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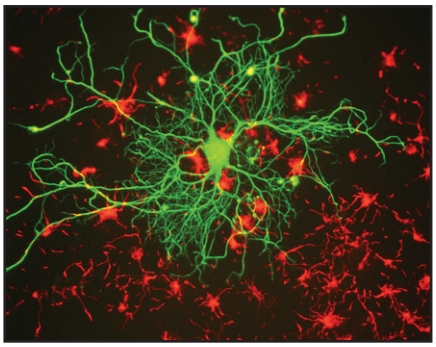

# CMSC Consensus Statement on Neurofilament Biomarkers in Multiple Sclerosis



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# CMSC Consensus Statement on Neurofilament Biomarkers in MS

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## Target Audience

This activity has been designed to meet the educational needs of physicians, nursing professionals, PAs, and other members of the healthcare team involved in the management of patients with multiple sclerosis (MS).

## Learning Objectives

Upon completing this activity, participants should be better able to:

- Review techniques used to measure neurofilament biomarkers in serum and cerebrospinal fluid of patients with MS
- Define neurofilament light chain (NfL) values and timing of NfL measurements and their clinical relevance in MS
- Discuss the influence of comorbid medical conditions on NfL outcomes
- Outline available strategies for applying NfL as a biomarker to aid in the clinical management of MS

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**About the cover:** Image shows rat brain cells grown in tissue culture and stained (green) with an antibody to neurofilament light (NfL) revealing a large neuron. Red stain indicates alpha-internexin in neuronal stem cells surrounding the large neuron. Image courtesy of EnCor Biotechnology Inc.

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# Consensus Statement on Neurofilament Proteins in Multiple Sclerosis

## CMSC Consensus Panel on Neurofilament Biomarkers in MS

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Neurofilaments are intracellular cytoskeletal proteins that leak into cerebrospinal fluid (CSF) and blood as a result of neuronal damage.[1] CSF and blood neurofilament levels are elevated, compared with age-matched controls, in a number of neurologic diseases such as multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and Parkinson's disease, as well as traumatic brain injury (TBI).[2,3] In MS, baseline levels are predictive of long-term prognosis.[4] Change in serum or cerebrospinal fluid (CSF) neurofilament levels is emerging as an important biomarker to predict or detect disease progression and even response to disease-modifying therapy (DMT).[5] Such blood and CSF biomarkers are significantly lacking and much needed in MS care and research.

Serum and CSF assays for neurofilament light chain (NfL) are now readily available, and their use will be expanded in many countries. The goal of this document is to provide universally applicable guidance on how to apply and interpret these biomarkers in MS research and clinical practice, based on current knowledge.

This consensus document was developed by an international panel of experts on neurofilament biomarker research and clinical applications in MS, in partnership with the Consortium of Multiple Sclerosis Centers (CMSC). The panelists met in two virtual consensus conferences held on September 3, 2020 and October 16, 2020 to comprehensively review available data, determine practical applications of the data for MS, and debate conflicting information and unanswered questions. The key questions and goals of the consensus conference are outlined in **Table 1.**

***Table 1.* Neurofilament Consensus Statement in MS: Key Questions and Goals**

| | |
|---|---|
| Overview of neurofilament proteins | • Relevance of neurofilament subunits (Heavy, medium, and light chain) |
| Neurofilament assays | • Which assays to use<br>• What is known about their sensitivity, specificity, and reproducibility<br>• Is neurofilament light (NfL) the primary biomarker of interest in MS?<br>• What is the role of heavy and medium chain? |
| Serum versus cerebrospinal fluid (CSF) | • Is there a role for both CSF and serum?<br>• If so, when would CSF assays be preferred over serum? |
| When/how often to measure NfL levels | • Cost of assays<br>• How often should resampling be done after baseline? |
| Confounders/ influencing factors that can raise or lower NfL levels | • What baseline levels mean<br>• What increments constitute clinically meaningful change? |
| Clinical use of NF in multiple sclerosis | • How to assess NF measures in combination with other disease characteristics and clinical information |
| Role of NF in research, and goals of ongoing/ future research | • Standardizing NF measures and values<br>• How can NfL be combined with other existing and emerging biomarkers |

## Neurofilament Proteins and Their Role in Neuronal Degeneration

Neurofilaments are the major cytoskeletal components of neurons, and cellular injury leads to their release into the surrounding area. Research on neurofilaments

is not new, but in fact has been ongoing since pioneer neuroscientist Santiago Ramón y Cajal first described these proteins in the early 1900s.[6] Neurofilaments are classified as light (NfL), medium (NfM), and heavy chain (NfH) based on their molecular weights. NfL is the predominant intermediate filament in the central nervous system (CNS), followed by α-internexin, NfM, and NfH. NfL is also the most common intermediate filament in the peripheral nervous system (PNS), followed by peripherin.[7,8]

The neurofilament molecule is composed of the head or domain section, the rod, and the tail **(Figure 1)**. As the axons in the CNS mature, they acquire more NfH subunits and thicken through phosphorylation. Both NfH and NfM undergo phosphorylation, which adds tail regions with radial enlargement of the neurofilament structure, leading to a highly stable cytoskeletal construct.[9,10] Hyperphosphorylation has been associated with neurodegenerative diseases such as ALS.[11]

Neurofilaments have a robust half-life. Although their elimination half-life is still unknown, they are thought to remain in the blood and CSF for many months following neurologic injury.[12] We know that phosphorylation and other cell modifications of neurofilaments play an important role in axonal transport.[13] Experiments with NfH knockout mice show that large myelinated axons have a significant decrease in conduction velocity, leading to a disruption of the electrical current through the axon.[14] In other studies, knocking out NfL and NfM in mice results in severely inhibited axonal radial growth.[15]

## Significance of Medium- and Heavy-Chain Neurofilaments in MS

### Neurofilament Medium Chain (NfM)

The role in MS of NfM is currently unclear. Few studies have focused on this neurofilament type in neurologic disease, and none have been done in MS. In patients with traumatic brain injury (TBI), NfM concentrations were shown to be increased in CSF and serum samples, especially in those with polytrauma.[16] It is possible that with further research NfM measurements may prove to be informative in MS.

### Neurofilament Heavy Chain (NfH)

NfH is less studied than NfL, but as we learn more, this marker may provide a wealth of information in MS and other neurologic diseases. In patients with ALS, phosphorylated NfH is increased in blood and CSF compared with healthy and neurological controls, and has been found to correlate with disease progression. Serum phosphorylated NfH appears to be elevated well before the time of diagnosis in patients with sporadic ALS.[17] NfH levels have been measured in experimental autoimmune encephalomyelitis (EAE) mouse models of MS. Throughout the EAE disease course, higher levels of NH were released into the blood compared with pre-disease induction. At the chronic stage, NfH release dropped off, likely because the animal had lost a significant portion of its spinal cord.[18]

In humans, elevated NH in the CSF occurs in patients with clinically isolated syndrome (CIS) and optic neuritis, providing evidence of early and ongoing neuroaxonal damage.[19] In a study on the prognostic value of baseline NfH, a high serum phosphorylated NfH titer was detectable in 9% of patients with relapsing-remitting MS (RRMS) and CIS versus 38.5% of those with secondary progressive MS (SPMS). High phosphorylated NfH levels correlated with a higher Multiple Sclerosis Severity Score (MSSS) and T2 lesion volume.[20] Serum NH levels may also offer prognostic value in MS. A trial of the sodium channel blocker lamotrigine as a neuroprotective agent also investigated the value of serum NfH as a predictor of prognosis

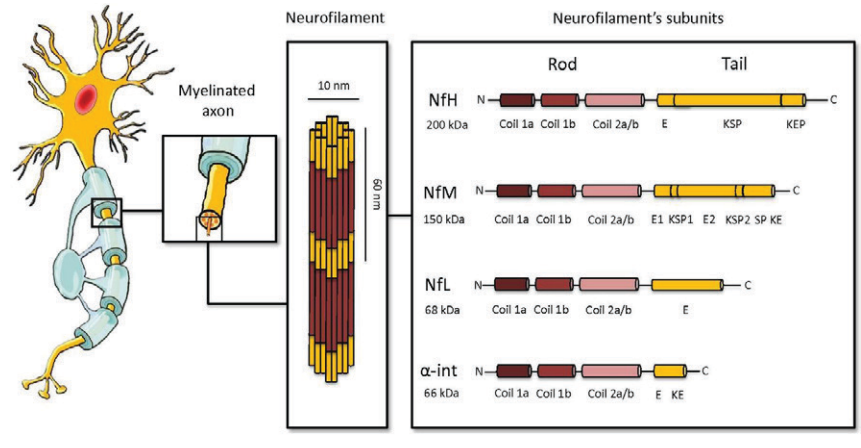


***Figure 1.* Neurofilament structure**

Neurofilament proteins add to the diameter of the axon and therefore influence its function. Structurally, all have a central, very highly conserved alpha helical rod domain in the middle, abutted by 2 variable regions: the head domain on the end terminus and the tail domain at the C terminus. Reprinted with permission from: Gaetani L, et al. *J Neurol Neurosurg* Psychiatry. 2019 Aug;90(8):870-881.

# Summary of Panel Recommendations

## Consortium of Multiple Sclerosis Centers (CMSC) Consensus on Neurofilament Proteins in Multiple Sclerosis

The goal of the consensus panel was to establish recommendations for integrating the measurement of neurofilament light chain (NfL) into multiple sclerosis (MS) research and clinical practice. NfL can be used to guide prognostic and treatment decisions and to evaluate the effects of disease-modifying therapy (DMT). Information derived from cerebrospinal fluid (CSF) or blood NfL is most informative when considered in the context of other clinical, radiographic, and biologic markers validated for MS. As information about NfL as an MS biomarker continues to expand, these recommendations will need to be updated accordingly.

### Overall Significance of Neurofilaments as a Biomarker in MS

- Neurofilament proteins are markers of neuronal degeneration that can serve as important biomarkers of disease activity in MS.
- Elevated levels of NfL in blood or spinal fluid are most likely markers of both inflammation and neurodegeneration in MS.
- Because it can indicate neuronal degeneration from a wide range of causes, NfL offers limited diagnostic value in MS and is useful mainly for prognosis.
- Neurofilament heavy chain (NfH) warrants further investigation as a potential biomarker in MS. A ratio of NfL-to-NfH levels may be informative.
- Too little is known about neurofilament medium chain (NfM) in MS to make conclusive statements at this time.

### Assays for Measuring NfL in CSF (CSF-NfL) and Serum (sNfL)

- High-sensitivity enzyme-linked immunoassay (ELISA) assays are appropriate for measuring NfL in CSF.
- The single-molecule array (Simoa) is currently the predominant method for measuring in blood (serum or plasma). Simoa assays must be processed on specialized laboratory equipment. Siemens also has a sensitive assay for sNfL that can be run on an automated immunoassay platform.
- The Uman Diagnostics monoclonal antibodies 47:3 and 2:1 are used in most sNfL assays. These highly non-competitive antibodies recognize NfL without cross-reactivity for NfM, NfH, or glial fibrillary acidic protein (GFAP).
- Coordinated efforts are underway to standardize assay platforms for sNfL and their interpretation in MS. As new assay systems are developed, they should be aligned for cross-comparison purposes.

### Use of CSF Versus Serum

- NfL is 10 times more highly concentrated in CSF than in serum, making it a more robust biomarker. However, the invasiveness of lumbar puncture limits the practicality of using CSF-NfL for routine monitoring in MS.
- CSF-NfL should be a part of the analysis from an initial diagnostic lumbar puncture in patients with suspected MS. NfL levels should be analyzed at other time points if other indications for lumbar puncture arise. Atraumatic needles should be used to reduce complications when lumbar puncture is indicated.
- After obtaining baseline NfL values using CSF, blood (serum or plasma) should be used for subsequent NfL monitoring in MS.

### Timing of CSF and Serum NfL Analysis in MS

- Baseline NfL levels (either serum or CSF) are a valuable contribution to the initial workup in patients diagnosed with or suspected of having MS and can be interpreted in the context of other clinical information.
- During periods of perceived clinical quiescence, the panel's recommendation for obtaining updated baseline sNfL levels are as follows:
  - Following relapse: at the 3- to 6-month follow-up visit
  - Magnetic resonance imaging (MRI) with gadolinium-enhancing (Gd+) lesion: at the 3- to 6-month follow-up visit
  - MRI with new or enlarging T2 lesion: no new baseline sample
- To evaluate the impact of a DMT in the absence of clinical or MRI change, re-sampling of sNfL may be done at 3-month intervals.
- In people with or without neurologic disease, sNfL levels increase gradually with age, with a marked increase around age 60. To take into account the impact of aging on sNfL levels, re-sampling is recommended as follows:
  - CSF-NfL: at 5- to 10-year intervals
  - Serum/plasma NfL: after age 60

### Quantification of NfL Levels in MS and Influence of Dynamic Change in NfL

- For interpretation of sNfL and CSF-NfL in MS, there is a need for robust, stratified reference ranges and cutoffs, potentially using Z-scores based on normative data from healthy subjects (soon to be available).
- There is still a need to identify disease-specific cutoffs to aid with prognostic and treatment decision-making;
  - What degree of change in sNfL would suggest worsening MS?
  - What degree of change in sNfL warrants consideration for a change in therapy?
- When evaluating treatment efficacy, the greatest drop in NfL values would be expected in treatment-naïve patients started on a new therapy. With subsequent monitoring, these changes may be more subtle.

- Very high NfL levels might support a decision for treatment escalation, and very low or normal NfL levels would suggest staying with the current therapy.
- Intermediate ranges offer less decisive decision-making support. In these cases, other clinical and MRI parameters should be factored into the decision to escalate therapy or monitor more closely. These management decisions require prospective validation studies.

**Value of NfL as an Adjunct to Imaging Biomarkers in MS**

- NfL is an objective, quantitative measure of recent neuronal loss, offering real-time disease activity.
- Using a combination of biomarkers provides additional information. NfL adds an element of tissue specificity over MRI (which measures mostly water change), especially since the clinical implications of brain atrophy are difficult to assess in individual patients.
- NfL captures spinal cord pathology that may be absent on brain MRI.
- NfL can provide additive information with optical coherence tomography (OCT) in MS. OCT is a marker that correlates well with brain atrophy, whereas NfL correlates well with Gd+ acute lesions as well as T2 burden of disease, but is also highly predictive of brain atrophy.

**Prognostic Value of NfL in MS**

- NfL has both short-term (within 2 years) and long-term prognostic value in MS (within 2 years). In CSF, this has been shown at the group level and in individual patients, while prognostic values of blood NfL (serum or plasma) are seen mainly at the group level.
  - Higher sNfL level correlates with development of more Gd+ lesions and new T2 lesions in the subsequent year
  - sNfL levels correlate with longer-term outcomes (5 years), including time to Expanded Disability Status Scale (EDSS) > 3.5 and time to clinically definite MS (CDMS)
  - sNfL levels correlate with brain atrophy measures
  - Brain atrophy and sNfL together predict time to EDSS 6 over 8 years
- Lowered NfL levels can be seen as a result of DMT. Short-term change as a result of treatment is associated with longer-term MRI and clinical outcomes. On a group level, reduction of sNfL 6 months after starting treatment is associated with:
  - Fewer new T2 lesions at year 2
  - Less brain atrophy at year 2
  - Less EDSS change after year 4
- Persistently high sNfL levels despite treatment are associated with worse MRI outcomes at 4 years.
- Interpretation of NfL levels is most informative when combined with clinical, MRI, and inflammatory markers, and when corrected for confounding factors such as age, obesity, and diabetes.
- Due to individual variations and a potential for crossover with healthy controls, we need to identify cutoff points and to correct for confounders in order for sNfL to be more informative in daily clinical practice.

**Potential Confounding Factors in NfL**

- Potential confounding factors should be recognized and controlled for when interpreting NfL in healthy controls or persons with MS.
- Age is an important confounder affecting NfL interpretation. Mean NfL levels in a healthy person are approximately 10 pg/mL at age 20 and rise steadily over the years, then increase sharply after age 59. To counter the age phenomenon, NfL may be calculated based on a Z-score, which is more reliable and sensitive to change.
- People with higher body mass index (BMI) tend to have lower sNfL levels, possibly due to increased blood volume. Further research will help to determine how to adjust for this confounder.
- Based on available data, race does not appear to influence baseline NfL or NfL change. More data are needed from large databases and in people with MS.
- Diabetes may cause elevated NfL levels due to nerve damage. Elevated sNfL has been associated with diabetes, but it is not clear how much those influence NfL variations in people with MS and comorbid diabetes.
- Available data have not shown associations with other comorbidities such as hypertension, hyperlipidemia, or renal dysfunction.
- Any drug treatment that causes neurotoxicity could potentially lead to transient elevations in NfL, even if MS disease activity is suppressed by the therapy.
- In autologous hematopoietic bone marrow transplant, CNS toxicity immediately after the procedure may be mediated by the chemotherapy. This contributes to transient increases in MRI atrophy and in elevated NfL levels.

**Ongoing Research Trials in MS and Future Research Goals**

- NfL is appropriate for use in all phases of MS clinical trials, and in clinical practice where available. Outcomes based on CSF-NfL and blood NfL (serum or plasma) will be further refined with coordinated research efforts and ongoing advances in the field.
- Large-scale studies are underway to assimilate NfL information from databases of people with MS and healthy controls. The goal is to study the cross-sectional relationship of sNfL levels with demographics and comorbid conditions, MS clinical characteristics, disability status, and imaging measures
- These coordinated research efforts will help to answer questions such as:
  - factors associated with sNfL in healthy controls
  - whether characteristics such as age, gender, race, or body weight are associated with sNfL > 97.5th percentile of control reference range
  - Relationship of NfL levels to MS clinical measures such as PDDS, walking speed, manual dexterity, and processing speed
- In addition to prognostic studies, CSF-NfL and sNfL have been adopted as outcome measures in many phase 3 studies of MS DMTs.

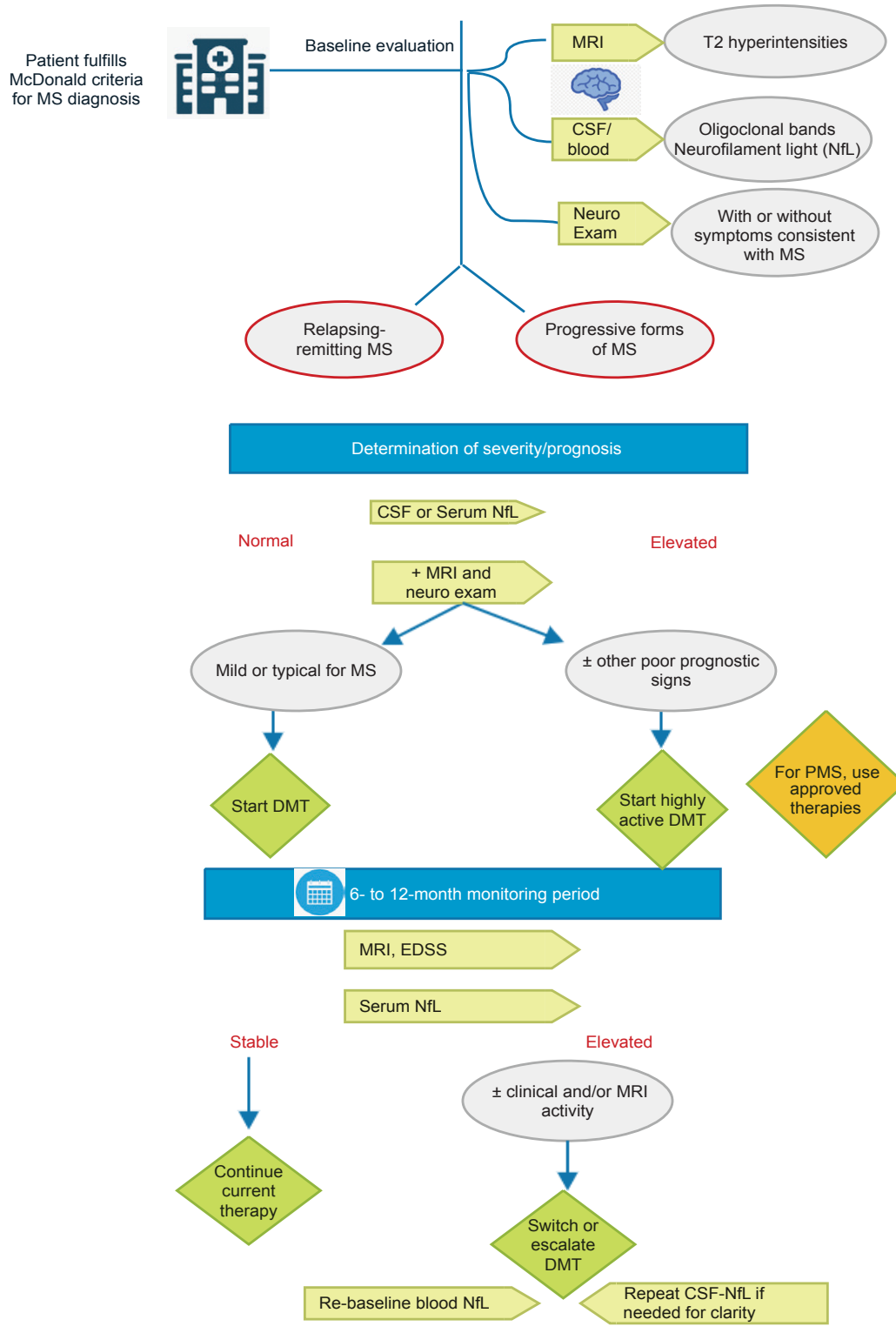


***Figure 2.* Algorithm for Use of Serum and CSF-NfL in Clinical Decision-Making for Patients With Multiple Sclerosis**

The panel recommends that evaluation of NfL be used in conjunction with other measures of MS severity and prognosis, including MRI, other imaging biomarkers, and findings of neurologic examination. If a patient shows clinical worsening and/or MRI changes while on therapy, elevations in sNfL levels may signal the need to perform further study or consider a change in therapy. For a patient who appears to be clinically stable but has elevations in sNfL, this may warrant closer monitoring and/or escalation of therapy.

and response to treatment in secondary progressive disease.[21] In this cohort of 120 patients with SPMS, serum NfH levels correlated with a number of disability measures including 24-foot walk, 9-hole peg test, Paced Auditory Serial Addition Test (PASAT), EDSS, cerebral atrophy on MRI, and magnetization transfer ratio (MTR).[21] However, a systematic review of 76 studies on the value of neurofilament proteins in progressive MS found NfL to be a better predictor than NfH of current inflammatory activity, future brain atrophy, and treatment response.[22]

Does measuring NfH contribute anything beyond NfL in patients with MS? This remains unknown. Biologically, NfH has greater presence in heavily myelinated axons.[23] This may prove useful in evaluating progressive disease, whereas NfL may be a more valuable marker early in the disease.

Neurofilament research is certain to advance significantly in the coming years. Further research and clinical experience will continue to refine the role of these biomarkers in MS and other forms of neurodegeneration. It is important for the MS community to determine best practices for applying these tools, with regular updates as the light about NfL becomes clearer. □

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# Measurement of Neurofilament Light Chain in Cerebrospinal Fluid and Blood

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Neurofilaments (Nf) are proteins expressed almost exclusively in neural tissue. When neurons are damaged or destroyed, Nf, predominantly the light chain (NfL), is released into the cerebrospinal fluid (CSF) in proportion to the extent of the damage.[1] While NfL release is not specific for any single neurologic injury or disorder, it is specific for neuronal damage. In this regard, NfL could be considered a "troponin for the brain," as a marker of neuronal loss.[2] In healthy controls, median concentrations of CSF-NfL range from 300 pg/L to > 700 pg/mL depending on the cohort and assay methodology. Blood concentrations are approximately 50 to 100 times lower than CSF, with median values ranging from 5 pg/mL to 11 pg/mL depending on assay and serum vs plasma. These require highly sensitive methods for accurate measurement.[2] Due to less invasive collection, blood has become the preferred biofluid for measurement of NfL over CSF.

Evaluation of a biomarker such as NfL for clinical use requires extensive clinical and analytical validation prior to routine use in patients. This section will briefly discuss the various assays currently available for measurement of NfL in serum or plasma and their potential for clinical utility.

## Analytical Methods

Due to the very low concentrations of NfL in blood, one must assess the ability of analytical methods to accurately measure these low values and establish at what concentrations the method is no longer able to be used. The clinical laboratory defines these lower limits as the lower limit of quantitation (LLOQ) or limit of detection (LOD). Lower limit of quantitation refers to the lowest value at which an accurate quantitative value (CV < 20%) can be reported, while the LOD is the value at which the assay is able to distinguish between the presence or absence of an analyte. In the case of blood NfL, the LLOQ is the most relevant and useful unit of measure.

## ELISA for NfL

The initial assays developed for NfL were enzyme-linked immunoassays (ELISA), which have a lower LOD of approximately 0.1 ng/L (100 pg/mL). This LOD is sufficient for measuring NfL in CSF; however, it is not sensitive enough to quantify NfL in blood. Therefore, more sensitive bioassays have since been developed, which will be further discussed. These include:

- Electrochemiluminescent (ECL) assays
- Enzymatic chemiluminescent assays (CLIA)
- Single-molecule array (Simoa) assay
- Aptamer-based assay

## ECL and CLIA

Chemiluminescent immunoassays produce light through either enzymatic (commonly alkaline phosphatase or horseradish peroxidase among others) or electrochemical methods to provide an increased sensitivity over traditional ELISAs. The electrochemiluminescent (ECL) assays are based on oxidative reduction reactions with Ruthenium complexes, while chemiluminescent assays (CLIA) that do not use Ruthenium are often acridinium-based ester or enzyme, linked to secondary detector antibodies. The level of sensitivity for chemiluminescent assays is higher than that of standard ELISA assays. Chemiluminescent methods are commonly used in routine clinical diagnostic laboratories, which can facilitate the routine use of NfL clinical practice.

## Simoa

The single-molecule array, or Simoa, assay is a novel fluorescence-based immunoassay method for detection of very low concentration antigens in biofluids. The Simoa assay uses a novel combination of "digital" and analogue methods to quantitate a broad range of analyte concentrations. Briefly, antibody-coated fluorescent beads are mixed with patient specimens where target antigens (NfL in this case) are captured on the beads, as with many automated enzyme immunoassays. Following traditional wash and secondary antibody

steps, beads settle into a specially designed micro-well array, where each micro-well can accommodate only a single bead. Detection is either digital for low concentrations (by counting the number of fluorescent beads) or analogue for higher concentrations (by capturing total fluorescence). Because of the high sensitivity of the assay, it is capable of detecting a single molecule per bead. The instrument is ready for routine clinical use and can accept specimens in clinical blood tubes of 96-well microtiter plates.

Compared with ELISA, the Simoa immunoassay has analytical sensitivity measurable down to the picogram per milliliter (pg/mL), with an LLOQ of about 0.1 pg/mL. The high dynamic range of the Simoa assay (about 1,800 pg/mL) offers accurate measurement of both the upper and lower concentrations from a relatively small volume. Simoa is currently the predominant method for measuring NfL in blood (serum or plasma), and is the current candidate instrument for medium- to high-volume routine use in clinical laboratories.

### Comparison of Simoa With Other Assays

An analysis by Kuhle and colleagues compared the LLOQ (precision of < 20% CV and accuracy of ±20%) for ELISA, ECL, and Simoa NfL assays in 33 paired CSF and serum samples **(Table 1)**.[3] LLOQ was 0.62 pg/mL for Simoa versus 15.6 pg/mL for ECL and 78.0 pg/mL for ELISA. Correlations between paired CSF and serum samples were strongest for Simoa ($r = 0.88$, $P < 0.001$) and ECL ($r = 0.78$, $P < 0.001$) and weaker for ELISA assays ($r = 0.38$, $P = 0.030$).[3]

## Siemens CLIA and Simple Plex Ella assays

Two additional newer assays with LLOQ low enough to be used for measurement of NfL in blood are the Siemens CLIA assay and the Simple Plex Ella assay.

- The Siemens assay is run on the Siemens Centaur, a currently available high-volume clinical diagnostic immunoassay analyzer. This assay is reported to have an LLOQ of approximately 1.62 pg/mL, sufficient for measuring NfL in blood or CSF. It uses the same Uman Diagnostics monoclonal antibodies as Simoa and has shown good correlation with the Simoa commercial assay. It is currently in the pre-clinical development phase.
- The Simple Plex Ella assay is a fully automated and fully self-contained "next-generation" ELISA. It is a microfluidic immunoassay with assay-specific cartridges allowing for detection of up to 4 different analytes. It is a low-volume analyzer. More work is required to determine how this assay compares with other NfL assays.

## Detection antibodies

Ideally, antibodies used in immunoassays should have a high degree of affinity and specificity for the target molecule. The antibodies used in many NfL assays are the monoclonal antibodies 47:3 and 2:1 produced by Uman Diagnostics. These antibodies have a high specificity for NfL and recognize the conserved rod domain of NfL without cross-reactivity for neurofilament medium chain (NfM), neurofilament heavy chain (NfH), or glial fibrillary acid protein (GFAP).[4] Because they are reactive against NfL in human, bovine, rat, mouse, sheep, and macaque samples, assays using these antibodies can be used for animal studies as well as humans. Currently, the Simoa Ella and Siemens assays utilize these antibodies, which should allow for comparable NfL results across platforms.

## Factors that May Influence Simoa Assay Findings for NfL

### Home-brew vs. Commercial Simoa Assays

Two different versions of the Simoa assay have been used in clinical trials. The original home-brew Simoa assay was developed in the research laboratory of Henrik Zetterberg in Sweden. This early NfL assay used Uman Diagnostics' monoclonal antibodies (47:3 and 2:1) in

***Table 1.* Comparison of sensitivity for NfL between ELISA, ECL, and Simoa assays**

| | ELISA | ECL | Simoa |
|---|---|---|---|
| LLOQ | 78 pg/mL | 15.6 pg/mL | 0.62 pg/mL |
| Mean CSF | 1074 (426.0–3051.5) | 965 (345–2727) | 1649 (558.5–4997.5) |
| Mean serum | 78 (78.0–252.0) | 51.6 (15.6–62.5) | 22.0 (12.5–54.5) |
| # serum below LOQ | 18 (54.5%) | 20 (60.6%) | 0 |

Kuhle J, et al. *Clin Chem Lab Med*. 2016;54(10):1655-1661.
LLOQ, lower limit of quantitation; LOQ, limit of quantitation; CSF, cerebrospinal fluid; ECL, electrochemiluminescence; NfL, neurofilament light chain; ELISA, enzyme-linked immunoassays

combination with bovine NfL calibrators, whereas the commercial Simoa assay utilizes the same antibodies with recombinant human NfL as calibrators. The different assay formulations produce slightly different NfL values, therefore caution must be used when reviewing and comparing clinical studies using the different assays. One comparative study showed a lower LOQ for the human versus the bovine calibrator and a significant bias between the assays.[5] Using matched specimens, Hendricks et al showed the home-brew assay using bovine calibrators generated results approximately 5 times greater than those of human calibrator assay (slope of 4.75). Another difference that may have contributed to variability of trial results is that the early kits used lyophilized NfL calibrators that required reconstitution by the customer, possibility contributing to bias between studies.[5] The current-generation Simoa NfL kits utilize pre-aliquoted calibrators with lot-specific concentrations and lot-specific quality control material to achieve greater consistency.

**Stability of Neurofilament in CSF and Serum Samples**

Neurofilaments have been shown to be remarkably stable in both serum and CSF. NfL is stable in serum, both at room temperature and at 4 degrees centigrade, for up to 24 hours. In both blood and CSF samples, NfL levels remain remarkably stable over multiple freeze-thaw cycles—indeed, up to 5 freeze-thaw cycles of CSF did not significantly alter the quantitation.[6] Even 5-day-old mailed-in samples did not show reduced stability.

**Significance in Patients Taking High-Dose Biotin**

The Simoa assay uses a streptavidin biotin-labeled link. Because of this, it is important to consider whether taking high-dose biotin—or even lower doses of biotin as a routine supplement—may interfere with Simoa assay results for NfL. Manufacturer data show that biotin of up to 80 µM in samples can be tolerated without significant impact on the measurement of NfL when using the Simoa assay. Practically, among patients taking biotin at 5,000 or 10,000 µg/day, 100% and 97.5%, respectively, are below the 80 µM biotin threshold 3 hours post dose. Furthermore, by 8 hours post dose, all patients had blood biotin below the 80 µM threshold.[7]

**Influence of Hemolysis or Lipemia on Simoa Assay For NfL**

Hemolysis and lipemia are the most frequently encountered endogenous causes of interference in clinical laboratories.[8] The Simoa NfL assay does not seem to be grossly affected by the presence of hemolysis or lipemia, and measured values tend to be mildly decreased (10% to 23%). This warrants additional study to determine the significance of these interferences. □

## PRACTICE POINTS

- The enzyme linked immunoassay (ELISA)—with a lower limit of detection of approximately 0.1 ng/L (100 pg/ mL)—is sufficient for measuring NfL in CSF, but is not sensitive enough to quantify NfL in blood.
- More sensitive bioassays for measuring from NfL in serum or plasma include electrochemiluminescent (ECL) and enzymatic chemiluminescent assays (CLIA). The single molecule array (Simoa) is currently the predominant method for measuring NfL in blood.
- Simoa is a novel fluorescence-based immunoassay which can detect very low antigen concentrations in biofluids. Compared with ELISA, Simoa has analytical sensitivity measurable to the picogram per milliliter (pg/mL), with an LLOQ of about 0.1 pg/ mL.
- Two additional newer assays with lower limit of quantitation (LLOQ) low enough to be used for measurement of NfL in blood are the Siemens CLIA assay and the Simple Plex Ella assay.
- Neurofilaments have been shown to be remarkably stable in both serum and CSF. In both blood and CSF samples, NfL levels remain remarkably stable over multiple freeze-thaw cycles. NfL is stable in serum, at room temperature and at 4 degrees centigrade, for up to 24 hours.

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# Potential of NFL as a Biomarker in MS: Cerebrospinal Fluid Versus Serum

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Cerebrospinal fluid (CSF) is rich in biomarkers of axonal damage and inflammation, including neurofilament light (NfL). Lumbar puncture to obtain CSF is an invasive procedure with risks including post-puncture headache, which can be reduced somewhat with less traumatic techniques using smaller needles. If NfL is to go mainstream as a biomarker of multiple sclerosis (MS) prognosis and treatment response, using serum or plasma instead of CSF has many advantages. This chapter will analyze the pros and cons of CSF versus serum for NfL analysis and review available data on the benefits of measuring CSF neurofilament levels in MS for prognosis and treatment efficacy.

NfL concentrations in CSF are approximately 50 to 100 times greater than NfL in serum.[1, 2] Additionally, NfL concentrations are approximately 20% higher when measured in serum compared to plasma, indicating that serum and plasma levels are not directly interchangeable within the same study. Studies have consistently shown good correlation between concentrations in serum, plasma, and CSF, as well as good correlation for serum versus plasma, as shown in **Figure 1**.[1, 3]

## Applications of Neurofilaments in MS

Potential applications for neurofilament measures in MS may include:

- Short-term prognosis (up to 2 years)
- Long-term prognosis (5 to 10 years)
- Aid in deciding potency of initial therapy based on prognostic factors
- Response to therapy and indicator for switching therapy
- Aid to decision making when clinical and magnetic resonance imaging (MRI) signs are unclear
- Expanding no evidence of disease activity (NEDA)-4 concept to NEDA-5

### Short-Term and Long-Term Prognosis in MS

Investigators from Linkoping University in Sweden studied a number of CSF biomarkers to evaluate change from baseline over a 2-year period in 41 patients with clinically isolated syndrome (CIS) or relapsing-remitting MS (RRMS) and 22 healthy controls.[4] Among a wide range of biomarkers examined (CXCL8, CXCL10, CXCL13, CCL20, CCL22, NfL, NfH, glial fibrillary acidic protein, chitinase-3-like-1, matrix metalloproteinase-9, and osteopontin), NfL was shown to be the best marker for detecting new disease activity and NEDA, followed by CXCL13.

NfL has been shown to have long-term prognostic value at a group level, but studies thus far suggest that it is less useful for individual prognosis. In a 2010 study

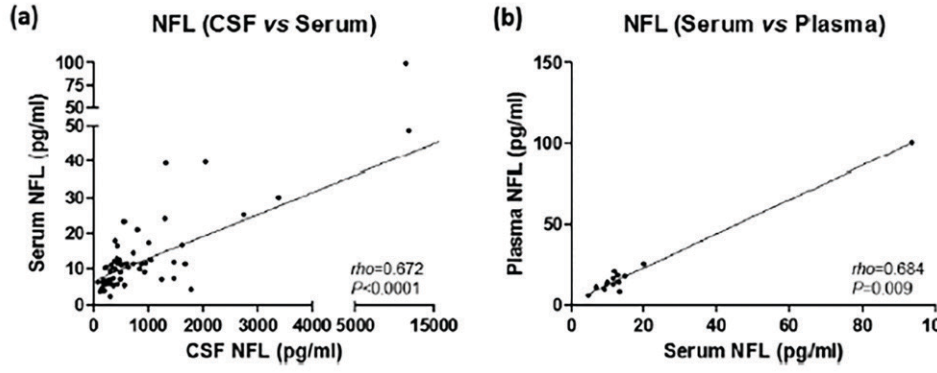


***Figure 1.* Correlation of NfL levels in cerebrospinal fluid/serum (A) and serum/plasma (B)**

Paired samples of cerebrospinal fluid and serum were obtained during diagnostic procedures from patients with MS and non-inflammatory neurological disease controls and show a high degree of correlation between the 2 compartments. Plasma samples were available in a subset of patients, also demonstrating a high degree of correlation between plasma and serum. Reprinted with permission from Piehl F, et al. *Mult Scler.* Jul 2018;24(8):1046-1054.

that analyzed group data from 99 patients with MS, having higher CSF-NfL levels early in the disease correlated with faster conversion to secondary progressive MS (SPMS) over an 8- to 20-year time period.[5]

A prospective longitudinal cohort study by the aforementioned Linkoping group analyzed a variety of neurodegenerative and neuroinflammatory markers (including NfL, CXCL markers, and MMP-9) in repeated CSF samples from 41 patients with CIS or RRMS and 22 healthy controls. Serum NfL was also analyzed using single-molecule array (Simoa).[6] NEDA-3 status and brain volume were evaluated and recorded over 4 years of follow-up. NfL levels in both CSF and serum correlated significantly ($P < 0.001$), but CSF-NfL was more strongly associated with NEDA-3 status, new T2 lesions, and brain volume loss. Compared to serum, the differential between healthy controls and patients with MS was significantly clearer when CSF was used. This study was one of the first examples that showed an overlap of NfL values between controls and some patients with MS.[6]

Another group from the Karolinska Institute investigated the association between plasma NfL levels and the risk of developing sustained disability worsening on Expanded Disability Status Scale (EDSS) and progression to SPMS. Concentrations of plasma NfL analyzed via the Simoa method were compared among 4,385 persons with MS and 1,026 randomly selected, population-based, sex- and age-matched controls.[7] This study also showed some overlap between controls and persons with MS in plasma NfL levels. However, there was a highly significant association between elevated plasma NfL levels and EDSS score increases of 1.4 to 1.7 and a sustained EDSS score of 3.0 (all $P < 0.001$). In contrast, the risks of reaching a sustained EDSS score of 6.0 and conversion to SPMS were not consistently significant (**Figure 2**).[7] Unpublished data from this group show similar correlations with cognitive function measured via Symbol Digit Modalities Test (SDMT).

On a group level, CSF-NfL clearly indicates prognosis in MS over both short- and long-term intervals. At the group level, serum or plasma NfL also indicates prognosis, although with less sensitivity than CSF. It is still unclear how to make prognostic predictions for individuals based on change in NfL. CSF-NfL ELISA assays are widely accessible, while serum NfL requires more advanced equipment. At an initial diagnostic lumbar puncture, baseline CSF-NfL should be included as part of the workup along with clinical and MRI markers, preferably along with a CSF inflammation marker such as CXCL13.

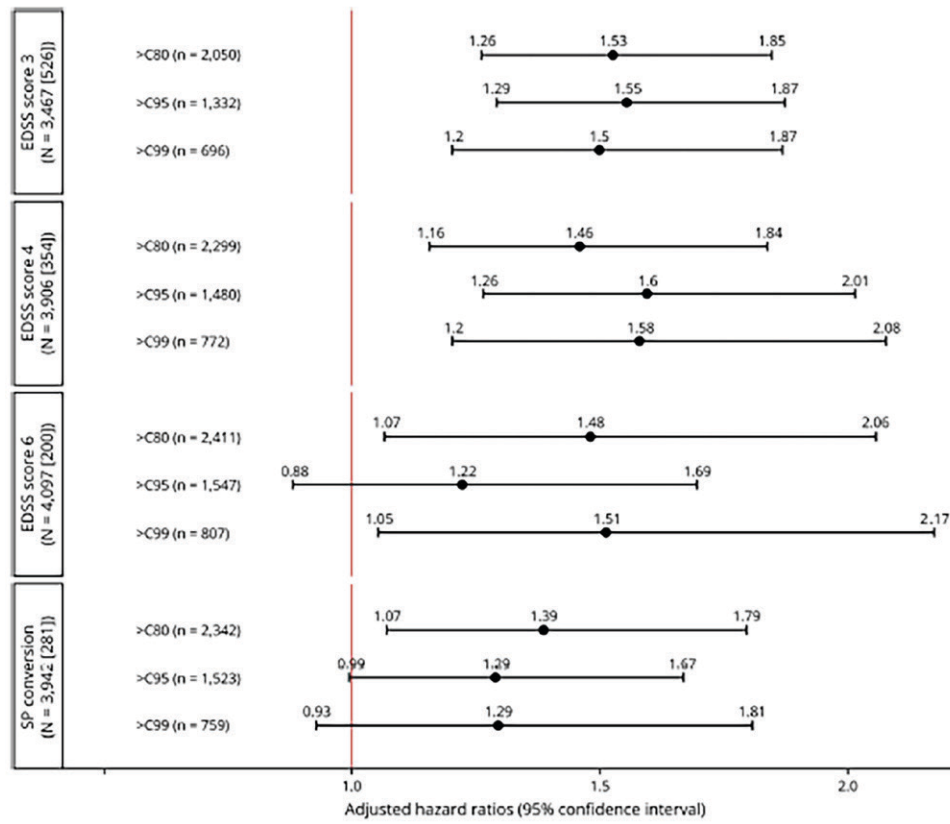


***Figure 2.* Risk of reaching major disability milestones, stratified by baseline plasma NfL (pNfL) levels**

Highly significant associations are shown between elevated plasma NfL levels and EDSS worsening— ranging from 1.4 to 1.7—and the risks of reaching a sustained EDSS score of 3.0 (all $P < 0.001$). The risk of reaching a sustained EDSS score of 6.0 and for conversion to SPMS were not consistently significant. Reprinted with permission from: Manouchehrinia A, et al. *Neurology*. Jun 9 2020;94(23):e2457-e2467.

In January 2019, the International Progressive MS Alliance convened an expert panel to consider the utility and validity of NfL as a biomarker for MS in general and progressive MS specifically.[8] The panel concluded that serum NfL (sNfL) "may provide a plausible biomarker of progressive MS, addressing some of the limitations of current imaging biomarkers." The panel identified contexts of use, which included: 1) to accelerate drug development; 2) as a pharmacodynamic/treatment response biomarker; 3) as an endpoint/outcome measure in clinical trials of progressive MS; 4) as a prognostic biomarker to predict disease progression; and 5) to be used for the selection of patients with progressive MS into clinical trials.[8]

The group also identified knowledge gaps relating to the use of NfL as a biomarker for progressive MS, including: 1) need for standardization of sample collection and assay methods; 2) need for a normative database of sNfL concentrations in healthy volunteers, including the effects of age and comorbidities; and 3) a deeper analysis of legacy clinical trial data to help clarify the predictive value of baseline concentrations of sNfL, define the response of sNfL to different therapies, and clarify the relationship between NfL and clinical and imaging outcomes. Furthermore, we need more information about how much inflammatory activity—including activated microglia and other disease processes—contributes to changes in NfL levels.[8]

**Evaluating Response to Disease-Modifying Therapy**

A study at the Karolinska Institute looked at the effect of natalizumab treatment on the release of CSF-NfL and another marker of neuronal damage, glial fibrillary acidic protein (GFAP).[9] CSF samples from 92 patients with relapsing forms of MS were collected prospectively before starting treatment with natalizumab and after 6 or 12 months of treatment. In nearly all cases, natalizumab was being used as a second-line agent due to breakthrough disease activity. Natalizumab treatment led to a 3-fold reduction of NfL levels, from a mean value of 1,300 ng/L (standard deviation [SD] 2,200) to 400 ng/L (SD 270) ($P < 0.001$). The value of 400 ng/L was not significantly different from NfL levels in healthy control subjects (350 ng/L).[9]

Another study evaluated change in CSF-NfL levels along with other CSF biomarkers in patients with MS taking a first-line DMT (usually an interferon) and after a switch to a second-line agent (natalizumab or fingolimod).[10] Reduced inflammatory activity (CXCL13, CHI3L1, and CHIT1) and reduced axonal damage (NfL) were shown in patients after switching to the second-line therapies. These studies establish that CSF-NfL can be useful for assessing efficacy. In a comparison of CSF and serum NfL for monitoring disease activity, both are useful, but CSF has greater specificity and sensitivity than does serum (**Figure 3**).[11]

Researchers from Barts and The London School of Medicine and Queen Mary University of London assessed the interactions between elevated NfL, clinical activity, and MRI findings in a cohort of 203 patients with RMS (58%) or progresive MS (42%).[12] Disease activity was most frequently indicated by elevated CSF-NfL (n = 85), followed by clinical (n = 81) and MRI activity (n = 65). CSF-NfL measurements were independently associated with clinical ($P = 0.02$) and MRI activity ($P < 0.001$). In some cases (mainly in patients with progressive MS, n = 22), elevated CSF-NfL was the sole indicator of disease activity (n = 22), 77% had progressive MS. The presence of elevated CSF-NfL was significantly more likely to prompt a treatment escalation than MRI, clinical indicators, and normal CSF-NfL ($P < 0.001$).[12]

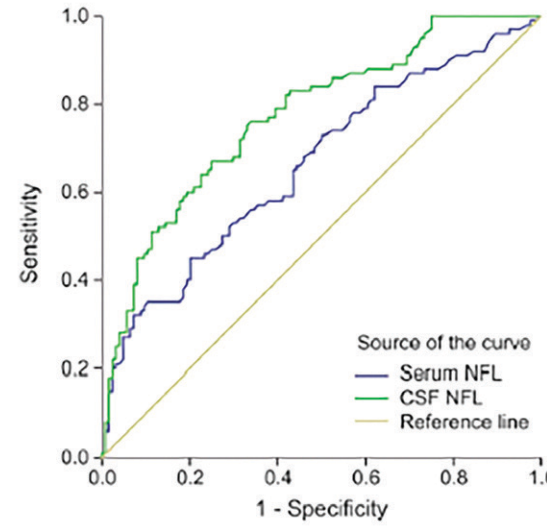


***Figure 3.* Specificity and sensitivity of NfL in serum and CSF for monitoring MS disease activity**

ROC curve with AUC for NfL in serum and CSF indicating specificity and sensitivity to discriminate patients with MS disease activity from patients without disease activity. AUC, area under the curve; CSF, cerebrospinal fluid; NfL, neurofilament light; ROC, receiver operating characteristic. Reprinted with permission from Novakova L, et al. *Neurology*. 2017;89(22):2230-2237.

## PRACTICE POINTS

- NfL in both CSF and plasma represents an important development in MS as a non-specific biomarker of axonal and neuronal damage. This development may be as important or even more important than MRI in monitoring disease activity.
- CSF neurofilaments have value in measuring therapeutic efficacy, probably also on the individual level, preferably in combination with an inflammatory marker. While repeated lumbar puncture may be problematic, less traumatic techniques with thin needles, can be used in selected cases.
- In comparison with CSF, serum and plasma NfL have a lower dynamic range and a large overlap with population-based controls or values. Serum and plasma NfL have prognostic value on the group level and in determining response to therapy at an individual level. Because of this, NfL levels are very useful in clinical trials.
- For progressive MS, the prognostic value of NfL is less clear. We understand too little about whether PMS is a problem with adaptive immunity, accessible for therapy with current drugs, or some form of slow, age-dependent neurodegeneration.

## Conclusions

In the future, technological development with multiomics (biological analysis in which the data sets use multiple "omes" including the genome, proteome, transcriptome, epigenome, metabolome, and microbiome) will be important to build on the evidence such as blood and CSF-NfL.[13, 14] We should expect a larger set of biomarkers that could reflect more aspects of MS pathology, such as types of inflammation, damage to myelin and oligodendrocytes, and other information. □

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# Considerations for Timing of NfL Measurement in Multiple Sclerosis

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As neurofilament light (NfL) is adopted as a biomarker of neuronal loss for evaluating MS disease course and treatment response, an important consideration is when and how often NfL should be measured. Beyond baseline measures of cerebrospinal fluid (CSF) or serum NfL, key questions include:

- During periods of perceived clinical or magnetic resonance imaging (MRI) quiescence, how often should re-baselining be done? How often when there is clinical or MRI activity?
- How often should re-sampling be done to evaluate the impact of a disease-modifying therapy (DMT) in the absence of clinical or MRI change?
- How often should re-sampling be done to take into account the impact of aging on NfL levels?
- Are any treatments for MS known to have a paradoxical effect on NfL (e.g., toxic effects that may lead to NfL elevation)?

## NfL as a Marker of Clinical Disease Activity in MS

NfL levels in healthy individuals and patients with MS are dynamic and vary under a number of conditions. NfL levels are increased during all clinical courses of MS, but are highest in patients with active relapsing-remitting disease (RRMS).[1] In patients with RRMS, NfL levels are up to 10-fold higher during relapse versus remission **(Figure 1)**.[1]

High NfL levels at disease onset correlate with disease progression, as measured with the Expanded Disability Status Scale (EDSS), in patients with an active relapse ($r = 0.49$; $P < 0.01$) and in clinically stable patients ($r = 0.29$; $P < 0.05$).[2]

Our 1998 study was the first performed on NfL in MS.[3] CSF was obtained from RRMS patients in a trial, to determine if treatment with acyclovir had an impact on the disease course and relapse activity. In place of MRI, repeated lumbar punctures were done every fourth month over 2 years. Increased NfL concentrations were seen in 78% of patients with MS. NfL levels were highest close to the time of relapse and peaked at 2 to 3 weeks after relapse onset, and were reduced to low levels about 3 months into the remission period.[3]

NfL levels correlate well with CSF inflammatory biomarkers, including CHI3L1, CXCL13, and osteopontin.[4, 5] To determine whether NfL and other CSF-derived proteins reflect different pathologic processes of MS, we performed serial lumbar puncture in 66 patients with MS and 50 healthy control subjects.[1] Compared with controls, mean NfL levels were increased during all stages of MS ($P < 0.001$), peaking almost 10 times higher during acute relapses. In contrast, glial fibrillary acidic protein (GFAP) showed the highest levels during

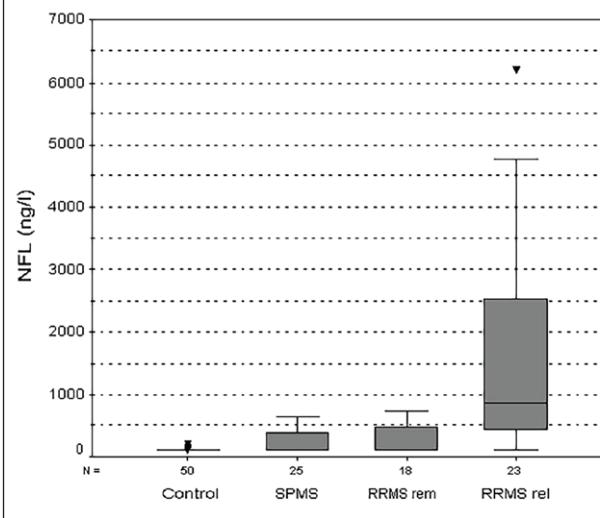


***Figure 1.* Concentration of NfL in CSF during relapse[1]**

Concentrations of NfL in cerebrospinal fluid (CSF) of patients with relapsing-remitting MS during relapse (RRMS rel) and in remission (RRMS rem); patients with secondary progressive MS (SPMS); and healthy controls. Boxes include median, 25th, and 75th percentiles; bars indicate 10th and 90th percentiles. Triangles indicate individual values. N=number of subjects.

secondary progressive MS ($P < 0.001$), with strong correlation to neurologic deficits on EDSS.[1]

### Real-World Studies of NfL Levels During Relapse or MRI Activity

Our MS clinic at the University of Gothenburg has assessed CSF-NfL routinely in clinical practice since 2001. In a real-world study, we evaluated data from 769 patients with RMS treated in our clinic between 2001 and 2018 who had lumbar punctures at diagnosis and during relapse or other clinical events. We confirmed higher NfL levels during relapse, with the highest in patients with severe relapses, and we also confirmed higher NfL lesions were seen in patients with contrast-enhancing lesions (unpublished data). DiSanto and colleagues showed similar findings using the Simoa assay for serum NfL (sNfL).[6] In addition to showing strong association between CSF-NfL and sNfL ($P < 0.001$), this study showed that patients who had either brain or spinal gadolinium-enhancing (Gd+) lesions or both had higher sNfL than those without.

To detect residual disease activity in patients with no signs of clinical or ongoing radiological activity, we recently conducted a real-world study of 90 patients with RRMS and 47 with progressive MS (PMS) (either primary or secondary).[7] CSF-NfL and CXCL13 concentrations were determined at baseline, before initiating or switching DMT, and after 12 and 27 months of follow-up. Even patients with no ongoing disease activity had elevations of NfL and CXCL13, while after 12 months of treatment with a DMT (mostly second line), 80% to 90% of the patients had decreased biomarkers **(Table 1)**.[7] We concluded that these markers seemed considerably more sensitive to disease activity than clinical and MRI measures.

***Table 1.* Residual disease activity in patients with RRMS and PMS without clinical/radiological signs of activity[7]**

**Before DMT**

- All patients with ongoing disease activity (relapse or contrast-enhancing lesions on MRI) had increased NfL or CXCL13
- RRMS (n = 90) and PMS patients (n = 47) without ongoing disease activity:
  - 39% of RRMS and 50% of PMS had elevations of either NfL or CXCL13
  - 11% of RRMS and 16% of PMS had elevations of both NfL and CXCL13

**After 12 months of DMT**

- DMT reduced CXCL13 and NfL in 80% to 90%
- 22% of RRMS and 19% of PMS still had elevated CSF-NfL despite no relapses or Gd+ lesions

Source: Novakova L, Axelsson M, Malmeström C, et al. NFL and CXCL13 may reveal disease activity in clinically and radiologically stable MS. *Mult Scler Relat Disord*. Nov 2020;46:102463.

## Influence of Timing on Conditions Associated with Elevated NfL

Any condition that causes axonal damage may lead to increased NfL spillage into CSF and blood. For some neurodegenerative diseases, NfL may have diagnostic or predictive value, while also serving as a marker of disease severity. CSF-NfL levels are elevated in persons with mild cognitive impairment and further increased in Alzheimer's disease. Diseases with higher axonal degenerative rates have higher values of NfL, particularly amyotrophic lateral sclerosis (ALS) and Parkinson's-like diseases with dementia and Lewy body disease.[8] Movement disorders with the highest NfL levels are progressive supranuclear palsy (PSP) and cortical basal degeneration, probably due to their high rate of neurodegeneration **(Figure 2)**.[9]

### Neuronal Damage Due to Athletic Activity

NfL is gaining attention as a biomarker of head trauma in sports such as boxing, soccer, football, and others.[10, 11] In these situations, timing of NfL elevations

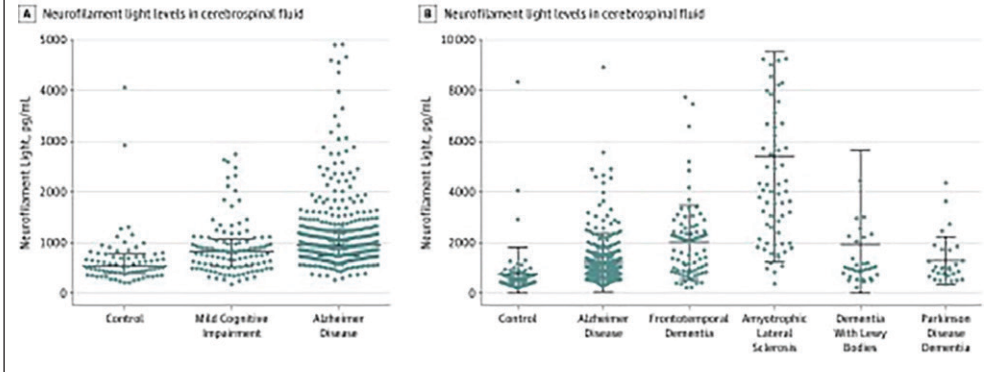


***Figure 2.* CSF-NfL levels for diagnostics and disease severity in neurodegenerative disorders[9]**

Source: Olsson B, Portelius, E, Cullen, NC, et al. Association of cerebrospinal fluid neurofilament light protein levels with cognition in patients with dementia, motor neuron disease, and movement disorders. *JAMA Neurol.* 2019;76(3):318-325.

can be informative as to the pattern of neuronal damage after trauma. A study in amateur boxers showed increased CSF-NfL 7 to 10 days after a bout, with normalization after 3 months.[11] MRI was normal in these boxers, and NfL was shown to be much more sensitive than Tau protein and GFAP measures. Another study in soccer players revealed increased levels of serum NfL just 1 hour after a match involving 40 headers (directing the ball with one's head) in 20 minutes.[10]

**Stroke**

Following stroke, a steady increase in CSF-NfL and sNfL is observed, peaking at 2 to 3 weeks.[12] Three to 5 months later, there is still evidence of elevated NfL. A study of 136 patients (101 with acute ischemic stroke and 35 with transient ischemic attack) showed a correlation between NfL elevations and infarct size.[13] A prospective study measuring sNfL in patients with stroke showed acute-phase sNfL levels to be at their highest concentrations 3 months post stroke. High sNfL correlated with stroke severity and poor outcomes; both associations were strongest for sNfL at 3 months.[14]

**Neural Damage Due to Neurosurgical Trauma**

In a phase 1b study on intrathecal administion of rituximab for PMS, an intraventricular catheter was inserted for drug delivery.[15] CSF and serum samples were obtained from 12 patients before and after catheter insertion. One month after this limited neurosurgical trauma of essentially white matter, NfL peaked 5 fold in CSF and 3 fold in serum compared to baseline, and both returned to baseline levels within 6 months.[15]

## Can NfL be Used to Monitor Individual Patients in MS?

Studies evaluating the potential of NfL to predict treatment response in patients with MS included an investigation of 15 patients with MS after immune reconstitution treatment with alemtuzumab.[16] Monthly sNfL measurements correlated with EDSS, MRI, and relapse activity over a period of up to 102 months after initiation of treatment. sNfL levels were significantly increased before treatment with alemtuzumab but decreased quickly within the first 6 months. In patients classified as NEDA-3, sNfL declined and persisted at an individual low steady-state level of <8 pg/mL.[16]

Another recent study looked at sNfL levels before and after relapse or formation of Gd+ lesions.[17] In the 3 months after appearance of a Gd+ lesion, the researchers observed an average 35% elevation in sNfL ($P < 0.0001$) compared to samples taken during remission. At the time of or prior to a Gd+ lesion, sNFL elevations averaged 32.3% ($P = 0.002$) compared with remission. In this study, significant elevations in sNfL after a clinical relapse occurred only when associated with a Gd+ lesion. The authors concluded that sNfL peaks in a 3-month window around the appearance of Gd+ lesions.[17]

## What is the Influence of Age on NfL?

In healthy controls, sNfL levels do not vary significantly by sex, but do increase with age by about 2.2% annually.[18] Studies conducted in our lab show average CSF-NfL levels in healthy persons as shown in **Table 2** (unpublished data). These values suggest neuronal degeneration is 5 times higher for persons over 59 years versus those under 30 years, based on CSF levels. In serum, the differential is lower. In our practice we do not factor in age with neurofilament samples from patients under age 60.

## What Can We Learn From Timing of NfL Elevations?

NfL elevations in CSF and blood are unspecific and may occur in a wide range of neurologic disorders, including brain and spinal cord injuries. In MS, NfL elevations are mostly associated with inflammatory disease activity. Confounding factors to NfL elevations in MS include age, head or spinal cord trauma, and other comorbidities (e.g., stroke, diabetes mellitus, other neurodegenerative diseases. Age dependence seems to be more important in CSF. We need to learn more about intra-individual day-to-day NfL fluctuations. Covariates in MS may include lesion volume on MRI, location of lesions, and whether there are cortical or spinal lesions.

**Do Treatments Used in MS Have a Potential Neurotoxic Effect That May Be Reflected in NfL?**

There is limited information on whether potentially toxic effects of MS treatments might lead to elevated NfL in either serum or CSF. The few studies available suggest that use of high-dose vitamin D had no influence on sNfL levels.[17, 19] Mesenchymal stem cell transplantation had no effect on sNfL.[20] Autologous hematopoietic stem cell procedures using busulfan as a

***Table 2.* CSF-NfL levels by age in healthy individuals**

| Age (healthy subjects) | CSF-NfL levels (ng/L) |
|---|---|
| < 30 | < 380 |
| 30 – 39 | < 560 |
| 40 – 59 | < 890 |
| > 59 | < 1,850 |

conditioning agent resulted in NfL increases within 3 months post transplantation.[21]

## Summary and Conclusions

Revisiting the questions posed in the beginning of the paper, best practices based on current knowledge can be summarized as follows:

**During periods of perceived clinical quiescence, how often should re-baselining be done? How often when there is clinical activity or MRI activity?**

- Following relapse: at 3- to 6-month follow-up
- MRI with Gd+ lesion: at 3- to 6-month follow-up
- MRI with new or enlarging T2 lesion: no new baseline sample

**How often should re-sampling be done to evaluate the impact of a DMT in the absence of clinical or MRI change?**

- 3-month intervals

**How often should re-sampling be done to take into account the impact of aging on NfL levels?**

- CSF-NfL: 5- to 10-year intervals
- Serum/plasma NfL: not before age 60?

**Other than bone marrow transplant, are any other treatments known to have a paradoxical effect on NfL post treatment?**

- Potentially, any treatment causing neurotoxicity □

## PRACTICE POINTS

- In healthy individuals and in people with MS, NfL levels vary under a number of conditions.
- NfL levels are increased during all clinical courses of MS, but are highest in patients with active relapsing-remitting disease (RRMS). NfL levels are up to 10-fold higher during relapse versus remission.
- High NfL levels at disease onset correlate with faster disease progression (EDSS) in patients with active relapses and in clinically stable patients.
- NfL levels correlate well with CSF inflammatory biomarkers, including CHI3L1, CXCL13, and osteopontin.
- In real-world studies, even patients without ongoing disease activity had elevated NfL, but after 12 months of treatment with a second-line DMT 80% to 90% had decreased NfL levels. NfL appears to be considerably more sensitive to disease activity than are clinical and MRI measures.

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# Confounders Affecting Interpretation of Neurofilament Biomarkers in MS

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Neurofilament levels rise with neuroaxonal damage in both cerebrospinal fluid (CSF) and blood, indicating neuroaxonal injury that is independent of causal pathways.[1] Neurofilament light chain (NfL) is a promising biomarker for assessing disease activity, monitoring treatment response, facilitating treatment development, and determining prognosis in multiple sclerosis (MS) and other neurologic conditions.[2] In order to understand the significance of changes in NfL levels among individuals with MS and with MS treatments, it is important to recognize what other factors influence NfL levels.

Key questions to be considered on NfL confounders include:

- How can we correct for the significant age-related increase in NfL in healthy controls?
- Are body weight and blood volume relevant confounders, and if so, should reference ranges reflect populations with higher body mass index (BMI)?
- How do comorbid neurologic conditions influence measurement of NfL in MS?

Age-adjusted reference values and profound information on physiological levels of NfL will most likely be needed in order to assign meaning and changes in these levels.[3, 4]

Concentrations of NfL in people with neurologic diseases may overlap to a great degree with those of age-matched persons without disease, even when measured at high concentrations in CSF. This is especially true for NfL levels in the lower ranges, as shown in a meta-analysis involving pooled data from more than 10,000 individuals. The data sets included healthy controls and a population with 35 neurologic or inflammatory conditions (**Figure 1**).[5]

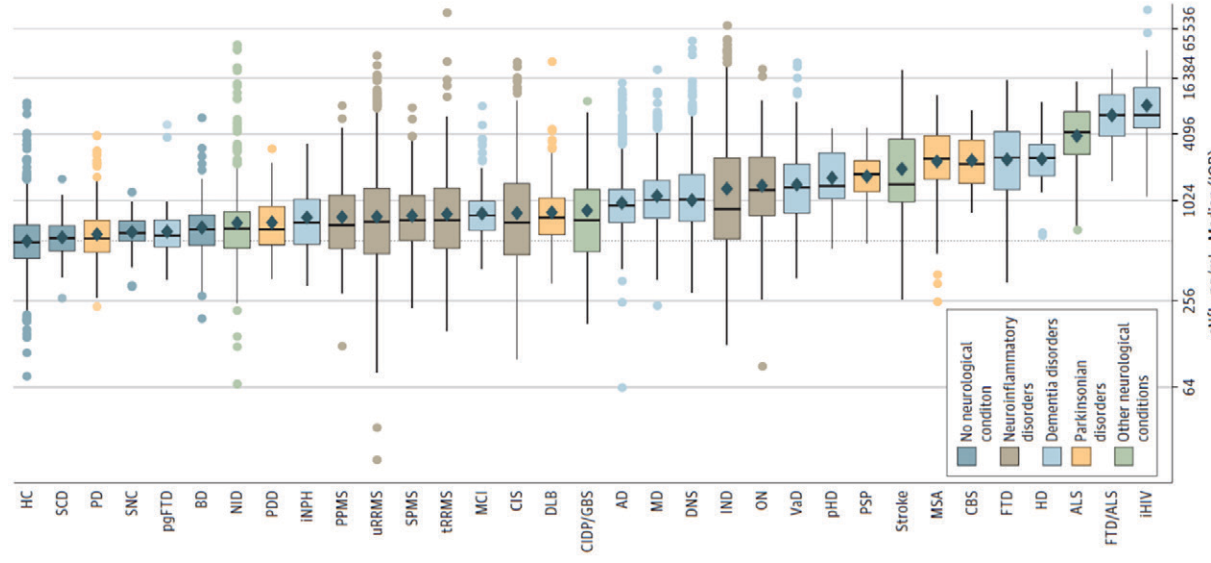


***Figure 1.*** **Neurofilament levels in neurologic diseases**

Data were collected for 10,059 individuals (mean age, 59.7 years; SD 18.8 years; 54.1% female). Thirty-five diagnoses were identified, including inflammatory diseases of the central nervous system (n = 2,795), dementias and predementia stages (n = 4,284), parkinsonian disorders (n = 984), and HC (n = 1,332). Source: Bridel C, et al. *JAMA Neurol.* 2019;76(9):1035-1048.

## Influence of Age on NfL

Age is a significant confounder affecting neurofilament levels. NfL levels in CSF and serum are higher and show a broader distribution of values among persons age 60 and over, both in healthy controls and in people with neurologic conditions or injuries.[6] This suggests an acceleration of neuronal injury with age. This could be driven by comorbid pathologies in older individuals that remain subclinical. In a study comparing NfL in people with and without human immunodeficiency virus (HIV), analysis of CSF-NfL in the healthy controls (n = 359) revealed a yearly NfL increase of 3.1%.[7] **Figure 2** shows the regression analysis from this healthy control group.[7] Age-related upper normal cutoff levels for CSF were suggested from this analysis, as shown in **Table 1**.[7]

Close associations have been observed between serum NfL (sNfL) and age-related changes in brain volume.[6] When we compared NfL with magnetic resonance imaging (MRI) measures in healthy individuals who had no neurologic impairments, we found that baseline sNfL and change in sNfL over 5 years correlated to change in brain volume. This correlation was strongly driven by the population over age 60.[6] Defining cutoff levels by age might allow a determination of whether NfL levels are within normal range or could be pathological. However, we don't know what degree of change in NfL levels would represent a pathologic process, versus a change that is still within the range of normal. This question will require more experience with NfL and studies in larger cohorts.

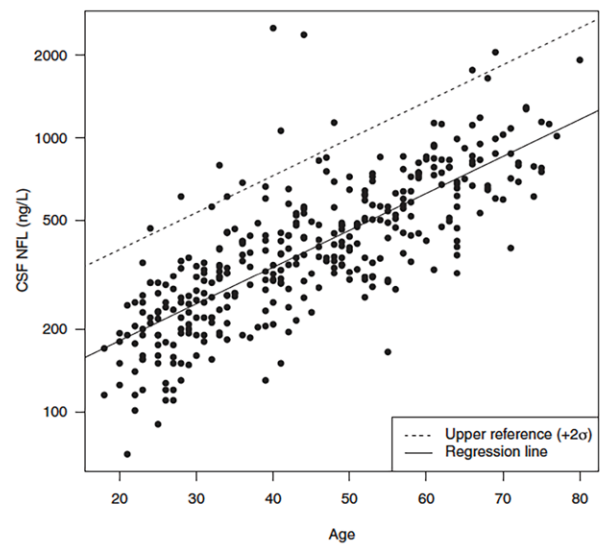


***Figure 2.* Cerebrospinal fluid NfL reference values in healthy controls, by age**

Source: Yilmaz A, et al. Neurofilament light chain protein as a marker of neuronal injury: review of its use in HIV-1 infection and reference values for HIV-negative controls. *Expert Rev Mol Diagn*. Aug 2017;17(8):761-770.

***Table 1.* Age-related upper normal CSF-NfL**

| Age | CSF-NfL upper reference values (ng/L) |
|---|---|
| 20 | 387 |
| 30 | 525 |
| 40 | 713 |
| 50 | 967 |
| 60 | 1313 |
| 70 | 1781 |
| 80 | 2417 |

CSF-NfL = $210.22 \times 1.031^{age}$
CSF, cerebrospinal fluid; NfL, neurofilament light
Source: Yilmaz A, et al. Neurofilament light chain protein as a marker of neuronal injury: review of its use in HIV-1 infection and reference values for HIV-negative controls. *Expert Rev Mol Diagn*. Aug 2017;17(8):761-770.

If an absolute value is used for NfL, such as 10 pg/mL, then a higher proportion of people over age 60 will be above that number. This is why use of percentiles or Z-scores appears to correct for the wide variations seen in absolute NfL values. For example, for brain volume correlation, a cutoff level might be set at 80%, 90%, 97.5% or 99%. Patients with NfL levels above these percentiles had markedly increased brain atrophy measures compared to those with lower levels.[8]

## Sex- or Race-Related Variances in NfL

Thus far, studies looking at neurofilament levels have not suggested a sex-related difference.[2, 5, 9] The same is true of race and ethnicity, although further study is warranted on both of these questions.

## Elevations of NfL in Other Neurologic Conditions

A wide range of neurologic conditions are associated with an increase in NfL. A large body of evidence links increased NfL levels to dementia, stroke, traumatic brain injury (TBI), amyotrophic lateral sclerosis (ALS), and Parkinson's disease (PD).[2] Other diseases could be associated with elevated NfL, but they have not yet been systematically studied **(Table 2)**.[2]

### Dementias

Neurofilament levels may be a useful biomarker in predicting neurodegeneration and clinical progression in Alzheimer's disease (AD), even prior to symptom onset.[10] A recent longitudinal study showed that

***Table 2.* Diagnostic and prognostic value of neurofilaments in neurological disorders**

| Evidence supports NfL value in diagnosis, prognosis, and/or monitoring treatment response | NfL could be of relevance, but associations have not been established |
|---|---|
| Multiple sclerosis | Epilepsy |
| Dementia | Encephalitis |
| Stroke | Meningitis |
| Traumatic brain injury | Hypoxic brain injury |
| Amyotrophic lateral sclerosis | Optic neuropathics |
| Parkinson's disease | Intracranial pressure |
| Huntington's disease | Neurotoxicity |
| Bipolar disorder (limited evidence for clinical utility) | Peripheral neuropathies (Guillain-Barré syndrome, chronic inflammatory demyelinating neuropathy, Charcot-Marie-Tooth disease) |

Khalil M, et al. Neurofilaments as biomarkers in neurological disorders. *Nat Rev Neurol.* Oct 2018;14(10):577-589.

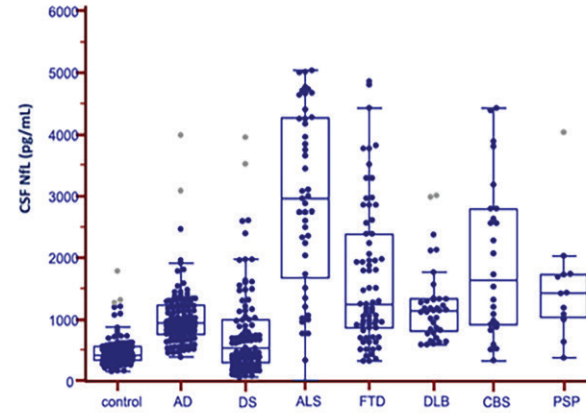


***Figure 3.* NfL levels in neurologic diseases**

CSF-NfL levels are higher in ALS relative to other neurologic diseases. AD, Alzheimer's disease; DS, Down syndrome; ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia; DLB, dementia with Lewy bodies; CBS, corticobasal syndrome; PSP, progressive supranuclear palsy. Source: Delaby C., et al. Differential levels of neurofilament light protein in cerebrospinal fluid in patients with a wide range of neurodegenerative disorders. *Sci Rep.* Jun 8 2020;10(1):9161.

CSF-NfL and sNfL levels are elevated in persons who carry mutations consistent with familial AD, even at the presymptomatic stages. Change in sNfL could be used to differentiate mutation carriers from non-mutation carriers almost a decade earlier (16.2 years before symptom onset) than the emergence of cross-sectional absolute NfL levels (6.8 years).[10] Because NfL is also increased in other dementia types such as frontotemporal dementia, this biomarker is less useful in distinguishing between different dementia types. However, this goal might be accomplished using NfL in conjunction with other biomarkers such as plasma phosphorylated tau181 (phospho tau181).[11]

**ALS**

Neurofilaments are present at higher levels in patients with ALS relative to other neurologic conditions **(Figure 3)**.[12] Measures of NfL and phosphorylated heavy-chain neurofilament may be useful diagnostically to differentiate between early ALS and other neurologic diseases. A large multicenter European study measured serum and CSF levels of NfL and phosphorylated NfH in patients with early ALS, as well as patients with motor neuron disease (MND) and MND mimics.[13] CSF and serum levels of both types of neurofilament were increased in early and later symptomatic phases of ALS ($P < 0.0001$) and could be used to discriminate patients with ALS with early symptom onset from those with other neurologic diseases and MND mimics with high sensitivity and specificity.[13]

**Parkinson's Disease**

Relatively few studies have assessed the role of NfL in PD. A recent longitudinal study measured sNfL levels in subjects who converted from prodromal PD to manifest sporadic PD (converters), at-risk subjects, and matched controls.[14] NfL levels were not increased at the prodromal stage, but those who converted to the manifest motor stage of PD had significant acceleration of age-dependent increases in NfL levels. However, there was some overlap observed in sNfL levels between those with PD and healthy controls.[14]

**Subcortical Infarction**

NfL might be a biomarker for cerebral small vessel disease (CSVD). Our research group measured sNfL levels at baseline, 3 months, and 15 months in people who experienced small subcortical infarctions and found increased levels compared to healthy controls.[15] These levels remained increased at the 3-month follow-up period, but returned to normal 15 months after the stroke. NfL correlated with infarction size and severity of baseline white matter hyperintensities. NfL levels were especially high in patients with new, clinically silent, CSVD-related lesions at follow-up. This suggests that NfL may serve as a valuable blood biomarker for active CSVD.[15]

### Traumatic Brain Injury

TBI has demonstrated the significant detrimental impact on neuronal tissues from certain contact sports such as boxing and hockey. Blood levels of NfL are elevated 7 to 10 days following a boxing match and decrease gradually over a 3-month rest period. However, even after recovery the NfL remains higher than that of controls ($P < 0.0001$).[16] Boxers who received many (> 15) hits to the head during a bout or were groggy after a bout had higher concentrations of sNfL. Neurofilament levels could potentially be used to separate athletes with rapidly resolving post-concussion symptoms (PCS) from those with prolonged PCS.[16, 17]

### Guillain-Barré Syndrome

A study from Vienna looked at sNfL levels in patients with Guillain-Barré syndrome and found increased levels compared to controls, correlating to a severity score, time of hospitalization, and the risk of admission to an intensive care unit.[18] Although NfL is exclusive to neurons, it is not exclusive to the central nervous system (CNS). A study in Guillain-Barré syndrome concluded that in this disease, most of the NfL released into the peripheral blood came from the peripheral nervous system and not from the CNS.[19]

## Other Potential Confounders Affecting NfL

### Neurotoxicity

CNS toxicity (e.g., due to chemotherapy) might affect NfL levels and could be an important confounding factor when applying this biomarker in MS and other diseases.[20] Thebault and colleagues studied 22 patients with MS undergoing autologous hematopoietic stem cell transplantation (AHSCT) involving immunoablation.[21] Three months after AHSCT, levels of sNfL and serum glial fibrillary acidic protein (sGFAP) increased from baseline by 32.1% and 74.8%, respectively. sNfL increases correlated with total busulfan dose, EDSS worsening at 6 months, and MRI grey matter volume. NfL levels returned to baseline, but these findings suggest that CNS toxicity immediately after AHSCT may be mediated by chemotherapy and contributes to transient increases in NfL levels and MRI atrophy.[21]

### Body Weight

Differences in BMI or blood volume (BV) might be reflected in NfL levels and affect its correlation to other biomarkers and disease outcomes. One study showed a significant negative correlation, with higher BMI and BV equating to lower sNfL. This was not seen for the CSF-NfL, but correlations between CSF and sNfL levels were improved when the investigators corrected for BV and BMI.[4] Similarly, in a study of patients who had recovered from anorexia nervosa at least 1 year prior, plasma NfL was negatively associated with BMI, suggesting that anorexia nervosa is associated with neuronal damage, which partially normalizes with weight recovery.[22]

### Childbirth

An interesting study looked at the potential impact of parturition on maternal cardiovascular and neuronal integrity.[23] Samples taken before the mother gave birth and the first postpartum day showed increased levels of NfL in the serum postpartum, suggesting some effect on neuronal integrity related to childbirth. No correlations were found for the type of anesthesia used or method of birth. This study suggests a possible confounding factor in this population that warrants further investigation.[23]

### General Anesthesia

Another question is whether general anesthesia for surgery may have an impact on NfL. A study of patients

## PRACTICE POINTS

- A wide range of neurologic conditions are associated with increased NfL, including dementia, stroke, traumatic brain injury, amyotrophic lateral sclerosis, and Parkinson's disease.
- Concentrations of NfL in people with neurologic diseases may overlap to some degree with those of aged-matched persons without disease, even when measured at high concentrations in CSF. This is especially true when NfL values are in the lower ranges.
- Age is a significant confounder affecting neurofilament levels. sNfL levels increase with age by about 2.2% annually. Neuronal degeneration may be 5 times higher for persons over 59 years versus those under 30 years. Close associations have been observed between sNfL and age-related changes in brain volume.
- No links have been shown between NfL and sex, race, or ethnicity, although further studies are ongoing. Differences in body mass index or blood volume might be reflected in NfL levels and affect its correlation to other biomarkers and disease outcomes.
- CNS toxicity (e.g., due to chemotherapy) might affect NfL levels and could be an important confounding factor when applying this biomarker in MS and other diseases.

undergoing knee surgery clearly showed that NfL levels increased 6 hours after the surgery and continued to increase through a 48-hour time period. In contrast, tau levels increased transiently after 6 hours but declined more quickly.[24]

### No Significant NfL Variations Seen in Sleep Loss, Vitamin D, Macular Degeneration

In a study of healthy male volunteers, NfL was found to be unchanged in subjects with acute sleep loss versus those without.[25] Another study in people with MS compared NfL levels at different months of the year along with vitamin D levels, and showed that natural variations in serum 25(OH)D values do not seem to be associated with alterations in serum NfL concentrations.[26] In a study of macular degeneration, elevations in NfL were attributed to age and did not differ between persons with and without macular degeneration.[27]

## Conclusion

Age is the most significant confounding factor influencing NfL as a biomarker in neurologic disease. To determine cutoff levels that correct for age, calculating Z-scores or using a percentile model appears to be the best approach, using a validated reference population. This work is ongoing; a study was recently completed in more than 10,000 NfL samples from a database of more than 5,000 persons from the general population without signs of CNS disease.[28] A number of confounding factors exist related to other diseases that occur comorbidly with MS. Gender and race or ethnicity do not seem to affect NfL, but BMI and BV do seem to be affected. This suggests that regional differences in the body weight of populations studied may lead to additional refinement when determining what cutoff levels could be considered an elevation of NfL. □

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# Significance of Dynamic Change in Neurofilament Levels in MS

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For neurofilament light (NfL) to be a useful biomarker of multiple sclerosis (MS) disease activity and treatment effect, it is essential to understand what degree of change in NfL is meaningful. Key questions to be addressed include:

- Are there differences depending on anatomical location of disease (e.g., brain, spinal cord, or optic nerve)?
- How much of NfL change is variability or "noise" in the assay, versus meaningful or disease-related change?
- Are there relevant enough controls to use as comparators in order to judge change?
- What is the typical impact of relapse in MS?
  - Sustained Expanded Disability Status Scale (EDSS) progression
  - Brain atrophy or other magnetic resonance imaging (MRI) metrics

## Interpreting Dynamic Change in NfL Levels

Variability in NfL levels across different neurodegenerative diseases contributes to the difficulty of interpreting measurements on the individual level.[1] While statistically significant changes in NfL can be meaningful on a group level, the large value spread makes it difficult to identify how important these changes may be on an individual level. A study by Piehl et al looked at correlations of cerebrospinal fluid NfL (CSF-NfL) with that of serum and plasma, as well as change in plasma levels when the patient was switched from an injectable MS therapy to fingolimod.[2] Mean NfL levels for controls and patients with MS are shown in **Table 1**.[2]

***Table 1.* CSF and serum NfL levels: comparison of MS and healthy controls**

| Mean NfL Levels (standard deviation) | CSF | Serum |
|---|---|---|
| Controls | 341 pg/mL (267) | 8.2 (3.58) |
| Patients with MS | 1,475 (2,358) | 17.0 (16.94) |

Source: Piehl F, et al. *Mult Scler*. Jul 2018;24(8):1046-1054.

## Change in NfL Levels on Treatment and Correlation to Other Markers in MS

In the above study, CSF/serum and plasma/serum levels were highly correlated ($P < 0.0001$), but the smaller degree of differential in serum measures between patients with MS and healthy controls illustrates the potential for overlap when evaluating serum NfL (sNfL). In patients starting fingolimod (n = 243), mean plasma NfL was reduced from baseline (20.4 pg/mL, standard deviation (SD) 10.7) at 12 months (13.5 pg/mL, SD 7.3) and remained stable at 24 months.[2]

A study based on data from the EXPAND study of siponimod correlated baseline serum NfL and brain atrophy in 1,452 patients with secondary-progressive MS (SPMS) and 378 with primary progressive MS (PPMS).[3] In this analysis by Kuhle and colleagues, patients' NfL levels were categorized as either low (< 30 pg/mL), medium (30–60 pg/mL), or high (> 60 pg/mL). Mean baseline NfL levels were higher for patients with SPMS (32.1 pg) versus those with PPMS (22.0 pg/mL). Gadolinium-enhancing (Gd+) lesion counts and T2 lesion volume at baseline correlated well with baseline NfL levels. Decrease in sNfL with siponimod treatment was more pronounced among patients who had relapses in the prior year. Patients with higher NfL levels had more brain atrophy at 12 and 24 months, with or without a prior relapse. NfL was able to predict brain volume loss in patients with and without prior relapses, suggesting that NfL may be a biomarker of neurodegenerative changes.[3]

In the ASCEND study of natalizumab in progressive MS presented at ECTRIMS 2019 by Kapoor and colleagues, patients with and without relapses were categorized by whether they had or did not have Gd+ lesions at

baseline.[4] Baseline sNfL concentrations were significantly associated with the number of Gd+ lesions, T2 lesion volume, other clinical measures, and brain atrophy over 96 weeks ($P < 0.0001$ for all). There was a small change from 17 pg/mL to 10 pg/mL in patients treated with natalizumab. This was less pronounced in patients who had no evidence of acute inflammatory activity, but the degree of dynamic change was determined to be clinically meaningful.[5] In this study, patients with no evidence of progression over time had lower NfL levels than those who experienced progressive events.

**Influence of Acute Brain Injury**

Placement of an intraventricular catheter for intrathecal rituximab administration would be expected to cause minimal trauma to the central nervous system (CNS), yet a study has shown it can lead to sustained release of NfL.[6] At 30 days post surgery, there was a distinct peak in CSF and serum NfL concentrations, which returned to baseline after 6 to 9 months. In contrast, other biomarkers such as S100 calcium binding protein B (S100B), glial fibrillary acidic protein (GFAP), and microtubule-associated protein tau did not show any significant changes. This suggests that NfL in both CSF and serum can be a sensitive marker for axonal white matter injury in MS.[6]

We used an animal model of traumatic brain injury called delayed-type hypersensitivity (DTH) to shed some light on what is happening behind the blood-brain barrier. We simulated Gd+ lesion activity starting on day 12 and peaking on day 18. After day 28 the acute inflammation ceased, but higher levels of NfL were sustained over 2 weeks, indicating that inflammatory and neurodegenerative activity continued despite the absence of acute inflammation. Our hypothesis is that there is ongoing brain injury beyond what we think is a relapse or a traumatic injury, leading to increased levels of NfL. (Anthony D, Leppert D, Kuhle J, unpublished)

**NfL Half-Life**

How long after NfL has been released into the blood and CSF does it remain in the system? From an analysis of current evidence on NfL, the elimination half-life time is still unknown, as no in vivo studies have yet been done. Available data suggest that NfL increases in the first 1 to 2 weeks after injury and may remain elevated for as much as 1 year after trauma (**Figure 1**), which reflects the "effective half-life time" or "fall rate," which is distinct from the elimination half-life. The long effective half-life time observed is due to constant release of NfL from injured neurons, indicating that the process of neuronal injury is long-lasting. Compared with other brain biomarkers such as S100B, neuron-specific enolase (NSE), GFAP, and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), NfL appears to have the longest half-life.[7]

**Comparison of MRI and NfL**

**Table 2** compares qualities of NfL with MRI as a measure of dynamic change in MS. While MRI is a retrospective measure of atrophy or structural damage, NfL offers a real-time readout of what is happening in the CNS. Much of what we call progression in MS occurs in the brain, but about 25% of patients have inflammation occurring exclusively in the spinal cord.[8] Across a number of studies, we see that patients without Gd+ lesions (about 25% of patients with relapsing MS, 50% of SPMS, and 20% of PPMS) have high NfL levels. This indicates that NfL covers an aspect of the disease pathology that escapes routine MRI.

**Timing of Dynamic Change in NfL Levels Following Treatment**

NfL findings from the FREEDOMS and TRANSFORMS studies of fingolimod have shown that change in serum NfL levels from baseline occurs about

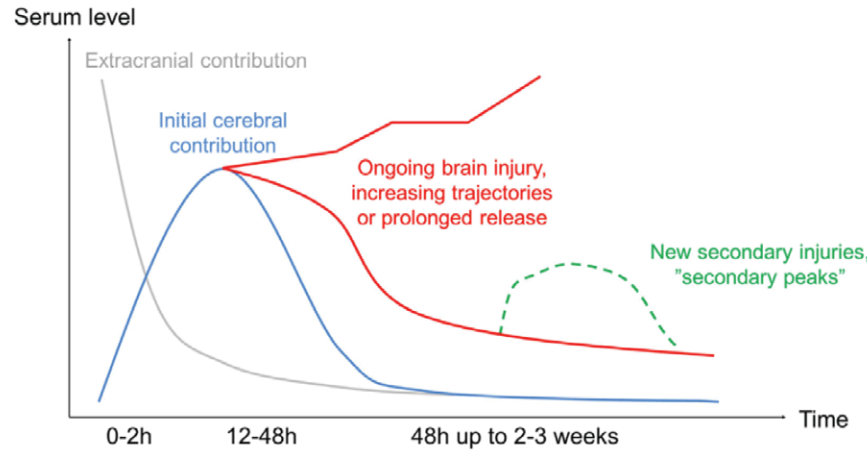


***Figure 1.*** **Prolonged elevation of NfL levels after trauma[7]**

Source: Thelin EP, et al. *Front Neurol.* 2017;8:300.

***Table 2.* MS disease activity: MRI versus NfL**

| | MRI | NfL |
|---|---|---|
| Relation to progression of MS | Brain atrophy | Continuous axonal injury, predominantly in spinal cord |
| Time | Retrospective (Gd+ > T2 > atrophy) | Real-time readout |
| Space | Restricted mainly to brain imaging | Captures damage in entire CNS |

3 to 6 months after treatment initiation, followed by a ceiling effect over the remaining 24-month period.[3] Remaining elevations in NfL may reflect new degeneration outside the reach of anti-inflammatory therapy. In a study of patients receiving alemtuzumab, sNfL levels decreased quickly within the first 6 months.[9] In patients classified as having no evidence of disease activity (NEDA) based on NEDA-3 criteria, sNfL decline persisted at an individual low steady-state level of < 8 pg/mL.[9] When sNfL peaks occurred, these were directly associated with clinical or MRI disease activity. sNfL elevations even appeared to predict relapse activity for patients who reported suspicious symptoms. In relapse, sNfL levels began to increase about 5 months before, peaked at clinical onset, and recovered within 4 to 5 months. Higher sNfL levels were seen in patients with active disease who required retreatment with alemtuzumab, compared with responder patients who did not require retreatment.[9]

NfL appears to be an appropriate method for comparing efficacy of drugs—such as oral versus injectable therapies—consistent with other clinical approaches. The degree of change can be considered statistically meaningful on a group level. The challenge remains how to transition these findings to treatment decision-making on an individual level.

### Assay "Noise" Versus Clinically Meaningful Change

A question that has arisen with use of sNfL is how much "noise" or biological/analytical variability, may occur with the single-molecular array (Simoa) assay, possibly clouding any meaningful clinical change. The evidence appears to support Simoa as a highly stable technique. NfL measurements in a given individual remain stable over time, as shown in **Figure 2**, with higher levels associated with advanced age. There is little intra-individual change over time, while inter-individual variation suggests pathologic activity.[10] We believe that small changes in sNfL are meaningful on a group level, but feel more work is needed to define what is clinically meaningful on an individual level.

Do we have relevant control sNfL values to use as comparators, in order to judge clinical change? To determine how sNfL predicted disease outcomes in MS, we studied 2,183 serum samples as part of an ongoing cohort study from 259 patients with MS (189 relapsing and 70 progressive) and 259 healthy controls. Clinical assessment, serum sampling, and MRI were done annually, and the median follow-up time was 6.5 years.[11]

- sNfL levels above the 90th percentile of values of healthy controls was an independent predictor of EDSS worsening in the subsequent year ($P$ < 0.001).
- The probability of EDSS worsening gradually increased by higher sNfL percentile category. Gd+ and new/enlarging lesions were independently associated with increased sNfL.
- Higher sNfL percentile levels were associated with more pronounced future brain and cervical spinal volume loss.

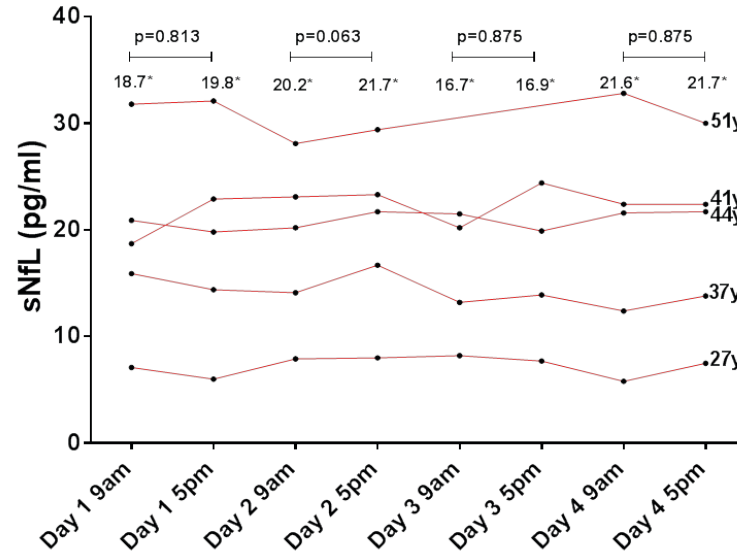


***Figure 2.* Stability of NfL over time in an individual**

Kuhle lab. Unpublished data.

- sNfL also correlated with concurrent and future clinical and MRI measures of disease activity and severity.

In the Swiss MS Cohort study, we showed how Z-scores can be used to reflect the deviation of a patient's sNfL value from the mean value of same-age healthy controls. In a population of patients with MS exhibiting NEDA-3, or no evidence of disease activity, higher Z-scores predicted EDSS worsening or relapse in the following year.[12] This has clinical value by suggesting that a patient with a very high Z score for sNfL warrants escalation of therapy.

## Conclusion

In summary, changes are meaningful on the group level. The sensitivity of NfL is sufficiently high to detect activity, such as smoldering lesions, even when no activity is evident on conventional MRI. We do need more normative data. Relative to absolute values using pg/mL, percentiles or Z-scores may offer a more meaningful comparison, as the latter discern the signal due to pathological NfL increase from the physiological age-related increase, and hence allow valuating intraindividual-levels over time and inter-individual levels without age as a confounding factor. Over time, measurement of NfL may lead to decreased use of monitoring MRIs during the routine follow-up of patients with MS. □

## PRACTICE POINTS

- The sensitivity of NFL is sufficiently high to detect activity, such as smoldering lesions, even when no activity is evident on conventional MRI.
- Gadolinium enhancing (Gd+) lesion counts and T2 lesion volume at baseline correlate strongly with baseline NfL levels.
- NfL is able to predict brain volume loss in patients with or without prior relapses. This suggests that NfL may be a biomarker of neurodegenerative changes.
- More normative data are needed to determine the degree of change in NfL that is clinically meaningful in MS. Rather than absolute NfL measurements using pg/ mL, percentiles or Z-scores may offer a more meaningful comparison.
- The elimination half-life time of NfL is still unknown. Available data suggest that NfL increases in the first 1 to 2 weeks after acute brain injury and may remain elevated for as much as 1 year after trauma.
- While MRI is a retrospective measure of atrophy or structural damage, NfL offers a real-time readout of what is happening in the CNS. NfL appears to cover aspects of MS disease pathology that are not seen on standard MRI.

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# Role of Neurofilament Light Chain in MS Clinical Trials and Clinical Practice

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In patients with multiple sclerosis (MS), serum neurofilament light chain (sNfL) is a biomarker of neuroaxonal loss that can be used as a measure of 1) acute disease activity; 2) therapeutic response and 3) to predict the course of disability progression.[1, 2] Area under the curve (AUC) measures of sNfL can potentially serve as an outcome measure for screening effective neuroprotective drugs for late-phase development. These advances in research suggest that peripheral blood NfL may prove to be as important a biomarker in neurology as C-reactive protein is in cardiology or rheumatology.[3]

NfL represents a "first in class" blood-based biomarker in MS that measures current disease activity. The rationale for using sNfL as a biomarker in multiple sclerosis (MS) clinical practice is based on the following proven concepts:[4]

- NfL in cerebrospinal fluid (CSF), plasma, and serum are highly correlated;
- sNfL levels are increased in all stages of MS compared to controls. Baseline measurements of NfL are predictive of outcomes in MS;
- sNfL levels are consistently associated with clinical and magnetic resonance imaging (MRI) disease activity, including T2 lesion volume and gadolinium-enhancing (Gd+) lesions;
- Change in sNfL is observed in response to MS disease-modifying therapy (DMT).

## Clinical Trials of sNfL

Clinical trials using sNfL have greatly expanded what can be learned about MS disease progression and treatment efficacy. sNfL remains highly stable in blood samples over many years and can be obtained more easily and less invasively than CSF. In addition, serum and CSF samples drawn from patients with MS participating in clinical trials of MS therapies are stored at −70°C and can later be analyzed for sNfL or CSF-NfL values.[5] Research using serum rather than CSF paves the way for implementation of sNfL into the clinical care of MS.

Validation of Serum Neurofilament Light Chain as a Prognostic and Monitoring Biomarker in Multiple Sclerosis (NINDS 1U01NS111678) is an ongoing study underway in conjunction with the National Institute of Neurological Disorders and Stroke (NINDS). This is a natural history cohort using the MS Paths database to examine the cross-sectional relationship of neurofilament with other aspects of MS, with 2 principal aims:

1) Assess the cross-sectional relationship of sNfL levels with demographics and comorbid conditions, MS clinical characteristics, disability status, and imaging measures;
2) Assess the relationship between baseline and/or serial sNfL levels with short-term and longer-term clinical and/or imaging outcomes in MS therapeutic efficacy: clinical outcomes, imaging outcomes and composite clinical and imaging outcomes,

The MS Paths MS population database is derived from 7 US cities and 3 European Union cities and is now up to 7,288 participants.[6] This and other populations in the analysis are outlined in **Table 1**. MS Paths will capture patient data from the electronic medical record, including real absence and switches in drugs—traditionally difficult data to capture due to delays in prior authorization and other factors. The group with NfL levels rising to about the 97.5th percentile will be compared with those patients who do not have high NfL levels.[7] Preliminary data from the study, presented in April 2021, showed that factors associated with elevated sNfL levels included: progressive MS, non-White race, diabetes mellitus, smoking.

Compared with those who did not have elevated sNfL, patients with MS and elevated sNfL levels had poorer clinical outcomes in measures such as walking speed and manual dexterity, higher T2 lesion volume, and lower brain volume measures.[7]

## Relevance of sNfL Z-Scores in Research and Clinical Practice

Ongoing studies can help clinicians better determine how changes in sNfL measurements over time predict outcomes in MS such as relapse, disease progression, and atrophy. To interpret sNfL in MS clinical practice, we need to correct for the effects of aging on neurofilament

***Table 1.* Research approach: serum neurofilament light chain as a prognostic and monitoring biomarker in multiple sclerosis**

| | Source of patient data | N |
|---|---|---|
| People with MS | MS PATHS 001, 002: 7 U.S. cities, 3 E.U. cities | > 7,000 participants followed over 5 years |
| | TREAT-MS Trial (PCORI): high-efficacy therapy vs first-line therapy | 700 participants followed over 4 years |
| Healthy controls | MS PATHS 005 | 200, followed over 2 years |
| | Johns Hopkins MS Center | 200, followed over 5 years |
| | CDC US population reference cohort | 2,300 |

levels. A population-based study of sNfL in healthy controls from baseline (n=335) and after a mean follow-up time of 5.9 years (n=103) has shown that sNfL levels increased at a rate of 2.2% per year, then accelerated beginning at age 60.[8] Change in sNfL levels from baseline correlated with brain volume loss in healthy control subjects as they aged (**Figure 1**).[8]

One way to correct for age and similar to percentiles, is to use the Z-score calculation, which represents a measure of degrees of deviation from mean levels in normal controls. In comparison to absolute sNfL levels (pg/mL), sNfL Z-scores do not increase with age in patients with MS. This was demonstrated in Z-scores from the Swiss MS Cohort.[9] Our research group has generated a normative database which now includes more than 10,000 sNfL samples from more than 5,000 healthy control subjects. These data have been submitted for publication in the medical literature and will recommend a normative database to calculate Z-scores for sNfL.[9]

Factors associated with elevated sNfL Z-scores (i.e., higher sNfL vs healthy controls of the same age) include:

- **Shorter disease duration.** A higher proportion of pathological sNfL levels (high Z-scores) are seen in younger patients with MS (i.e., those with more active disease).
- **Relapse.** Recent relapse increases sNfL.
- **T2 lesion volume.** Higher T2 lesion volumes independently drive higher Z-scores.
- **Suboptimal therapy.** More effective treatments typically lead to lower sNfL Z-scores.[9]

Numerous studies have shown that sNfL is associated with clinical and MRI disease activity as well as treatment response.[1, 2, 10, 11] Correlations between sNfL and conventional outcomes of MS treatment response have been confirmed in a series of retrospective phase 3 studies (e.g., FREEDOMS, TRANSFORMS, ADVANCED, ASCEND, CARE-MS 1, EXPAND, INFORMS) and patient registries using plasma or serum.[4]

On the group level, the reduction in Z-scores for sNfL was more profound with the use of highly active monoclonal antibody (mAb) therapies, more moderate for oral therapies, and lowest for injectable DMTs.[9] This is consistent with previous MS research findings and clinical experience. For patients on DMT, significant reductions in sNfL can be seen in the first 12 to 18 months. The change is seen more quickly with the use of an mAb, which may allow sNfL to reach close to normal levels.[9]

## Use of sNfL as a Prognostic Indicator in MS

In addition to reflecting current neuroaxonal injury, data show that NfL is actually a dynamic predictor of future outcomes in MS.

- Baseline sNfL predicts percentage of brain volume change over 2 and 5 years;[2]
- When patients in the FREEDOMS trial were classified as having either high, medium, or low baseline NfL levels, more pronounced brain volume loss was seen in the high NFL group, with a clear treatment effect observed for fingolimod;[4]

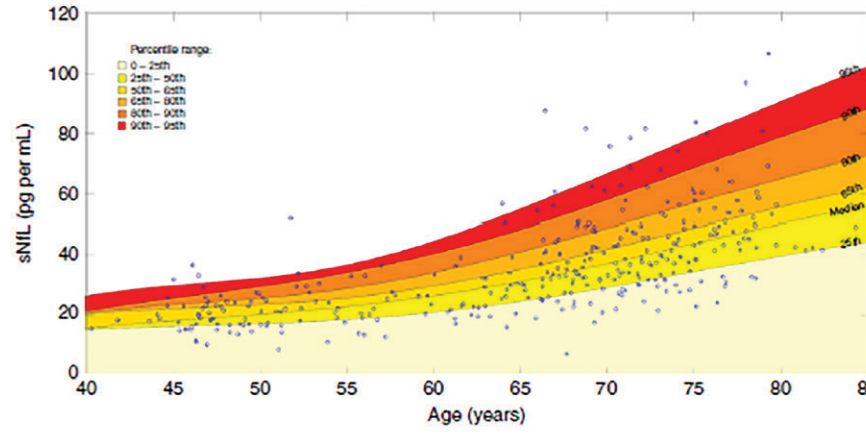


***Figure 1.* Serum neurofilament levels in healthy controls by age**

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- In the EXPAND trial of siponimod in patients with SPMS, elevated NfL (≥ 30 pg/mL) increased the risk of disability progression by 32%. In the INFORMS trial of fingolimod in PPMS, elevated NfL (≥ 30 pg/mL) was associated with a 49% increase in risk of disability progression.[12]
- In the ADVANCE study, baseline sNfL was a predictor of brain atrophy at 4 years and development of new T2 lesions.[13]

At the recent virtual meeting of ECTRIMS in 2020, we presented data on sNfL as a predictor of confirmed EDSS progression independent of relapse activity (PIRA).[14] We included patients of all MS subtypes enrolled in the Swiss MS Cohort who had at least three prospective follow-up visits and no relapses during the median follow-up period of 4.7 years. PIRA was defined as:

- EDSS increase of ≥ 1.5 steps from baseline EDSS of 0
- ≥ 1.0 step from baseline EDSS of 1.0 to 5.5
- ≥ 0.5 steps from baseline EDSS > 5.5

Of the 1,400 patients, 800 met study criteria and 4,000 serum samples were taken. sNfL levels were observed to increase with age (1.7% per year) and baseline EDSS (7.6% per step). PIRA occurred in 153 patients (19.0%). Those experiencing PIRA had 11.6% higher sNfL levels, compared with stable patients. sNfL levels were lower during mAb therapy (–10.8%) and oral DMT (–10.4%), compared with untreated time points.[14]

- In terms of predicting future disability progression, the hazard of future PIRA increased by 23.5% for every 1 standard deviation from baseline sNfL Z-score (95% CI: 8.3–40.8%, $P$ = 0.002). This finding was confirmed after adjusting for age, sex, EDSS score, and treatment at baseline.
- Patients with a baseline sNfL Z-score >2 had a 2.5-fold higher risk for future PIRA compared to patients with a sNfL Z-score ≤2 (95% CI 1.7-3.9, $P$ < 0.001 **(Figure 2)**.[14]

## Blood NfL as a Measure of Suboptimal Treatment Response

A few studies have shown that measuring sNfL in a patient receiving DMT can identify those likely to have breakthrough disease activity or progression despite therapy. In LONGTERMS, a 14-year open-label extension of the FREEDOMS trial of fingolimod, high NfL levels at month 6 and month 12 predicted future risk of confirmed disability progression. Patients with high NfL at both 6 and 12 months had a 2-fold higher risk of 6-month disability progression, compared with those with low levels at both time points ($P$ = 0.0373).[15]

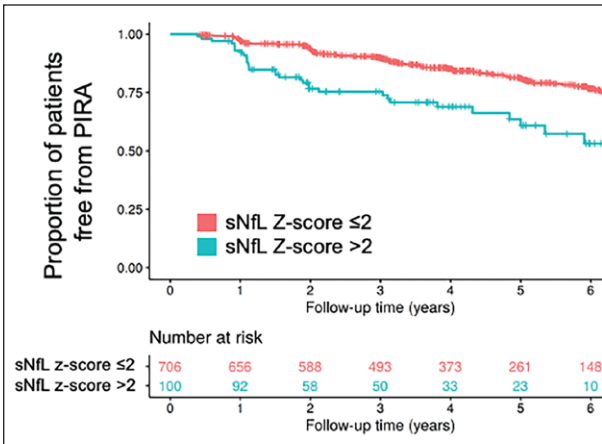


***Figure 2.* sNfL scores predict patients with disability progression in the absence of relapse**

The Swiss MS Cohort looked at sNfL patterns in patients treated with fingolimod over a 3- to 24-month period.[16] Having a high sNfL while on treatment was associated with 2 to 6 times higher relapse rates in the following 12 or 24 months, and 2 to 5 times more T2 white matter lesions. Additionally, this analysis showed an association between high serum NfL in treated patients and accelerated brain volume loss. Patients with sNfL levels above the 99th percentile had *an additional 0.95% yearly brain volume loss* compared with patients below the 99th percentile. These findings indicate that sNfL can be used to predict suboptimal response in patients on DMT.[16]

## Can sNfL Predict Future Clinical Events in Patients with NEDA?

Another interesting question is, does the current sNfL value in patients with NEDA-3 (no evidence of disease activity) predict clinical events in the following year? This was explored in patients from the Swiss MS Cohort with relapsing MS, on DMT for at least 3 months, with MRIs done every 6 or 12 months. Outcomes were reported for 1,062 patients with 5,000 serum samples and 3,573 MRIs:[16]

- sNfL Z-score predicted relapse or EDSS worsening in the following year
- sNfL Z-score predicted new/enlarging T2 in the following year

The discriminative capacity of sNfL for the degree of drug response could serve as an endpoint in phase 2 trials for specific features of disease progression or confirmed disability progression in the absence of relapse. This represents an aspect of MS where currently no trial paradigm is established.

## Conclusion

sNfL levels in individual patients with MS correlate with symptoms of activation of disease. This has been demonstrated even when routine clinical and MRI assessments have shown false negative results. Thus the use of NfL may have immediate consequences for initiation or escalation of DMT.

The algorithm on page 6 suggests how, using the current state of knowledge, NfL can be used for therapeutic decision-making in MS clinical practice. A newly diagnosed patient with clinically active or highly active MS on MRI can be placed on a high-efficacy disease-modifying therapy (DMT), as shown in the top row of the algorithm. In a clinically stable patient for whom active disease is not observed on the MRI, the presence of high NfL levels would help to inform the decision for escalation to a high-efficacy DMT.

Some caveats to consider:

1. sNfL shows strong age dependency with increase in older adults. However, physiological levels in children—specifically early childhood—are not well defined.
2. Medical comorbidities (e.g., increased body weight) or neurological comorbid diseases (e.g., Alzheimer's disease) correlate with an increase of sNfL.
3. While there is clear distinction of sNfL levels between MS and controls, and between different stages of MS on the group level, the range of levels among individual patients shows relevant overlap.[1] Interpretation of individual sNfL values to monitor MS in a routine diagnostic setting can only be valid when these confounding factors are well controlled, and when levels are interpreted in relation to age-matched physiological values.
4. An effort to establish a large normative database of age-related reference values in normal controls is ongoing,[7,9] specifically also in the typical age range of progressive MS, is prerequisite to establish sNfL as a standard biomarker of MS in clinical practice.
5. Commutability of values across the different assay protocols and platforms needs to be established in order to be on common ground with what can be considered normal versus pathologic levels of sNfL. □

## PRACTICE POINTS

- Data from more than 1,600 patients across 5 Phase 3 studies demonstrate the feasibility of establishing clinically relevant sNfL levels for disease severity stratification and treatment monitoring in relapsing-remitting MS.
- sNfL levels >16 pg/mL (using Simoa NF-light Advantage Kit) predict a high probability of disease activity and worse short- and long-term outcome (clinical, MRI, and OCT) but are confounded by the known strong age effect on sNfL.
- sNfL levels are lowered with the efficient use of DMT.
- **Elevated sNfL was associated with poorer neurologic function** including slower walking speed and manual dexterity, poorer cognitive performance, and increased self-reported disability.
- In order to implement sNfL into MS clinical practice, we need to have a standardized, widely accessible assay; generate comprehensive normative data; and validate sNfL in prospective real-world cohorts.

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## Post-test

# CMSC Consensus Statement on Neurofilament Biomarkers in MS

To receive credit, please read the program in its entirety, answer the following post-test questions, and complete the program evaluation. A certificate will be awarded for 5 correct answers or better. A certificate will be emailed (or mailed) to you within 2 weeks. There is no charge for CE credit.

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### PLEASE SELECT THE BEST ANSWER

1. **Which of the following best describes the significance of neurofilament markers in the diagnosis of multiple sclerosis (MS)?**
   - A. Neurofilament light (NfL) is an important marker for initial MS diagnosis but the significance of medium- and heavy-chain neurofilaments in MS diagnosis is unknown.
   - B. NfL may be useful in distinguishing progressive MS from relapsing MS in the early diagnostic stages.
   - C. Neurofilament levels in cerebrospinal fluid (CSF) have diagnostic value in MS; the diagnostic value of NfL in serum is unknown.
   - D. Because neurofilaments are elevated in a range of neurologic conditions, they have limited diagnostic value in MS.
2. **To evaluate a patient's blood NfL levels, the appropriate assay would be:**
   - A. Single-molecule array (Simoa)
   - B. High-sensitivity ELISA
   - C. Complete blood count with differential
   - D. These tests are not yet available outside of research studies
3. **The recommended sequence for evaluation of CSF and serum NfL in patients with MS is:**
   - A. CSF is not recommended because of the invasiveness of lumbar puncture; use serum at baseline and for follow-up.
   - B. Serum NfL is less reliable than CSF so MS patient follow-up should be based on CSF levels.
   - C. Evaluate baseline CSF level with initial (diagnostic) lumbar puncture; obtain baseline blood NfL at start of treatment and at follow-up unless a repeat lumbar puncture is indicated.
   - D. Evaluate baseline CSF and serum NfL and then re-check levels if patients have clinical or MRI activity.
4. **To account for normal age-related changes in NfL, the panel recommends:**
   - A. Re-baseline CSF levels every 1 to 2 years to account for impact of age.
   - B. After baseline, CSF re-sampling may be done every 5 to 10 years to account for impact of age.
   - C. Re-baseline serum or plasma NfL level at around age 60
   - D. Both B and C above
5. **Which of the following statements is most accurate regarding the prognostic value of NfL in MS?**
   - A. Higher serum NfL (sNfL) levels predict risk of developing gadolinium-enhancing lesions and new T2 lesions in the coming year.
   - B. NfL levels correlate with short-term outcomes (<2 years) but not with longer-term outcomes (> 5 years).
   - C. sNfL levels correlate well with T2 lesion burden but not with brain atrophy measures.
   - D. Correlation of NfL levels with clinical milestones such as time to EDSS 6.0 is unclear based on available data.
6. **Which of the following is TRUE when comparing NfL as a biomarker of MS with MRI and other imaging studies:**
   - A. NfL is an excellent marker of acute inflammation but does not predict subclinical disease activity to the extent that advanced grey matter imaging can.
   - B. Compared to MRI, NfL is a real-time marker of disease activity with greater tissue specificity.
   - C. NfL is capable of capturing brain atrophy and other changes but does not capture spinal pathology.
   - D. NfL can replace optical coherence tomography (OCT) since both offer the same types of information.
7. **In addition to age, the most important confounding factors that may affect NfL levels are:**
   - A. Cardiovascular disease, including hypertension and dyslipidemia
   - B. Body weight and diabetes
   - C. Racial differences that may influence MS prognosis
   - D. Kidney dysfunction that slows clearance of NfL fragments from the blood
8. **A patient with MS is on a standard disease-modifying therapy and appears to be stable clinically. A recent Simoa assay shows a significant rise in serum NfL compared with 6 months ago. What do the available data suggest about this patient's prognosis?**
   - A. Because the patient is clinically stable, the elevation in NfL is most likely related to a minor, transient condition.
   - B. The elevated NfL level is a definite indicator that the DMT is not working; the therapy should be escalated to a high-efficacy agent.
   - C. Elevated NfL in a clinically stable patient is likely an indicator of subclinical disease activity and should be evaluated along with other findings to consider a switch in therapy.
   - D. The patient should be monitored again in 6 months with MRI and repeat sNfL.

# Evaluation Form
# CMSC Consensus Statement on Neurofilament Biomarkers in MS

**Please answer the following questions by circling the appropriate rating:**

5 = Outstanding 4 = Good 3 = Satisfactory 2 = Fair 1 = Poor

**Extent to Which Program Activities Met the Identified Objectives:** *After completing this activity, participants should be better able to:*

1) Review techniques used to measure neurofilament biomarkers in serum and cerebrospinal fluid of patients with multiple sclerosis (MS) 5 4 3 2 1
2) Define NfL values and timing of NfL measurements and their clinical relevance in MS 5 4 3 2 1
3) Discuss the influence of comorbid medical conditions on NfL outcomes 5 4 3 2 1
4) Outline available strategies for applying NfL as a biomarker to aid in the clinical management of MS 5 4 3 2 1

**To what extent was the content:**

5) Well-organized and clearly presented 5 4 3 2 1
6) Current and relevant to your area of professional interest 5 4 3 2 1
7) Free of commercial bias 5 4 3 2 1
8) Clear in providing disclosure information 5 4 3 2 1

**General Comments**

9) As a result of this continuing education activity (check only one):

❒ I will modify my practice. (If you checked this box, how do you plan to modify your practice?) _____

_____

❒ I will wait for more information before modifying my practice.
❒ The program reinforces my current practice.
❒ No, I will not modify my practice.

Please indicate any barriers you perceive in implementing these changes:

❒ Cost
❒ Lack of time to assess/counsel patients
❒ Lack of administrative support
❒ Cultural or language barriers
❒ Reimbursement/insurance issues
❒ Concerns about patient safety/well being

10) This activity will assist in the improvement of my (check all that apply):

❒ Competence ❒ Performance ❒ Patient outcomes

Suggestions for future topics/additional comments: _____

_____

**Follow-Up**

As part of our continuous quality-improvement effort, we conduct postactivity follow-up surveys to assess the impact of our educational interventions on professional practice. Please check one:

❒ Yes, I would be interested in participating in a follow-up survey.
❒ No, I would not be interested in participating in a follow-up survey.

There is no fee for this educational activity.

| Post-test Answer Key | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |

**Request for Credit** ***(Please print clearly)***

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