Neurofilaments as biomarkers in neurological disorders

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Key points:

- Neuronal damage and loss are the pathological substrate of permanent disability in various acute and chronic neurologic disorders.
- Levels of neurofilament proteins rise upon neuroaxonal damage in cerebrospinal fluid (CSF) and in the blood.
- 1st generation (immunoblot) and 2nd generation (enzyme-linked immunosorbent assay) neurofilament assays only captured the tip of the ice-berg in disease.
- 3rd generation (electrochemiluminescence) and 4th generation (single molecule array) assays permithighly sensitive longitudinal detection of blood neurofilament levels even in mild disease and from normal controls. **[Au: Edited to reduce key point to word limit (30 words). OK?]**
- Multicentre studies are underway to consolidate neurofilaments as biomarkers that reflect brain tissue damage, enabling longitudinal monitoring of disease activity and drug effects in clinical trials in neurological diseases. **[Au: Edited to reduce key point to word limit (30 words). OK?]**

Competing interests

The authors declare no competing interests related to this article.

Abstract:

[Au: Most of my edits to the abstract have been made to reduce the length - the limit is ~200 words, and the original was 240. Please check that you are happy with how it reads and that no crucial information has been removed.]

Neuroaxonal damage is the pathological substrate of permanent disability in various neurological disorders. Reliable quantification and longitudinal follow-up of such damage is important for assessing disease activity, monitoring treatment responses, facilitating treatment development and prognostic purposes. The neurofilament proteins have promise in this context because their levels rise upon neuroaxonal damage not only in the CSF, but also in blood, and they indicate neuroaxonal injury independent of causal pathways. First-generation (immunoblot) and second-generation (enzyme-linked immunosorbent assay) neurofilament assays were of limited sensitivity. Third-generation (electrochemiluminescence) and especially fourth-generation (single molecule array) assays enable reliable measurement of neurofilaments throughout the range of concentrations found in blood samples. This technological advancement has paved the way to investigate neurofilaments in a range of neurological disorders. Here, we review what is known about the structure and function of neurofilaments, discuss analytical aspects and knowledge of age-dependent normal ranges of neurofilaments and provide a comprehensive overview of studies on neurofilament light as a marker for axonal injury in different neurological disorders, including multiple sclerosis, neurodegenerative dementia, stroke, traumatic brain injury, amyotrophic lateral sclerosis and Parkinson disease. We also consider work needed to explore the value of this axonal damage marker in managing neurological diseases in daily practice.

[H1] Introduction

Neuroaxonal damage and loss are the pathological substrate of many acute and chronic neurological disorders that result in accrual of permanent disability. **[Au: Edited to avoid repetition. OK?]** The ability to readily detect and follow such damage would be a great advantage in the assessment of disease activity, monitoring of treatment responses and prognosis. **[Au: Edited for flow. OK?]** Therefore, a biomarker that accurately reflects neuroaxonal injury would be invaluable for reaching individual therapeutic decision and measuring drug effects in clinical trials. Attempts to discover such a biomarker have involved investigation of several avenues, **[Au: Beginning of the previous sentence edited to make clear that the avenues relate to the biomarker. OK?]** from cerebrospinal fluid (CSF) proteins to MRI, magnetic resonance spectroscopy **[Au: Changed to group imaging techniques together. OK?]** and metabolic imaging, and have provided different insights with different limitations.

Neurofilaments **[Au: We prefer not to abbreviate one-word terms, so I think it's best to not use the abbreviation for neurofilament when it's used alone, but use the abbreviations for NfL, NfM and NfH. OK?]** are gaining increasing attention as candidate biomarkers of neuroaxonal injury **[Au: From here to the end of the paragraph, I have rearranged the information so that it's clear that it's the combination of the specificity and abnormal levels in CSF and serum that makes neurofilaments so attractive as biomarkers. Please check you are happy with how this reads.]** because they are abundant structural scaffolding proteins that are exclusively expressed in neurons and that reach pathological levels as a result of axonal damage in neurodegenerative, inflammatory, vascular and traumatic diseases not only in the CSF, but also in serum. The specificity of neurofilaments in terms of cellular source and indication of pathomechanisms means they are highly specific for neuronal cell damage and eventual neuronal cell death, offering a key advantage over other possible biomarkers.

Many, if not all, pathological processes that cause axonal damage release neurofilament proteins into the extracellular fluid, CSF and peripheral blood, depending on the extent of damage. High levels of neurofilaments, **[Au: Addition of "high levels of" OK?]** therefore, are general indicators of axonal damage irrespective of its cause and any clinical diagnosis, and blood levels of neurofilaments are useful for monitoring and prediction of progression in various acute and chronic neurological diseases and for assessing the efficacy and/or toxicity of treatment.
Until recently, measurements of the most promising of the neurofilament proteins, neurofilament light levels in patients with neurological disorders could only be performed with CSF samples, mainly because assay sensitivity was insufficient for reliable quantification of neurofilament light

levels in the blood. Several studies of CSF **[Au: Addition of "of CSF" OK, to relate to the previous sentence more clearly?]** have demonstrated that levels of neurofilament proteins are increased in a wide range of neurological diseases[1]. However, given that lumbar puncture is a relatively invasive procedure, longitudinal analyses have been rare and not performed systematically. For the same reasons, neurofilaments have rarely been measured in diseases in which diagnostic lumbar punctures are infrequently indicated. Neurofilament levels in the blood can be quantified with enzyme-linked immunosorbent assay (ELISA)[2, 3] and more-sensitive electrochemiluminescence (ECL) assay technology in many different diseases[4, 5], but neither technique can detect small, disease-related changes. Only the introduction of single molecule array (SiMoA) **[Au: This format has been used by other Nature journals for this abbreviation]** assays has enabled reliable detection of neurofilament light proteins in blood samples across the whole range of concentrations, including those in healthy individuals[6-8]. Consequently, the past 2 years have witnessed a surge in the number of publications on neurofilament blood levels in a broad range of neurological disorders.

In this Review, we provide background on the structure and function of neurofilaments, consider the analytical aspects of neurofilament measurements and discuss current knowledge on age-dependent normal ranges of neurofilament concentrations. We also review the main neurological disorders in which neurofilament measurements could play a role in research or clinical settings, and highlight aspects that need to be addressed in future studies.

[H1] Neurofilaments — structure and function **[Au: Heading shortened to fit our character limits. OK?]**

Neurofilaments are classified as intermediate filaments according to their diameter (~10 nm), which is between that of actin filaments (6 nm) and myosin filaments (15 nm). Neurofilament heavy chain (NfH, 111 kDa), **[Au: Molecular weights added here to avoid the need for repetition of the subunits later in the paragraph. OK?]** neurofilament medium chain (NfM, 102.5 kDa), neurofilament light chain (NfL, 61.5 kDa) and α-internexin (55.4 kDa) belong to the class IV intermediate filaments, and peripherin (53.7 kDa) is a class III intermediate filament (Fig. 1). The molecular weights are higher in vivo owing to an abundance of negatively charged amino acids (glutamic acids) in their sequences and to post-translational modifications[11]. **[Au: Statement about molecular weights in vivo moved to here to avoid it being disconnected from the molecular weights after they have been moved to the previous sentence. OK?]**

The neurofilament proteins **[Au: Correct, or do you did you mean that class III and IV intermediate filaments contain intrinsically unstructured regions?]** contain intrinsically unstructured regions. One key feature of these unstructured regions is that a high proportion of residues are lysine[9, 10]; **[Au: Additions for clarity. OK?]** lysine and serine are the dominant amino acids in the neurofilament tail domain[9]. A relatively conserved, central α-helical rod region, a short variable head domain at the amino-terminal end, and a tail of highly variable length at the carboxy-terminal end are highly characteristic for the Nf protein subunits[11] (Fig. 1). **[Au: Subunit list deleted to avoid repetition of what is above. OK?]** The head domain contains serine and threonine residues and O-linked glycosylation and phosphorylation sites. The tail domain contains abundant glutamic and lysine-rich stretches of variable length with multiple serine phosphorylation sites. The central rod domain contains hydrophobic repeats that facilitate formation of coil-to-coil dimers.

Formation of neurofilament protein dimers is the first step in heteropolymer assembly. **[Au: Sentence added to enable addition of a paragraph break. OK?]** Antiparallel aggregation of these dimers leads to formation of tetramers, and eight laterally associated tetramers form the cylindrical unit-length filament (UFL) structure[11,12]. Annealing of UFLs leads to longitudinal elongation of neurofilaments, which is followed by radial compaction to form the final long neurofilaments with diameters of 10 nm (Fig. 1)[12].

Post-translational modifications of neurofilaments include addition of *O*-linked *N*-acetylglucosamine (O-GlcNAc) to individual serine and threonine residues, nitration, oxidation, ubiquitination and most importantly phosphorylation[11, 13]. All subunits are phosphorylated on their head domain, but only NfM and NfH are extensively phosphorylated on their carboxy-terminal domains, and this phosphorylation increases the resistance of these subunits to proteases[14]. **[Au: Does this edit retain your meaning? Or do you mean that the fact these subunits are phosphorylated makes them more resistant to proteases than the other subunits?]** Under normal conditions, neurofilaments are highly stable within axons, and their turnover is low. The filaments form a liquid crystal gel network with in diseases like ALS, Lewy-body-based dementia or Parkinson's disease, neurofilament accumulation **[Au: Please clarify what you mean by compartmental accumulation - where is this accumulation, and what compartments are being referred to?]** related to subunit stoichiometry and the degree of phosphorylation[14].

The precise functions of neurofilaments remain unknown, but they are thought to be critical for radial growth and stability of axons, thereby enabling effective, high-velocity nerve conduction[15,16]. Several reports indicate that neurofilaments interact with other proteins and

organelles, including mitochondria and microtubules[11], suggesting that they have important functions beyond preserving axonal stability.

Several mutations identified in the genes that encode neurofilament proteins can lead to abnormal neurofilament aggregation and accumulation with the consequence of axonal dysfunction and neurodegeneration. For example, mutations in the *NEFL* gene, which encodes NfL, lead to Charcot-Marie-Tooth Neuropathy Type 2E/1F (CMT2E/1F) **[Au: Please make clear what the two abbreviations mean specifically]** disease. Mutations of the genes that encode peripherin (*PRPH*), NfH (*NEFH*) and NfM (*NEFM*) have been associated with increased susceptibility to amyotrophic lateral sclerosis (ALS) and familial Parkinson disease (PD). Mutations in genes other than those that encode neurofilament proteins can have secondary effects on neurofilament aggregation; such mutations include those in heat-shock 27-kDa protein 1 in CMT2F, gigaxonin in giant axonal neuropathy and superoxide dismutase 1 (SOD1) in ALS[11,17].

[H1] Assays to detect soluble neurofilaments

In the past three decades, impressive advances have been made in the development of sensitive immunoassay technologies. With these advances, detection of neurofilaments has improved (Fig. 2), **[Au: See my suggestion for making Figure 1c a separate Figure 2]** moving towards evermore clinically useful capabilities. **[Au: I have added the previous sentence firstly because I felt it was helpful to relate the advances to neurofilament in a general sense at the outset, and also because I think it would be nice to have each generation discussed in its own paragraph, but breaking the paragraph here left one sentence in the first paragraph, which is not ideal. Addition OK?]**

First-generation **[Au: Unfortunately, our style does not allow use of italics for emphasis]** immunoassays were semi-quantitative at best. Immunoblots based on electrophoretic protein separation or dot blots were, however, consistent in that they reliably demonstrated the presence of neurofilament isoforms in the CSF and blood of patients with a range of diseases[10].

Second-generation sandwich ELISA technology produced the first reliable quantitative data that enabled assessment of the prognostic and diagnostic value of NfH and NfL in the CSF in human disease[2,18-20]. Human body fluid compartments that were analysed with this technique extended to the interstitial and extracellular fluid[21], serum and plasma, amniotic fluid and the vitreous body[22]. Meta-analyses and international validation studies demonstrated that high precision could be achieved in expert laboratories, but also highlighted the need for improved assay standardization[23,24]. **[Au: Standardization of what? Please clarify]**

Third-generation ECL technology led to a substantial improvement in the analytical sensitivity[4, 5, 25-27]. ECL based assays are known to be highly sensitive, exhibit a broad dynamic range and require small sample volume, however we found the SiMoA technology to be 126- and 25-fold more sensitive than ELISA and the ECL assay, respectively, to quantify NfL[7]. **[Au: Is it possible to expand on this statement to explain more about the technology, why it improved sensitivity and the limitations it still had?]**

Finally, fourth-generation SiMoA technology improved analytical sensitivity to an extent that reliable quantification of NfL levels in blood became possible across the range of concentrations that are observed in disease and in physiological conditions[6-8,28]. This cutting-edge method is based on single-molecule arrays and the simultaneous counting of singulated capture microscopic beads (2.7 µm diameter) carrying sandwich antibody complexes. **[Au: Please clarify for non-experts what these microbeads are - presumably neurofilament proteins bind to them?]** The analytical sensitivity is manifold higher than with use of the same antibodies in the ELISA format designed for CSF measurements[19], and enables reliable measurement of the low NfL concentrations in blood samples from young healthy individuals[6, 8] so that minor changes in levels of this protein that occur in normal ageing or after mild injury **[Au: Change from "concussion" to mild injury OK?]** can be monitored. Close correlation between NfL levels in the serum or plasma with levels in the CSF, which has been demonstrated in numerous studies and various neurological diseases, allows conclusions about the degree of ongoing neuroaxonal injury to be drawn from blood levels without the need to obtain CSF by lumbar puncture[4, 8, 29-36]. Investigations of NfM have been sparse[37], but commercial SiMoA kits for detection of NfL and phosphorylated NfH are available.

[H1] Neurofilaments in ageing **[Au: Edited to fit our character limits for headings and also because only ageing is discussed in this section. OK?]**

Normal ageing is associated with neurodegenerative processes that can be detected with various markers such as volumetric loss of brain tissue **[Au: Here, I think the wording raises the question for the reader of why neurofilaments are needed as a marker if neurodegeneration can be detected with other markers. I assume the existing markers, such as imaging, are more difficult and more costly and the neurofilament could be detected with a simple blood test - correct? If so, I think it would be helpful to explain this so that the reader is clear about the need for neurofilament.]** but also by increased levels of a range of fluid biomarkers comprising neurofilaments. The advantage of an easy to access body fluid biomarker, such as neurofilament in

the blood is to provide a real-time signal on neuro-axonal damage of the entire CNS, paralleled by lower costs and the ability of repeated measurements in a relatively non-invasive manner (Barro et al. Brain 2018). In CSF, the normal upper reference value for NfL levels increases 2.5-fold between the ages of 20 years and 50 years, and doubles further by the age of 70 years[38]. This age-related increase in levels in the lumbar CSF could be due to reduced CSF turnover[39], as a general physiological phenomenon[39] **[Au: Did you mean that reduced CSF turnover is a general physiological phenomenon? I have removed on the basis of this, as I felt it was not necessary, but if you meant a general physiological phenomenon to be a separate item in the list, please clarify]** but could also indicate slow, ongoing axonal injury. The latter possibility is supported by the finding that CSF levels of NfL in cognitively healthy elderly individuals correlate with hippocampal atrophy independently of age and AD biomarkers[40]. However, a detailed understanding of the mechanisms that underlie age-related increases in neurofilament levels is lacking. Besides structural damage and loss of neurons, metabolic alterations in the turnover of neurofilament proteins might play a role: experimental evidence demonstrates complex changes in the expression of mRNA, post-translational mRNA modification **[Au: "post translation" removed from here because it did not seem to relate to anything. If it is necessary, please clarify what it meant.]** and neurofilament protein turnover[41].

A highly significant correlation is also seen between age and NfL blood levels: use of **[Au: "fourth generation" removed from here because it implied that there have been four generations of SiMoA technology.]** SiMoA technology has shown that NfL levels in the blood increase by 2.2% per year between the ages of 18 years and 70 years[8,42]. The strong correlation between CSF and blood levels of NfL at the group level suggests that the two measures reflect similar physiological processes[4, 8, 29-36]. **[Au: Change from "factors" to "physiological processes" OK?]** Nevertheless, important to acknowledge is the possibility that degenerative processes in the PNS contribute to neurofilament levels in peripheral blood[4, 43, 44].

Important for further development of neurofilaments as a biomarker is to establish universal reference values for healthy controls by analysis of samples under standardized and controlled conditions in reference laboratories. These reference values would enable correct interpretation of levels seen in various pathological conditions, thereby maximizing the potential of neurofilaments in the management of **[Au: Addition of "the management of" OK?]** diseases that involve neuroaxonal injury.

[H1] Neurofilaments in neurological disease [Au: Edited to fit our character limits for headings. OK?]

CSF and blood levels of neurofilament proteins have been measured in various neurological disease (Box 1), and evidence has accumulated that they can be clinically useful biomarkers in many of these. Below, we discuss the evidence in each of the studied diseases. **[Au: I have added this paragraph to provide a short introduction to this main section and also to provide an opportunity to cite Box 1. Please see my suggestion for converting Figure 2 to Box 1 below.]**

[H2] Multiple sclerosis

Multiple sclerosis (MS) is a chronic disease of presumed autoimmune origin that is, at least initially, characterized by episodes of focal inflammation in the brain and spinal cord that predominantly involve the white matter but can involve the grey matter[45]. Formation of new lesions can be visualized with MRI, the only established biomarker of disease activity used in routine clinical practice today. However, MRI primarily detects lesions in the white matter, and grey matter damage is largely missed with standard imaging techniques[46, 47]. In addition, MRI does not allow selective detection of neuroaxonal degeneration, which seems to be the most important determinant of long term disability[48-50]. Several MRI-based volumetric measures, including analysis of cortical thickness, have been used to assess neuronal degeneration, but the specificity and sensitivity of these measures at the individual level are limited[51].

Use of second-generation immunoassays to measure NfL, pioneered by Rosengren et al.[18,52], revealed three key aspects of disease associated with CSF levels of NfL: **[Au: Edited for clarity about what the relationships are between. OK?]** the degree of disability, disease activity, and the time since the last relapse in patients with relapsing–remitting MS (RRMS)[52]. These initial findings were replicated and extended by subsequent, larger studies of clinical aspects associated with CSF levels of NfL[53-56] and NfH[26, 54, 57, 58].

Use of third-generation immunoassays further revealed that CSF levels of NfL reduce as a consequence of disease-modifying therapy (DMT). For example, initiation of the high-efficacy DMT natalizumab resulted in normalization of CSF NfL levels to those seen in healthy controls within 6–12 months[59], suggesting that NfL can be used to monitor therapeutic efficacy. Similar observations were made in placebo-controlled[60] and observational[61, 62] studies of fingolimod in patients with RRMS and in studies of mitoxantrone and rituximab[63] and of natalizumab[64] in progressive MS.

Despite the promising results in MS, a major barrier to widespread adoption of NfL analysis in MS research and clinical practice has been the requirement of CSF sampling, but this problem has finally been overcome by use of fourth-generation immunoassays. Of particular interest is the demonstration that serum levels of NfL can be used to separate not only patients with MS from healthy controls, but also patients with MS who have enhancing MRI lesions from patients without such lesions[8]. Furthermore, serum NfL levels in patients with MS have been independently associated with disability and relapse status[8, Barro et al., Brain 2018], **[Au: Please cite the appropriate reference(s) to support this statement]** and the risk of future relapses and disability worsening is higher among patients with high serum levels of NfL than those with lower levels[8, Barro et al Brain 2018] . **[Au: Please cite the appropriate reference(s) to support this statement]** Finally, patients with ongoing DMT had lower serum NfL concentrations than did untreated patients[8]. Yet another study found that patients who switched from injectable therapies to **[Au: Edited because it wasn't clear what injectable therapies are less effective in comparison to until you continued, whereas this wording makes clear that fingolimod is more effective than injectables. OK?]** fingolimod had significantly lower serum NfL levels than when they were on injectables **[Au: Lower than levels when they were on injectables, or lower than patients who continued on injectables? Please clarify]** over a 2-year period[30]. Associations with disease activity and treatment-related reductions in serum NfL levels were confirmed by another observational study in which a fourth-generation immunoassay was used in a large, independent cohort of patients with RRMS[36]. Recently, a longitucinal observational study demonstrated that patients with increased serum NfL at baseline, independently of other clinical and MRI variables, experience significantly more brain and spinal cord volume loss over 2 and 5 years of follow-up (Barro et al., Brain, 2018).

Collectively, the findings described (Table 1) make a strong case for bringing fourth-generation serum NfL assays from the bench to the clinics in the management of MS. Further studies are required to show how these assays can be used for monitoring disease activity and for therapeutic decision-making.

[H2] Dementias

Dementia — defined in this context as cognitive disturbances that are severe enough to interfere with activities of daily living — can be caused by several different neurodegenerative disorders, of which Alzheimer disease (AD) is the most prevalent, frontotemporal dementia (FTD) is the second-most prevalent among people aged <60 years, and dementia with Lewy bodies (DLB) is the second-most prevalent among patients aged >60 years. **[Au: Edited to define "older" on the basis**

of the definition of "younger" in the previous sentence. OK?] Currently, clinical diagnosis of the different types of dementia relies largely on documenting cognitive decline or on post-mortem evaluation. However, it is becoming clear that early brain damage occurs decades before the onset of clinical symptoms. This observation opens a window of opportunity for secondary prevention and suggests the value of **[Au: Change of wording OK?]** a shift from using clinical hallmarks for diagnosis to monitoring of biological measures that reflect ongoing pathological processes. Several studies have addressed the question of whether neurofilaments can provide such a biological measure (Table 2). **[Au: Sentence added to enable citation of Table 2. OK?]**

An early study in 1999 demonstrated a mild increase in CSF levels of NfL in AD, and substantially higher levels in FTD[66]. These findings were confirmed in a subsequent study that also showed that the increase in CSF levels of NfL in AD is seen only in patients with late-onset disease, **[Au: Edit to avoid referring to patients as "cases"]** whereas NfL levels are not significantly different from normal controls **[Au: Edited to specify controls - correct?]** in patients with early onset AD[67].

Subsequent meta-analyses of findings obtained with second-generation immunoassays consistently demonstrated that CSF levels of NfL are increased in the mild cognitive impairment and dementia stages of AD[23, 68] and are independent of Aβ load[68,69]. **[Au: Edited for clarity. OK?]** The diagnostic specificity of NfL levels was lower than the hallmark AD biomarkers of $A\beta_{1–42}$ levels, **[Au: OK?]** $A\beta_{1–42}$:$A\beta_{1–40}$ ratio and phosphorylated tau levels[68,70]. Nevertheless, evidence indicates that NfL levels correlate with and are predictive of brain atrophy and worsening of cognition independently from Aβ pathology[69, 71]. Moreover, NfL levels in the blood have some predictive value for progression to AD dementia in patients with subjective memory complaints[42], so the potential for use of NfL levels in combination with clinical evaluation and other biomarkers to detect the earliest stage of the disease should be assessed. Furthermore, the greatest value of NfL in AD dementia could be in monitoring responses to treatment, as in MS, as reductions in plasma NfL **[Au: Please explain what was observed - presumably NfL levels decreased?]** were observed in animal models of AD when treated with a BACE-inhibitor[31].

Measurement of NfL levels is also likely to be of value in the diagnosis of FTD, in which CSF[23,67] and serum[35,75] levels of NfL **[Au: CSF levels of NfL correct?]** are high and approach those observed in ALS (see Amyotrophic lateral sclerosis section below). Indeed, among the chronic dementias, the highest CSF NfL concentrations are observed in FTD and vascular dementia, followed by AD[23, 72]. Among patients with FTD, CSF levels of NfL are higher in those with TAR DNA-binding protein 43 (TDP-43) inclusions than in those with tau pathology (confirmed by genetic

testing or post-mortem evaluation)[73]. Moreover, CSF levels of NfL increased when symptoms developed in patients with genetic FTD, and these levels were inversely correlated with survival[35]. Several studies have confirmed a strong relationship between CSF and serum levels of NfL and the time to death in patients with FTD[35,73].

The results of fourth-generation immunoassays[74] for detection of neurofilaments reflect neuroaxonal damage in neurodegenerative dementias, including FTD[75], familial and sporadic AD[42, 76] and atypical parkinsonian disorders[77]. In sporadic AD, plasma NfL concentrations are already increased in the mild cognitive impairment stage, and correlate with cognitive, biochemical and imaging hallmarks of the disease[42]. In familial AD, blood NfL concentrations start to increase ~10 years before the expected onset[76].

Very high CSF and blood levels of NfL **[Au: This is a little confusing, because CJD has not been mentioned earlier in the section, and earlier in the section, it's stated that the highest levels of NfL are seen in FTD among the dementias. This conflict either needs to be resolved, or the discussion of CJD needs to be omitted. Also, does "highest" here mean within dementias, or the highest in any condition?]** have been observed in patients with sporadic and familial Creutzfeldt–Jakob disease. In this condition, CSF levels of NfL were increased before symptom onset, and the sensitivity and specificity of serum NfL concentration for diagnosis of Creutzfeldt – Jakob disease **[Au: Addition correct, to ensure it's clear what the sensitivity and specificity relate to?]** were 100% and 85.5%, respectively[78]. Elevated serum levels of NfL have also been described in patients with primary progressive aphasia[79]; **[Au: Please cite the appriopriate reference(s) to support the previous statement]** higher levels were identified in patients with the non-fluent or agrammatic and semantic variants than in those with the logopenic variant. NfL levels correlated with clinical progression and brain volume loss in all patients with primary progressive aphasias[79]. **[Au: Does the last sentence relate to all three variants, or only the non-fluent and semantic variants? Please clarify.]**

[H2] Stroke

Most existing data on neurofilaments in stroke are CSF measurements in subarachnoid haemorrhage (SAH). Studies have shown that NfH and NfL levels are higher among patients with aneurysmal SAH than among healthy controls or patients free of neurological disease[80-82]. **[Au: Please define the controls more specifically - were they healthy?]** The exact causes of neurofilament elevation in SAH in the absence of associated focal lesions (parenchymal haematoma or ischaemia owing to vasospasm) are not entirely clear, but are presumably

attributable to diffuse neuroaxonal injury or iatrogenic following for eaxample placement of an external ventricular drain. **[Au: Please clarify what you mean by "neurosurgical procedures"]** Regardless, evidence suggests that neurofilament levels consistently correlate with the clinical severity and extent of morphological brain damage[80, 81].

The ability to analyse neurofilament light levels in blood samples with fourth-generation immunoassays has facilitated the study of this marker in stroke in which a lumbar puncture is usually not indicated. **[Au: Please clarify. Do you mean that lumbar puncture is not indicated in some subtypes, or do you mean that lumbar puncture is not indicated in stroke generally? And by "not indicated", do you mean it is contra-indicated, or there is just no reason to do it?]** This approach has been used to show that serum levels of NfL are higher among patients with spontaneous cervical artery dissection who had an ischaemic stroke than among those with transient ischaemic attacks or isolated local symptoms[83]. **[Au: Change to "isolated" OK?]** Similarly, serum NfL concentration was found to be increased in patients with a single, recent, small subcortical infarct compared with concentration in age-matched and sex-matched healthy controls[84]. In the same study, assessment of the temporal dynamics of NfL at 3 months and 15 months after stroke revealed especially high levels in patients with new, clinically silent brain lesions related to small vessel disease detected with MRI during follow-up, suggesting that NfL levels indicate active small vessel disease. Interestingly, serum NfL levels increased during the first few days after stroke onset and remained elevated in a follow-up assessment at 3 months. Comparable findings of neurofilament dynamics have been reported in other studies[5, 83, 85]. Prolonged release of NfL into the blood after acute neuronal injury might be caused by persistent blood–brain barrier breakdown, but ongoing post-ischaemic immunological or inflammatory processes could also explain these findings.

[H2] Traumatic brain injury

Mild traumatic brain injury (TBI), also called concussion, is caused by non-penetrating head trauma and is increasingly recognized as a major health problem[86]. Most patients with mild TBI recover within hours to days, but a percentage have symptoms for weeks to months after the head impact, a condition called post-concussive syndrome. Furthermore, an unknown proportion of people who are exposed to repeated concussions, primarily contact sports athletes such as boxers and American football players and soldiers who are exposed to explosive blasts, develop a chronic neurodegenerative disease called chronic traumatic encephalopathy (CTE)[87], previously known as dementia pugilistica[88].

Mild TBI and post-concussive syndrome are vaguely defined clinical entities and their diagnosis is based only on the presence of one or several unspecific symptoms (such as loss of consciousness, dizziness, headache and poor concentration), causing a major issue in research, clinical management and drug development in this field[86]. Consequently, sensitive biomarkers are needed to identify and grade neuronal injury in individuals with mild TBI and post-concussive syndrome. Furthermore, biomarkers that enable grading of severity of neuronal injury after a mild TBI might be important as objective tools for guiding sports physicians with return-to-play decisions for their athletes.

Studies of contact sports athletes with mild TBI show that CSF levels of NfL increase more than levels of tau, suggesting that minor head injuries affect long myelinated white-matter axons more than they affect shorter cortical axons[89,90]. In severe TBI, fourth-generation NfL immunoassays[6] have demonstrated a marked increase in blood NfL levels that also predicted clinical outcome[91], thereby confirming earlier findings from third-generation immunoassay studies of CSF and blood samples[92]. Interestingly, marked increases in blood NfL levels have been detected in amateur boxers after a bout; higher NfL levels were seen in boxers who had received more head impacts, and levels approached normalization after 3 months of rest from boxing[93]. Similarly, blood levels of NfL were found to increase during the course of a season in American football players[94]. Taken together, these results (Table 3) support the idea that the blood level of NfL is a sensitive indicator of axonal injury after mild TBI and is a promising candidate for clinical application and contact sports medicine.

[H2] Amyotrophic lateral sclerosis

Motor neuron diseases are neurodegenerative disorders characterized by degeneration of the upper and lower motor neurons, and the most common form is ALS[95]. Given that axonal impairment can be seen early in the disease, measurement of neurofilaments in the CSF of patients with ALS was an obvious and straightforward experiment and led to the observation that NfL levels are increased in this condition[18,96,97].

Several independent studies have confirmed that neurofilament levels are significantly elevated in patients with ALS compared with several other disorders (Table 4); the largest prospective study included 455 patients[34, 98-103]. Diagnostic sensitivities and specificities were up to ~80%. Higher levels were also associated with faster disease progression. Increases in NfL and NfH levels were also observed in the early clinical phase of patients with genetic ALS and in patients with sporadic ALS[33, 104-107]. The first clinical sign of the disease seems to be associated with a

massive increase of neurofilament levels in the CSF[33], and a corresponding increase in NfL levels has been observed in the blood[33]. Furthermore, increased levels of blood NfH have been seen in patients with sporadic ALS [100, 102, 108].The difference in dynamics of higher NfL levels if compared to NfH levels in ALS and controls may partly be explained by earlier assay sensitivity issues. A new hypothesis, adaptive protein stoichiometry, suggests that the neurodegenerative process itself alters the quantitative relationship of neurofilament subunits. This leads to a relative over expression of NfL compared to NfM and NfH in order to minimise ATP requirements for subunit translation in the motorneuron [Zucchi et al. Journal Neurochemistry, 2018]. **[Au: Edited for clarity. OK?]**

Although helpful for diagnostic purposes, the reason for the very high CSF levels of neurofilaments in ALS is still not entirely clear, even under the assumption that neurofilament levels reflect neuroaxonal damage. One small study showed a correlation of NfL levels with axonal impairment assessed with diffusion tensor imaging (DTI)[109], **[Au: Edited to streamline. OK?]** but this correlation was not seen in a similar study that included 75 patients[101]. **[Au: Edited to clarify and simplify. Is there a significance to the number of patients in the second study - is it much larger than the number in the first study? Please make this clear, because I think it is important to indicate to the reader which of these two studies is likely to be more reliable.]** NfL concentrations in the blood seem to be stable at a very high level during follow-up of patients with ALS, whereas DTI values increase[34, 79]; only **[Au: Addition of "only" OK?]** a slight increase in blood levels of NfH has been described[99]. One mechanistic explanation is based on evidence that TDP-43, the major neuropathological hallmark of ALS, directly interacts with neurofilament production and causes **[Au: This wording suggests that the protein causes the release. Do you mean the aggregation of TDP-43 causes the release, or another aspect of TDP-43 function/pathology?]** the sudden and massive release of neurofilaments in ALS[110]. More prospective studies of neurofilament levels in ALS, especially in the blood, are needed. **[Au: Please expand on this sentence to say why they are needed - to determine the mechanisms, or for translation of the findings to clinical use?]**

[H2] Parkinson disease

Although Parkinson disease (PD) is one of the most common neurodegenerative disorders, no validated neurochemical biomarkers are currently available to aid clinical diagnosis. In PD and other synucleinopathies, α-synuclein is the main component of **[Au: OK?]** neuronal inclusions. Many studies have been performed to assess whether α-synuclein in the CSF could be an effective

biomarker of PD; ELISA has been used in most of these studies, which have produced contradictory results[112,113]. **[Au: Sentence edited for clarity. OK?]**

In 1998, NfL was first investigated in the CSF of 49 patients **[Au: Number of patients added here. OK?]** undergoing differential diagnosis for a Parkinsonian syndrome, including patients with atypical parkinsonian syndromes such as progressive supranuclear palsy (PSP) and multiple system atrophy (MSA). These investigations demonstrated increased CSF NfL levels in PSP and MSA compared with the PD group patients[114]. **[Au: Correct that the levels were increased in all of these patients?]** This increase in PSP and MSA versus PD was also seen for NfH[115]. In a larger study that included >450 patients with PSP, MSA or PD, almost no overlap was seen between CSF levels of NfL in patients with atypical parkinsonian disease and those with PD; NfL levels were increased mainly in the atypical disorders[116]. The finding was validated in an independent cohort[117].

In a study published in 2016, high levels of NfL were observed in blood of patients with PSP, and this difference persisted at one year follow-up. **[Au: Correct that the follow-up was after treatment? How long was the follow-up period? How were they treated? Please add more detail so that findings are clearer]** Patients with higher NfL levels had more severe neurological, functional, and neuropsychological deterioration over 1 year. Higher baseline NfL predicted greater whole-brain and superior cerebellar peduncle volume loss[118]. On the basis of these findings, the investigators concluded that NfL could be used not only to aid diagnosis, but also to monitor pharmacodynamic effects, especially in clinical trials. The findings of this study were also validated in three independent cohorts, leading to the suggestion that NfL could be used in both primary care and specialized clinics[77].

[H2] Huntington disease **[Au: I didn't see any reason to group HD and bipolar disorder together.]**

Huntington disease (HD) is a progressive neurodegenerative disorder caused by CAG repeat expansions in the *HTT* gene, leading to the formation of mutant huntingtin (mHTT). No proven disease-modifying treatments yet exist[119]. The slow and insidious progression of neurodegeneration in HD has made it challenging to detect disease-related changes in the levels of neurofilament proteins in the blood[120]. However, increased CSF levels of NfL have been demonstrated in patients with HD[121, 122], and fourth-generation technology has revealed a strong relationship between plasma levels of NfL, HD onset and subsequent progression of neurodegeneration[119]. If confirmed, blood NfL levels could be included as a secondary outcome measure in future clinical trials in HD.

[H2] Bipolar disorder **[Au: We don't generally cover psychiatric conditions, but I do feel it is interesting to include this because the fact that there are some changes in a disease associated with some degeneration reinforces the association of the marker with neuronal injury. However, I have suggested some changes to wording to emphasize the aspects that will probably be of most interest to our audience. Please check that you are happy with these changes.]**

Some evidence suggests that neurodegeneration and neuroaxonal injury can be associated with some subtypes of bipolar disorder[123]. Although these aspects are not prominent features of the condition, CSF levels of NfL were slightly increased in a subset of patients, particularly those who are treated with atypical antipsychotics[124], presumably reflecting a not yet fully understood disease-associated or treatment-associated effect. **[Au: Addition made to emphasize the focus on the biological/mechanistic aspects. OK?]** However, no clear relationship was seen between NfL levels and clinical outcomes, such as manic or hypomanic and depressive episodes (cross-sectional data), suicide attempts, psychotic symptoms or inpatient care[125]. Although the current evidence for detection of neuroaxonal injury in bipolar disorder by measuring neurofilaments is limited, the available results warrant longitudinal studies of well-characterized patients to examine how neurofilament concentrations change over time in relation to disease activity and phase (depression and mania) and whether neurofilaments can indicate adverse effects of treatments. Fourth-generation measurement technology will facilitate such studies by enabling measurements to be taken from blood samples.

[H1] Conclusions and future aspects **[Au: The last section of all our Reviews must contain the word "conclusions". Change of heading OK?]**

In summary, highly sensitive neurofilament measurements have the potential to fill a gap in the assessment of neuroaxonal damage in various neurological disorders. For the first time, this approach provides a sensitive assessment of the consequences of brain tissue damage with only a blood sample, an important advance to aid research and towards use of the assays in clinical practice. **[Au: Edited to give the importance of the advance a little more weight?]** In relation to clinical trials, the reviewed characteristics of neurofilaments, especially of NfL, make these proteins optimal candidates as markers of outcome in phase II trials in neurological disorders. Definitive phase III trials must use clinical endpoints (clinical events with a clear effect on the duration or quality of life) to confirm a clinical benefit, but the aim of phase II trials is to identify

drugs with sufficient activity to continue to phase III, so earlier end points are preferable. **[Au: Information about trial end points edited to improve flow. OK?]** **[Au: The previous sentence has been removed, as this passage felt a bit repetitive. OK?]**

To validate neurofilament measurements as phase II trial end points, two additional properties must be verified: a correlation with the clinical end points used in phase III trials, and an ability to detect a treatment effect. To verify these properties, a promising approach is retrospective analysis of data from randomized clinical trials in which blood samples suitable for measurement of neurofilaments have been collected. Comparison of neurofilament levels between subgroups of patients enrolled in the trials would determine whether the drug tested had an effect on the neurofilament biomarker. Moreover, neurofilament levels and their stoichiometry could be correlated with all the other relevant clinical and para-clinical measures collected in the trial.

The main factors limiting application of neurofilament measurements to disease monitoring individuals are the lack of normal values of neurofilament across all age groups, a detailed understanding of how comorbidities affect blood neurofilament measurements, and the need for thorough multicentre analytical assay validation to achieve standardized and reliable measurements across different sites. In light of the effect of ageing on neurofilament levels, generation of normative data in large collections of controls is a priority. Co-ordinated multicentre research activities are already ongoing to tackle these obstacles.

[Au: For references that are particularly worth reading (5-10% of the total), please provide a single bold sentence that indicates the significance of the work.]

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Figure 1 | Structure, assembly and release of neurofilaments. **[Au: The figure needs an overall title. Suggestion OK?]** **a |** Domain structure and post-translational modifications of neurofilament subunits[11]. Neurofilament light chain (NfL), neurofilament medium chain (NfM), neurofilament

heavy chain (NfH), α-internexin and peripherin are the subunits of neurofilaments in the mature nervous system. All neurofilament subunits include a conserved α-helical rod domain that comprises several coiled–coils, and variable amino-terminal globular head regions and carboxy-terminal tail domains. NfM and NfH subunits are unique among the intermediate filament proteins in that they have long carboxy-terminal domains with multiple lys–ser–pro repeats that are heavily phosphorylated. Phosphorylation and *O*-linked glycosylation sites on neurofilament subunits are shown. E segment, glutamic-acid-rich segment; E1, glutamic-acid rich segment 1; E2, glutamic-acid-rich segment 2. **[Au: We will use the three-letter amino acid codes in the figure, so the one-letter codes do not need to be in the legend.]** **b |** Neurofilament assembly. Neurofilament protein monomers form parallel coiled-coil heterodimers[11]. Two dimers form staggered antiparallel tetramers through interactions between coil domains 1a, 1b and 2a[12]. The lateral association of eight tetramers results in formation of cylindrical structures known as unit-length filaments that have a diameter of ~16 nm and a length of ~60 nm. Gradual end-to-end annealing of these unit-length filaments results in filament elongation, which is followed by radial compaction to form the mature, long neurofilament polymer with a diameter of ~10 nm. Tail domains of NfM and NfH radiate outwards from the filament core because of the extensive negative charges from large numbers of glutamic acid and phosphorylated serine and threonine residues.

[Au: I suggest that part c is a separate figure, because it deals with a slightly different aspect to the structure, and this avoids adding to what is already a very long figure legend.]

Figure 2 **|** Neurofilament release after axonal damage. When an axon is damaged, cytoskeletal proteins, including neurofilaments, are released into the extracellular space and subsequently into the CSF and, at lower concentrations, into the blood. First-generation (immunoblots) and second-generation (ELISA) immunoassays can typically detect neurofilament in the CSF. Third-generation (electrochemiluminescence) and, in particular, fourth-generation (Single molecue array) immunoassays can reliably measure blood levels of neurofilament light which was not possible from the blood with ELISA.

[Au: I think the information in your figure 2 would be better presented in a text box because it is really just text. I have suggested a format for this below.]

Box 1 | Relevance of neurofilaments to neurological disorders

Neurofilaments have been studied in several neurological disorders, and in many, good evidence indicates their diagnostic and prognostic value and/or their use for monitoring treatment responses. The disorders reviewed here are:

- Multiple sclerosis
- Dementia
- Stroke
- Traumatic brain injury
- Amyotrophic lateral sclerosis
- Parkinson disease
- Huntington disease
- Bipolar disorder (limited evidence for clinical utility).

In addition, neurofilaments could be of relevance in many other neurological disorders, but their association with these disorders has not been studied. Such disorders include:

- Epilepsy
- Encephalitis
- Meningitis
- Hypoxic brain injury
- Optic neuropathies
- Intracranial pressure
- Neurotoxicity
- Peripheral neuropathies including Guillain-Barré Syndrome, CIDP and CMT.