

# Neurofilaments: A Biomarker for Axonal Degeneration

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## 1 Aim

The aim of this chapter is to explain why *Neurofilaments (Nf)* are a useful *biomarker* for axonal degeneration and can be used as a *surrogate endpoint* in clinical and experimental research.

## 2 Definitions

- **Neurofilaments:** Nf are proteins which are exclusively expressed in neurons and their adjacent axons. Nf are particularly abundant in the axon, where they are key building blocks of the axonal cytoskeleton. The complex protein chemistry of Nf is briefly described.
- **Biomarker:** A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological responses to therapeutic intervention.
- **Surrogate endpoint:** defines a biomarker that is intended to serve as a substitute of a clinically meaningful endpoint and is expected to predict the effect of a therapeutic intervention or the evolution of disease.

## 2 Quantitative description

Nf are obligate heteropolymers that are composed of 4 subunits: a light (NfL), a medium (NfM), a heavy (NfH) [1] chain and also alpha-internexin [2, 3]. In some cases, peripherin may be added to the list [2]. These subunits differ not only in their molecular weight, but also in their functional properties, as discussed below.

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## **NfL**

The Nf light chain (NfL) is coded on chromosome 8p21 and consists of 543 amino acids. The molecular mass corresponds to 61 kDa, but due to phosphorylation and glycosylation, migration in sodium dodecyl sulfate (SDS) polyacrylamide gels (PAGE) is slow, and most authors refer to a molecular mass of 68 kDa as determined in SDS-PAGE. NfL forms the back-bone of the Nf heteropolymer and can self-assemble. Mutations in the NfL gene have been associated with Charcot-Marie Tooth disease.

## **NfM**

The Nf medium chain (NfM) is also coded on chromosome 8p21 and consists of 916 amino acids. The molecular mass is calculated as 102.5 kDa, and runs at 150 kDa in SDS gels. NfM is important for the radial axonal growth. One mutation in the NfM gene has been associated with Parkinsons disease.

## **NfH**

The Nf heavy chain (NfH) is coded on chromosome 22q12.2 and consists of 1020 amino acids. The molecular mass of the amino acids corresponds to 111 kDa. Most authors however refer to the molecular mass derived from SDS gels which is also influenced by the charge/weight of bound phosphate and therefore ranges from 190 to 210 kDa for the various phosphoforms. NfH is important for protein-protein interactions which is regulated locally in the axon by phosphorylation. Mutations in the NfH gene have been associated with Amyotrophic lateral sclerosis (ALS).

## **Alpha-internexin**

The 66 kDa alpha-internexin protein is coded on chromosome 10q24.33 and able to form homopolymers. Alpha-internexin has only recently been rediscovered as one of the Nf subunits and its role of alpha-internexin is still poorly understood [3]. Extracellular deposits of alpha-internexin are an important hallmark of a newly discovered neurodegenerative dementia named neurofilament inclusion disease (NFID).

## **Assembly of the Nf heteropolymer**

Figure 1 illustrated how NfL, NfM and NfH assemble to produce the Nf heteropolymer, which has a diameter of about 10 nm. Because of its size, which is *intermediate* between the smaller proteins, e.g. microfilaments (7 nm) and larger proteins such as microtubules (approximately 25 nm), the Nf heteropolymer belongs to the *intermediate filaments*.

## **Stoichiometry of the Nf subunits**

The estimated *in vitro* molar ratio of isolated Nfs from the mouse optic nerve and spinal cord is 4:2:2:1 (NfL:α-internexin:NfM:NfH) [3]. The *in vivo* stoichiometry of Nfs in body fluids remains unknown.

## **Classification of Nf**

Nf are type IV intermediate filaments (Table 1).

### **3 Nf are a biomarker for axonal degeneration**

Nf subunits are useful biomarkers for axonal degeneration, as illustrated in Figure 2. Any insult causing neuronal death or axonal degeneration will inevitably result in disintegration of the axonal membrane. Subsequently the contents of the axonal cytoplasm are released into the extracellular fluid (ECF). From the ECF Nfs diffuse into other body fluid compartments such as the cerebrospinal fluid (CSF), blood or amniotic fluid. As explained above Nf are a major structural protein component of the axon and the quantification of Nfs from body fluids therefore allows estimation of the degree of axonal degeneration.

#### **3.1 The measurement of Nf body fluid levels**

At present, high-throughput quantification of Nfs from body fluids and tissue homogenates is best achieved using enzyme linked immune assays (ELISA). In-house ELISAs have been developed for NfL and NfH [4, 5, 6, 7]. These assays are highly robust and have been cross-validated [8, 9]. A commercial NfH ELISA kit has recently been made available (Chemicon). Alternatively immunoblots or dot-blot assays have been used, but generally they are not high-throughput and only semi-quantitative.

#### **3.2 The diseases associated with high body fluid Nf levels**

Neuronal loss and axonal degeneration are a key feature in numerous disorders and frequently represent the endstage of a pathophysiological cascade. Not surprisingly, body fluid levels of Nf subunits have been used to estimate the degree of axonal damage in a number of diseases (Table 2). It is important to remember that body fluid Nf levels are not a diagnostic test for one single disease. In contrast Nf are a biomarker and surrogate endpoint according to the initial definitions.

## **4 Conclusion**

Neurofilaments are complex proteins composed of four subunits, expressed exclusively in the neuro-axonal compartment. Nf are released into the extracellular fluid from degenerating axons. From the extracellular fluid they diffuse into adjacent body fluid compartments. Using standard ELISA techniques Nf subunits have been quantified from the cerebrospinal fluid, the blood and the amniotic fluid. Because body fluid levels of Nf are related to the amount of neuronal death and axonal loss, they provide valuable prognostic information and correlate with disability in a number of diseases.

## **5 Acknowledgements**

I apologise to all colleagues whose work has not been cited due to space limitations. The biomarker definitions were adapted from a recent NIH meeting on biomarkers. A more complete list of references can be requested from the author (a.petzold@ion.ucl.ac.uk)

## **6 Glossary**

### **Neurofilament (Nf)**

Obligate polymer consisting of four proteins:  $\alpha$ -internexin, the neurofilament light (NfL), medium (NfM) and heavy (NfH) chains. Nf are a structural constituent of the neuronal and axonal cytoskeleton.

### **Alpha-internexin**

A 66 kDa protein encoded on chromosome 10q24.33.

### **Neurofilament light chain (NfL)**

A 68 kDa protein encoded on chromosome 8p21.

### **Neurofilament medium chain(NfM)**

A 150 kDa protein encoded on chromosome 8p21.

### **Neurofilament heavy chain(NfH)**

A 190 to 210 kDa protein depending on the degree of phosphorylation, encoded on chromosome 22q12.2.

### **Axonal degeneration**

There are two types: (1) Wallerian degeneration occurs distal to a lesion and effectively removes the damaged axon; (2) dying back neuropathy occurs proximal to the lesion and ultimately causes apoptosis of the neuron.

### **Biomarker**

A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological responses to therapeutic intervention.

### **Surrogate outcome**

A biomarker that is used as an outcome measure in e.g. a clinical trial. Surrogate outcomes are powerful tools increasingly used in large, multi-center trials.

### **Cerebrospinal fluid (CSF)**

The fluid surrounding the brain and spinal cord. In humans approximately 150 to 250 mL of CSF fill the ventricles and subarachnoid space all the way down into the lumbar sac. The CSF consists to 99% of water and has a much lower protein concentration (approximately 350 mg/L) than the serum (70,000 g/L). Of these proteins only about 10% originate from the ECF drained from the CNS parenchyma. These may be called “brain specific proteins” and are of particular interest for biomarker research.

### **Extracellular fluid (ECF)**

The extracellular or interstitial fluid surrounds the cells and is a component of the extracellular matrix.

### **Polymer**

A compound consisting of many repeated linked units.

Figure 1: Neurofilament assembly. The central rod domain of the Nf subunits is intertwined in order to form dimers. The dimers are arranged antiparallel to form tetramers. Tetramers combine to form protofilaments, which finally assemble to produce the 10 nm thick Nf (Figure reprinted with permission from reference [10]).

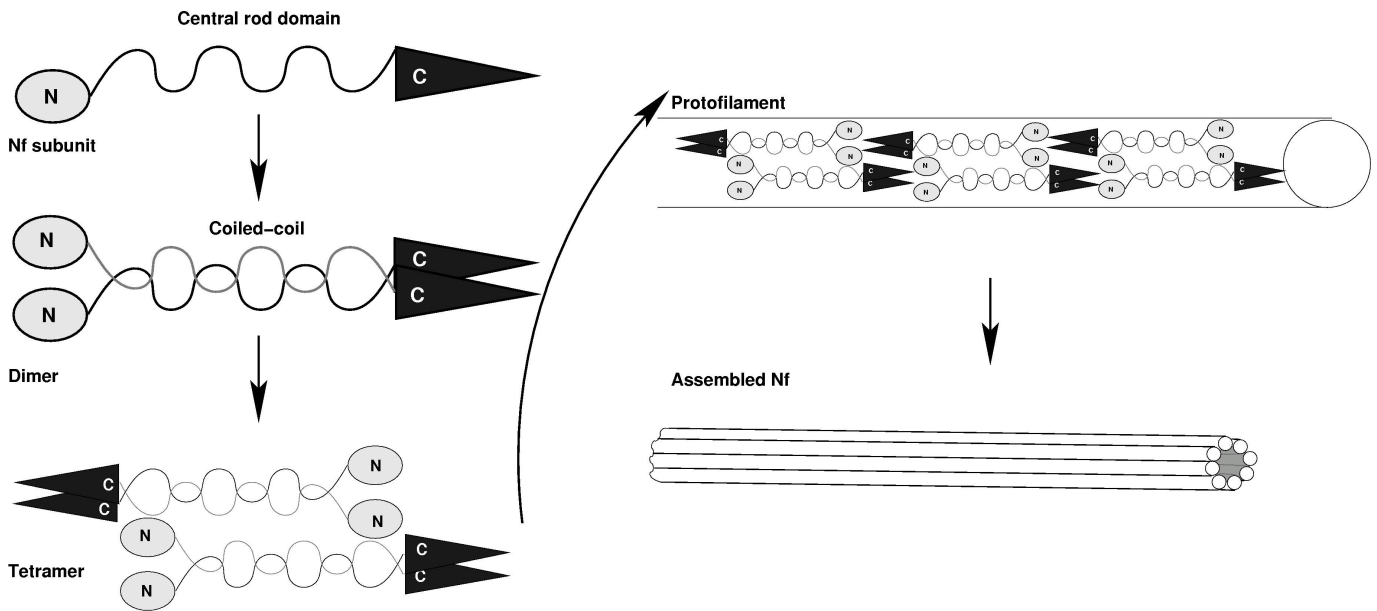


Figure 2: Neurofilaments are released into the extracellular fluid (ECF) following axonal disintegration. From the ECF Nfs equilibrate with the adjacent body fluid compartment. Quantification of Nfs is therefore possible from the cerebrospinal fluid (CSF), blood and amniotic fluid. The degree of axonal degeneration is related to the amount of Nf measured in these body fluids. For this reason body fluid Nf levels permit to estimate the amount of axonal degeneration. Axonal degeneration is extremely important because the loss of axons is irreversible and may therefore lead to persistent disability.

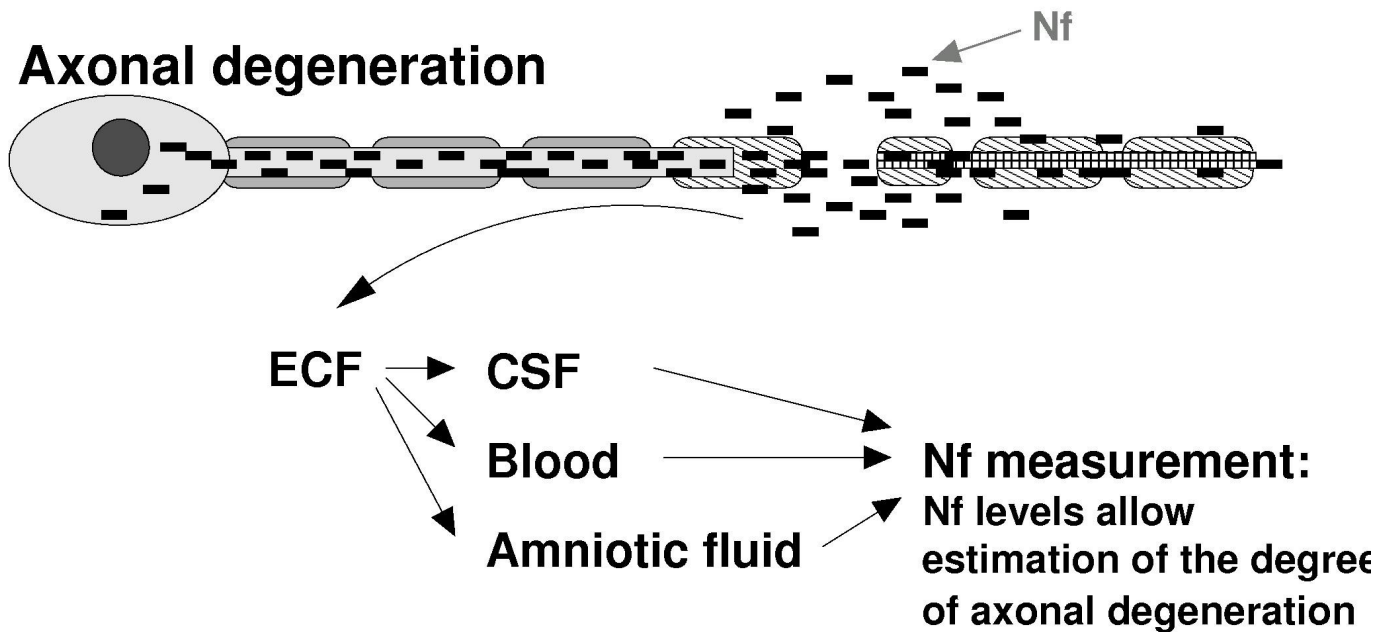


Table 1: *Classification of intermediate filaments and cell-type specificity. GFAP = glial fibrillary acidic protein.*

<b>Class</b>	<b>Identity</b>	<b>Cell-type specificity</b>
Type I	Acidic keratins	Epithelial
Type II	Neutral & Basic keratins	Epithelial
Type III	GFAP	<i>Astrocyte</i>
	Peripherin	Neuronal (peripheral)
	Vimentin	Mesenchymal
	Desmin	Muscle
Type IV	NfL, NfM, NfH	<i>Neuron &amp; Axon</i>
	Alpha-Internexin	<i>Neuron &amp; Axon</i>
Type V	Laminin A, B, C	Most cells
Type VI	Nestin	CNS stem cells



Table 2: Diseases in which Nf have been used as a body fluid biomarker for neuronal death and axonal degeneration. AD = Alzheimer's disease, ALS = amyotrophic lateral sclerosis, CBD = cortico–basal degeneration, DLB = Diffuse Lewy body disease, FTLT = fronto–temporal lobar degeneration, GBS = Guillain–Barré syndrome, ICH = intracerebral haemorrhage, MMC = meningo–myelocele, MS = multiple sclerosis, MSA = multiple system atrophy, NMO = neuromyelitis optica, ON = optic neuritis, PD = Parkinson's disease, PSP = progressive supranuclear palsy, SAH = subarachnoid haemorrhage.

Disease	Findings
AD	CSF NfL and NfH levels are elevated in AD. The difference from controls was marginal for CSF NfH levels and more impressive for NfL levels.
ALS	CSF NfL and NfH levels are considerably increased in patients with ALS. Rapidly progressing ALS patients had the highest CSF NfH levels.
CBD	CSF NfL and NfH levels are elevated in patients with CBD.
FTLD	CSF NfL is elevated and CSF NfH marginally elevated in patients with FLTD. The degree of NfH phosphorylation is increased in FTLT compared to AD and controls.
GBS	Elevated CSF NfH levels in patients with GBS are a poor prognostic sign, probably due to proximal axonal degeneration. Proximal axonal degeneration at the level of the nerve roots rapidly releases Nfs into the CSF. Proximal axonotmesis requires axonal regrowth over a long distance with the risk of losing chemical and anatomical guidance cues.
ICH	CSF NfH levels are high in ICH, probably indicating direct axonal degeneration due to rupture and ischemia.
DLB	CSF NfH but not NfL levels are elevated in DLB compared to AD and controls.
MMC	Amniotic fluid NfH levels are elevated in mice with MMC and correlated with the size of the lesion.
MS	CSF NfH and NfL levels are elevated in MS. CSF NfL levels are highest following a clinical relapse and return to baseline within about 3 months. CSF NfH levels are highest in the secondary progressive phase of the disease when axonal degeneration accumulates. The degree of NfH phosphorylation is increased in patient with more severe disease. High CSF NfH levels are a poor prognostic sign. Both CSF NfL and NfH levels correlate with disability.
MSA	CSF NfL and NfH levels are markedly elevated in MSA compared to controls and patients with PD. This may be related to the greater degree and more rapid disease progression in MSA. The highest levels are found in patients with the cerebellar variant of MSA, which may be of help for the differential diagnosis of patients with cerebellar syndromes.
NMO	CSF NfH levels are considerably elevated in NMO (synonymous Devic's disease) suggesting that these patients suffer from substantially more axonal damage than patients with MS or ON.
ON	Plasma NfH levels are increased in acute ON. CSF NfH levels were elevated in patients with subacute ON. Plasma and CSF NfH levels correlated with loss of visual function.
PD	CSF NfH and NfL levels are increased in PD compared to controls.
PSP	CSF NfL and NfH levels are elevated in PSP compared to controls and patients with PD. As with MSA this may be related to the greater degree of axonal loss and more rapid disease progression in PSP patients, who are also very resistant to pharmacological treatment.
SAH	CSF NfL and NfH levels are elevated in SAH and correlated with the outcome. Importantly CSF NfH levels showed a secondary increase during the high risk period of vasospasm, probably indicating secondary axonal degeneration following an ischemic insult.

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