of SMA therapies, NfL could be used to further inform treatment decisions. Finally, in the advanced stages of all SMA variants, the trajectory of NfL levels – whether they rise, decline, or plateau – has yet to be elucidated. A longitudinal assessment of NfL levels is needed to ascertain the long-term efficacy of SMA treatments and their corresponding impact on NfL concentrations.

Neurofilament light protein in monitoring of SMA treatment

Studies investigating the treatment effects of the approved therapies (i.e., risdiplam, nusinersen, and onasemnogene abeparvovec) consistently show that the majority of patients under treatment either achieved improved motor function or could maintain motor function levels [18–23]. The best results in terms of motor function improvement or maintenance were reached when treatment was started at an early age and less severe disease state [18–23]. Up to now, NfL has been mainly reported in studies investigating nusinersen treatment. The administration regimen of nusinersen starts with four initial loading doses. The initial three doses are administered at 14-day intervals, followed by the fourth dose administered 30 days after the third. Following the initial doses, maintenance doses of nusinersen are given three times annually [18–23]. Those studies demonstrated that upon administration of nusinersen, NfL levels rapidly decline with the largest decline seen in patients with SMA that carry ≤2 copies of *SMN2* [18–23]. For instance, research indicated that serum NfL levels began to decline after the third dose, dropping from an average of 519 pg/mL to 36.8 pg/mL by the seventh [19]. While patients with >2 *SMN2* copies exhibit lower serum NfL levels than those with 1 or 2 *SMN2* copies [19] and also experience a relatively slower disease progression due to the higher copy numbers, it has been validated by two studies that both average serum NfL [19] and CSF NfL [22] levels decreased over time when these patients were treated with nusinersen. Moreover, in patients under SMA treatments, improved motor function scores were strongly correlated with declined serum and CSF NfL levels in SMA patients carrying ≤2 *SMN2* copies [19, 20, 23]. No correlations were found between motor function scores and serum or CSF NfL levels in patients under treatment with ≥3 *SMN2* copies [19, 22]. It is important to highlight that one study observed considerable variations in motor function scores over time in children with 2 SMN2 copies, with even more pronounced fluctuations in children with more than 2 *SMN2* copies [19]. The fact that NfL levels were stable in these patients while the motor function scores fluctuated largely, suggests that NfL has the potential as a biological readout of disease severity to aid treatment continuation decision-making. This is illustrated by a case report that presented a 7-year-old child with the lowest possible motor function score (i.e., minor disability; and no possibility for detecting clinical improvement with this score), CSF NfL levels did decrease upon treatment [21] suggesting that treatment might be biochemically effective in this patient. Together, the NfL data show that NfL can be used to monitor nusinersen treatment response in patients with SMA, mainly in those who carry ≤2 *SMN2* copies. Thus far, one study has shown that patients who receive a combination of nusinersen and onasemnogene abeparvovec had lower serum NfL levels compared to untreated patients [18]. There is a need to evaluate and monitor the neurodevelopment and

treatment response in children receiving SMA therapy other than nusinersen or combinations of the different therapies. Nevertheless, given the observed treatment responses in patients administered nusinersen, we speculate that NfL levels will correlate with the responses to other types of treatment. A graphical summary of the results of clinical studies on NfL levels to monitor SMA therapy response is presented in Figure 4. Finally, there are no studies about the longitudinal fluctuations of NfL levels in untreated patients.

Normal reference serum NfL levels in children

To interpret NfL levels in the management of SMA, neurologically healthy reference levels in neurologically healthy children are needed. Like in adults [34, 63, 64], NfL levels vary with age and are age-dependent in children. Where NfL levels increase with age in adults, NfL levels were shown to be elevated early in life, after which the levels gradually decrease until the age of 18 years [19, 26, 55, 65]. To estimate age-specific reference levels for NfL in the serum of neurologically healthy children, we compiled single serum NfL measurements of 240 neurologically healthy children from four cohort studies (demographics presented in Table 1) [19, 26, 35, 36].

Across these cohorts, the children's ages ranged from 2 months to 18 years (median age 6.3 years). The NfL levels observed in these children spanned from 1.4 to 49.4 pg/mL (Table 1). The NfL levels did not differ by sex (p=0.128). However, there was a significant negative association between serum NfL and age of the neurologically healthy children (p<0.001). Table 2 shows the percentiles by age, indicating normal serum NfL levels (median [10th–90th percentile]) at different stages, going from 10 [6–17] pg/mL at age zero, to 5 [3–7] pg/mL at age 6 years, and 3 [2–5] pg/mL at age 16 years. We utilized quantile regression to estimate age-specific percentile ranges [53, 56]. Figure 5 provides a visual representation of the data fit.

As an illustration of how the provided NfL reference ranges can be implemented clinically (Table 2), we selected the data of 10 children with SMA for whom NfL data were available both at baseline and upon the 7th dose of nusinersen [19]. We assessed the baseline levels of NfL in correlation with age. Overall, at baseline, NfL levels in 8 children with SMA were above the 90th percentile of normal NfL levels, while in two children the levels were within the 75th percentile. NfL levels seemed to decline over time to levels below or around the medians estimated for the age groups in Table 2. Therefore, these reference percentiles of NfL in neurologically healthy children provide a practical visualization tool to estimate the relative increase of serum NfL levels in young children with SMA.

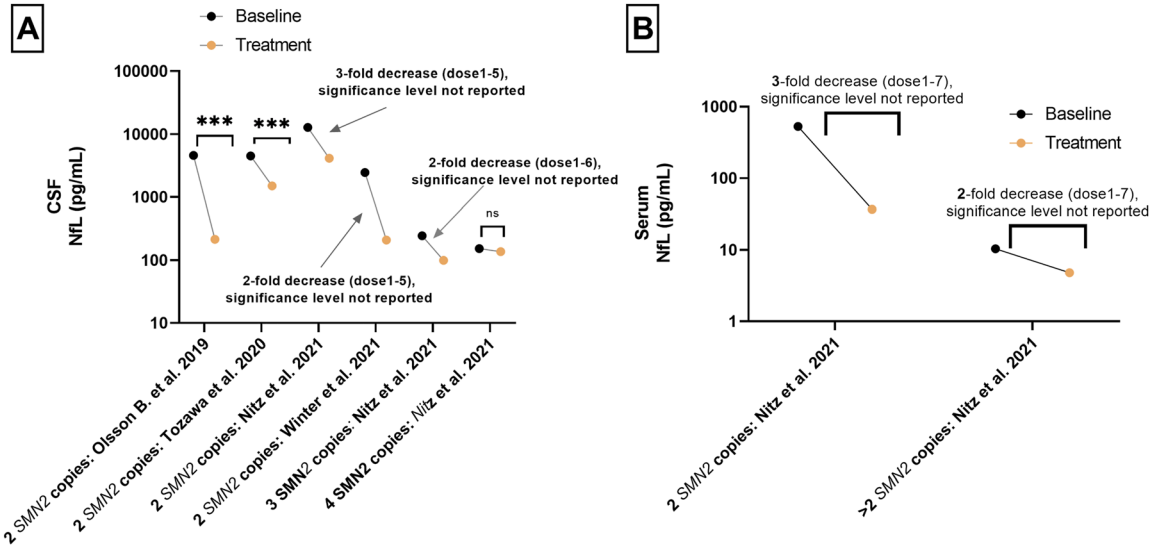


Figure 4: The potential of CSF and serum/plasma NfL as a biomarker to monitor treatment effects in SMA. (A) Demonstrates the decrease in CSF NfL levels in SMA 1 and SMA 3 upon treatment, as cited in the noted studies. (B) Demonstrates the decrease in serum or plasma NfL levels upon treatment of each disease compared to untreated patients, as cited in the noted studies. NfL medians or means were plotted based on the cited studies. The studies by Olsson et al. reported mean NfL ± standard deviation, Nitz et al., reported median NfL levels, and Winter et al. [20], and Olsson et al. [23], were case report studies.

Table 1: Characteristics of the neurologically healthy children included in our quantile regression analysis.

	Total	Stratified for cohort			
		Cohort 1 [35]	Cohort 2 [26]	Cohort 3 [19]	Cohort 4 [36]
Total, n	240	38	34	97	71
Sex male, %	136 (57 %)	16 (42 %)	22 (65 %)	54 (56 %)	44 (62 %)
Age, year (median [range])	6.8 [0.16–18.2]	11.5 [8.0–17.9]	2.7 [0.4–17.5]	6.3 [0.2–18.2]	5.9 [0.2–16.2]
NfL, pg/mL (median [range])	4.7 [1.4–49.4]	3.0 [1.5–5.9]	5.8 [1.9–19.9]	4.7 [1.8–25.0]	5.9 [1.4–49.4]

In cohorts 1, 2 and 3, healthy children were recruited as age and sex-matched controls in each study, while cohort 4 included samples from children who underwent small surgeries. NfL: neurofilament light chain.

Discussion

The reviewed data showed that plasma/serum and CSF NfL levels are the highest in children with SMA compared to those with other pediatric neurological diseases. Among patients with SMA, those with ≤2 *SMN2* copy numbers were compared to those with >2 *SMN2* copy numbers. The studies examined in our review demonstrate the potential of NfL measurements as a biomarker to monitor disease severity or treatment response in SMA. The data consistently indicate significantly elevated NfL levels in children with SMA, particularly in those with ≤2 copies of *SMN2* compared to patients with >2 *SMN2* copies. NfL levels correlate with the severity of SMA, and this correlation seemed notably stronger in patients with ≤2 *SMN2* copies compared to those with more copies, although long-term follow-up studies in patients with more *SMN2* copies in a more advanced SMA disease state are needed to confirm this observation. Furthermore, NfL levels rapidly decrease following the administration of SMA therapies, particularly in patients with ≤2 copies of *SMN2*, but also in those with more *SMN2* copies. Based on the available literature, we can therefore recommend using NfL as a biomarker in SMA to monitor disease severity and treatment responses, particularly in young children with ≤2 *SMN2* copies who receive treatment, but also in patients with more *SMN2* copies to monitor disease progression. While NfL may also hold promise as a biomarker in pediatric neurological diseases other than SMA, the available data in those diseases is still limited, precluding us from formulating a solid recommendation for the use of NfL as a biomarker in any pediatric neurological disease. In line, the EMA recently recognized the potential of NfL as a promising biomarker for quantifying axonal damage in pediatric neurological diseases in general, for various contexts of use [62].

To facilitate the interpretation of serum/plasma-based NfL levels in SMA, we provided age-related NfL normal reference ranges for neurologically healthy children. We compiled the NfL data from four cohorts totaling 240 neurologically healthy children, covering the age range from 2 months to 18 years. The CLSI guideline (EP28-A3c), recommends a minimum of 120 samples per age group for establishing reference intervals [66]. In our study, given the constraints posed by the limited sample size, we employed

Table 2: Reference percentile serum NfL levels in neurologically healthy children.

Age, years	5th percentile, pg/mL	10th percentile, pg/mL	25th percentile, pg/mL	50th percentile, pg/mL	75th percentile, pg/mL	90th percentile, pg/mL	95th percentile, pg/mL
0–1	5.0	5.6	7.4	9.5	12.0	16.9	18.2
1–2	4.5	5.1	6.7	8.5	10.8	14.7	16.6
2–4	4.1	4.7	5.9	7.5	9.6	12.7	15.0
4–6	3.5	3.9	4.7	5.9	7.7	9.6	12.4
6–8	2.9	3.2	3.8	4.7	6.2	7.3	10.2
8–10	2.4	2.7	3.0	3.7	5.0	5.5	8.4
10–12	1.9	2.1	2.9	3.8	4.3	5.5	6.0
12–14	1.8	2.0	2.9	3.6	4.3	5.5	6.2
14–16	1.8	1.9	2.9	3.5	4.3	5.4	6.5
16–18	1.7	1.9	3.0	3.4	4.2	5.3	6.7

The Table shows percentile levels of serum NfL in neurologically healthy children population by age groups. These data are based on a reference group of 240 patients and research participants without a neurological disease, aged 0.2–18.2 years. The reference data are based on plasma NfL measurements on Simoa with the Quanterix NF-Light kit.

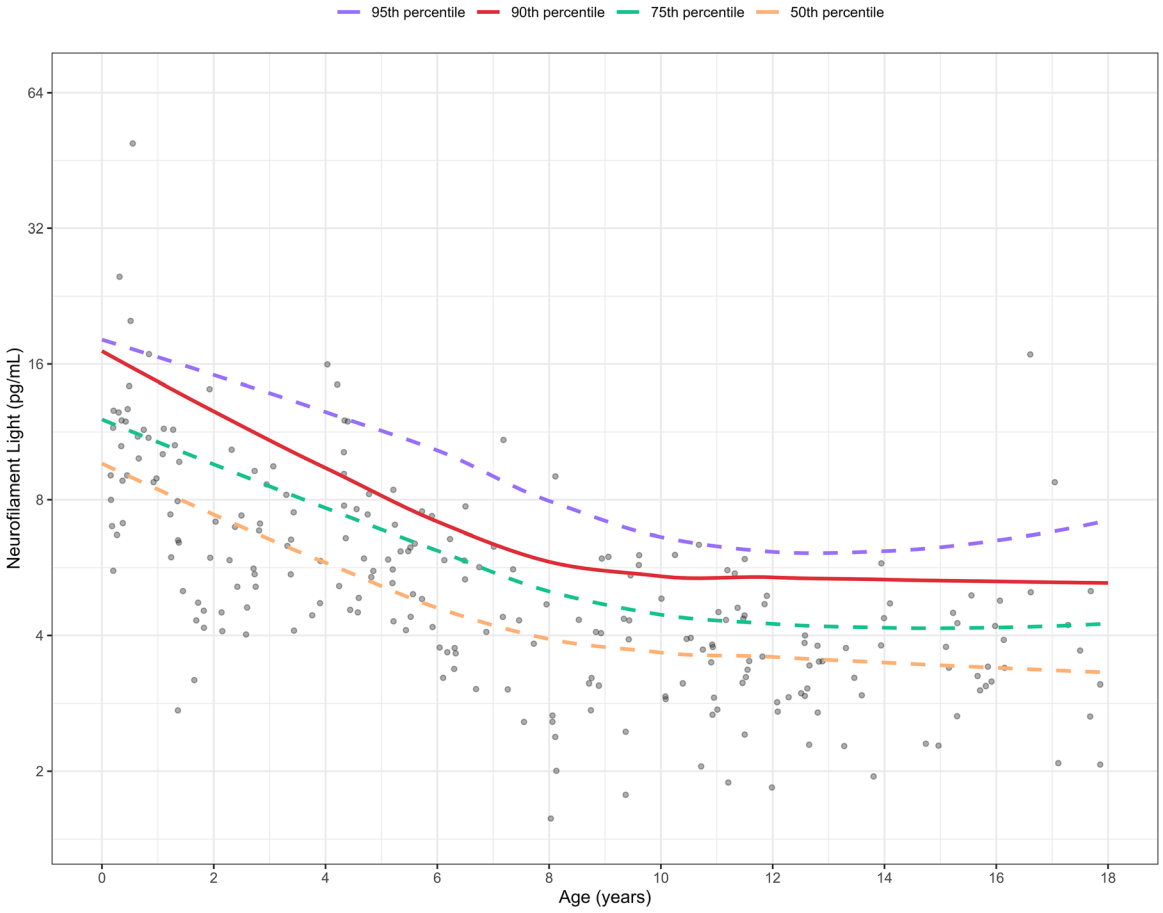


Figure 5: Serum NfL levels by age in neurologically healthy children, color-coded for percentiles. Percentiles were calculated using quantile regression. A total of 240 NfL measurements of neurologically healthy children were included in the analysis. The solid line represents the 90th percentile and the dashed lines represent the 50th, 75th and 95th percentiles. The reference percentiles of serum NfL levels are in Table 2.

non-parametric quantile regression, a statistical approach deemed suitable to the specific characteristics of our dataset. Our cohort fell short in terms of samples per age group, leading to our deviation from explicitly adhering to the CLSI guideline. In this dataset, we visualized a steady decline from birth to around age 9 years, after which the levels stabilized until age 18. Our reference NfL ranges align mostly well with those provided in a recent extensive study [65] as well as in other studies with smaller cohorts [55, 67, 68], where serum NfL levels showed similar age-dependency in healthy children. Estimating percentiles becomes increasingly challenging in the upper percentiles, due to the limited amount of data points, which results in a large influence of the small number of outliers for our 95th percentile estimate, where the 90th percentile and lower are better reflecting the data. The 90th percentile is comparable to the 95th percentile of a recently published large reference cohort [65]. There was no difference in serum NfL levels per sex, in line with previous findings in both children and adults [55, 63, 65, 67].

Elevated serum NfL levels in neurologically healthy newborns and early childhood may be attributed to high cell turnover during neuronal migration and differentiation, as well as developing blood-brain barrier and CSF flow rate influences. In neurologically healthy newborn children and early childhood, the elevated levels of serum NfL were hypothesized to be attributed to the high cell turnover that occurs during the migration and differentiation of neurons in the developing brain [55]. Additionally, it has been suggested that the developing blood-brain barrier and CSF flow rate may have an influence on NfL levels during this period [55]. Studies focusing on late adolescence until old age showed that NfL levels gradually increase with age [34, 64]. In those individuals, the gradual increase in NfL levels may be attributable to the mechanism of aging which

encompasses neurodegenerative diseases, which may start in late adolescence [8].

Our results indicate the relevance of tracking changes in NfL over time in children's diseases with reference to the natural dynamics of NfL changes by age. Nevertheless, owing to the reproducibility of the results obtained for NfL across studies and laboratories [69], largely attributed to the sensitivity characteristics and reproducible performance of the NF-light Advantage Simoa kit™ (Quanterix), the reference ranges we provide could be applied to assess NfL levels, monitor individual patients and perform cross-comparison studies. To enhance the clinical validity of these reference ranges, future studies should encompass diverse cohorts of neurologically healthy children and those with various neurological diseases. Adhering to the CLSI guideline C28-A3 for minimum samples per age group, these follow-up studies would contribute to fine-tuning the presented reference ranges. Furthermore, such efforts aim to broaden the utility of these ranges in neurological diseases beyond SMA, mirroring our NfL application in diseases like MS and several types of dementia.

Before NfL can be widely adopted as a biomarker for SMA, several facets of its application necessitate further exploration. The EMA's Letter of Support [62] underscored some of these concerns, particularly its use in SMA. Determining the precise context of use (CoU) for NfL in SMA demands additional research. Emphasis should be on real-world studies that gauge its prognostic value and ability to monitor treatment response in SMA. It is also relevant to compare the benefits of NfL measurements to existing clinical and biomarker assessments for different CoUs. The utility of NfL in patients having more than two *SMN2* copies remains unclear, especially regarding its relationship with SMA phenotypes and *SMN2* copy number [62]. While the relationship between NfL levels and clinical presentations in patients with milder SMA forms is less clear compared to those with a more severe disease trajectory, NfL may play a crucial role in monitoring disease severity. Longitudinal studies on untreated SMA patients are pivotal to assessing the link between NfL levels, SMA severity, and clinical outcomes. For instance, untreated children with MLD exhibited a decline in NfL levels [26]. Given such observations, understanding the trajectories of NfL levels in untreated SMA patients is invaluable for refining clinical assessments and guiding therapeutic interventions.

From a technical and logistical standpoint, the development of ultrasensitive serum/plasma NfL assays on high-throughput *in vitro* diagnostic (IVD) platforms would further facilitate the implementation of NfL measurements for clinical care. This could be complemented by the development of NfL assays on point-of-care technologies to enable near-patient frequent and efficient monitoring. Additionally, adopting dry blood spots (DBS) as sampling tools in infants could enable the collection of lower volumes of blood, compared to venipuncture, and direct NfL testing in newborns genetically confirmed to have SMA after newborn screening. DBS could further facilitate NfL measurements in daily practice because it is a reliable option in resource-limited settings such as rural clinics [70]. However, such analysis using DBS still requires more extensive technical and clinical validation. In addition, an ongoing effort to develop NfL reference materials to calibrate all clinical NfL assays is crucial to harmonize NfL levels obtained across different platforms and laboratories [71]. This approach will further facilitate the implementation of NfL in clinical practice [71].

In conclusion, although further clinical validation, as well as technical laboratory advancements, are needed to accelerate the wide implementation of NfL as a biomarker in SMA and other pediatric neurological diseases, based on the reviewed literature and outlined in a letter of support by the EMA on the added value of NfL in monitoring childhood neurological disease, we can recommend the use of NfL in patients with SMA, especially in those who carry ≤2 *SMN2* copies.

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