



# The potential of serum neurofilament as biomarker for multiple sclerosis

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Multiple sclerosis is a highly heterogeneous disease, and the detection of neuroaxonal damage as well as its quantification is a critical step for patients. Blood-based serum neurofilament light chain (sNfL) is currently under close investigation as an easily accessible biomarker of prognosis and treatment response in patients with multiple sclerosis.

There is abundant evidence that sNfL levels reflect ongoing inflammatory-driven neuroaxonal damage (e.g. relapses or MRI disease activity) and that sNfL levels predict disease activity over the next few years. In contrast, the association of sNfL with long-term clinical outcomes or its ability to reflect slow, diffuse neurodegenerative damage in multiple sclerosis is less clear.

However, early results from real-world cohorts and clinical trials using sNfL as a marker of treatment response in multiple sclerosis are encouraging. Importantly, clinical algorithms should now be developed that incorporate the routine use of sNfL to guide individualized clinical decision-making in people with multiple sclerosis, together with additional fluid biomarkers and clinical and MRI measures.

Here, we propose specific clinical scenarios where implementing sNfL measures may be of utility, including, among others: initial diagnosis, first treatment choice, surveillance of subclinical disease activity and guidance of therapy selection.

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**Abbreviations:** CIS = clinically isolated syndrome; EDSS = Expanded Disability Status Scale; RRMS = relapsing-remitting multiple sclerosis; sNfL = serum neurofilament light chain; SPMS = secondary progressive multiple sclerosis

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## Introduction

Multiple sclerosis is a chronic inflammatory CNS disorder in which neuroaxonal damage is closely related to clinical and MRI events and prognostication.<sup>1,2</sup> Neurofilament light chain (NfL) is a major component of neuronal and axonal cytoskeleton proteins, providing structural support in the central and peripheral nervous systems. Apart from inflammatory diseases, elevated NfL has among others been reported in neurodegenerative, traumatic and ischaemic brain diseases.<sup>3–5</sup> A comparison of CSF NfL levels across more than 30 different neurological disorders revealed increased levels compared with healthy controls in most cases.<sup>6</sup> High NfL levels are therefore a general reflection of axonal damage, independent of underlying aetiology. However, the absolute values and/or temporal dynamics seem to reflect different competing aetiologies. In patients with multiple sclerosis, especially in cases where serum NfL (sNfL) values are higher than expected in otherwise stable patients, alternative causes and comorbidities such as head trauma, polyneuropathy or microvascular CNS lesions need to be considered. Thus far, neuropsychiatric or cognitive symptoms are not related to sNfL levels according to results from a smaller cohort.<sup>7</sup> With axonal damage, NfL proteins are not only released into the CSF compartment, but also subsequently in low amounts (~2%) into the peripheral blood.<sup>8,9</sup> Research on neurofilament proteins has been performed for more than 20 years,<sup>10,11</sup> but was initially limited with respect to translational suitability due to the necessity of obtaining CSF samples. However, because of novel highly sensitive analytic methods (namely single molecule array, SIMOA, technology),<sup>12</sup> minimal concentrations in the single-digit picogram/millilitre range can now be detected in serum or plasma samples by specialized laboratories<sup>13</sup> and, since c. 2017, are increasingly being used in multiple sclerosis research<sup>8</sup> (Box 1). Correlations between CSF and blood values of NfL are high,<sup>14,15</sup> and thus assessment in the peripheral blood is presumed sufficient, although a certain amount of uncertainty still exists as to whether this assumption can be generalized to all concentrations and patient subgroups.<sup>16</sup> The minimally invasive detection in blood samples can be considered a breakthrough for the potential broader application of this marker in clinical practice. Herein, we will critically discuss the current progress and view regarding the role of sNfL in answering essential clinical questions in patients with multiple sclerosis (Box 2).

## Serum NfL as a diagnostic biomarker in multiple sclerosis: distinct diagnostic situations

Without considering any additional clinical context, sNfL alone is insufficient for a diagnosis of multiple sclerosis or for differentiating multiple sclerosis from other neuroinflammatory disorders with neuroaxonal damage and elevated sNfL levels,<sup>32</sup> such as neuromyelitis optica spectrum disorders or myelin oligodendrocyte glycoprotein (MOG)-encephalomyelitis.<sup>33–35</sup> However, in specific clinical situations, sNfL can contribute to differential diagnostics. As one example, patients developing progressive multifocal leukoencephalopathy (PML) on natalizumab treatment had a steeper sNfL increase than those with multiple sclerosis-related relapses.<sup>36</sup> A recent study took advantage of a unique prospective longitudinal serum biobank effort with detailed clinical and demographic data in the US Department of Defense Serum Repository. Sixty individuals were identified who subsequently developed multiple sclerosis and had previous serum collection in the years preceding diagnosis. sNfL levels were already elevated 6 years (range: 4–10 years) prior to disease onset and showed a further increase in the years leading up to the first clinical symptoms.<sup>37</sup> Other studies have investigated patients with radiologically isolated syndrome, who are clinically asymptomatic patients undergoing MRI examinations for other reasons with incidental multiple sclerosis-like CNS lesions. In patients with radiologically isolated syndrome, elevated sNfL levels indicate a higher risk for developing either clinically isolated syndrome (CIS) or multiple sclerosis in the future.<sup>38</sup> These studies from presymptomatic patients with multiple sclerosis underscore the long prodromal phase of this disease prior to the first clinical relapse with evident ongoing neurodegenerative processes.

MRI lesions and oligoclonal bands in the CSF are established risk factors for a diagnosis of multiple sclerosis.<sup>39</sup> In several studies performed in patients with CIS, elevated NfL values in the CSF<sup>40–42</sup> or serum<sup>43,44</sup> were an additional predictor of future relapses. Importantly, studies mostly corrected for other known risk factors (such as age, oligoclonal bands and T<sub>2</sub> lesion numbers), highlighting the added value of sNfL as an independent risk factor. In a cohort of more than 800 patients of the German Clinical Competence Network for Multiple Sclerosis (KKNMS), the role of early sNfL values for the diagnostic evaluation of patients was

### Box 1 Neurofilament in a nutshell

Blood levels of sNfL, a neuron-specific cytoskeletal protein, have emerged as a biomarker able to capture neuronal damage in multiple sclerosis and a wide variety of neurological conditions. Following neuronal damage, sNfL is released into the CSF and subsequently into the blood, where it can be measured with current ultrasensitive assays (SIMOA), overcoming the problem of the inherently invasive lumbar punctures needed for CSF-based markers. This simple approach to assessing the degree of ongoing neuronal damage in the peripheral blood during standard patient care could greatly enhance clinical decision-making. One major advantage of sNfL is that it shows high stability at room temperature and in frozen blood samples, and it is not affected by thawing cycles or storage time,<sup>8,17,18</sup> opening the door for broad application. Although many candidate biomarkers were in the past found to correspond to existing clinical information, to add no additional information to MRI<sup>19,20</sup> or to be too technically challenging to implement in clinical practice, sNfL is not hampered by these issues. It is important to note when comparing different studies that the commercially available SIMOA assay<sup>21,22</sup> is known to produce lower absolute sNfL concentrations (by about 50%) compared to previously used assays with different protocols in earlier studies.<sup>23,24</sup> Importantly, a recent multicentre study analysing identical serum samples across 17 different international sites reported excellent inter-assay (<6%) and inter-site (<9%) coefficients of variation for the most widely used commercial NF-light™ assay.<sup>25–29</sup> Inter- and intra-batch assay variability, as well as variability across different newly emerging technical platforms (e.g. the ELLA system)<sup>30</sup> are issues that still need to be addressed. Further international efforts to standardize sNfL measures are ongoing.<sup>27,31</sup> See Table 2 for recommendations on quality controls to be reported in publications. Furthermore, sNfL has thus far been investigated on a group level, whereas prospective use on an individual patient level has not yet been established (see Fig. 1 for proposed clinical algorithms).

## Box 2 Overview on the role of sNfL in multiple sclerosis

- Role in preclinical multiple sclerosis? Serum NfL is increased up to 6 years prior to first clinical symptoms and indicates a risk for a first clinical event in patients with radiologically isolated syndrome, when it is increased.
- Role in diagnosis? At a group level, sNfL is higher in patients with RRMS than in healthy controls. sNfL thus indicates disease versus functional symptoms and might improve differentiation between CIS and RRMS patients when included in current diagnostic criteria.
- Role for prognosis? Elevated sNfL levels have predictive value for future relapses, new gadolinium-enhancing or T<sub>2</sub> lesions and future brain and spinal cord atrophy. With regard to clinical outcome, long-term predictions according to high sNfL levels is still controversial, while the predictive value of short-term EDSS-deterioration is undisputed.
- Role for monitoring of disease activity? Levels of sNfL are associated with clinical and MRI parameters that indicate inflammatory disease activity. Low or stable sNfL levels can exclude clinical or subclinical disease activity. Small increases in sNfL levels may indicate progression in relapse-free phases.
- Treatment response? Levels of sNfL are decreased by effective treatment initiation in both clinical trials and real-world cohorts. First studies suggest that sNfL levels might be able to differentiate between low and high efficacy treatments.

assessed.<sup>21</sup> The inclusion of sNfL levels as an additional parameter into the current 2017 version of the McDonald criteria<sup>45</sup> increased the sensitivity and specificity of differentiating patients with CIS and relapsing-remitting multiple sclerosis (RRMS).<sup>21</sup>

## Serum NfL as a biomarker of disease activity in multiple sclerosis

### Serum NfL, clinical activity and MRI: short-term

A broad spectrum of clinical and MRI parameters linked to inflammatory processes have demonstrated correlations to concurrently assessed sNfL levels (Table 1). Large studies in 814 and 607 patients clarified that the current Expanded Disability Status Scale (EDSS) score and sNfL levels are weakly, yet significantly, associated (12% and 8% sNfL increase per EDSS step, respectively).<sup>21,23</sup> Furthermore, sNfL levels were shown to correlate with concurrent relapses,<sup>8,21,23</sup> the presence of gadolinium-enhancing lesions,<sup>8,9,21,43</sup> the number of gadolinium-enhancing lesions,<sup>14,15,23,47</sup> the occurrence of new T<sub>2</sub>-weighted lesions,<sup>9</sup> the number of new T<sub>2</sub>-weighted lesions,<sup>21,23,43,46</sup> the number and volume of cortical lesions,<sup>57</sup> the presence of T<sub>1</sub>-hypointense lesions in patients with CIS<sup>41</sup> and T<sub>1</sub> lesion volume<sup>46</sup> as well as normalized brain volume, a cross-sectional measure of brain atrophy.<sup>23</sup> Here it should be noted that sNfL levels are significantly increased after a relapse or detection of a gadolinium-enhancing lesion and can persist for some time (a few weeks to several months).<sup>9,15,23,58</sup> Other parameters thus far have demonstrated mixed results [e.g. (i) sNfL with T<sub>2</sub> lesion volumes with significant<sup>8,47</sup> and not significant<sup>23</sup> correlations; and (ii) sNfL and deep grey-matter structures with significant<sup>14,44,51</sup> and not significant correlations] or showed no correlation with sNfL values (e.g. the presence of oligoclonal bands or vitamin D3 levels).<sup>43,47</sup> See also Table 1 for a summary of the relationships between sNfL and different clinical/MRI parameters. Despite differences in methodological approaches across studies, all findings support that sNfL levels provide a good reflection of ongoing inflammation-driven neuroaxonal damage. This is in line with positive correlations between the inflammatory activity of multiple sclerosis lesions and axonal damage.<sup>59</sup>

Over a relatively short period, high sNfL levels were associated with an increased risk for relapses and/or EDSS deterioration over the next 1–3 years.<sup>8,21,23,48,49</sup> In a study of patients with multiple sclerosis and healthy controls (*n* = 259 each), sNfL levels above the 90th percentile of healthy controls predicted EDSS worsening in the subsequent year [odds ratio (OR) 2.8, confidence interval (CI) 1.61–4.83],<sup>23</sup> which confirmed findings from an earlier study in 241 patients with repeated serum sampling (OR 2.1, CI 1.03–4.29).<sup>8</sup> The

probability of EDSS deterioration gradually increased with each category of higher sNfL level percentile.<sup>23</sup> Notably, in a multivariable model, only sNfL predicted future brain volume loss in contrast with other parameters (T<sub>2</sub> lesion volume, baseline normalized brain volume and contrast-enhancing lesions).<sup>23</sup> Importantly, the central messages of these studies only apply to a relatively small portion of patients with multiple sclerosis who have the highest sNfL levels. Of note, studies reported 46% of samples<sup>23</sup> and 49% of samples<sup>8</sup> from patients with multiple sclerosis were above the 80th percentile and showed significantly more EDSS worsening in the following year.

### Serum NfL, clinical activity and MRI: long-term

A number of studies have confirmed that high sNfL levels have predictive value for future MRI-based brain atrophy over the next 2–5 years<sup>23,46,49,51</sup> and two studies have found predictive value for brain atrophy in the longer-term at 10 and 12 years.<sup>24,52</sup> In contrast, data concerning the longer-term predictive value of sNfL for disability progression are thus far less convincing. sNfL levels predicted the transition from RRMS to secondary progressive multiple sclerosis (SPMS) in two 5-year follow-up studies,<sup>51,53</sup> whereas other studies did not find a significant relationship between sNfL and the risk of SPMS conversion.<sup>48,54</sup> In three studies, sNfL was not associated with EDSS-progression over 5 years<sup>48</sup> and 10 years<sup>24,53</sup>, whereas a correlation was observed in other 5-year studies.<sup>53,60</sup> In a study of more than 120 patients, initial sNfL levels were not correlated with EDSS values after 10 years, but did predict T<sub>2</sub> lesion load and brain atrophy rates.<sup>52</sup> The patient cohort in this study, however, was rather benign, with only 11% of patients with multiple sclerosis reaching an EDSS score of 3.0 or more by 10 years (mean disease duration at first visit: 1.6 years). In contrast, another study with a more aggressive disease cohort recruited in the era before modern disease-modifying drugs (43% of patients reaching an EDSS score of 3.0 by 10 years; mean disease duration at first visit: 3.1 years) showed that patients with the highest sNfL levels progressed most rapidly with an annual rate of increase in the EDSS score of 0.16 over a median follow-up of 19 years.<sup>55,56</sup> In one large study in >4000 patients and a median follow-up of 5 years, high sNfL levels were associated with the risk of reaching an EDSS score of 3.0 and 4.0, but not 6.0.<sup>54</sup> One plausible explanation for the discrepancies observed across these studies might be the fact that sNfL levels strongly reflect acute, focal inflammatory neuronal injury due to relapses or subclinical MRI lesions in RRMS, and that this might mask slowly progressing neurodegenerative processes. At this point, our assessment is based on the size of the cohort studies and comes with the knowledge that technical differences

**Table 1** Current evidence on the correlation and prediction of clinical and MRI parameters by sNfL levels

| Parameter  | Level of evidence | Key results   |
|--|-------------------|---|
| <b>Cross-section correlation</b>                           |                   |   |
| Relapses and T <sub>1</sub> -gadolinium enhancing lesions  | +++               | Relapses and gadolinium-enhancing lesions causing acute neuronal damage are the most important driver of sNfL peaks. It is currently unclear whether blood-brain barrier damage in acute lesions facilitates efflux of sNfL proteins into the peripheral blood thereby resulting in higher absolute levels. <sup>8,9,15,21,23,43,44</sup> |
| EDSS   | ++                | Large well-powered studies have clarified that sNfL and current EDSS scores are weakly, yet significantly correlated. Furthermore, multiple studies have confirmed higher levels at later disease stages compared to earlier stable patients. Studies showing no correlation are most likely underpowered. <sup>8,21,23</sup>             |
| New T <sub>2</sub> lesions                                 | ++                | Both the occurrence and number of new T <sub>2</sub> -weighted lesions raise sNfL levels. <sup>9,21,23,43</sup>   |
| T <sub>1</sub> -hypointense lesions                        | +                 | Not as well studied, but was positively correlated in a few smaller studies. <sup>9,46</sup>  |
| Existing T <sub>2</sub> lesion load                        | +                 | sNfL and number or volume of existing T <sub>2</sub> lesions were significantly correlated in some studies, whereas no correlation was found in others. As sNfL indicates acute ongoing axonal damage, existing lesions without ongoing pathology are less likely to contribute to sNfL level increase. <sup>8,23,47</sup>                |
| Relapses and EDSS increase in the next 1–3 years           | +++               | High sNfL levels were consistently associated with an increased risk for relapses in the next years. Some studies indicate that the sNfL percentile category reflects the strength of this prediction. <sup>21,23,48–50</sup>   |
| <b>Prediction</b>  |                   |   |
| Brain and spinal cord volume loss in the next 2–5 years    | ++                | High sNfL levels are associated with future brain and spinal cord volume loss on a group level. It is plausible that high sNfL levels precede visual structural alterations in MRI, while exact time frames are still unclear. <sup>46,49,51</sup>  |
| Long-term EDSS progression (> 5 years) and SPMS conversion | +                 | The long-term predictive value of sNfL values is so far not consistent in all studies. While it is likely that investigations from further studies will bring more clarity, sNfL will probably be more useful in clinical situations with regards to prediction of the next 1–3 years. <sup>24,48,51–56</sup>                             |

+ = non-replicated observations that require further study or conflicting evidence.

++ = observations that have been replicated and/or supported by independent methods.

+++ = high level of evidence from larger studies, consistently replicated.

in laboratories may still influence results (Box 1). Precisely defined progression states in large patient cohorts may clarify the exact value of sNfL, reflecting gradual degenerative processes, since even patients classified as having RRMS can suffer from disability increase in relapse-free phases of the disease (so-called ‘progression independent of relapse activity’, PIRA<sup>61</sup>). Overall, sNfL is most likely a predictor for brain atrophy and a milder predictor for long-term EDSS development over several years; however, so far, it is not an irrefutable predictor for conversion to SPMS.

## Relationship between serum NfL and cognitive impairment

A limited number of studies thus far have investigated the association between NfL and cognition in multiple sclerosis. A small study in 27 patients did not find correlations between sNfL levels and symbol digit modalities test (SDMT) scores after 1 and 10 years, while other studies demonstrated an association between serum and CSF NfL levels and lower verbal fluency performance or SDMT scores.<sup>52,62,63</sup> In one larger study using BICAMS (Brief International Cognitive Assessment for Multiple Sclerosis), cognitively impaired patients with multiple sclerosis had higher sNfL levels and a greater longitudinal sNfL increase compared with non-cognitively impaired patients.<sup>51</sup> Limitations of this study include the heterogeneous study cohort (mixed CIS, RRMS and SPMS patients) with quite low inflammatory activity, the use of binary categorizations of both BICAMS and sNfL based on cut-off values (‘normal’ versus ‘not-normal’), and the fact that cognitively impaired and non-cognitively impaired patients varied in a number of parameters that potentially impact sNfL levels (e.g. age and EDSS).

Nevertheless, another study confirmed the main finding that CSF NfL is higher in multiple sclerosis patients with cognitive impairment and especially in those with impaired information processing speed and verbal fluency, assessed by the Brief Repeatable Battery of Neuropsychological Tests (BRBN).<sup>64</sup> Another interesting concept is that high sNfL might (probably due to relapses or new gadolinium-enhancing lesions) precede short-term changes in cognitive parameters: data from the phase III EXPAND trial (siponimod versus placebo in patients with SPMS) showed that patients with high baseline sNfL levels had a ~40% greater risk of 6-month SDMT worsening than patients with low sNfL values.<sup>65</sup> Overall, while initial data do indeed point towards an association between sNfL and measures of cognitive impairment, sNfL as a marker of axonal damage will probably not be useful as a singular specific marker for cognitive damage in patients with multiple sclerosis. This notion is further supported by pathological concepts preferentially linking grey matter damage and network dysfunction to cognitive impairment.<sup>66,67</sup> Further studies should therefore focus on developing multi-modal approaches integrating sNfL levels with MRI data and other molecular biomarkers indicative of grey matter damage (see also below).

## Combination of serum NfL with other markers

As sNfL specifically reflects neuroaxonal damage, the addition of one or several other markers might give a broader view of the pathophysiological processes in multiple sclerosis. Indeed, a number of studies have started to investigate whether sNfL composite scores are able to outperform single biomarkers.

Glial fibrillary acidic protein (GFAP) is the major cytoskeleton protein in astrocytes and released upon changes in cellular integrity. GFAP is drawing increased research interest as a second major blood biomarker that can reliably be measured in serum samples and that is moderately correlated with sNfL.<sup>68,69</sup> Early studies in patients with multiple sclerosis suggested that GFAP is not elevated in association with acute relapses and focal inflammatory infiltrates, and hence could be used to elucidate the ongoing glial-driven neurodegenerative pathology.<sup>68,70</sup> Indeed, using diffusion tensor imaging as a means to assess diffuse neuroaxonal damage not visible in conventional MRI, it has recently been shown that both T<sub>2</sub> lesions and diffuse damage contribute to sNfL levels, and that the latter was preferentially found in older patients with more advanced disease course.<sup>71</sup> These concepts were further expanded by efforts combining sNfL with GFAP, emphasizing that a combination of both markers might be useful in differentiating RRMS from SPMS patients.<sup>69</sup> This notion is supported by an independent report that assessed GFAP and chitinase-3-like protein 1 (CHI3L1) as markers of astrocytic and microglial activation. A simplified 'glia score' (GFAP × CHI3L1 / sNfL) was higher in SPMS versus RRMS patients and correlated with EDSS values only in SPMS patients.<sup>72</sup> These studies are also interesting as they are in line with the concept that glia activation is closely linked with axonal damage and disability progression in multiple sclerosis. Assessing both sNfL and GFAP simultaneously might be useful for differentiating multiple sclerosis activity across different stages of the disease.

Other approaches have assessed the combination of sNfL and markers of B cell activity in light of the recent appreciation of (intrathecal) B cells as drivers of multiple sclerosis pathology.<sup>73</sup> Patients that are positive for oligoclonal bands have higher serum and CSF NfL levels in comparison with oligoclonal band-negative patients.<sup>74,75</sup> In one study of 142 patients, sNfL values were associated with CSF total CD80<sup>+</sup> (i.e. B cells and myeloid cells) as well as CD80<sup>+</sup>CD19<sup>+</sup> (i.e. B cells) frequency.<sup>47</sup> Patients with early multiple sclerosis were stratified into probable benign and aggressive disease courses based on MRI criteria, and the investigators found that combining sNfL with CD20<sup>+</sup>/CD14<sup>+</sup> ratios in the CSF (i.e. increased B cell frequency) considerably improved distinction between the groups compared to sNfL alone.<sup>76</sup>

As elaborated above, sNfL strongly reflects (focal) acute inflammatory axonal damage of the white matter and its clinical value might be extended by combination with other biomarkers indicating gradual grey matter damage. Parvalbumin is a protein expressed in GABAergic interneurons and has been proposed as a marker of cortical grey matter neurodegeneration in patients with multiple sclerosis.<sup>77,78</sup> A reduction in CSF parvalbumin correlated with meningeal inflammation, cortical lesion load, cortical thickness and cognitive impairment; all of which possibly showed a better correlation of gradual degenerative pathology than NfL levels. Extending these findings in larger, multicentre longitudinal studies is warranted to assess a possible combination of both biomarkers.

In summary, combining sNfL with other biomarkers reflecting glial activation, intrathecal inflammation or grey matter pathology is highly promising. The integration of a multi-modal biomarker assessment of multiple sclerosis pathology must still be investigated in large cohorts and in international efforts.

## Serum NfL in multiple sclerosis: considerations of age as a potential confounder

An important point that needs to be considered when implementing the use of sNfL in clinical practice is the difficulty in developing normative values. Reassuringly, consistent reports have demonstrated

that sNfL is not impacted by sex in healthy cohorts or in patients with multiple sclerosis.<sup>25</sup> However, the physiological increase in sNfL levels seen with ageing in healthy individuals<sup>54,79,80</sup> needs to be taken into consideration when interpreting sNfL data. The strength of the association between age and sNfL levels seems to be dependent on the specific age of the investigated cohort as well as the underlying disease. In both a meta-analysis and independent cohort studies in multiple sclerosis, no clear association between CSF NfL levels and age was observed for patients with multiple sclerosis, in contrast with healthy controls or people with most neurodegenerative disorders.<sup>6,21,47,54</sup> It is plausible that the increase in sNfL levels observed in healthy controls (e.g. due to age-related neuronal loss or preclinical age-related disorders) is masked by the higher baseline inflammatory activity regularly observed in younger patients with multiple sclerosis. Thus, compared with inflammatory-associated elevations, the age-dependency of sNfL may not be a relevant confounder in clinical practice in younger patients without comorbidities. Some studies took the approach of modelling the 'normal' sNfL distribution across different ages based on a matched healthy donor control cohort,<sup>8,23,79</sup> but unfortunately, different normative sNfL values were reached in each study despite being from the same laboratory.

## Serum NfL as a biomarker of treatment response in multiple sclerosis: towards personalized immunotherapy

In an early study, CSF NfL levels in patients with multiple sclerosis were reduced to the levels of healthy controls 6 to 12 months after treatment initiation with natalizumab, providing for the first time evidence that that: (i) NfL levels increase upon acute inflammatory attacks; and (ii) a subsequent control of inflammatory disease activity with an immunomodulatory drug reduces NfL levels back to baseline levels.<sup>81</sup> Since then, a broad number of studies have confirmed that sNfL values from patients receiving immunomodulatory drugs are generally lower than untreated patients and that initiation of any treatment is associated with a decrease in sNfL levels.<sup>8,9,15,21,24,82,83</sup>

Early studies suggest that sNfL levels might be able to differentiate between different treatments at a patient group level. In one study, patients who changed treatment between disease-modifying therapies with similar efficacy had stable sNfL concentrations, while patients who escalated to therapies with higher efficacies had decreased sNfL concentrations after a median follow-up of 12 months.<sup>15</sup> However, it should be noted that this study cohort of multiple sclerosis patients was quite heterogeneous and that no data on T<sub>2</sub> lesions was available. Confirming and extending these findings, patients starting highly active immunotherapies have higher sNfL levels at treatment initiation than those starting on mild/moderate therapies, leading to a larger relative decrease after commencing therapy.<sup>21,24,63</sup> The number of future therapy changes, as well as treatment escalations were predicted by baseline sNfL levels.<sup>21,24</sup> Another well-powered study assessed registry data from 1261 patients on different disease-modifying therapies and after using inverse propensity score weighting to correct for differences in baseline factors, confirmed a similar picture: patients starting alemtuzumab displayed the highest reduction and lowest on-treatment sNfL levels, while patients on teriflunomide started from lower levels, had a smaller decrease in sNfL and higher on-treatment levels.<sup>63</sup> These studies underscore that repetitive measurements and evaluating longitudinal changes in sNfL will likely be an important part of supporting and managing therapy decisions.

After broadly introducing SIMOA assays, sNfL levels were analysed retrospectively from stored samples of completed randomized clinical

trials. In phase 3 trials of fingolimod, natalizumab and alemtuzumab, sNfL reflected the same benefit with therapy initiation as clinical and MRI parameters.<sup>56,84</sup> Subsequently, a recent study simulated whether sNfL could serve as an end point in phase 2 studies in patients with multiple sclerosis. Assuming typical features of a phase 2 trial in RRMS (6 months, 90% power, 5% significance level) and taking into account sNfL data from the FREEDOMS trial, between 28 and 143 subjects per arm would have been needed to show a 20–40% reduction in sNfL levels.<sup>85</sup> Although the calculated numbers sound realistic, no trials utilizing such a design have been performed to date, but this may change in the near future. In the ASCLEPIOS trial (ofatumumab versus teriflunomide), sNfL was included for the first time prospectively as a secondary end point in a phase 3 multiple sclerosis trial. Interestingly, sNfL levels were significantly different between both groups, whereas brain atrophy rates were not, raising questions about the underlying causes for this discrepancy.<sup>86</sup> It is plausible that short-term sNfL changes might rather reflect inflammatory processes in multiple sclerosis and changes are therefore evident earlier than MRI-based brain atrophy. As sNfL is included in most upcoming larger clinical trials, more insight will be obtained in the future.

## Conclusions and future directions

In recent years, numerous studies have linked sNfL with outcomes related to disease activity, disability progression, treatment response and prognosis in patients with multiple sclerosis, generating convincing evidence that sNfL may soon be broadly used as the first blood-based biomarker monitoring disease activity and treatment responses in clinical practice. One major advantage of

sNfL is that it is stable in fresh and frozen blood samples and is not affected by thawing cycles or storage time, opening the door for a broad range of applications. From a technical viewpoint, the next challenges in the sNfL field are to establish age- and comorbidity-adjusted normative values and to ensure methodological harmonization across different laboratories (see **Box 3** and **Table 2** for recommendations on quality controls). From a clinical point of view, the two questions ‘When’ and ‘How’ are of utmost relevance: When should sNfL be measured and how should findings be integrated into clinical decision-making? Because of the high inter-individual distribution of sNfL levels, we postulate longitudinal intra-individual changes being the most appropriate application for assessing clinical activity and treatment responses. Given the ongoing expansion of the therapeutic landscape in multiple sclerosis, sNfL could support individualized decision-making. From a clinical standpoint, longitudinal sNfL assessments of patients with RRMS can support therapeutic decisions in key areas including: (i) initial classification of CIS versus RRMS; (ii) choice of initial treatment; (iii) evaluation of subclinical disease activity in parallel with MRI measurements; (iv) treatment escalation in clinically active patients; and (v) treatment de-escalation or treatment cessation (**Fig. 1** and **Table 3**). Importantly, sNfL measurement needs to be considered in a comprehensive and context-specific manner together with clinical information and other MRI markers of disease activity. While sNfL may indeed become the first blood biomarker with relevance in multiple sclerosis monitoring, multimodal composite indices integrating existing or other emerging markers could enable increasingly precise individualized treatment decisions.

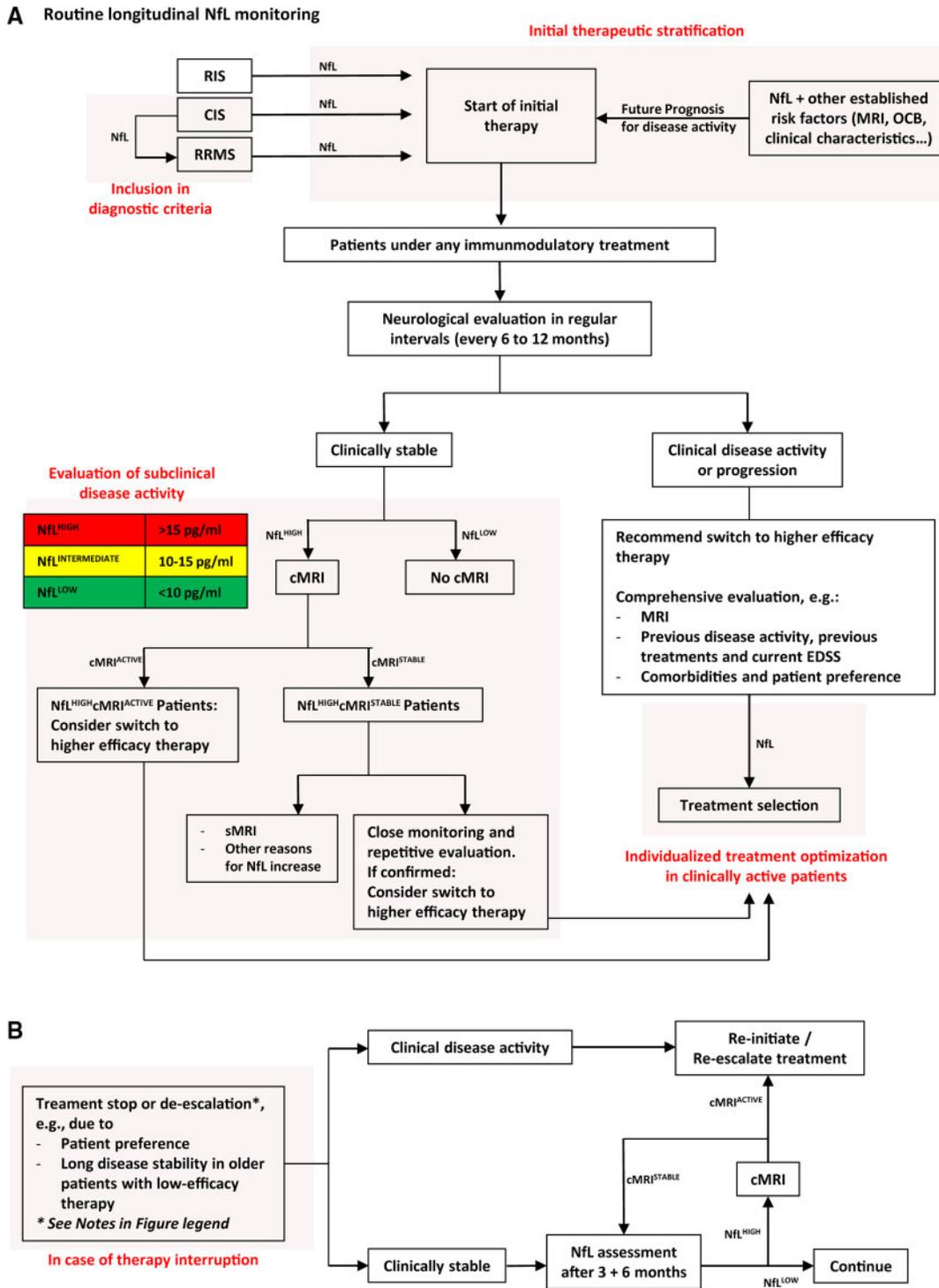
### Box 3 Key challenges

- To clarify when and how often sNfL should be measured to assess subclinical disease activity and guide therapeutic decisions.
- To define the threshold that constitutes a clinically meaningful change in longitudinal measurements.
- To clarify whether absolute values are comparable in standardized investigations.
- To implement the standardization of neurofilament measures and values across different assays and laboratories.
- To take confounding factors (e.g. age and other comorbidities) into account. It should be clarified whether it is sufficient to consider age exclusively in elderly cohorts (>60 years) as the association with sNfL is weak in younger patients with RRMS.

**Table 2 Suggested quality criteria to support the validity of measurements**

| Checkpoint                       | Quality criteria   |
|----------------------------------|--|
| Replicate measurements           | Calibrators and samples should be measured at least in duplicates. Samples with a missing result for a replicate or a CV of duplicate determination > 20% should be measured again. The number of samples with repeated measurements due to quality criteria should be reported in the method section. |
| Intra-assay precision            | Mean CV of duplicate determinations should be reported. Intra-assay CVs below 10% can usually be achieved.   |
| Control samples                  | Three (pre-characterized) control samples with low, medium and high NfL concentrations should be included in each run to monitor any matrix effects and to determine the inter-assay CV. Control samples should preferably be derived from the same material as samples (e.g. serum, plasma or CSF).   |
| Inter-assay precision            | Inter-assay CV should be reported. Values below 10% can usually be achieved and may reduce the risk of reporting plate effects instead of true group effects.  |
| Different LOTs or assay versions | Inter-LOT effects should be negligible. However, caution is advised with different assay versions. If different LOTs were used this should be announced in the method section and the inter-LOT CV should be reported.   |
| Blinding                         | Individuals performing the NfL measurements should be blinded to clinical data.  |

Note that the recommendations apply to the first broadly used commercially available platform (NfL-light™ assays, Quanterix, HD-1/HD-X). CV = coefficient of variation.



**Figure 1** Potential sNfL decision concepts in clinical practice. (A) Longitudinal sNfL assessment algorithm guiding treatment optimization in RRMS. Red fields mark four areas using sNfL for guiding decisions in (i) initial diagnosis of multiple sclerosis; (ii) choice of initial treatment; (iii) evaluation of subclinical disease activity; and (iv) treatment optimization in clinically active patients. (B) An sNfL assessment algorithm in patients undergoing treatment cessation or de-escalation, stratifying patients with stable disease course and those in need of therapy reinitiation or re-escalation. Note that First, no randomized controlled trials have directly addressed the question of whether or when to discontinue treatment in RRMS patients who have no evidence of relapses, no disability progression and stable MRI parameters. Especially in patients on higher-efficacy therapies (e.g. natalizumab or fingolimod), the risk of return of disease activity or rebound when stopping treatment has been well documented. In agreement with the European and American Academy of Neurology (AAN) guidelines,<sup>87</sup> this algorithm is not suggesting treatment cessation in specific patient cohorts, but rather is an approach to implement sNfL in ongoing follow-ups and periodic re-evaluations when treatment cessation occurs for clinical reasons. Second, the suggested differentiation into NfL<sup>HIGH</sup>, NfL<sup>INTERMEDIATE</sup> and NfL<sup>LOW</sup> is a rough estimation based on our datasets and data from Table 3. These values apply to RRMS patients (age 18 to ~40–50 years) without comorbidities and are currently only partially validated within international efforts. Older age groups still have to be compared to normal cohorts, since the age-associated sNfL increase seems to be markedly steeper beyond about 50 years of age and is less studied up to date. cMRI = cranial MRI; NEDA = no evidence of disease activity; OCB = oligoclonal bands; RIS = radiologically isolated syndrome; sMRI = spinal cord MRI.

**Table 3** Corrected reference values for sNfL levels for two clinically relevant scenarios

| Parameter   | Reported values  | Corrected values   |                              |
|---|--|--|------------------------------|
| MS versus healthy controls  | 9.7 pg/ml (age 18–40 years; sNfL >95th percentile of healthy cohort) <sup>26</sup>             | 9.7 pg/ml  |                              |
|   | 29.3 pg/ml (age 30 years, sNfL > 95th percentile of healthy cohort) <sup>23</sup>              | 14.7 pg/ml <sup>a</sup>  |                              |
|   | 27.9 pg/ml (age 30 years, sNfL > 95th percentile of healthy cohort) <sup>8</sup>               | 14.0 pg/ml <sup>a</sup>  |                              |
|   | 14.4 pg/ml versus 8.5 pg/ml (pooled study data patients versus healthy controls) <sup>84</sup> | 14.4 pg/ml versus 8.5 pg/ml  |                              |
|   | 11.4 pg/ml versus 7.5 pg/ml (MS patients versus healthy controls) <sup>54</sup>                | 14.3 pg/ml versus 9.4 pg/ml <sup>b</sup>                                     |                              |
|   | 17.0 pg/ml versus 8.2 pg/ml (MS patients versus healthy controls) <sup>88</sup>                | 17.0 pg/ml versus 8.2 pg/ml  |                              |
|   | 10.1 pg/ml versus 7.3 pg/ml (MS patients versus healthy controls) <sup>55</sup>                | 10.1 pg/ml versus 7.3 pg/ml  |                              |
|   | Serum NfL comparisons indicating disease activity in MS patients                               | 25.0–45.1 pg/ml (median; presymptomatic to symptomatic) <sup>37</sup>        | 12.5–22.5 pg/ml <sup>a</sup> |
|   |  | 29.6–43.4 pg/ml (median; no Gd + lesion to Gd + lesion) <sup>8</sup>         | 14.8–21.7 pg/ml <sup>a</sup> |
|   |  | 28.9–39.3 pg/ml (median; no relapse to recent relapse <60 days) <sup>8</sup> | 14.5–20.0 pg/ml <sup>a</sup> |
| 9.9–16.1 pg/ml (median; no Gd + lesion to Gd + lesion) <sup>21</sup>  |  | 9.9–16.1 pg/ml   |                              |
| 28.1–63.2 pg/ml (median, no Gd + lesion to Gd + lesion) <sup>44</sup> |  | 14.1–31.6 pg/ml <sup>a</sup>   |                              |

Note that when considering published data, there is a significant variation in data analysis procedures, making it difficult to systematically compare different studies. To name a few challenges: published datasets have used mean, median, geometric, different parametric or non-parametric tests and cross-sectional or longitudinal analyses. Analyses are performed either on raw data (non-parametric tests) or in log-transformed data in order to use parametric tests. While age-adjusted z-scores of log-transformed data might indeed be the optimal statistical approach,<sup>26</sup> this is challenging to implement in a broad clinical setting. Therefore, only reports publishing absolute values with cut-offs were included. The presented data are only meant as a simplified approach for a rough range of expected values in two clinically relevant scenarios and not as validated cut-offs. Gd + = gadolinium-enhancing lesions; MS = multiple sclerosis.

<sup>a</sup>Because of technical differences between different protocols, values are reduced by 50% to give a rough estimation. For full values and more details (e.g. interquartile range), see the original publications.

<sup>b</sup>Plasma concentrations are ~25% lower than serum concentration (Thebault et al.<sup>26</sup> and own experience), values were increased accordingly.

## Search strategy and selection criteria

We searched PubMed and Web of Science for articles on neurofilament published between 1 January 2018 and 15 May 2021. Search terms were ‘neurofilament’, ‘neurofilaments’, ‘neurofilament light chain’, ‘NfL’, ‘sNfL’, ‘PML’ and all combinations of these phrases with ‘multiple sclerosis’, ‘MS’ and ‘neuroinflammation’. The final reference list was generated based on novelty and relevance to this review.

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