Towards quantifying axonal damage in blood samples from patients with neurological diseases

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Abstract

Reliable biomarkers of axonal damage are urgently needed in neurological diseases. Neurofilaments (Nf) are specific structural elements of neurons composed of at least three subunits: Nf light chain (NfL), Nf medium and Nf heavy chain (NfH).
This PhD aimed to characterise NfL levels and their correlation with clinical features in patients with neurological diseases with a different rate of progression and following and under different treatment regimes. An important aim was also to develop a bioassay for NfL measurements in blood.
Cerebrospinal fluid (CSF) NfL levels discriminated patients with a clinically isolated syndrome (CIS) ($p=0.001$) or multiple sclerosis (MS) ($p=0.035$) from healthy controls more efficiently, and was more sensitive to change after natalizumab therapy ($p<0.0001$) than CSF NfH ($p=0.002$). Further, CSF NfL levels decreased in fingolimod-treated MS patients ($p=0.001$), but not in those receiving placebo ($p=0.433$). Based on these findings, a sensitive method for the detection of NfL in serum was developed and validated. Patients with neurological diseases had higher serum NfL values than controls. In acute spinal cord injury (SCI), serum NfL levels correlated with injury severity and long-term motor outcome, and Minocycline treatment was associated with decreased NfL levels in complete SCI patients compared to placebo. Finally, I found that serum NfL levels were higher in CIS patients than in healthy controls but did not predict conversion to clinically definite MS (CDMS). Independent predictors of CDMS were instead oligoclonal bands, number of T2 lesions and age at CIS. Lower 25-OH-vitamin D levels were associated with CDMS in univariate analysis, but this was attenuated in the multivariate model.
In conclusion, NfL proved to be an analytically stable protein which is an important prerequisite for biomarkers. The role of NfL quantification as a surrogate measure of neuroaxonal damage is corroborated by my findings and further supports the usefulness of NfL as a putative biomarker of axonal damage in various neurological diseases.

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Abbreviations

25-OH-D	25-hydroxy-vitamin D3 levels
AD	Alzheimer's disease
AIDP	Acute inflammatory demyelinating polyradiculoneuropathy
ALS	amyotrophic lateral sclerosis
ALSFRS-r	ALS functional rating scale
AMAN	Acute motor axonal neuropathy
AMSAN	Acute motor and sensory axonal neuropathy
AU	arbitrary units
AUC	Area under the curve
BS	brainstem syndrome
BSA	Bovine serum albumin
BvFTD	behavioural variant frontotemporal dementia
BL	baseline
CBS/D	corticobasal syndrome/degeneration
CCS	central cord syndrome
CDMS	clinically definite multiple sclerosis
CDRsb	clinical dementia rating
CHI3L1	chitinase-3-like-1
CI	cerebral infarction
CI	confidence interval
CIS	clinically isolated syndrome
CMT	Charcot-Marie-Tooth disease
CMV	Cytomegalovirus
CP	control patients
cSCI	complete spinal cord injury
CSF	cerebrospinal fluid
CV	coefficient of variation
CVA	cerebrovascular accident
DLB	dementia with lewy bodies
EAD	early onsed Alzheimer's dementia
EBNA1	EBV nuclear antigen 1
EBV	Epstein-Barr virus
ECL	electrochemiluminescence
EDSS	Expanded Disability Status Scale
EEG	electroencephalography
ELISA	enzyme-linked immunosorbent assay
F	female

FC	fast conversters (from CIS to CDMS)
FTD	frontotemporal dementia
GBS	Guillain-Barré-syndrome
Gd+	gadolinium enhancing MRI lesions
GFAP	glial fibrillary acidic protein
HC	healthy controls
HR	hazard ratio
HRP	horseradish peroxidase
IF	intermediate filaments
$_{IF}$	intrathecal fraction
Ig	Immunoglobulin
IND	inflammatory neurological diseases
IQR	interquartile range
iSCI	incomplete spinal cord injury
IU	international units
IVIg	intravenous immunoglobulin
kDa	kilo Dalton
LAD	late onset Alzheimer's dementia
Lys	lysine
M	male
M12	month 12
mAb	monoclonal antibody
MCI	mild cognitive impairment
MD	mixed type dementia
MMS	mini mental status
MRI	magnetic resonance imaging
MS	multiple sclerosis
MSSS	MS severity score
NAWM	normal appearing white matter
NC	non-converters (from CIS to CDMS)
NC	normal controls
ND	normal donor
NMO	neuromyelitis optica
Nf	neurofilaments
NfH	neurofilament heavy chain
NfL	neurofilament light chain
NfM	neurofilament medium chain
NIHSS	National Institute of Health stroke scale

NIND	non-inflammatory neurological diseases
NMO	neuromyelitis optica
NPH	normal pressure hydrocephalus
Ns	not significant
NTZ	natalizumab
OCB	oligoclonal bands in CSF
OD	optical density
ON	optic neuritis
OR	odds ratio
OPCA	olivopontocerebellar atrophy
PBS	phosphate buffered saline
PBVC	percentage of brain volume change
PD	Parkinson’s disease
PDD	Parkinson’s disease with dementia
PNFA	progressive nonfluent aphasia
PPMS	primary progressive MS
Pro	proline
PSP	progressive supranuclear palsy
Q_{alb}	Albumin quotient
Ref	reference
ROC	Receiver operating characteristic
RRMS	relapsing remitting MS
RT	room temperature
SAH	subarachnoid hemorrhage
SCI	spinal cord injury
sCJD	sporadic Creutzfeld-Jakob disease
SD	standard deviation
SD	semantic dementia
SE	standard error
Ser	serine
Spinal	spinal cord syndrome
SPMS	secondary progressive MS
SVD	subcortical vascular dementia
SWD	subcortical white matter dementia
T2 lesions	T2 hyperintense lesions in MRI
TBI	traumatic brain injury
TIA	transient ischemic attack
TBS	tris buffered saline

VAD	vascular dementia
VD	vascular dementia
Vs	versus
WMC	white matter changes
y	year

1. Introduction

1.1. Intermediate filaments

The neuronal cytoskeleton is composed of three interconnected structures: microfilaments, microtubules, and intermediate filaments (IF). The diameter of IF (10 nm) is “intermediate” between the microfilaments (6-8 nm, mostly actin) and microtubules (24 nm, mostly tubulin). Based on molecular structural homology, five types of IF have been identified, and neurofilaments (Nf) belong to type IV (type I: acid keratins, type II: basic keratins, type III: desmin, GFAP, peripherin/vimentin, type V: nuclear lamins) (1). Neurons differentially express several IF proteins depending on their developing stage and their localisation in the nervous system: for example nestin (200 kDa), three Nf subunits (Nf light chain, NfL, 68 kDa; Nf medium chain, NfM, 160 kDa and Nf heavy chain, NfH, 205 kDa), α-internexin (66 kDa), peripherin (57 kDa) and synemin (41 kDa) (2).

1.2. Neurofilament function and structure

Nf are highly specific to the neuro-axonal compartment. Their main role is to increase axon calibre of myelinated axons and consequently their conduction velocity. Numerous rodent studies have shown that both number of Nf and a precise stoichiometry of their subunits are essential in the expansion of the axonal diameter (2). Nf account for approximately 13% of total and 54% of Triton-insoluble proteins in some neurons, representing the most abundant structures in large myelinated axons (3, 4). Their number relative to axonal cross-sectional area does not change during development or after axonal injury, leading to the proposal that Nf density is essential in determining axonal diameter (5). This has also been demonstrated in genetically engineered animals that either contain enhanced levels or lack axonal Nf (6). Despite being present also in perikarya and dendrites, Nf are particularly abundant in the axons. They have exceptionally long half-lives and their elastic fibrous properties enable them to maintain the shape of neurons (7).

All Nf subunits share a common structure, with non-helical amino and carboxy-terminal regions (the head and tail domains) flanking a central α-helical rod domain of about 310 amino acids (**figure 1.1**) (2, 8). The central rod domains, including regions 1a, 1b, and 2, contain highly conserved motifs and every seventh residue is hydrophobic facilitating the formation of coiled-coil parallel dimers. The tail domain is the distinctive feature of the Nf and contains numerous repeats of the phosphorylation sites Lys-*Ser*-Pro (KSP) in both NfM and NfH. These sites are called KSP repeats: 51 are present in

mice NfH, 7 in mice NfM and 43-44 in human NfH. Most of the serines in the KSP repeats are phosphorylated and it is suspected that phosphorylation contributes to Nf spacing by inducing charge repulsion and prevention of protease degradation during the long lifespan of these proteins (9, 10).

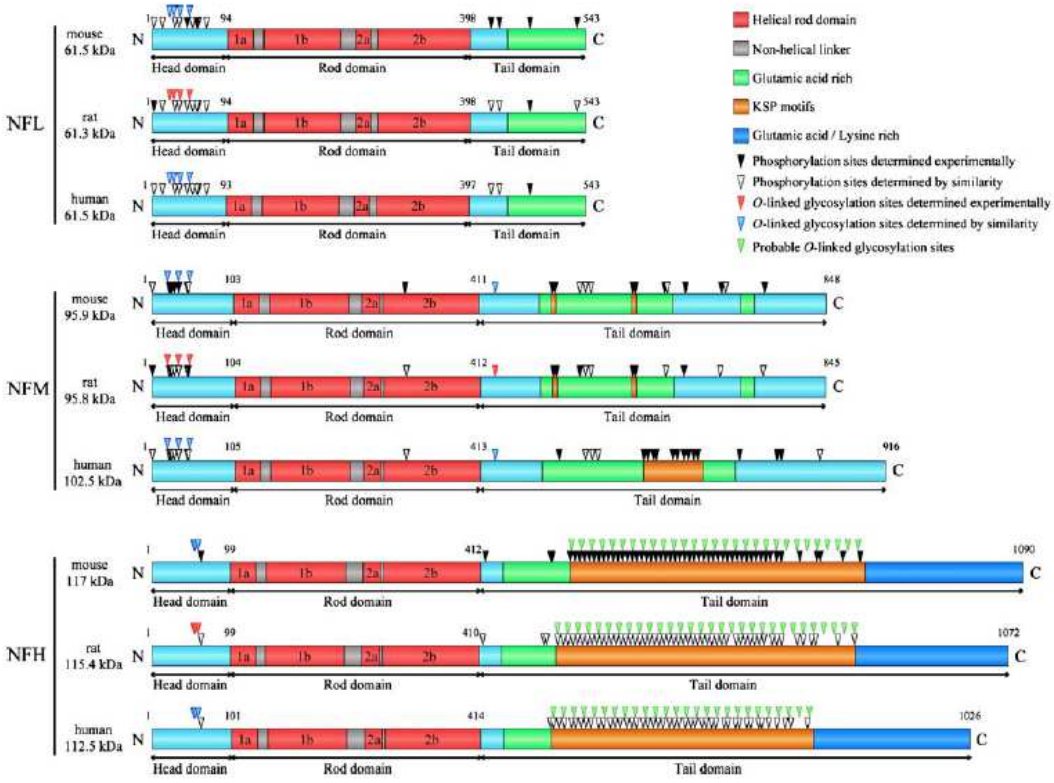


Figure 1.1. Mouse, rat and human Nf subunits and predicted molecular weights from the DNA sequences. The three subunits share a highly conserved central α-helical domain of approximately 310 amino acids that is flanked by the amino and carboxy-terminal head and tail regions. The tail domains are of variable size and in case of NfM and NfH contain multiple KSP repeats (orange) which can be heavily phosphorylated (arrow-heads) (2).

1.3. Nf assembly, transportation and degradation

It was originally thought that Nf were composed only by NfL, NfM and NfH, but later studies indicated that other proteins such as α-internexin and peripherin are also co-assembled with Nf (2, 11, 12). Nf are obligate heteropolymers requiring NfL with either NfM or NfH (13, 14); the exact mechanisms leading to Nf assembly are not known yet. The first step of Nf formation is the dimerisation of NfL with either NfM or NfH to form parallel head-to-tail coiled coil dimers (13-15). Two dimers line up forming half-staggered anti-parallel tetramers. These tetramers form protofilaments, which finally assemble to constitute the 10 nm filament (16) (**Figure 1.2.**). The C-termini of NfM and NfH are not in the coils, but they form the side arms of Nf (1). NfL is known to be able

to self-assemble, while NfM and NfH cannot perform self-assembly, but only co-polymerize with assembled NfL (14, 17) with a stoichiometry of 4 (NfL):2 (NfM):1 (NfH) (14, 18, 19). In vivo, these partly understood processes are strongly influenced by phosphorylation of several Ser residues in the KSP repeats.
In numerous animal studies only mice overexpressing >3-fold NfL and NfM/NfH double knockout mice showed severe phenotypes. From these experiments it was concluded that NfL is responsible for Nf assembly, and NfM/NfH subunits are also required, whereas NfM is more critical for axonal growth than NfH (1).

Nf are the most extensively phosphorylated proteins in neurons, and the phosphorylation state in different neuronal compartments depends on a dynamic balance between the activities of a complex network of kinases and phosphatases (2). It is assumed that phosphorylation also plays an important role in regulating Nf transport, formation and function. Most phosphorylation sites are in KSP motifs of the tail domains of NfM and NfH and myelination promotes Nf phosphorylation and radial growth of axons, while demyelination of motor neurons causes loss of phosphorylation of NfM and NfH (20, 21). Interestingly, phosphorylation also plays a critical role in inhibiting Nf assembly in the perikaryon, which explains why Nf only form filaments in axons, and in protecting the neuron from abnormal accumulation of phosphorylated Nf aggregates in cell bodies (1, 4, 22-24). Abnormally phosphorylated Nf in the cell bodies represent a characteristic pathological finding in several neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Lewy bodies in Parkinson's disease (PD), progressive supranuclear palsy (PSP), Charcot-Marie-Tooth disease (CMT), diabetic neuropathy and giant axonal neuropathy (25-29).
Whereas phosphorylation has been intensely studied, the role of another common modification, the attachment of O-linked N-acetylglucosamine (GlcNAc) to individual serine and threonine residues, is hardly understood. O-linked glycosilation regulates signaling events related to nutrient sensing and stress responses, among other functions (30). It has been shown that the amount of glycosilated NfM, which is highest in axons of human neurons, is decreased in brains from patients with Alzheimer's disease (AD) (31), similarly in a rat model of ALS (32). The functional significance of Nf glycosilation awaits further investigation; this also needs to take into account potential technical artefacts like altered glycosylation in post-mortem tissue (32).

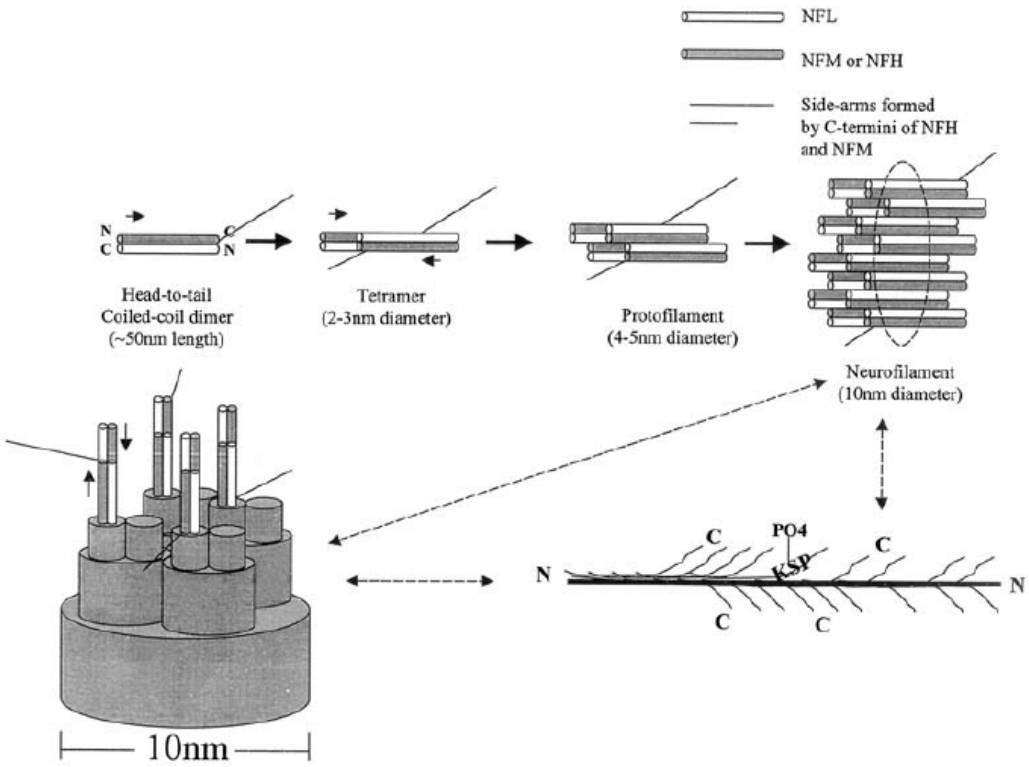


Figure 1.2. Model of Nf assembly. Two Nf subunits (NfL, and either NfH or NfM) form head-to-tail coiled-coil dimers, anti-parallel half-staggered tetramers, protofilaments, and 10-nm Nf. Side arms formed by the C-termini of NfH and NFM stick out of the stem of NF (1).

After their assembly in the perikaryon, NFs are transported by the slow axonal transport (0.2-1 mm/day) towards the nerve terminal where they are degraded by non-specific proteases such as lysosomal cathepsin D, trypsin and α-chymotrypsin (33). The mechanism by which Nf are transported has been under debate for many years. It is also not clear whether assembled Nf or subunits are transported (34).
Many neurons in humans extend their axons up to one meter. During the passage through the axon, the cytoskeletal proteins are metabolically stable and do not undergo degradation. Therefore, it is believed that Nf degradation takes place mainly at the level of synapses (35). This process is preceded by Nf dephosphorylation by phosphatases, further suggesting that phosphorylation has a protective function (10).

1.4. Neurological diseases studied in this thesis

In this thesis I studied CSF and serum samples from patients with neurological diseases with a different rate of progression and following and under different treatments. The main focus of the work is multiple sclerosis (MS) (chapters 2., 3., and

6.), but I also investigated samples from patients with AD, Guillain-Barré-syndrome (GBS), ALS (chapter 4) and acute spinal cord injury (SCI, chapter 5).

1.4.1. Multiple Sclerosis (MS)

MS is a disseminated, chronic, inflammatory demyelinating disease of the CNS with progressive neuroaxonal degeneration. The phenotype is very heterogeneous and the course unpredictable. MS is the most frequent cause of nontraumatic neurological disability in young adults (36).

Approximately 85% of MS patients show a relapsing-remitting course, in which acute exacerbations are followed by periods of remission of symptoms (relapsing-remitting MS (RRMS)). The first clinical episode of RRMS patients is often referred to as clinically isolated syndrome (CIS). Most of RRMS cases will enter in the long term the secondary progressive phase of MS (SPMS), which is characterized by progressive deterioration of neurological function, independent of relapses (37). In approximately 15% of MS patients, disability progression occurs from onset of first symptoms. This is called primary progressive MS (PPMS) and up to approximately 10% can also have relapses (also termed progressive relapsing MS) (38). Importantly, these categories are based on subjective views of MS experts, lack objective biological support and have recently been revised (39-41). Interestingly, while the length of the relapsing remitting phase is very variable, the rate of neurological decline at some stage of the disease course appears less heterogeneous, regardless of the preceding disease course and severity (42, 43). These findings suggested that the underlying neurodegenerative process in MS depends more on the patient's age and not on number of relapses which have previously occurred (44). This led to the hypothesis that MS could be a two-stage disease, starting with an inflammatory phase and later entering a distinct neurodegenerative phase. However, more recent imaging and neuropathology studies in early MS have shown that axonal injury and neurodegeneration start since the very early phases of the disease, and that the transition from RRMS to SPMS is likely to be a point at which compensatory mechanisms of neuronal injury fail (45).

Highly efficacious immunomodulatory treatments for RRMS have been developed; however, no effective treatment has been identified for the progressive forms of MS (PPMS; SPMS). This is a significant unmet need as the accrual of disability, loss of quality of life and socioeconomic costs predominate in the progressive stage of the disease.

MS is unsurpassed for patient variability in terms of disease progression. However, no robust prognostic markers regarding the individual disease course have been established. Optic neuritis (ON), isolated sensory symptoms, long interval to second

relapse, no disability after 5 years (46, 47) and a normal initial brain MRI (48-51) predict a good prognosis in CIS. Whereas a multifocal CIS (52), involvement of efferent systems (47), a high relapse rate in the first 2 - 5 years (53), disability after 5 years (47) and an abnormal MRI with large lesion load (49-51, 54-56) all have been associated with a poor prognosis (57). Early motor, cognitive, cerebellar and sphincter dysfunction and cigarette smoking have been described as additional factors predicting an unfavorable course (58, 59). Progressive disease (SPMS and PPMS) has a worse prognosis than RRMS, and in RRMS, incomplete recovery from relapses is associated with a worse outcome (60).

1.4.2. Alzheimer's dementia (AD)

AD is the most common form of dementia, accounting for 50 - 60% of all cases. The prevalence of AD among people aged 85 years or older ranges between 24 and 33% in the Western world (61). Slowly progressive impairment of episodic memory is the key feature, accompanied by aphasia, apraxia and agnosia, together with more general symptoms such as impaired judgment, decision-making and orientation.
The differential diagnosis of dementia is broad and the diagnostic criteria keep evolving in order to improve sensitivity and specificity (62-66). Mild cognitive impairment (MCI) describes an intermediate stage between normal ageing and dementia. The conversion rate of MCI to dementia is 10 - 15% per year and approximately 80% at 6 years of follow-up (67).

1.4.3. Guillain-Barré Syndrome (GBS)

The diagnosis of GBS is mainly clinical, based on a combination of progressive symmetrical weakness and areflexia. Acute inflammatory demyelinating polyradiculoneuropathy (AIDP) is the most common subtype in western countries, whereas forms primarily affecting the axons are less frequent (acute motor axonal neuropathy [AMAN] and acute motor and sensory axonal neuropathy [AMSAN]) (68, 69). Cerebrospinal fluid (CSF) findings are characterized by a normal cell count and increased protein concentration (i.e. 'albuminocytological dissociation'). Most patients reach their maximum weakness within 2 weeks; by definition this should be reached within 4 weeks followed by a variable plateau phase from days to several months (70, 71). Up to 20% of the most severe GBS cases remain wheelchair-bound after 6 months despite treatment with intravenous immunoglobulin (IVIg) or plasma exchange (72). Older

age at onset, longer time to nadir, necessity for ventilator support, preceding diarrhea or axonal degeneration in electrophysiological examinations are related with poor outcome (73-75).

1.4.4. Amyotrophic Lateral Sclerosis (ALS)

ALS, the most common (> 80%) and most severe form of motor neuron disease, is a fatal neurodegenerative disorder of large motor neurons of unknown etiology. Approximately 10% of ALS cases are familial with mutations in the gene encoding cytosolic copper-zinc superoxide dismutase 1 (SOD1) in about 15 - 25% of these cases (approximately 1.5 - 2% of all ALS patients) (76). ALS has a considerable variability in outcome. The median age of onset is 55 years, and the median survival from onset to death in ALS is reported to vary from 20 to 48 months (77-79). Importantly, 5 - 10% of the patients are consistently reported to survive more than 10 years; nevertheless, 50% die within 3 years and approximately 90% within 5 years (80).

1.4.5. Acute spinal cord injury

See chapter 5.1.

1.5. Pathophysiological mechanisms of axonal degeneration in MS

The exact mechanisms driving MS pathogenesis are currently unknown. However, current knowledge indicates that environmental exposures in genetically susceptible individuals lead to an immune mediated demyelination process within the central nervous system. It is thought that the immune cell invasion across the blood-brain barrier leads to continuous activation of CNS-homing and CNS-resident innate immune cells (macrophages and microglia) in the brain and spinal cord with resulting demyelination and neurodegeneration (45, 81). Axonal loss is thought to be the pathological substrate that results in the acquisition of irreversible disability in MS and appears to occur by at least two principle mechanisms. Firstly, as a result of axonal transection in acutely inflamed focal lesions (82). For example, the inflammatory process in acute optic neuritis leads to axonal transection in the optic nerve and to retinal nerve fiber layer thinning, that can be detected using optical coherence tomography (83). Secondly, axonal loss can occur as a delayed consequence of earlier and chronic damage that renders axons vulnerable to degeneration when compensatory mechanisms fail (84). In comparison to axonal loss in acute focal lesions, this process is less understood. Focal inflammation occurring early in the course of the disease could prime the damaged, but surviving, axons for degeneration

in the future (85). Several additional mechanisms have been proposed to cause this delayed axonal loss and include persistent demyelination (despite axonal injury also occurs without demyelination) (86-89), oxidative stress, free radical damage, mitochondrial dysfunction, microglial activation, hypoxia, ageing and energy deficits secondary to excessive sodium loading of axons (90).

1.6. Overview of NfL findings in different neurological diseases

Nf subunits are among the most abundant proteins of the nervous system and are concentrated in neurons and axons.

NfL is the most abundant and also most soluble Nf subunit. Therefore, it is expected that NfL would be released in relatively large amounts from damaged neurons and particularly their dying axons. I thought that this could eventually provide a unique opportunity to reliably measure axonal degeneration in the peripheral blood compartment of neurological patients. In this chapter I summarize findings from previous studies on NfL levels in neurological diseases. Noteworthy, these have so far been performed exclusively in CSF.

Table 1.1. *Studies describing NfL assays.*

Ref. no.	**Healthy control groups studied (n): NfL: [median (IQR), pg/ml]**	**Investigated compartment (CSF/serum/ plasma)**	**Type of disease studied (n): NfL [median (IQR), pg/ml]**	**[NfL] in disease different to controls**	**Correlations of [NfL] with clinical or laboratory measures**	**Assay characteristics NfL: [median (IQR), pg/ml]**	**Special features: e.g. longitudinal design**
1.) Rosengren et al., 1996 (91)	Neurologically healthy individuals (34): **138**	CSF	(mean levels) AD (11): 346 RRMS (5): 463 NPH (6): 582 VD (6): 597 OPCA (2): 892 CI (5): 958 ALS (12): 1743	- all patient groups higher than controls	- higher levels in ALS with upper motor neuron involvement than lower (<0.05) - age in controls (0.41, <0.05)	"Gothenburg assay" - sensitivity 85	
2.) Norgren et al., 2003 (92)	(mean levels) Neurologically healthy individuals (11): **2 above detection limit: 31**	CSF	(mean levels) PD (5): 200 AD (5): 300 VD (5): 1400 PSP (3): 1900 RRMS (5): 2500 ALS (5): 3600 CI (6): 19800 MSA (2): -	- all patient groups higher than controls	-	"Umea assay" (mAb 47:3 and mAb 2:1) - sensitivity 60 pg/ml	
3.) van Geel et al., 2005 (93)	No neurological disease (110): <15 y (39): **8** (5-17) 15-50y (40): **5** (0-6) >50y (31): **9** (0-25) (no signif. correlation	CSF	Mild TBI (3): 5 TIA (2): 30 SAH (13): 67 (32-136) CVA (13): 142 (7-276) Severe TBI (3): 470 (n.a.)	Higher levels in SAH, severe TBI and CVA (no statistical analysis)	-	"Nijmegen assay" - detection limit: 5 - two overnight incubation steps - 76/110 controls measurable	- Commercially available antibodies - compared with (91) levels by factor 34.4 higher

	with age)						
4.) Gaiottino et al., 2013 (94) (see chapter 4)	(geometric means) Serum/CSF: HC (67): 3/- CP (68): 4/**324** (median age 35y)	CSF and serum	(geometric means) Serum/CSF: ALS: 95/5513 GBS: 79/1361 AD: 31/1396	- serum levels higher in ALS (<0.0001) and GBS (<0.0001) versus HC and CP - CSF levels higher in ALS (<0.0001) and GBS (<0.0001) versus CP - higher CSF levels in ALS (<0.0001) than AD and GBS	- CSF levels in CP and age (0.68, <0.0001)		- CSF levels 97-fold higher than serum levels - CSF/serum correlations: AD (0.48, 0.033), GBS (0.79, <0.0001), ALS (0.7, <0.0001)

For correlations the coefficient r and p-values are given (r, p-value).

AD: Alzheimer's disease; ALS: amyotrophic lateral sclerosis; CI: cerebral infarction; CP: control patients; CVA: cerebrovascular accident; HC: healthy controls; NPH: normal pressure hydrocephalus; OPCA: olivopontocerebellar atrophy; SAH: subarachnoid hemorrhage; TBI: traumatic brain injury; TIA: transient ischemic attack; VD: Vascular dementia; y: year; -: not done.

Three different ELISAs for NfL in CSF have been developed previously. The "Umea assay" has been used most widely. Chapter 4 describes the development and validation of an ECL-based NfL assay for measurements in human serum samples employing the identical monoclonal antibodies (mAb) used in Norgren et al., 2003 ("Umea assay": mAb 47:3 and mAb 2:1) (92, 94, 95).

Table 1.2. *Overview of studies on NfL in multiple sclerosis (MS) or neuromyelitis optica.*

Ref. no.	**Healthy control groups studied (n): NfL: [median (IQR), pg/ml]**	**Type of disease studied (n): NfL [median (IQR), pg/ml]**	**[NfL] in disease different to controls**	**Correlations of [NfL] with clinical or laboratory measures**	**Assay**	**Special features: e.g. longitudinal design**
1.) Lycke et al., 1998 (96)	Healthy subjects (11): **below detection limit in all**	RRMS (60): **265**	- higher in RRMS than controls	- EDSS (0.27, <0.05) - exacerbation rate during the 2-year trial (0.38, <0.01) - time of lumbar puncture (-0.48, <0.001)	(91)	- 2-year acyclovir trial with two CSF samplings, no influence of acyclovir on NfL levels
2.) Malmeström et al., 2003 (97)	Healthy blood donors (50): **measurable in n=4**; (mean age: 35y)	RRMS relapse (23): 91% detectable RRMS remission (18): 44% detectable SPMS (25): 48% detectable	- higher in all stages (<0.001) - RRMS relapse higher than RRMS remission and SPMS (<0.001)	- no relationship with age or gender, EDSS, disease duration	(91)	
3.) Norgren et al., 2004 (98)	Thunderclap headache (25): **detectable in one**	(means) PRMS (5): **0** PPMS (15): **240** RRMS (58): **248** SPMS (21): **367** (partially extrapolated from figure 2a)	- (stable) RRMS (<0.01), SPMS (<0.001) and PPMS (0.04) higher than controls - trend for SPMS higher than (stable RRMS)	- higher in relapse (3 months, n=39) than stable (n=60): mean 754 versus 266, 0.004 - after 14 years: correlation with MSSS (0.3, 0.005), higher levels predictive of SPMS (=0.01) and higher MSSS score (OR=3.2) (99)	(92)	- NfL (relapse patients excluded) correlated with EDSS at clinical FU examination (r=0.28, p=0.03) and progression index: stable patients: r=0.29, p=0.023; relapse patients: r=0.49, p=0.002 - CSF cell count (patients in

						relapse): r=0.52, p=0.001
4.) Teunissen et al., 2009 (100)	NIND (18):1000 (mean age: 47y) IND (40): 1300 (mean age: 53y) ND (28): **800** (mean age: 40) (extrapolated from figure 3)	CIS (38): **1000** PPMS (6): **1200** SPMS (28): **1400** RRMS (42): **1800** (extrapolated from figure 3)	- NIND (<0.006) and IND (<0.0001) higher than ND - ND vs CIS: <0.001 - ND vs RRMS: <0.0001 - CIS vs RRMS: <0.04 - ND vs SPMS: <0.0001 - ND vs PPMS: <0.004	- number of T2 lesions (0.35, 0.024) - number of Gd+ lesions (0.50, <0.001) - EDSS (0.19, <0.05) - higher in CIS and MS with OCB (<0.01) or in relapse (<0.05) - higher in CIS with conversion to RRMS (<0.05) - NfL with NfH (0.49, <0.0001)	Uman Diagnostics NF-light (only reagents provided)	
5.) Gunnarsson et al., 2010 (101)	(mean) Controls (28, blood donors, university students): **350** (mean age 43y)	(means) RRMS (92): **1300** (after NTZ: 400) RRMS relapse (30): 2300 (after NTZ: 350) RRMS stable (62): 860 (after NTZ: 430) SPMS with relapses (9): **1600** (after NTZ: 630) (partly extrapolated from figures 1 and 2)	- higher levels in MS (<0.001) than in controls - reduced levels after NTZ (<0.001), no difference to controls after NTZ	-	Uman Diagnostics NF-light	- longitudinal sampling before and after NTZ
6.) Khalil et al., 2013 (102)	NIND (15): **500** (mean age 35y) (extrapolated from figure 1)	CIS (47): **1200** (extrapolated from figure 1)	- higher in CIS (<0.001) than controls	- no difference between relapse and remission - age (0.93, <0.001) - no difference between converters to CDMS and non-converters	Uman Diagnostics NF-light	- clinical follow-up in 46, follow-up MRI in 28 patients available

				- NfL with NfH (0.55, <0.001) - CSF cell count (0.65, <0.001), IgG index (0.46, <0.001) and qAlb (0.35, <0.01)		
7.) **Romme et al., 2013 (103)**	NIND (7, lower back pain, headache, psychosomatic complaints): **250** (mean age 53) (extrapolated from figure 1)	SPMS (40): **500** PPMS (21): **500** RRMS in relapse (36): **1100** (extrapolated from figure 1)	- RRMS (0.002), SPMS (0.04), PPMS (0.001) higher than controls	- osteopontin (0.49, <0.0001) - CXCL 13 (0.35, 0.007) - MMP9 (0.15, 0.25)	Uman Diagnostics NF-light	- repeat sampling in 22 SPMS after 1 year: no change in NfL (p=0.08)
8.) **Kuhle et al., 2013 (104) (see chapter 2)**	(geometric mean) No objective clinical or paraclinical finding (72): **272**; (median age: 38y)	(geometric means) CIS (62): **766** SPMS (25): **785** PPMS (23): **1007** RRMS (38): **1201**	- CIS (<0.0001), RRMS (<0.0001), SPMS (0.001), PPMS (<0.0001) higher than controls - RRMS (0.025) higher than SPMS	- age in controls (0.61, <0.0001) - CSF cell count in CIS (0.27,0.016) and RRMS (0.43, 0.01) - qAlb in CIS (0.28, 0.025), RRMS (0.49, 0.002) and SPMS (0.67, <0.0001) - EDSS in CIS and RRMS (0.31, 0.002) - relapse higher than remission (1070 versus 734, p=0.054)	Uman Diagnostics NF-light	- differences larger than for NfH - correlation with NfH (after age correction): controls (0.058, 0.627), CIS (0.46, <0.0001), RRMS (0.56, <0.0001), SPMS (0.31, 0.128), PPMS (0.41, 0.054)
9.) **Modvig et al., 2013 (105)**	Healthy controls (27): **414** (262-630) (median age 33y)	CIS (optic neuritis, 56): **1476** (1024-3036)	- higher in CIS (<0.0001) than controls	- age in controls (-, <0.0001) - dissemination in space in MRI (-, 0.0512) - time from onset to CSF sampling (0.46,	Uman Diagnostics NF-light	- no correlation with severity of visual function, or Gd+ lesions on MRI

				0.0024) - CHI3L1 (0.39, 0.073) - osteopontin (0.5, 0.0023)		
10.) **Wang et al., 2013 (106)**	(mean) OND (18, schizophrenia, sciatica, cervical spondylosis): **703** (mean age 38y)	(means) RRMS (25): **991** NMO (32): **1570**	- higher in NMO than RRMS (0.001) and OND (<0.0001) - higher in RRMS (0.0003) than OND	- EDSS in NMO (0.48, 0.006) and RRMS (0.47, 0.017)	Uman Diagnostics NF-light	- sampling during relapse
11.) **Axelsson et al., 2014 (107)**	(mean) Healthy controls (14, blood donors): **577** (mean age 42y)	(mean) PPMS (5) and SPMS (30): **1780** (after treatment: **870**)	- higher levels in MS than controls (<0.001)	- reduced levels (0.007) after treatment - higher levels after treatment than controls (0.045) - higher levels in previously untreated (2462) than treated patients (874, 0.019) - higher baseline levels in Gd+ patients (2925, n=12) versus non Gd+ patients 1184 (0.013) - CXCL 13 (0.53, <0.01) - GFAP (0.47, <0.01) - no correlation with EDSS or MSSS	Uman Diagnostics NF-light	- patients treated with mitoxantrone (29) or rituximab (5) - repeat sampling after 12-24 months - clinical measures unchanged over follow-up
12.) **Burman et al., 2014 (108)**	Controls (15, other non-inflammatory neurological diseases: e.g. thunderclap	RRMS Gd+ (25): **1700** RRMS Gd- (19): **400** SPMS Gd+ (3): **1950** SPMS Gd- (17): **900**	- Gd+ RRMS higher than controls (<0.001) - Gd+ RRMS higher than Gd- RRMS (<0.01)	- increased in patients with Gd+ lesions - 2 controls with iih above reference: after exclusion: Gd- SPMS versus controls: <0.01	Uman Diagnostics NF-light (normal values established by this group:	

	headache, idiopathic intracranial hypertension (iih) or no objective signs of MS): **300** (mean age: 40y)	(extrapolated from figure 2)		- number Gd+ lesions (0.51, <0.0001) - number T1 lesions (0.31, 0.013) - number T2 lesions (0.27, 0.033) - age in controls (0.83, 0.0004)	<30y: <380; 30-39y: <560; 40-59y: <890; > 59y: <1850)	
13.) Trentini et al., 2014 (109)	NIND (15, headache, vertigo, hydrocephalus secondary to tumor, trigeminal neuropathy, polyradiculopathy, epilepsy): **532** (273-812)	SPMS (10): **697** (495-1380) PPMS (21): **839** (723-1514)	- higher in PPMS than NIND (0.003)	- NfL predictor of EDSS annual increase (0.44, <0.05) - NfH in patients (0.71, <0.01), in controls (0.54, <0.01)	Uman Diagnostics NF-light	- 23 patients followed-up for a median of 9 years
14.) Romme et al., 2014 (110)	-	(mean) SPMS (12) : - PPMS (12) : - SPMS and PPMS : **657**	-	- MTR in NAWM (-0.73, 0.003) - MTR in grey matter (-0.66, 0.01)	Uman Diagnostics NF-light	- mean decrease by 243 pg/ml after 1 year of natalizumab treatment (p=0.03)
15.) Villar et al., 2014 (111)	NIND (37, headache, benign intracranial hypertension, cranial nerve palsy, epilepsy, vitamin B1 deficiency): **335**	RRMS (127): -	- (55 patients with <900 and 72 >900)	- higher levels in relapse/Gd+ lesions (0.03) - not age in patients or controls - T2 lesions (0.44, <0.0001) - Gd+ lesions (0.50, <0.0001) - MSSS (0.54, <0.0001)	Uman Diagnostics NF-light	- more lipid specific IgM bands in patients with high NfL (<0.0001)

				- T1 black holes (0.47, <0.0001) - CD4+ cells (0.33, 0.002), CD8+ (0.42, 0.0007), CD19+ (0.48, <0.0001)		
Summary	**457** (292-672)	(only studies using Uman Diagnostics NF-light assay): **CIS: 1100** (825-1407) **RRMS: 1200** (461-1675) **SPMS: 950** (400-1550) **PPMS: 754** (305-1152)				

For correlations the coefficient r and p-values are given (r, p-value).

CIS: Clinically isolated syndrome; CHI3L1: chitinase-3-like-1; EDSS: Expanded disability status scale; GBS: Guillain-Barré syndrome; Gd+: gadolinium enhancing MRI lesions; IND: Inflammatory neurological disease; MSSS: Multiple Sclerosis severity score; NAWM: normal appearing white matter; ND: normal donors; NIND: non-inflammatory neurologic diseases; NMO: neuromyelitis optica; NTZ: natalizumab; OCB: oligoclonal bands in CSF; PPMS: primary progressive MS; RRMS: relapsing remitting MS; SPMS: secondary progressive MS; T2 lesions: T2 hyperintense lesions; -: not done.

Most of the studies in CIS/MS were done using the Uman Diagnostics NF-light ELISA (12/15 studies and all published after 2004). I also used the Uman Diagnostics NF-light ELISA (chapters 2 and 3) and the mAB it is based upon (chapters 4-6).

NfL levels in controls showed considerable variation (250 pg/ml to 800 pg/ml) between the different studies and this clearly complicates their comparison. Different criteria have been used to define control groups (healthy controls versus patients without objective clinical or paraclinical findings versus patients with “other” neurological diseases) (112) and variability in assay performance between different sites (113) probably adds to this complexity. In summary all stages of MS showed increased CSF NfL levels, with CIS and RRMS patients having slightly higher concentrations than SPMS and PPMS. Also, levels correlated with clinical and paraclinical measures of disease activity to a variable extent. Three studies showed CSF NfL responds to immunomodulatory treatment in RRMS (101) and in progressive MS (107, 110), (chapter 3) and no studies on blood NfL levels in CIS or MS have been published so far.

Table 1.3. *Overview of NfL studies in different forms of dementia.*

Ref. no.	Healthy control groups studied (n): NfL: [median (IQR), pg/ml]	Type of disease studied (n): NfL [median (IQR), pg/ml]	[NfL] in disease different to controls	Correlations of [NfL] with clinical or laboratory measures	Assay characteristics NfL: [median (IQR), pg/ml]	Special features: e.g. longitudinal design
1.) Rosengren et al., 1999 (114)	(mean) Control subjects (minor surgery, spinal anesthesia, 39): 156 (mean age: 72y)	(means) AD (37): 348 VAD (20): 674 FTD (5): 997	- higher in all patient groups than controls (<0.001)	- age in controls (0.41, <0.05), but not patients (0.18, ns) - no difference between mild and moderate dementia (455 vs. 559)	(91)	
2.) Sjögren et al., 2000 (115)	Control subjects (18): 241 (mean age 71y)	FTD (18): 1442 EAD (21): 498 LAD (21): 1006	- higher in FTD (<0.05) and LAD (<0.001) than controls - higher in LAD (<0.05) than EAD	- tau in EAD (0.58, <0.01) and in controls (0.71, <0.01) - degree of cognitive impairment in FTD (0.59, <0.05) and LAD (0.61, <0.01)	(91)	
3.) Wallin et al., 2001 (116)	Healthy controls (18): 241 (median age 71y)	SWD (25): 1316	- higher in SWD (<0.001) than controls	- no difference for CSF tau	(91)	
4.) Andreasen et al., 2001 (117)	(mean) Subjects without symptoms or signs of brain disorders (19): 295 (mean age 71)	(mean) AD (35): 615	- higher in AD than controls (0.002)	- little additional value as diagnostic biochemical marker for AD	(91)	

5.) **Sjögren et al., 2001 (118)**	Healthy volunteers (20): 156 (mean age: 66y)	(means) AD (22): 569 SVD (9): 1977 Insignificant WMC (37): 394 Extensive WMC (14): 1347	- higher in AD (<0.001) and SVD (<0.001) than controls	- higher in extensive WMC than insignificant WMC (<0.001) - higher in SVD than AD (<0.05)	(91)	Study on “white matter changes”
6.) **Pijnenburg et al., 2006 (119)**	(mean) Nondemented controls (19 nonprogressive subjective memory complaints, 3 cognitively healthy spouses, 3 other, 25): 390 (mean age: 59y)	(means) BvFTD (17): 510 EAD (20): 950	- higher in EAD than controls (<0.001) (not seen for NfH)	- age in controls (0.68, <0.001) - NfH (FTD: 0.71, 0.001; controls: 0.83, 0.001) - Tau (AD: 0.73, <0.001)	Uman Diagnostics NF-light	- large variation in patients with FTD
7.) **Petzold et al., 2007 (120)**			- higher levels in AD than controls (OR 1.27, 95% CI 1.03-1.51) - higher in FTD than controls - higher in SVD than controls - higher in FTD than AD			Meta-analysis
8.) **De Jong et al., 2007 (121)**	Controls (underwent CSF examination for various reasons, no	EAD (37): 6.1 LAD (33): 15.2 DLB (18): 10.4 FTD (28): 16.9	- higher in FTD than EAD (<0.01) and controls (<0.001)	- no correlation with MMS, duration, age	(93)	

	neurological disorder, 26): 5.0; (median age 60y)					
9.) Mattson et al., 2008 (122)	-	Rapidly progressing FTD (13): 410 Slowly progressing FTD (11): 125	- no difference between rapid and slow progressors (p=0.186)	- no correlation with CDR or MMS	(91)	
10.) Bjerke et al., 2009 (123)	Healthy controls (senior citizen's organizations, spouses, 51): 250 (250-250) (median age: 66y)	MCI - SVD (8): 424 (255-1414) MCI - MCI (113): 250 (250-250) MCI – AD (18): 250 (250-406) MCI – MD (15): 250 (250-406)	- MCI – SVD higher than controls (<0.001) and MCI-MCI (<0.001) - MCI – MD higher than controls (<0.01) and MCI – MCI (<0.001) - MCI – AD higher than controls (<0.05) and MCI – MCI (<0.001)	- for separation between MCI – SVD and stable MCI, NfL was the most influential marker, while Aß42, T-tau and P-tau provided more discriminating power for the other comparisons	(91) (detection limit: 250)	- 4-year longitudinal study -large number of samples below detection limit
11.) Van Eijk et al., 2010 (124)	Non-demented controls (no neurological disease, 23): 5 (0-20); (mean age 58y)	sCJD (21): 78 (33-123) AD (55): 7 (6-18)	- sCJD higher than AD (<0.0001) - sCJD higher than controls (<0.0001) - higher in AD than controls (0.044)	(- 14-3-3 present in 20 sCJD and 5 AD) - Age (AD: 0.30, 0.022) - NfH (0.47, <0.001) - GFAP (0.45, <0.0001)	(93)	
12.) Bjerke et al., 2011 (125)	Healthy controls (30): 600 (median	AD (30): 1000 SVD (9) + MD (17): 1700	- higher in SVD than controls (<0.0001) and	- Age in controls (0.60, 0.001) - contributing most to	Uman Diagnostics NF-light	

	age 68y) (extrapolated from figure 1)	(extrapolated from figure 1)	AD (<0.05) - higher in AD than controls (<0.0005)	SVD versus controls - contributed third most for AD versus controls (1. Aß1-42, 2. T-tau)		
13.) Skillbäck et al., 2013 (126)	-	Overall study population (clinical routine samples, 5542): 1530		- T-tau (0.42, <0.001) - P-tau (0.23, <0.001) - Aß42 (-0.13, <0.001) - age (<0.001), sex (<0.001) and AD biomarker profile (0.001) predict NfL - only 29% with positive AD biomarker profile had normal NfL levels	Uman Diagnostics NF-light	- defined threshold value for subcortical axonal degeneration: 108 healthy volunteers (median age 38y): 95th percentile: 1400
14.) Landqvist et al., 2013 (127)	Healthy controls (26): 250 (median age 70y)	FTD (34): BvFTD (23): 770 SD (7): 1340 PNFA (7): 455 Post mortem verified FTD (10): higher in tau neg. cases than tau-positive cases (1620 vs. 665, 0.017) AD (20): 415	- higher in FTD than AD (<0.001) and controls (<0.001) - SD higher than AD (<0.001) - bvFTD higher than AD (<0.001)	- no correlation with neuropathological severity of degeneration or brain weight	(91) (17 healthy controls below detection limit)	
15.) Scherling et al., 2013 (128)	(mean) NC (29/17): 1197/1107 (mean age 66y/65y)	(means) BvFTD (22/22): 6227/4267 PNFA (8/10): 4304/6347 SD (10/6): 6726/6405 AD (31/17): 2828/2972 probable or possible PSP (11/10):	- all FTD higher than NC (<0.001; validation: <0.001) and AD (<0.03; validation: SD and PNFA: <0.001, not bvFTD: 0.795) - SD higher than PSP (<0.003;	- CDRsb (all FTD: 0.41, 0.008 and 0.36 and 0.002) - MMS (-0.33, 0.039 and -0.55, 0.002) - CDRsb (bvFTD: 0.41, 0.008; SD:0.64, 0.019; PNFA: 0.63, 0.011) not in AD and PSP - backward digit span (-0.47, 0.005)	Uman Diagnostics NF-light	- included validation cohort - CSF NfL levels correlated with disease severity in FTD - tendency for higher levels in SD versus other FTD phenotypes

		1899/2843 CBS (9/7): 5524/3950	<0.007) - higher in AD, PSP, and CBS than NC (<0.001; <0.001)	- phonemic fluency (-0.54, 0.002/-0.44, 0.019) - category fluency (-0.56, 0.001/-0.65, 0.001) - stroop color naming (-0.41, 0.038/-0.53, 0.012) - interference (-0.49, 0.016/-0.55, 0.01) - gray matter density (-0.35, <0.05)		
16.) Skillbäck et al., 2014 (129)	Clinically ascertained healthy (107): **275** (mean age 69)	EAD (223): **360** LAD (1194): **510** FTD (146): **855** DLB (114): **436** AD+VAD (517): **660** PDD (45): **360** CVD (465): **701**	- all groups except DLB and PDD had significantly higher values than controls - FTD significantly higher than all other groups	- age (LAD 0.28, <0.001; DLB 0.26, 0.05; PDD 0.45, 0.002; VAD 0.19, <0.001; controls 0.6, <0.001 - MMS (all <0.001; LAD <0.001; VAD 0.018; not FTD) - survival (<0.001)	3 methods for CSF NfL measurements: (91), adapted version from (91), Uman Diagnostics NF-light	

For correlations the coefficient r and p-values are given (r, p-value).

AD: Alzheimer's disease; BvFTD: behavioural variant frontotemporal dementia; CBS/D: Corticobasal syndrome/degeneration; CDRsb: Clinical dementia rating; DLB: dementia with Lewy bodies; EAD: early onset AD; FTD: frontotemporal dementia; LAD: late onset AD; MCI: Mild cognitive impairement; MD: mixed type dementia; MMS: Mini mental status; NC: normal controls; PD: Parkinson's disease; PDD: Parkinson's disease with dementia; PNFA: Progressive nonfluent aphasia; PSP: Progressive supranuclear palsy; sCJD: sporadic Creutzfeld-Jakob disease; SD: Semantic dementia; SVD: Subcortical vascular dementia; SWD: subcortical white matter dementia; VAD: vascular dementia; WMC: white matter changes.

There is variability in the assays used to quantify NfL in patients with different forms of dementia. Noteworthy, there are no studies published on measurements in the blood compartment so far in this condition. Nf show particularly high expression in large myelinated axons. In line with this observation, high CSF NfL levels were primarily found in disorders with subcortical pathology such as vascular dementia, while less increased levels were typically found in more cortical pathologies such as AD. Nevertheless, CSF NfL levels in AD were in general higher than in the various control groups and correlated with MMS in LAD and VAD. Also, NfL levels correlated with white matter lesion load and were particularly high in FTD.
It is generally thought that tau protein (reflecting neuronal degeneration) and Aß42 (reflecting disturbances in Aß metabolism and possibly Aß deposition in the senile plaque) are more sensitive and specific biomarkers for differentiating AD from controls than Nf. However, the specificity of these molecules in dementias other than AD is not optimal. It is still unclear whether blood NfL measurements can add to the diagnosis and the assessment of treatment effects in patients with dementia.

Table 1.4. *Overview of NfL studies in amyotrophic lateral sclerosis.*

Ref. no.	Healthy control groups studied (n): NfL: [median (IQR), pg/ml]	Type of disease studied (n): NfL [median (IQR), pg/ml]	[NfL] in disease different to controls	Correlations of [NfL] with clinical or laboratory measures	Assay characteristics NfL: [median (IQR), pg/ml]	Special features: e.g. longitudinal design
1.) Zetterberg et al., 2007 (130)	Healthy control individuals (40): 175 (mean age: 63) Unrelated patients with other neurological disorders (206): 277 (mean age 63)	ALS (79): 2110	- higher in ALS than healthy controls (<0.001) and reference patients (<0.001)	- age in reference (0.022) and control (<0.001) group - sensitivity 93%, specificity 91% for diagnosis ALS versus healthy controls - survival time (-0.52, 0.001) - shorter survival with levels above median (0.002)	(91)	- lower in patients with SOD1 mutation (0.001)
2.) Reijn et al., 2009 (131)	-	(means) ALS (32): 62 ALS-mimic disorders (26): 24	- ALS higher than ALS-mimics (0.005)	- disease duration (0.74, -)	(93)	
3.) Tortelli et al., 2012 (132)	Other neurological disorders: CIDP (25): 1000; (mean age: 61) OND (21, eg AD, MCI): 1800; (mean age: 62) (extrapolated	ALS (37): 5500 (extrapolated from figure 1)	- higher in ALS than in CIDP (<0.0001) or OND (<0.0001) - no difference between CIDP and OND	- no correlation with age - higher in rapidly progressive patients (<0.006) - diagnostic delay (-0.55, <0.0001) - ALSFRS-r score (-0.41, 0.014) - progression rate (0.65, <0.0001)	Uman Diagnostics NF-light	

	from figure 1)					

For correlations the coefficient r and p-value is given (r, p-value).

OND: other neurological disease; AD: Alzheimer's disease; MCI: Mild cognitive impairment; ALSFRS-r: Amyotrophic Lateral Sclerosis Functional Rating Scale

CSF NfL levels were found consistently higher in ALS than in controls in three studies (all using different assays). CSF NfL levels also correlated with measures of disease activity and disease progression. The high CSF NfL levels in ALS may be related to the higher content of axonal proteins in large myelinated motor neurons compared to other neuronal populations. In conclusion, NfL appears a sensitive biomarker of disease severity, progression and potentially treatment response in ALS that clearly deserves further investigation.

Table 1.5. *Overview of NfL studies in vascular diseases.*

Ref. no.	**Healthy control groups studied (n): NfL: [median (IQR), pg/ml]**	**Type of disease studied (n): NfL [median (IQR), pg/ml]**	**[NfL] in disease different to controls**	**Correlations of [NfL] with clinical or laboratory measures**	**Assay characteristics NfL: [median (IQR), pg/ml]**	**Special features: e.g. longitudinal design**
1.) Nylen et al., 2002 (133)	CNS vasculitis unlikely (40): 855	(means) CNS vasculitis (32): 1639	-	- higher levels in patients with CNS injury than without (<0.001) - higher in patients with >5/ul CSF cells (<0.01) and increased IgG index (<0.01)	(91)	
2.) Nylen et al., 2006 (134)	-	SAH (44): 9035 pg/ml	-	- no difference between neurosurgical clipping and endovascular coiling - higher in surgical versus no intervention group (<0.01), unfavorable versus favorable outcome (<0.01), parenchymatous versus no parench. lesion (<0.001) - long-term outcome (-0.56, <0.001), MMS (-0.52, 0.001), and NIHSS (0.50, <0.001)	(91)	- CSF sampling 10-14 days after SAH
3.) Jonsson et al., 2010 (135)	-	Mild WML (15): 250 Moderate WML (23): 300	-	- volume of WML (0.48, <0.001)	(91)	

		Severe WML (15): 630				
4.) Zanier et al., 2011 (136)	Non-neurological patients (13): undetectable	SAH (35): 643	-	- higher in patients with cerebral ischemia than without (<0.01) - no significant difference regarding outcome	adapted from (93)	

For correlations the coefficient r and p-value is given (r, p-value).

SAH: Subarachnoid hemorrhage, MMS: Mini mental status; NIHSS: NIH stroke scale; WML: white matter lesions

Few studies have investigated CSF NfL levels in patients with cerebrovascular ischemia. An obvious reason for this is the fact that lumbar punctures are not indicated in patients with stroke or transient ischemic attacks (TIA). No studies in blood samples have been performed. Interestingly, none of the mentioned investigations used the Uman Diagnostics NF-light assay. Two studies demonstrated increased CSF NfL levels in patients with SAH, partly also associated with clinical outcome.

1.7. Types of biomarkers and surrogate endpoints

An expert working group convened by the National Instutute of Health defined a biological marker (biomarker) as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic response to a therapeutic intervention (137).

Biomarker applications include the following:

1. Use as a diagnostic tool for the identification of those patients with a disease or abnormal condition (eg, blood glucose levels in diabetes mellitus).
2. Use as a tool for staging of disease or classification of the extent of disease (eg, prostate-specific antigen concentration in prostate cancer).
3. Use as an indicator of disease prognosis.
4. Use for prediction and monitoring of clinical response to an intervention (137).

The expert working group also proposed important definitions for “clinical endpoint”: a characteristic or variable that reflects how a patient feels, functions, or survives; and “surrogate endpoint”: a biomarker that is intended to substitute for a clinical endpoint. A surrogate endpoint is expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence (137). Although all surrogate endpoints can be considered biomarkers, it is likely that only a few biomarkers will achieve surrogate endpoint status (137). For a surrogate endpoint to be used in a clinical trial, it needs to be shown that the effect of the intervention on the surrogate end point predicts the effect on the clinical outcome (138). Prentice developed criteria to validate surrogate endpoints in phase 3 clinical trials:

1. The treatment must have an effect on the surrogate
2. Treatment must have an effect on the clinical outcome
3. The surrogate and the clinical outcome must be correlated
4. Intervention effect on the true clinical outcome must disappear when adjusting for the surrogate (139).

Several factors may explain the potential failure of surrogate endpoints:

a) the surrogate may not be in the causal pathway of the disease process.
b) of several causal pathways of disease, the intervention affects only the pathway mediated through the surrogate.
c) the surrogate is not in the pathway of the intervention’s effect or is insensitive to its effect.
d) the intervention has mechanisms of action independent of the disease process (138).

These factors explain why effects on surrogate endpoints often do not predict the true clinical effects of an intervention (140-142). Nevertheless, the successful use of a

surrogate endpoint in a clinical trial could allow reduction in sample size or trial duration (139).

1.8. Preliminary work of the student leading to the experiments detailed in the thesis

Electrochemiluminescence (ECL) based assays are known to be highly sensitive, exhibit a broad dynamic range and require small sample volume; the technology has demonstrated the ability to quantify levels of nucleic acids, recombinant proteins and bacterial and viral components in the sub-picogram range with increased precision compared to conventional enzyme-linked immunosorbent assay (ELISA) (143-148).

Highly sensitive methods for detecting soluble biomarkers for neuro-axonal damage are needed in neurodegenerative diseases. I have previously developed an ECL solid-phase sandwich immunoassay on the Meso Scale Discovery (MSD, Gaithersburg, MD, USA) to measure the soluble fraction of neurofilament heavy chain (NfH^{SMI35}) in cerebrospinal fluid (CSF) employing the same commercially available antibodies used in a conventional ELISA (cELISA) (149-151). Adhering to a previously proposed nomenclature, the soluble fraction of NfH measured is indicated with the capture antibody in the superscript (149).
The NfH^{SMI35} assay protocol was optimised and validated for reproducibility, precision, accuracy and parallelism. The analytical sensitivity (background plus three standard deviation (SD)) of this assay was 2.4 pg/ml. The mean intra-assay coefficient of variation (CV) was 4.8% and the inter-assay CV 8.4% (151). Patients with ALS (160.1 pg/ml, n=50), mild cognitive impairment (MCI)/AD (65.6 pg/ml, n=20), GBS (91.0 pg/ml, n=20) or subarachnoid haemorrhage (SAH) (345.0 pg/ml, n=20) had higher CSF NfH^{SMI35} values than the reference cohort (27.1 pg/ml, n=73, $p<0.0001$ for each comparison). The reference cohort included patients who, based on extensive diagnostic evaluation, had no objective clinical or paraclinical signs of a neurological disease: tension type headache (n=17), lower back pain (n=5), psychiatric disorders (n=30) or miscellaneous diseases for which no neurological explanation could be found (n=21) (151).

In a next step, employing this ECL-based immunoassay, we measured levels of the NfH^{SMI35} protein in the CSF of healthy controls (HC) and in patients with a clinically isolated syndrome (CIS) or Multiple Sclerosis (MS) (151, 152). In particular, we examined whether NfH^{SMI35} levels differ between MS patients and controls and between specific stages (relapsing-remitting versus progressive forms) or states (relapsing

versus stable) of disease. The main findings of this study were that CSF levels of NfH^{SMI35} increase in the course of disease evolution from CIS to definite MS and correlate with the Expanded Disability Status Scale score (EDSS) as clinical measure of disability in CIS and relapsing remitting MS (RRMS), but not in progressive stages (secondary progressive MS (SPMS) and primary progressive MS (PPMS)). In contrast, none of the CSF measures related to the immune response (CSF cell count, intrathecal IgG, IgM or IgA production, CSF total protein or albumin quotient as measures of the blood CSF barrier integrity) correlated with EDSS at any time point of MS evolution. In summary our results supported the utility of NfH^{SMI35} as a specific biomarker for on-going neuroaxonal damage that can be quantified with high sensitivity and a broad dynamic measuring range, a pre-requisite for use in clinical practice (153).

These findings were important, because we anticipated that further development of the assay in serum/plasma samples could have provided tools to measure longitudinally NfH^{SMI35} levels during disease progression as well as in clinical trials of potential neuroprotective drugs in diseases like MS and ALS.

To date there are only very few studies on NfH in the blood compartment (154-156). These results await validation and I and others have previously experienced analytical difficulties with reliable and reproducible quantification of NfH levels in blood samples (characterised by the so called “hook effect” or other matrix interferences and thus lack of parallelism between plasma samples and standards in serial dilutions). This effect is most likely based on either the formation of aggregates or endogenous binding of Nf by antibodies and poses an important pre-analytical problem for a quantitative immunoassay of Nf levels (157, 158). A method for solubilising Nf aggregates by urea preincubation of samples has been recently proposed (159).

Unfortunately, during the following months in 2011 we were not able to adapt the NfH^{SMI35} ECL immunoassay to measurements in serum or plasma samples due to insufficient recovery and lack of dilutional linearity. Despite several attempts to reach acceptable analytical performance, I finally decided not to follow this development for NfH^{SMI35} further.

Nevertheless, an assay including the benefits of the ECL technology seemed a promising approach forNf measurements in the blood compartment. Such an assay could potentially provide a sufficiently sensitive tool for blood measurements in several chronic neurodegenerative diseases.

1.9. Specific aims

Chapter 2 aims to compare CSF levels of NfL (UmanDiagnostics NF-light® assay) with those of (previously determined) NfH^{SMI35} in a well characterised group of 148 CIS/MS patients and 72 controls (152). Second, I evaluated the analytical and clinical performance of the UmanDiagnostics NF-light® assay and stability of its analyte (160).

In chapter 3A, I aimed to determine CSF NfH^{SMI35} levels using the NfH^{SMI35} assay I have developed in a subset of MS patients who had previously shown reduced NfL (UmanDiagnostics NF-light®) levels after natalizumab treatment (101).

In chapter 3B, I aimed to assess the ability of CSF NfL (by UmanDiagnostics NF-light®) as a therapeutic biomarker in RRMS, by comparing levels in fingolimod-treated patients versus placebo, and correlating NfL levels with clinical and MRI outcomes.

In chapter 4 I aimed to develop and validate a sensitive ECL-based NfL assay suitable for the quantification of NfL in serum at concentrations relevant to clinical settings.

In chapter 5, I aimed to analyse NfL levels in longitudinally collected serum samples from subjects enrolled in a phase II clinical trial investigating the utility of minocycline to attenuate neurological deficits after spinal cord injury (SCI) (161). I report the correlation of serum NfL with acute and long-term clinical outcome in these patients. Further, I investigated the potential of serum NfL as drug response marker of the therapeutic effect of minocycline in SCI.

Finally, in chapter 6, my objective was to assess the ability of serum NfL to predict the risk of conversion from adult CIS to CDMS (defined by occurrence of a second clinical attack) using the largest cohort of adult CIS cases ever studied to date (n=1,047). I decided to use a two steps strategy by initially measuring serum NfL in the 100 patients with the shortest time to conversion to CDMS (fast converters (FC)), the 100 patients with the longest follow-up time in the absence of conversion to CDMS (non converters (NC)) and 100 healthy controls. I decided that only if I saw a difference in serum NfL levels between FC and NC serum NfL, I would have measured serum NfL in the rest of the cohort.

2. A comparative study of CSF NfL and NfH protein in MS (104)

2.1. Introduction

In this chapter, I first evaluated the analytical and clinical performance of the UmanDiagnostics NF-light® assay and the stability of its analyte (160). Second, I compared the ability to discriminate between MS patients and controls of CSF levels of NfL (as measured with the UmanDiagnostics NF-light® assay) with previously determined CSF NfH^{SMI35} levels in a well characterized group of 148 CIS/MS patients and 72 controls (152).

2.2. Patients and Methods

2.2.1. Patients and CSF samples

Samples were collected in the Department of Neurology, University Hospital Basel in the course of routine diagnostic measures as indicated by the treating physicians and after patient informed consent. The sample collection procedure, clinical measurement methodology and immunomodulatory treatment have been described in reference: (152). Eighty six patients with MS and 62 patients with a CIS were included. Patients were classified as having clinically definite RRMS (n=38), SPMS (n=25), or PPMS (n=23) by a trained neurologist (152, 162).

The control group consisted of 72 patients who, based on extensive diagnostic evaluation, had no objective clinical or paraclinical signs of a neurological disease. Due to lack of CSF, samples from one CIS, one RRMS and one control used in NFH^{SMI35} could not be assayed for NfL (**table 2.1**) (152).

Table 2.1. *Demographic and clinical characteristics of patients and controls.*

	Controls	**CIS**	**RRMS**	**SPMS**	**PPMS**	**All**
N	72	62	38	25	23	220
Females (n [%])[a]	44 (61.6)	50 (80.6)	25 (65.8)	13 (52.0)	10 (43.5)	142 (64.5)
Age [years, median (IQR)][b]	38.2 (26.6-46.4)	34.2 (26.3-43.0)	34.3 (28.8-46.2)	52.8 (46.0-58.4)	57.8 (40.5-64.6)	39.8 (29.5-49.0)
Disease duration [years, median (IQR)][b]	-	0.1 (0.0-0.2)	2.3 (1.0-7.0)	14.9 (10.0-22.8)	3.0 (1.0-6.0)	1.0 (0.1-7.0)
EDSS [median, IQR][c]	-	2 (1.5-2.5)	2 (1.5-3)	4 (3.5-5.75)	3 (2.5-4.5)	2.5 (2-3.5)
Relapse at LP (n [%])[d]	-	33 (53.2)	20 (52.6)	8 (32.0)	-	61 (48.8)

[a] There were more female CIS patients compared to controls (p=0.015), SPMS (p=0.015) and PPMS patients (p=0.002).

[b] Controls, CIS and RRMS were younger and had a shorter disease duration compared to SPMS (p<0.0001 for each) and PPMS (p<0.0001 for each comparison). PPMS had a shorter disease duration compared to SPMS (p<0.0001).

[c] Lower EDSS in CIS versus SPMS (p<0.0001) and PPMS (p<0.0001). Lower EDSS in RRMS compared with SPMS (p<0.0001) and PPMS (p<0.0001) and in PPMS compared with SPMS (p=0.022).

[d] chi-square test: ns

2.2.2. UmanDiagnostics NF-light® ELISA and ECL-NfH^{SMI35} assay

The UmanDiagnostics NF-light® ELISA was performed at room temperature. CSF samples were diluted 1:1 with sample dilution buffer to a total volume of 100µl and incubated with agitation (800rpm) for 1 hour in precoated anti-NFL ELISA plates. Thereafter, a 100 µl solution of tracer antibody (biotin anti-NfL) was added to each well and incubated for 45 minutes. Washing cycles were performed after all incubations. Detection was performed using 100µl of streptavidin-HRP incubated for 30 minutes, followed by another incubation with 100µl 3,3',5,5'-tetramethylbenzidine for 15 minutes. A volume of 50µl stop solution (8% v/v sulphuric acid) was added to each well, and absorbance was read at λ490nm. The sensitivity of the NfL assay was 31pg/ml.
The ECL-NfH^{SMI35} assay and basic CSF analysis has been described in my published work on developing a sensitive ECL based immunoassay for NfH^{SMI35} (151). Baseline demographics, disease and CSF characteristics were not significantly influenced by omission of one control, one CIS and one RRMS samples due to lack of material (**table 2.1., and table 2.2.**).

Table 2.2. *Distribution of CSF parameters in patients and healthy controls.*

	Controls	**CIS**	**RRMS**	**SPMS**	**PPMS**
N	72	62	38	25	23
cell count [a] (n/mm^3) ¶ ref: ≤5	1.0 (0.6-1.7)	4.0 (2.3-8.9) *	6.2 (3.0-12.5) *	2.0 (0.9-5.4) p=0.015	1.6 (0.7-3.3) ns
Q_{alb} [b] ref: age adjusted¶[#]	4.5 (3.5-5.6)	4.8 (3.8-6.0) ns	5.7 (4.3-8.5) p=0.002	5.9 (4.6-7.5) p=0.01	6.0 (4.1-7.9) p=0.012
IgG-Index¶ ref: <0.7	0.48 (0.45-0.5)	0.87 (0.6-1.3) *	0.92 (0.6-1.4) *	0.86 (0.6-1.2) *	0.87 (0.7-1.1) *
IgG_{IF} (%)¶	<10%	19.0 (0.0-46.9) *	22.2 (0.0-49.6) *	22.0 (0.0-39.3) *	20.5 (0.0-33.0) *
IgA_{IF} (%)¶	<10%	0.0 (0.0-0.0) p=0.008	0.0 (0.0-0.0) *	0.0 (0.0-0.0) p=0.001	0.0 (0.0-0.0) p=0.012
IgM_{IF} (%)¶	<10%	0.0 (0.0-0.0) ns	0.0 (0.0-0.0) *	0.0 (0.0-0.0) p=0.001	0.0 (0.0-0.0) p=0.015

OCB[+ c] (n (%))	0 (0)	48 (77.4) *	35 (92.1) *	18 (72.0) *	18 (78.3) *

¶ Values represent median (IQR), p values versus controls are displayed, * denotes p<0.0001; ns: not significant

Cell count: white cell count in CSF, Ref: upper reference value. OCB+: oligoclonal bands in CSF, evidence for intrathecal IgG synthesis.

[#] age-dependent upper limit of Q_{alb} calculated by the formula: Q_{alb} ref: (age/15+4) $x10^{-3}$.

[a] Higher CSF cell count in CIS versus SPMS and PPMS (p=0.003 and <0.0001). Higher cell count in RRMS versus SPMS and PPMS (p<0.0001 for both).

[b] Lower Q_{alb} in CIS versus RRMS and SPMS (p=0.025 and 0.021). Lower Q_{alb} in stable RRMS (5.7 (3.2) versus RRMS in relapse (7.8 (3.7), p=0.009).

[c] OCB were less frequently detected in SPMS compared to RRMS (p=0.042).

2.2.3. Precision of the NF-light® ELISA and stability of NfL

I evaluated reproducibility (intra-assay variability) and repeatability (inter-assay variability) of the NF-light® ELISA using 4 native CSF samples in 5 consecutive assays on independent days. I tested the stability of NfL at room temperature (RT), 4°C and compared this to samples stored at -80°C. Three aliquoted CSF samples were frozen at -80°C. The aliquots were thawed on day 0, 3 hours, 1, 4 and 8 days in advance of measurement and stored at RT or 4°C until analysis. I normalized the measured signals to the signal of the day 0. I analyzed three CSF samples for stability during freeze-thawing cycles (151). The samples underwent 1, 2, 3, or 4 freeze-thawing cycles and I normalized the signal to the sample freeze-thawed once. Samples were refrozen for 24 hours after each thawing step. Due to limited volume, samples for these experiments were chosen based on their CSF NfL levels (high, medium and low NfL levels) from patients with other diagnoses than CIS, MS or control patients.

2.2.4. Statistical evaluation

Continuous variables were described by their median and interquartile range (IQR), and categorical variables by numbers and percentages. Comparison of basic quantitative CSF parameters across groups was performed using the Kruskal-Wallis test, and pair wise post-hoc comparisons using the Mann-Whitney U test. Comparisons of categorical variables were done using the chi-square test. CSF levels of NfL, Q_{alb} and other basic CSF parameters were log-transformed to achieve a normal distribution for subsequent analyses. Yet, for simplicity of notation, I used the original terms of CSF parameters when reporting and discussing results. To control for age as a potential confounding factor an analysis of covariance with age as a covariate and disease stage group as a fixed factor was performed. Group-specific levels of NfL and other biomarkers were expressed as geometric means with 95%-confidence intervals. For log-normal variables, the geometric mean equals the median. Partial correlations adjusted for age were computed by first regressing the two variables on age and then determining the Spearman rank correlation coefficient (r_s) of the respective residuals. Receiver operating characteristic (ROC) curves were derived from logistic regression (with age as a covariate) to compare the discriminatory power of NfL and NfH^{SMI35} between CIS/different stages of MS and healthy controls. The area under the curve (AUC) was calculated for NfL and NfH^{SMI35} and compared using the method of DeLong et al (163). A two-sided p-value < 0.05 was considered as significant. P-values of post hoc comparisons were adjusted using a Bonferroni correction. All statistical analyses and graphs were prepared using SPSS (Version 15.0 SPSS, Chicago, IL) and Graph Pad Prism 5.02 for Windows (GraphPad Software, San Diego, CA).

2.3. Results

2.3.1. Analytical performance of the NfL assay and stability of the analyte

The mean coefficients of variation (CV) of duplicates within given assays were 5.6% (5680 pg/ml), 3.1% (564 pg/ml), 5.5% (242 pg/ml) and 3.0% (156 pg/ml). In-between-assay variation was 8.9% (5680 pg/ml), 7.3% (564 pg/ml), 11.3% (242 pg/ml) and 13.5% (156 pg/ml).

Then, I tested the stability of the NfL protein at room temperature (RT) and 4°C as compared to reference aliquots stored at -80°C. There was no significant change in measured concentration in samples stored at RT and at 4°C for up to 8 days (RT: day 8: 1.04±0.053 (mean normalized ratio between day 0 and day 8±SD), p=1.0 and 4°C: day 8: 1.05±0.051, p=0.5) (**figure 2.1.**)

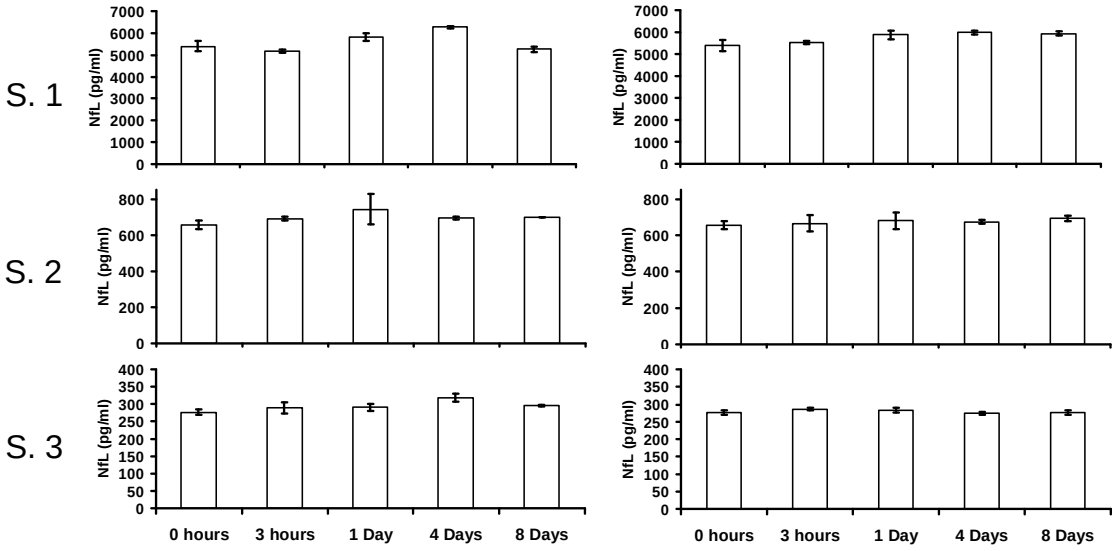


Figure 2.1. Stability of the NfL protein at RT (left) and 4°C (right).
Three CSF samples (S. 1-3) were thawed on day 0 (0 hours, reference), 3 hours in advance of measurement (3 hours), and 1 (1 Day), 4 (4 Days) and 8 days (8 Days) before the experiment and stored at RT and 4°C. There was no significant change in measured concentration in samples stored at RT and at 4°C up to 8 days (RT: day 8: 1.04±0.053 (mean normalized ratio between day 0 and day 8±SD), p=1.0 and 4°C: day 8: 1.05±0.051, p=0.5). Mean calculated pg/ml of duplicates (SD) are displayed

Next, I analyzed three CSF samples for stability during freeze-thawing cycles. There was again no significant effect of freeze-thawing up to 4 times on the measured concentrations in three CSF samples (4 freeze-thawing cycles: 1.03±0.026, p=0.25) (**figure 2.2.**).

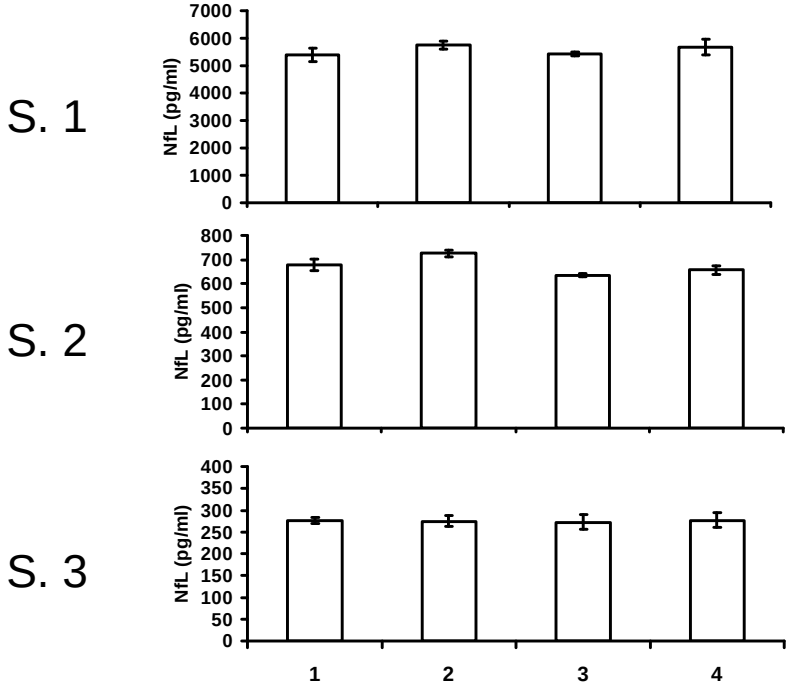


Figure 2.2. Stability of CSF NfL during freeze-thawing cycles.
Three CSF samples (S. 1-3) underwent 1, 2, 3 or 4 thawing cycles and the measured concentrations were normalized to the sample freeze-thawed once, without any relevant effect of freeze-thawing (4 freeze-thawing cycles: 1.03±0.026, p=0.25). Mean calculated pg/ml of duplicates (SD) are displayed.

2.3.2. NfL levels in CSF as a function of clinical features and age

CSF NfL levels were increased ($F_{4, 215}$=26.89, p<0.0001) in all forms and stages of MS compared to controls (p<0.0001 for all comparisons). Levels of CSF NfL were 2.8, 4.4, 2.9 and 3.7 fold higher in CIS, RRMS, SPMS and PPMS, respectively, compared with controls. A strong correlation with age was seen for NfL in controls (r=0.61, p<0.0001), while this association was absent in CIS (r=0.06, p=0.778), RRMS (r=0.11, p=0.417), SPMS (r=0.13, p=0.444) and PPMS (r=-0.08, p=0.694).

Subsequent analysis of covariance with age confirmed the previous highly significant group differences between CIS and all stages of MS in comparison to controls ($F_{4, 214}$=26.05, p<0.0001; p=0.001 for SPMS, p<0.0001 for CIS, RRMS and PPMS). Moreover, this analysis also revealed a difference between RRMS and SPMS (p=0.025) (**figure 2.3.**).

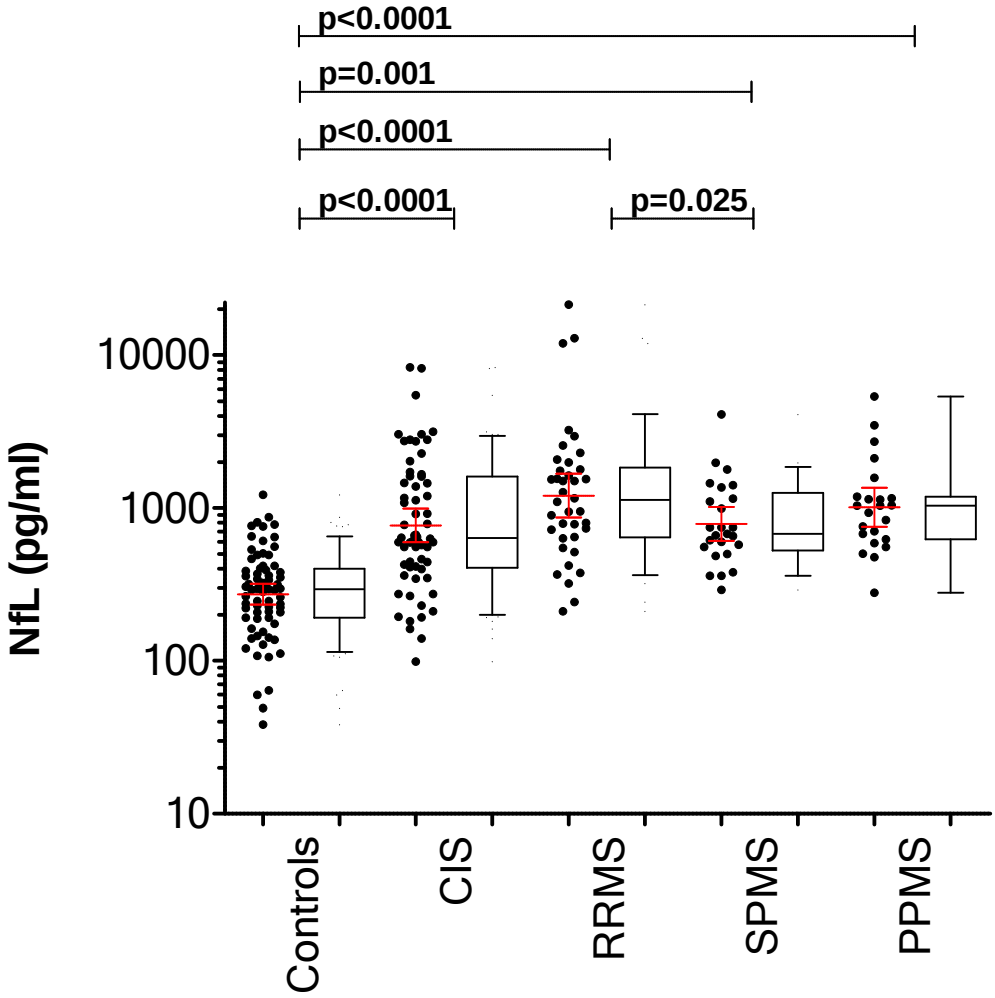


Figure 2.3. NfL levels in the controls, patients with CIS, and patients with MS. Geometric mean and 95% confidence interval are displayed (and box and whiskers: median and 10-90% percentile). CIS (765.8 pg/ml), RRMS (1200.8 pg/ml), SPMS (784.6 pg/ml), and PPMS (1007.0 pg/ml) showed higher CSF NfL levels than controls (271.9 pg/ml) and RRMS higher values than SPMS (p=0.025). Dots represent individual samples. P Values are adjusted for age and corrected by Bonferroni method.

2.3.3. Correlations of NfL with CSF markers of inflammation

In CIS and RRMS levels of NfL correlated with CSF cell count (r_s=0.27, p=0.016 and r_s=0.43, p=0.01) and albumin quotient (qAlb) in CIS, RRMS and SPMS (r_s=0.28, p=0.025, r_s=0.49, p=0.002 and r_s=0.67, p<0.0001). CIS and RRMS patients with a CSF cell count >5 cells/mm^3 showed almost twice higher NfL concentrations as compared to those with normal CSF cytosis (>5 cells/mm^3, n=47: 1252 pg/ml [899-1744] versus ≤5 cells/mm^3, n=53: 684 pg/ml [544-859]; p=0.0078). In contrast, no such correlation could be observed in progressive MS.

IgG index, and intrathecal fractions of the immunoglobulin subclasses were not related to CSF NfL levels. Similarly, levels of NfL were not influenced by presence or absence of OCB in CIS/MS (OCB^+, n=119: 908.3 pg/ml [770.1-1097.9]; OCB^- , n=29: 869.5 pg/ml [604.7-1250.6], p=0.983).

2.3.4. Correlations of NfL with disability and disease activity

Age-corrected NfL levels correlated with EDSS score in patients with relapsing disease (CIS and RRMS: r_s=0.31, p=0.002), but not in progressive stages of MS (SPMS and PPMS: r_s=-0.18, p=0.218). There was no significant correlation between any of the inflammation-related CSF markers (Q_{alb}, CSF cell count, OCB positivity, IgG index, IgG_{IF}, IgA_{IF}, IgM_{IF}) and EDSS (data not shown).

Patients with a relapse at the time of lumbar puncture tended to have higher NfL values (n=61: 1070 pg/ml [818-1401]) than those in remission (n=64: 734 pg/ml, [598-900]) (p=0.054). Similarly, the levels of NfL in patients with relapses due to spinal cord pathology was nearly double the levels seen in patients with relapses due to cerebral lesions (n=16: 1728 pg/ml [924-3229] vs. n=45: 906 pg/ml [674-1217], p=0.037). As previously reported, Q_{alb} levels were higher during relapses (7.0) than in stable disease (5.2) (p=0.012) only in RRMS, while all other inflammation-related CSF markers were not influenced by the presence of relapses at the time of spinal tap (152).

2.3.5. Discriminatory power of NfL and NfH^{SMI35} between CIS/MS and controls and relationship of NfL and NfH^{SMI35}

Figure 2.4 shows ROC plots of NfL and NfH^{SMI35} in CIS and MS (RRMS, SPMS and PPMS) patients. The discriminatory power of NfL was greater than that of NfH^{SMI35} in CIS patients (**figure 2.4.**, left) and RRMS, SPMS and PPMS together (**figure 2.4.,** right) (CIS: AUC 0.83 versus 0.67, p=0.001; all MS patients (RRMS, SPMS, PPMS): AUC 0.91 versus 0.85, p=0.035). In separate analyses for RRMS, SPMS and PPMS, the differences between NfL and NfH^{SMI35} did not reach significance (RRMS: p=0.273, SPMS: p=0.480 and PPMS: p=0.308).

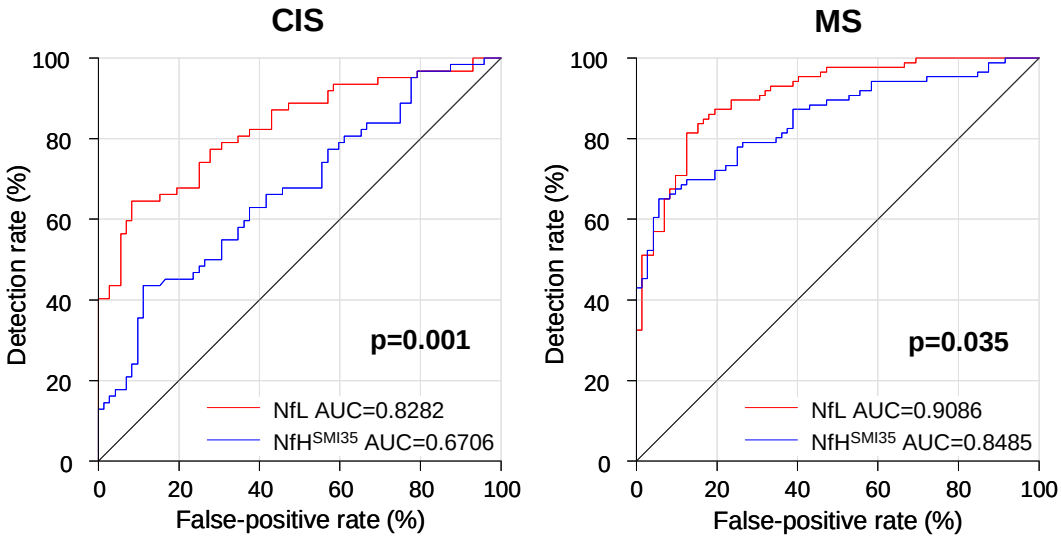


Figure 2.4. ROC plots of NfL and NfH^{SMI35} in CIS and MS patients versus controls. The discriminatory power of NfL was greater than that of NfH^{SMI35} in CIS patients (AUC 0.83 versus 0.67, p=0.001) and in all MS patients (RRMS, SPMS, PPMS) grouped together (AUC 0.91 versus 0.85, p=0.035).

There was a highly significant correlation of NfH^{SMI35} and NfL in controls (r=0.40, p<0.0001), CIS (r=0.44, p<0.0001), RRMS (r=0.57, p<0.0001) but not SPMS (r=0.23, p=0.163) or PPMS (r=0.40, p=0.061). After age correction, this relationship was no longer observed in controls (r_s=0.058, p=0.627), persisted in CIS (r_s=0.46, p<0.0001) and RRMS (r_s=0.56, p<0.0001) and was still absent in SPMS (r_s=0.31, p=0.128) and PPMS (r_s=0.41, p=0.054).

2.4. Discussion

The primary findings of this comparative study were that NfL proved to be a stable analyte, and its assay system used here is more sensitive than that for NfH^{SMI35}. NfL is considered to represent the most abundant and also most soluble subunit, but there have been concerns about its susceptibility to proteases, especially in the protease-rich CSF or blood (160). Several groups, have therefore previously concentrated on NfH, as a biomarker for axonal damage, as its phosphorylated state is assumed to be more stable (149, 164-166). In the present study, CSF NfL levels were demonstrated to be stable up to eight days at room temperature and for up to four freeze-thawing steps (93, 132). These results are well in line with my previous findings of NfH^{SMI35} levels in CSF being stable over prolonged storage times and repeated freeze-thawing steps (151). We conclude that there is no basis to prefer NfH over NfL as biomarker of axonal damage due to concerns of sample stability.

Persisting neurological deficits in MS likely emerge as a consequence of accumulating nerve injury starting in the very early phase of the disease. Confirming my previous

NfH^{SMI35} results, levels of NfL were increased already in CIS and in all other stages of the disease as compared with controls. Interestingly, differences between CIS and MS patients and controls were more pronounced for NfL than for NfH^{SMI35} (**figure 2.3.**) (152). The increased discriminatory power of the commercial NfL ELISA as compared with the ECL-NfH^{SMI35} assay, especially in CIS stages, was also reflected by the results of the ROC curve analyses. The higher abundance of NfL and/or better performance of the two monoclonal antibodies (versus the polyclonal detection antibody in the ECL-NfH^{SMI35} assay) included in the UmanDiagnostics NF-light® assay seem to outweigh the known high sensitivity and higher dynamic range of the ECL technology used in the NfH^{SMI35} assay (95) (see also chapter 1).

In my previous study, NfH^{SMI35} showed a strong correlation with age in controls (r_s=0.5, p<0.0001) and in patients with a CIS (r_s=0.5, p<0.0001); the correlation was weaker in RRMS (r_s=0.35, p=0.027) and absent in SPMS and PPMS (152). In this study, the correlation between age and NfL was even more pronounced in controls (r_s=0.61, p<0.0001), but absent in all other stages of disease. Both NfH^{SMI35} and NfL findings are well in line with a recent report on CSF NfH and NfL levels showing that NfH (r=0.71, p<0.005) and NfL (r=0.93, p<0.001) were strongly correlated with age in controls. In patients with CIS, this correlation was less strong for NfH (r=0.33, p<0.01) and absent for NfL levels (r=0.15, p>0.05) (102) (see chapter 1). I therefore hypothesize that disease related neurodegenerative processes outweigh physiologic, age-related changes of NfL clearance even in the earliest stages of MS and that this effect is more evident in NfL given the higher sensitivity of this assay compared to the NfH^{SMI35}.

In the above mentioned recent work by Khalil et al., NfH and NfL levels in CSF of CIS patients significantly correlated with CSF cell count, IgG index and Q_{alb}. Therefore, my results suggest that CSF NfL levels not only reflect chronic neurodegenerative processes but also are linked to more acute inflammatory processes (102). NfL levels correlated with the extent of blood-CSF barrier damage in CIS, RRMS and SPMS, and with CSF inflammatory cell counts in CIS and RRMS. Further, NfL levels determined at the time of a relapse, especially during spinal cord related relapses were highly increased. This further supports the concept that Nf release in MS reflects two parallel neurodegenerative processes: 1) a chronic *brain-diffuse* neuroinflammation; 2) an acute *focal* inflammatory activity in the course of plaque formation.

Similarly to my findings on NfH^{SMI35}, NfL levels correlated with disability in earlier (CIS and RRMS) but not in progressive (SPMS and PPMS) stages (152). Conversely, none of the other inflammatory CSF markers correlated with the EDSS. Likewise NfL and NfH^{SMI35} concentrations in CIS and RRMS (but not controls) showed a robust correlation, whereas this was not seen in progressive disease. It remains speculative, if

the relatively small sample number of patients in progressive MS, the difficulty in quantifying neurological deficits by the EDSS, or a dissociation of liberation of different Nf subunits in progressive MS have contributed to these findings. In addition, a number of biological mechanisms may act over time to change Nf bioavailability and disproportionally distort correlations between different Nf isoforms or clinical measures. These include: 1) the formation of aggregates which may reduce Nf detection and 2) the raising levels of autoantibodies against NfL which may have a clearing effect of circulating proteins (157-159).

NfL levels in CSF have been reported to be higher in different stages of MS compared to healthy controls and in relapse versus remission (92, 97) (see chapter 1). Previous studies also reported relatively weak correlations of NfL levels with the EDSS (96, 98, 167) and, in fewer studies, with age in controls (91). Teunissen and colleagues performed the only study so far investigating NfL and NfH levels in all stages of MS and control groups. NfL was determined by the Uman-Diagnostics NF-light® ELISA (reagents) and NfH by a modified conventional ELISA assay (100, 149). Similarly to my findings, NfL was increased in CIS and all stages of MS and, after age correction, correlated weakly with the EDSS in CIS and MS ($r=0.192$, $p<0.05$). Patients in relapse also displayed higher levels than those in remission ($p=0.04$). Differences for NfH were less pronounced than previously reported by our group. Information regarding correlation of NfL and NfH and/or age in controls and separate stages of MS were not given and performance of the NfL versus the NfH assay was not described (100, 152).

Limitations of this study include the relatively small number of patients (especially SPMS and PPMS) and lack of follow-up data and samples. Furthermore, I did not have access to CSF samples from healthy controls, but only to samples from individuals who did not have any objective clinical or paraclinical findings, but anyway needed to perform a lumbar puncture. Also I was not able to choose age matched controls and had to apply statistical correction for age. Finally, conventional and advanced MRI data were not available.

Taken together my results confirm and expand on previous findings of Nf as quantitative markers of neurodegeneration in CSF. NfL and NfH^{SMI35} are both stable proteins, an important prerequisite for biomarkers. It is important to note, that based on my findings, we cannot conclude that NfL is a superior analyte over NfH in general. Some of my results are likely to reflect the properties of the assays used and not wholly the properties of the proteins. Rather, in comparison to the very sensitive ECL-NfH^{SMI35} assay, the NF-light® ELISA differentiates better between health and disease, especially in the CIS stage. All my analyses were performed in CSF, conversely, serum Nf

measurements would be the most relevant for clinical practice, an aim so far reached more frequently for analyses of NfH (154, 159, 168, 169) as compared to serum NfL (170). Based on this, further development of an NfL assay including the benefits of the ECL technology seemed a promising approach towards NfL measurements also in serum/plasma samples. These findings support the role of Nf as a useful measure of neurodegeneration and their potential usefulness as surrogate measure for treatment studies in MS.

3. CSF NfL and NfH as therapy response biomarkers in multiple sclerosis

3A. NfL and NfH as therapy response markers to natalizumab (171)

3.1. Introduction

Increased NfL and NfH levels have been found in all stages of MS with the highest levels observed during relapses and in the presence of Gd+ lesions on MRI (96, 97, 100, 152). Natalizumab binds to α4β1-integrin, preventing lymphocyte transmigration into the CNS and is approved as monotherapy for RRMS (172).

The UmanDiagnostics NF-light® assay uses two highly specific non-competing monoclonal antibodies to quantify NfL in human body fluids (95). A recent study employed this assay in patients with RRMS showed that natalizumab treatment normalized CSF NfL levels (101). As pointed out in the introduction, we had developed a sensitive ECL-based solid-phase sandwich immunoassay for NfH^{SMI35} in CSF (151). The aim of this study was to measure CSF NfH^{SMI35} levels using our ECL based assay and compare these with CSF NfL levels in a subset of the patients who had previously shown significantly reduced NfL levels after natalizumab treatment (101).

3.2. Patients and Methods

3.2.1. Patients, CSF samples and Nf assays

CSF was consecutively collected from 30 patients by lumbar spinal taps before (preNat) and after (postNat) 12 months of natalizumab treatment (all samples from the Sahlgrenska Academy, University of Gothenburg that were included in (101)). All patients (median age 35.0 (IQR 28.8-40.5) years; disease duration 6.5 (4.0-9.3) years, EDSS 3.5 (2.0-6.0)) were in the RRMS stage of the disease. CSF NfH^{SMI35} levels were determined by ECL-based solid-phase sandwich immunoassay (151). CSF NfL levels were measured with the UmanDiagnostics NF-light® ELISA as described in (101). The investigator who conducted the NfH^{SMI35} measurements had no access to the clinical data.

3.2.2. Standard Protocol Approvals, Registrations, and Patient Consents

Samples were collected after written patient informed consent and the study was approved by the regional ethical board of Uppsala University, Sweden.

3.2.3. Statistical evaluation

Nf levels are described by median and IQR and compared by Wilcoxon matched pairs test or Mann-Whitney U test. Correlation analysis was done by Spearman rank

correlation coefficient (r). Partial correlations adjusted for age were computed by first regressing the two variables on age and then determining the Spearman rank correlation coefficient (r_s) of the respective residuals. A two-sided p-value < 0.05 was considered as significant. All statistical analyses and graphs were prepared using SPSS (Version 15.0 SPSS, Chicago, IL) and Graph Pad Prism 5.02 for Windows (GraphPad Software, San Diego, CA).

3.3. Results

3.3.1. Effect of natalizumab treatment

22/30 patients (73.3%) had lower NfH^{SMI35} levels in postNat compared with preNat (median 32.4 (IQR 22.5-44.8) pg/ml vs. 27.4 (21.0-34.7) pg/ml, p=0.002, **figure 3.1.**). In comparison 27/30 (90%) patients had lower NfL levels in postNat compared with preNat (820 (405-2130) pg/ml vs. 375 (293-575) pg/ml, p<0.0001, **figure 3.1.**).

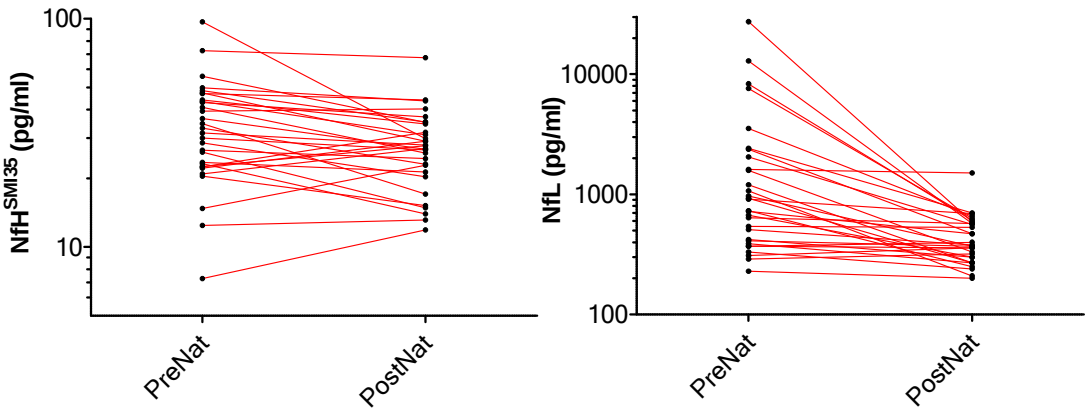


Figure 3.1. CSF NfH^{SMI35} (left) and NfL (right) levels before (PreNat) and after natalizumab treatment (PostNat).
22/30 patients (73.3%) had lower NfH^{SMI35} levels in postNat compared with preNat (median 32.4 (IQR 22.5-44.8) pg/ml vs. 27.4 (21.0-34.7) pg/ml, p=0.002) (left). In comparison 27/30 (90%) patients had lower NfL levels in postNat compared with preNat (820 (405-2130) pg/ml vs. 375 (293-575) pg/ml, p<0.0001) (right).

3.3.2. Age association

NfH^{SMI35} showed a moderate correlation with age at both time points (preNat: r=0.38, p=0.038; postNat: r=0.399, p=0.029). Conversely, this was not seen for NfL (preNat: r=-0.07, p=0.73; postNat: r=0.20, p=0.299).

3.3.3. Disability and disease activity

NfH^{SMI35} and NfL did not correlate with EDSS score after age correction at either of the two time points (NfH^{SMI35}: preNat: r_s=0.29, p=0.123; postNat: r_s=0.05, p=0.794; NfL: preNat: r_s=0.001, p=0.997; postNat: r_s=0.058, p=0.761).
Eight of the 30 patients experienced a relapse within 3 months prior to preNat and 3 patients had a relapse whilst receiving natalizumab prior to sampling (postNat) (one patient experienced a relapse prior to both samplings). NfH^{SMI35} levels were significantly higher in preNat patients experiencing a relapse (47.7 pg/ml, n=8) versus those in remission (27.6 pg/ml, n=22, p=0.001), whereas this difference was not significant for NfL (relapse: 1055 pg/ml, remission: 725 pg/ml, p=0.256). The duration since onset of relapse and NfH^{SMI35} correlated (R=0.73, p=0.04); this was less clear for NfL (R=0.64, p=0.091).
After exclusion of the 10 patients experiencing a relapse in the 3 months prior to or after starting natalizumab, median NfL levels dropped from 830 pg/ml to 365 pg/ml (p=0.0002) after natalizumab treatment. This was less clear for NfH^{SMI35} levels (preNat: 28.3 pg/ml; postNat: 26.9 pg/ml, p=0.086), i.e. 90% of the patients experienced a reduction of NfL, whereas this was only seen in 65% of the patients for NfH^{SMI35}. Both NfL and NfH^{SMI35} levels in the 10 patients experiencing a relapse were clearly lower in postNat compared with preNat (775 pg/ml versus 550 pg/ml, p=0.013 and 45.6 pg/ml versus 32.2 pg/ml, p=0.013).

3.3.4. Relationship of NfL and NfH ^{SMI35} in preNat and postNat

NfL levels in preNat correlated with NfL levels in postNat (r=0.59, p=0.001). This relationship was more pronounced for NfH^{SMI35} (r=0.73, p<0.0001). After age correction, NfL and NfH^{SMI35} levels in preNat correlated (r_s=0.38, p=0.037) while this was not seen in postNat (r_s=0.25, p=0.179).

3.4. Discussion

Persisting neurological deficits in MS likely emerge as a consequence of accumulating axonal injury starting in the very early phase of the disease. Due to the lack of reliable, quantitative biomarkers, the effect of immunomodulatory treatments on neuro-axonal damage and degeneration has been difficult to assess. Increased CSF levels of Nf reflect on-going axonal deterioration, the culprit of disability development in MS. Several other body fluid biomarkers mirror different parts of the actual immune activity in MS. In comparison with these inflammatory biomarkers, the therapeutic impact of immunomodulatory drugs on Nf levels adds essential information about the effects on neuro-axonal damage.

A recent study in 92 patients with RRMS showed that natalizumab treatment for 6-12 months reduced NfL levels from a mean of 1300 pg/ml to 400 pg/ml ($p<0.0001$). Post natalizumab treatment values were similar to levels from healthy subjects (350 pg/ml) (101). In this study the mean NfL concentration in patients with a recent relapse was 2300pg/ml (n=30), as compared to 860pg/ml in patients in remission (n=62, $p<0.038$). Importantly, when analyzing exclusively the patients in remission, NfL levels were still significantly reduced following natalizumab treatment ($p<0.001$). I confirmed the reduction in CSF Nf levels after natalizumab treatment by independently measuring CSF NfH^{SMI35} in a subset of 30 patients from this cohort ($p=0.002$). Despite losing significance for the reduction of NfH^{SMI35} after excluding 10 of the 30 patients that experienced a relapse within 3 months of sampling, these results support and confirm mitigation of neuro-axonal damage or degeneration by natalizumab treatment.
Of note, the NfL measurements seemed to be more treatment responsive than NfH^{SMI35}: the mean post-treatment reduction of NfL was 45% as opposed to 11% for NfH^{SMI35}. This suggests that measurements of NfL could be superior over NfH^{SMI35} to detect treatment effects in the CSF of MS patients.
Previous studies have reported relatively weak correlations of NfL with the EDSS (98) and with age in controls (91). Similar findings have been reported for NfH in CSF of MS patients (152). Sample access in this study was limited to 30 CSF pairs and the follow-up was limited to 12 months. Furthermore, we need to remember that measured Nf values likely reflect the rate of on-going axonal destruction rather than established cumulative damage, and this may well represent a possible explanation for the lack of correlation between the investigated Nf proteins and EDSS scores.
Limitations of this work include availability of only a relatively small subfraction of samples from the original study (101) and the lack of imaging data. Because high disease activity is the main indication for treating patients with natalizumab and a parallel placebo group was missing, regression to the mean cannot be excluded as an additional effect.
Taken together, I confirmed CSF Nf as promising candidates to measure neuro-axonal damage or degeneration in MS treatment trials. In comparison to the ECL-NfH^{SMI35} assay, the responsiveness of the NF-light ELISA to natalizumab treatment was more pronounced, suggesting that the UmanDiagnostics NF-light® assay should be considered the preferred assay for future investigations.

3B. Fingolimod reduces CSF NfL levels in relapsing multiple sclerosis (173)

3.5. Introduction

The effect of immunomodulatory treatments on neuroaxonal damage and degeneration in MS has been difficult to assess, in part, due to the lack of reliable, quantitative biomarkers.

Fingolimod (Gilenya™, Novartis Pharma AG), a sphingosine-1-phosphate receptor modulator, is the first oral therapy approved for the treatment of RRMS. Preclinical findings (174) and consistent effects on brain atrophy observed in MS clinical trials (175-177) suggest fingolimod has potential neuroprotective properties.

In this study I assessed the reliability of CSF NfL, as a therapeutic biomarker in RRMS, by comparing levels in fingolimod-treated patients versus placebo, and correlating NfL levels with clinical and MRI outcomes.

3.6. Methods

This was a post-hoc investigation of NfL in CSF samples collected at baseline and Month-12 in a subgroup of RRMS patients participating in the 2-year, placebo-controlled, phase III FREEDOMS study (ClinicalTrials.gov number, NCT00289978), that evaluated fingolimod at the doses of 0.5mg and 1.25mg, once-daily (176). Provision of CSF samples was an optional component of the FREEDOMS study protocol. Definitions and methodologies of clinical and MRI assessments, protocol approvals and registration details, have been previously described (176). The study was approved by the local Institutional review Boards. All patients provided written informed consent.

CSF samples were available from 36 consenting patients (0.5mg, n=9; 1.25mg, n=15; placebo, n=12). I measured CSF NfL levels using the Uman Diagnostics NF-light® ELISA kit (Umeå, Sweden). The assay was conducted blinded to the clinical data and treatment allocation (101, 104, 171). Inter- and intra assay variability (CV) in three longitudinal control samples were below 15%.

Statistical analysis

Variables are described as medians (interquartile range) or numbers and percentages. The Mann-Whitney test was used for between group comparisons (0.5mg, 1.25mg and pooled fingolimod-treated [0.5mg and 1.25mg] versus placebo). The Wilcoxon matched pairs test was used to evaluate longitudinal comparisons. Correlations were analyzed using the Spearman correlation methodology on the pooled fingolimod-treated (0.5mg or 1.25mg) patient group. A two-sided p-value <0.05 was considered significant. All

analyses were conducted using SPSS (version 20, Chicago, IL) and GraphPad Prism 5.04 (GraphPad Software, San Diego, CA).

One patient discontinued from the fingolimod 1.25mg group due to an adverse event (increased liver enzymes) after 6.4 months on treatment. Although month-12 CSF sample was available from this patient, considering the short duration on treatment, the data of this patient was included only in the primary but not the correlation analyses.

3.7. Results

Baseline and on study characteristics for the 36 patients evaluated in this study, are presented in **Table 3.1 (A-G)**. The median time since onset-of-the-last relapse to baseline was 159 days (122-277). During the study, three patients on placebo experienced a total of five relapses. Two patients in the fingolimod groups reported one confirmed relapse each, and one patient in the fingolimod 1.25mg group experienced two confirmed relapses. One patient in the placebo group had a relapse with onset 13 days prior to CSF sampling at Month-12. Fingolimod-treated patients had better clinical and MRI outcomes versus placebo at Month-12.

Table 3.1. *Baseline and month 12 results*

	Fingolimod 0.5 mg N=9	Fingolimod 1.25 mg N=15	Placebo N=12
A] Demographics			
Age, years	29.0 (25.5 – 39.0)	40.0 (32.0 – 42.0)	37.5 (27.8 –50.0)
Gender: females, n (%)	7 (77.8%)	8 (53.3%)	6 (50.0%)
B] Relapses[#]			
Previous 2 years	2.0 (1.0 –2.5)	2.0 (1.0 – 2.0)	2.0 (1.0 –3.0)
BL to M12 (n)*	1	3	5**
C] T2 volume (mm^3)			
BL	1966 (582 – 3754)	1957 (1170 – 5153)	1858 (479 – 6438)
M12	2040 (635 – 3554)	1828 (1127 – 5159)	2352 (830 – 6885)
Change	-52 (-210 – 53)	6 (-89 – 60)	318 (-30 – 1213)
D] new enlarging T2 lesion count			
M12	0 (0 – 3)	0 (0 – 1.0)	4.5 (0.3 – 11.0)
E] Gd+ T1 lesion count			
BL	0 (0 – 1.0)	0 (0 – 1.0)	0 (0 – 1.8)
M12	0 (0 – 0)	0 (0 - 0)	0 (0 – 1)
Change	0 (-1 – 0)	0 (-1.0 – 0)	0 (-0.8 -0.8)
F] Brain volume			
BL normalized brain volume (mm^3)	1596 (1507 – 1634)	1538 (1481 – 1585)	1522 (1481 – 1569)
PBVC (%): BL-M12	-0.6 (-0.8 – 0.5)	0.04 (-1.1 – 0.2)	-0.4 (-0.7 – -0.1)
PBVC (%): BL-M24	-0.3 (-1.1 – -0.2)	-0.2 (-1.3 – -0.1)	-0.8 (-1.6 – -0.0)

Values are median (interquartile range, IQR) unless indicated otherwise. BL: Baseline;; Gd+:gadolinium enhancing; M12: month 12; PBVC: Percentage brain volume change; * Confirmed relapses; ** Two patients experienced two relapses and one patient one relapse.# Acute relapses were defined as those starting within 30 days before CSF sampling

3.7.1. Effect of fingolimod treatment on NfL levels (**Figure 3.2.**)

NfL levels at baseline were comparable across the treatment groups (0.5mg: 644pg/ml; 1.25mg: 659pg/ml; pooled fingolimod 0.5/1.25mg: 652pg/ml; placebo: 886pg/ml, p-value [fingolimod vs. placebo] =0.619, 0.495 and 0.481 respectively). By Month-12, median NfL levels had decreased significantly, as compared to baseline in the fingolimod treated groups (0.5mg: 401pg/ml [37.7% reduction], p=0.028; 1.25mg: 321pg/ml [51.3% reduction], p=0.017; pooled fingolimod 0.5/1.25mg: 335pg/ml [48.6% reduction], p=0.001), while the reduction in the placebo group between baseline and month 12 was not significant (738pg/ml [16.7% reduction], p=0.433). At month-12, NfL levels were lower in the pooled fingolimod group than placebo (p=0.022).

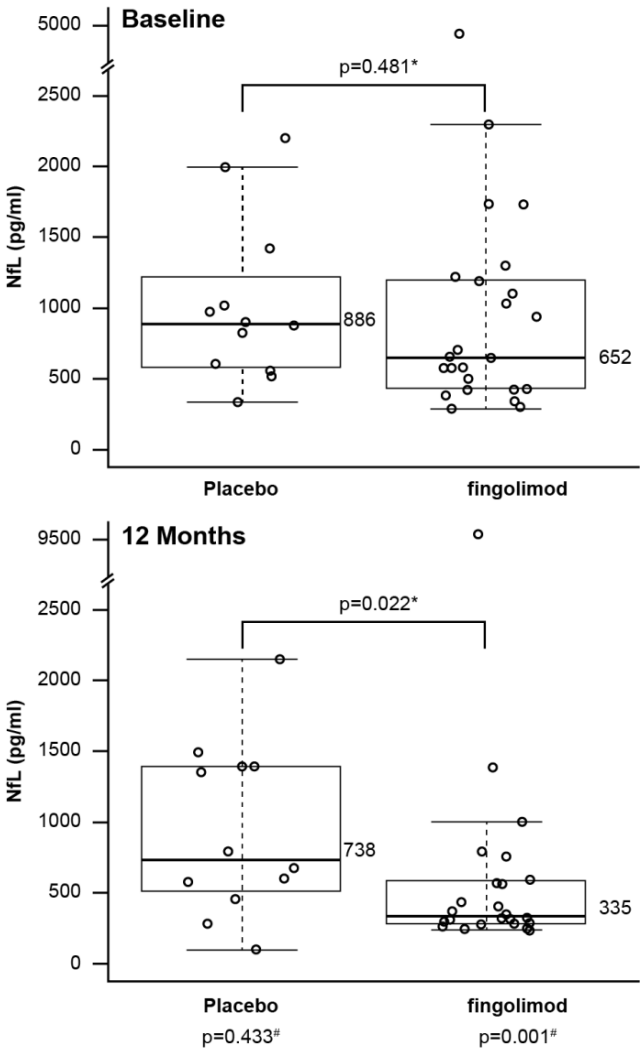
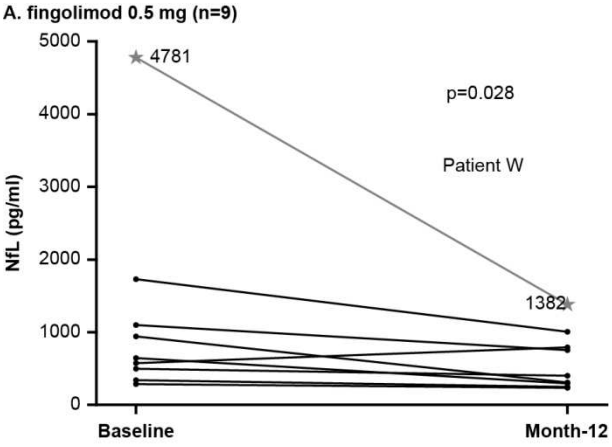
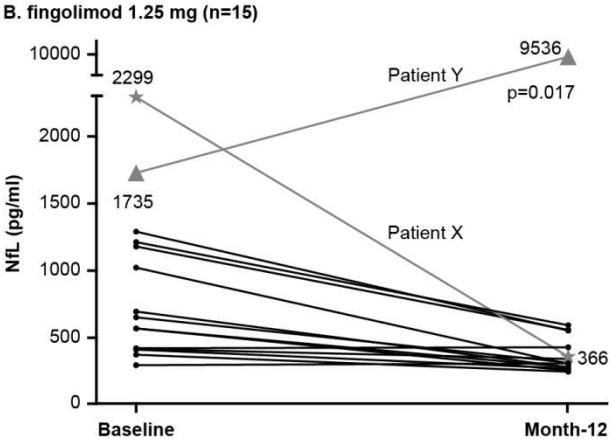


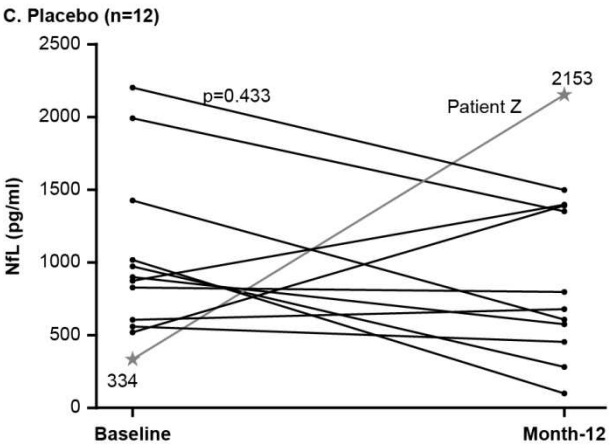
Figure 3.2. NfL levels at baseline and after 12-months, cross-sectional analysis. NfL levels at baseline; pooled fingolimod 0.5/1.25mg: 652pg/ml; placebo: 886pg/ml, p=0.481. At 12 months, NfL levels pooled fingolimod group: 335pg/ml; placebo: 738 pg/ml, p=0.022.

NfL: neurofilament light chain in CSF; * Mann-Whitney test; # Wilcoxon matched pairs test: baseline versus month-12. Dots represent individual samples. Box and whiskers plotted according to the Tukey method.

3.7.2. Evaluation of NfL outliers (**Figure 3.3. A-C**)







Clinical and MRI outcomes at baseline and Month-12 for outlier patients

Patient W: EDSS decreased from 2 to 1.5; reduction in Gd+lesions from 5 to 0; decrease in T2 lesion volume (12309 mm^3 to 11828 mm^3) and no relapses.

Patient X: stable EDSS score of 1.5; no Gd+ lesions (both timepoints); stable T2 lesion volume (5153 mm^3 to 5159 mm^3) and no relapses.

Patient Y: EDSS from 1.5 to 4.5; reduction in Gd+ lesions from 1 to 0; increase in T2 lesion volume (6512 mm^3 to 23794 mm^3); two relapses.

Patient Z: increase in EDSS (3 to 4.5); increase in Gd+ lesions from 1 to 7; increase in T2 lesion volume (5888 mm^3 to 6569 mm^3); two relapses.

Four patients, one patient each in the placebo and fingolimod 0.5mg groups and two patients in the fingolimod 1.25mg group, showed extreme changes in NfL levels at Month-12 compared with baseline. The overall results were not affected by inclusion or exclusion of these outlier patients in the analysis.

Patients W (**Figure 3A**) and *X* (**Figure 3B**) in the fingolimod groups showed a marked reduction in NfL levels at Month-12 versus baseline. These were paralleled by clinical and paraclinical improvements over the 12-month observation period. *Patient Y* (**Figure 3B**) experienced a marked increase in NfL levels, paralleled by two relapses, an increase in Expanded Disability Status Scale (EDSS) score and T2 lesion volume. This patient subsequently discontinued from the study drug after 6.4 months due to an adverse event. *Patient Z* (placebo; **Figure 3C**) experienced an increase in NfL levels at Month-12 compared with baseline and a corresponding increase in EDSS.

3.7.3. Correlation analysis (n=35 patients)

Baseline CSF NfL levels did not correlate with age (r=-0.08, p=0.63) or gender although trending to higher values in males (female: 576pg/ml [426-920], male: 1023pg/ml [634-1238], p=0.106).

Month-12 NfL levels were higher in patients who experienced relapses during the study (1398pg/ml [428-1826]) versus those who did not (384pg/ml [285-698], p=0.048). In patients receiving placebo, Month-12 NfL correlated with Month-12 EDSS (r=0.65, p=0.021, n=12) as well as EDSS change from baseline to Month-12 (r=0.58, p=0.047, n=12). Similar correlations did not reach statistical significance in the entire cohort or in patients treated with fingolimod.Across all groups, baseline NfL levels positively correlated with T2 lesion volume at baseline (r=0.37, p=0.027). Month-12 NfL levels and new/enlarging T2 lesions count (r=0.54, p=0.001) were also positively correlated. Reductions in NfL levels were associated with reductions in gadolinium enhancing (Gd+) T1 lesion count (r=0.44, p=0.008) and Gd+ lesion volume (r=0.44, p=0.008). NfL levels inversely correlated with normalized brain volume (nBV) (r=–0.38, p=0.025) across all groups at baseline. Correlations of percentage brain volume change from baseline to months (M)-12 and -24 with baseline NfL levels in both fingolimod (r_{M12}=–0.17, p=0.602; r_{M24}=–0.32, p=0.137) and placebo (r_{M12}=–0.17, p=0.602; r_{M24}=–0.47, p=0.124) groups did not reach statistical significance.

3.8. Discussion

The results of this study supported the premise that CSF NfL levels suitably indicate the treatment effect due to immunomodulatory drugs on ongoing neuroaxonal damage in MS. My data demonstrates that CSF NfL levels decrease markedly in fingolimod-treated patients, whereas a similar change is not seen in patients on placebo. The reduction in NfL levels correlated with the absence of any worsening in clinical and MRI measures of disease activity in treated patients, consistent with outcomes observed in the fingolimod trials (175, 176). Additionally, extreme changes in CSF NfL levels were reflected by the clinical and paraclinical disease course even in individual patients. These results are consistent with those of chapter 3A which showed that natalizumab treatment also considerably decreases CSF NfL levels in RRMS patients to levels observed in healthy controls. It is important to note that the methodology used by myself and Gunnarson et al to measure CSF NfL levels is the same (101).

The design of the FREEDOMS trial (176) allowed me to analyze clinical and MRI data longitudinally and to include placebo-treated patients. A reduction in CSF NfL levels was also observed in the placebo group, however this was not statistically significant and I believe this could potentially reflect a regression to the mean phenomenon for

CSF NfL. Indeed, all patients included in the study required a high level of disease activity to fulfill the inclusion criteria of the trial. However, changes in NfL levels were clearly more pronounced in both fingolimod groups than in placebo and this strongly suggests a valid fingolimod treatment effect, rather than spontaneous fluctuations in disease activity.

The relationship between CSF NfL and MRI measures in RRMS has been rarely investigated. A correlation between CSF NfL levels and the number of T2 ($r=0.35$, $p<0.024$) and Gd+ ($r=0.50$, $p<0.001$) has been reported (100). In partial contrast, one study in patients with a CIS found no association between CSF NfL levels and T2 lesion volume or nBV. However, higher CSF NfH levels correlated with brain volume loss over a median follow-up of one year ($r=-0.518$, $p<0.01$) (102). In my study, baseline CSF NfL levels and normalized brain volume showed an inverse correlation ($r=-0.38$, $p=0.025$). Further, in favour of NfL reflecting subclinical disease activity and ongoing axonal degeneration, I noted an association between M12 CSF NfL levels and MRI measures and confirmed relapses over the 12 months. In addition, baseline CSF NfL levels were associated with the PBVC over 24 months thus suggesting a potential of predicting future brain volume loss.

Limitations of this study are the relatively small sample size, due to the difficulty in obtaining paired CSF samples and the short duration of follow-up. The main strength is represented by the longitudinal and placebo controlled design.

To conclude, I present the first study evaluating the effect of fingolimod treatment on CSF NfL levels in RRMS patients. My results support CSF NfL quantification as a surrogate measure of subclinical disease activity related to ongoing CNS neurodegeneration and as a putative therapeutic biomarker in clinical trials of novel agents for MS therapy. In this context, it is foreseeable that CSF NfL levels could provide relevant information to facilitate treatment decisions in the future possibly also including tailored therapeutic regimens based on individual treatment response.

4. Increased NfL blood levels in neurodegenerative neurological diseases (94)

4.1. Introduction

Blood Nf levels could be useful for both predicting and monitoring disease progression and for assessing the efficacy and/or toxicity of future neuroprotective treatment strategies.

Several previous studies have demonstrated the presence of NfH and NfL in CSF, which has been assumed to reflect brain pathology more accurately than the peripheral blood compartment (92, 98, 100, 101, 149, 151-153, 178-180). However, obtaining longitudinal CSF samples is considered too invasive outside the clinical trial arena, precluding the broader clinical use of Nf. In contrast to CSF, serial blood samples can readily be collected, hence reliable quantification of NfL in blood would be a major stride towards a biomarker of the course of neurodegeneration. Several reports have suggested peripheral blood levels of NfH as a potential marker of neurodegeneration in ALS (168, 181), stroke (182, 183), subarachnoid hemorrhage (184), neurotoxicity after aggressive chemotherapy (169), or brain injury after cardiac arrest (185). In contrast, only one study has investigated serum NfL by examining its relation with neurological outcome following cardiac arrest (170).

So far, only a few studies investigated NfH levels in blood samples from MS patients, while NfL concentrations in serum or plasma of MS patients had never been assessed. An initial study showed that plasma NfH concentrations were significantly higher in patients with an acute episode of optic neuritis (170 pg/ml, n=18) as compared to healthy controls (5 pg/ml, n=14). NfH levels were inversely correlated with visual acuity at presentation ($r=0.67$; $p = 0.01$) and were higher in patients with poor compared to good visual recovery (0.25ng/mL vs. 0.09 ng/mL; $p=0.05$) (154). A recent study found slightly higher serum NfH levels in SPMS compared to healthy controls (HC) ($p=0.011$), CIS ($p=0.041$), and RRMS ($p=0.048$). RRMS and SPMS patients with NfH levels above the cut-off had a higher median MSSS score than patients with normal NfH levels ($p<0.05$), but did not have higher serum NfH levels than HC (156). Similarly, to my knowledge only one study had reported correlations between serum and CSF NfH levels (CSF and plasma: n=20, $r=0.47$; CSF and serum: $r=0.51$) (186).

The commercially available ELISA (UmanDiagnostics NF-light® assay) (see also previous chapters) uses two highly specific, non-competing monoclonal antibodies (47:3 and 2:1) to quantify soluble NfL in CSF samples but it cannot in its present form be used for analysis of blood samples (95).

The aim of this study was to develop and validate (both analytically and clinically) a sensitive ECL-based NfL assay suitable for the quantification of NfL in serum at concentrations relevant to clinical settings.

4.2. Materials and Methods

4.2.1. Antibodies and chemicals

The following mouse antibodies were used: Capture monoclonal antibody (mAB) 47:3, and the biotinylated detector mAB 2:1 (92, 95). MSD SULFO-TAG™ labelled streptavidin was used as detection reagent to generate ECL (MSD, Gaithersburg, MD). Bovine serum albumin (BSA), NaCl, phosphate buffered saline, pH 7.5 (PBS), tris base and Tween 20 were of analytical grade (Sigma-Aldrich, Saint Louis, MO).

4.2.2. Standards

Bovine lyophilized NfL was obtained from UmanDiagnostics (N Norgren). Standards were diluted in tris buffered saline (TBS) containing 1% BSA, 0.1% Tween 20, pH 7.5 and ranged from 0 to 10,000 pg/ml. Batch prepared standards were stored at -80°C.

4.2.3. Patients and Controls

Paired CSF and serum samples were collected during routine diagnostic investigations as indicated by the treating physicians. Samples were collected and processed at room temperature within two hours. Serum samples were spun at 2,000 g, CSF samples at 400 g at room temperature for 10 minutes, aliquoted in polypropylene tubes and stored at -80°C.

Serum samples from 67 healthy control subjects (HC) were included in the study. For ethical reasons CSF samples were not available from these subjects. The group of control patients (CP) (n=68) consisted of patients who, based on extensive diagnostic evaluation had no objective clinical, structural (cranial magnetic resonance imaging, MRI), laboratory (CSF analysis) or functional (electroencephalography, EEG) deficit. These patients suffered from tension type headache (n=21), lower back pain (n=7), psychiatric disorders (n=26) or miscellaneous non-specific symptoms for which no neurological explanation could be found (n=14). From two of these patients there was not enough CSF left for further analysis. In addition, 49 patients with probable or definite ALS (for three no serum and for one no CSF sample was available) (187), probable AD (63), or GBS (for one no serum sample was available) (n=20 each) were included (**table 4.1.**).

Table 4.1. *Demographic characteristics of healthy controls, control patients and patients.*

	HC	CP	AD	GBS	ALS
N	67	68	20	20	49
Females (n [%])[a]	38 (56.7)	41 (60.3)	13 (65.0)	10 (50.0)	14 (28.6)
Age [years, median (IQR)][b]	35.0 (28.0-42.0)	38.3 (27.5-46.4)	72.5 (70.1-80.2)	59.6 (39.1-71.7)	62.7 (54.5-70.7)

[a] There were less female ALS patients compared to HC (p=0.004), CP (p=0.001) and AD (p=0.007).

[b] HC and CP, were younger compared to AD, GBS and ALS (p<0.0001, respectively).

HC: Healthy controls; CP: Control patients; AD: Alzheimer's disease; GBS: Guillain-Barré syndrome; ALS: Amyotrophic lateral sclerosis; IQR: Interquartile range.

Samples for precision and accuracy experiments as well as for stability and parallelism were chosen based on their NfL levels (high, medium and low) irrespective of their diagnosis due to availability of limited sample volumes.

4.2.4. Analytical procedure

The 96-well plates (Multi-Array® plates, Meso Scale Discovery, Gaithersburg, MD) include integrated screen-printed carbon ink electrodes on the bottom of the wells. Coating was done overnight with 30 µl of capture antibody (mAB 47:3,1.25 µg/ml) diluted in PBS (pH 7.4) at 4ºC. All following incubation steps were done on a plate shaker (800 rpm) and were preceded by three wash steps with 200 µl of TBS, containing 0.1% Tween 20 (pH 7.5) per well. Non-specific binding sites were blocked with 100 µl of TBS, containing 3% BSA, per well for 1h. After washing, 25 µl of TBS containing 1% BSA and 0.1% Tween 20 was added as sample diluent to each well. 25 µl of standard, control or serum/CSF sample was then added in duplicate and the plate incubated at RT for 2h. After washing, 25 µl of the secondary antibody (mAB 2:1, 0.5 µg/ml) diluted in TBS containing 1% BSA and 0.1% Tween 20 was added to each well and the plate incubated for 1 h at RT. After washing, MSD SULFO-TAG™ labelled streptavidin (0.25 µg/ml), diluted in TBS containing 1% BSA and 0.1% Tween 20, was added and incubated for 1h at RT. Following a final wash, 150 µl of ECL read buffer (MSD) diluted 1:2 with distilled water was added and the ECL signal, detected by photodetectors, measured using the MSD Sector Imager 2400 plate reader. A four-parameter weighted logistic fit curve was generated, sample concentrations

extrapolated and analysed using the Discovery Workbench 3.0 software (MSD). If required, samples were appropriately diluted to fall in the range of the standard curve. Non measurable NfL samples were reported as 0 pg/ml.

4.2.5. Statistical analysis

Continuous variables were described by their median and interquartile range (IQR), and categorical variables by numbers and percentages. Comparison of demographic data was performed using the Kruskal-Wallis test, and pairwise post-hoc comparisons using Dunn's post-test or chi-square test as appropriate. Serum and CSF levels of NfL were log-transformed to achieve a normal distribution for subsequent analysis. To control for age as a potential confounding factor, an analysis of covariance with age as covariate and disease group as fixed factor, was performed (152). Group-specific levels of NfL were expressed as geometric means with 95%-confidence intervals. For log-normal variables, the geometric mean equals the median. Correlations were computed by determining the Spearman rank correlation coefficient (r). The cut-off (upper reference range of normal) providing optimal sensitivity and specificity in distinguishing ALS from HC by serum NfL was defined by ROC curve analysis (by maximizing the Youden index) (188). Proportions above and below this cut-off were compared with the Chi-Square test. A two-sided p-value < 0.05 was considered as significant. P-values of post-hoc comparisons were adjusted using a Bonferroni correction. All statistical analyses and graphs were performed using SPSS (Version 15.0 SPSS, Chicago, IL) and Graph Pad Prism 5.02 for Windows (GraphPad Software, San Diego, CA).

4.3. Results

4.3.1. Reproducibility of the standard curve

Figure 4.1. shows the mean raw counts of 20 consecutive standard curves in the range of 0-1,000 pg/ml and the resulting regression line. Individual standard curves showed a high degree of linearity (R^2 = 0.99) (**figure 4.1.**).

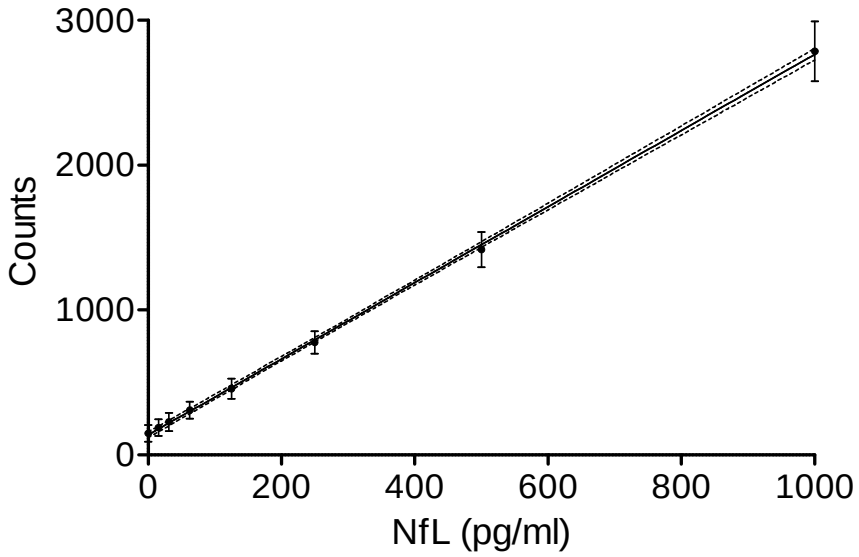


Figure 4.1. Reproducibility of the standard curve.
Reproducibility of 20 consecutive standard curves. The graph shows the mean counts (dots) ± SD (bars), linear regression line and 5% and 95% confidence interval curves (broken lines) (R^2=0.99).

4.3.2. Precision and accuracy

Reproducibility (intra-assay variability) and repeatability (inter-assay variability) of the assay was evaluated with native serum samples in 10 consecutive assays on independent days. In four independent samples of native serum the mean coefficients of variation (CV) of duplicates (intra-assay precision) for $NfL^{Umea47:3}$ were 4.9% (12.1 pg/ml, sample 1), 5.5% (39.6 pg/ml, sample 2), 4.1% (83.1 pg/ml, sample 3) and 3.8% (103 pg/ml, sample 4, average: 4.6%). In CSF the mean intra-assay CVs were 6.0% (569 pg/ml, sample 1), 6.4% (3,645 pg/ml, sample 2), 2.7% (7,501 pg/ml, sample 3) and 6.8% (12,762 pg/ml, sample 4) averaging at 5.5%. Inter-assay CVs for serum were 23.6% (sample 1), 16.9% (sample 2), 8.5% (sample 3), and 10.9% (sample 4, average: 15.0%). In CSF inter-assay CVs were 10.3% (sample 1), 10.4% (sample 2), 6.7% (sample 3) and 11.7% (sample 4, average: 9.8%).
Recovery rates were tested in 6 serum samples from healthy volunteers. Recovery of NfL (serum spiked with 50 pg/ml of HPLC purified bovine NfL) was 72% and 114%. For serum spiked with 100 pg/ml of NfL it was 81% and 96%, and for 1,000 pg/ml of NfL recovery was 82% and 116%.

4.3.3. Analytical sensitivity and stability of the analyte

Sensitivity (lowest standard above blank) was calculated as blank signal plus three SD from 32 assays. The mean blank signal was 138 counts (SD 20.9 counts). The mean

signal of the lowest standard (15.6 pg/ml) was 184.5 counts (SD 23.2): accordingly, analytical sensitivity was defined to be 15.6 pg/ml. We tested the stability of NfL at RT, 4 °C and compared this to samples stored at -80 °C. Four aliquoted serum samples were frozen at -80 °C. The aliquots were thawed on days 0, 3 hours before measurement, days 1, 4 and 8 and stored at RT or 4 °C until analysis. The measured signals were normalised to the signal of the day 0. There was no significant change in signal in samples stored at RT and at 4 °C (RT: day 8: 1.06 ± 0.08 (mean ± SD), p = 0.4063 and 4 °C: day 8: 1.01 ± 0.09, p = 0.1721). Four serum samples were analysed for stability during freeze-thawing cycles. The samples underwent 1, 2, 3, 4 or 5 freeze-thawing cycles and the signal was normalised to the sample freeze-thawed once, without any relevant effect of freeze-thawing on the measured signals (5 freeze-thawing cycles: 1.03 ± 0.03, p = 0.5076).

3.3.4. Parallelism

Parallelism between standards and samples was studied by reciprocal dilutions of three serum samples and three standard curves. The obtained signals were normalised to the highest value within this series (100%). The parallel relationship is demonstrated in **figure 4.2**., suggesting the absence of aggregate formation or endogenous binding between NfL and other blood substrates (159) (**figure 4.2.**).

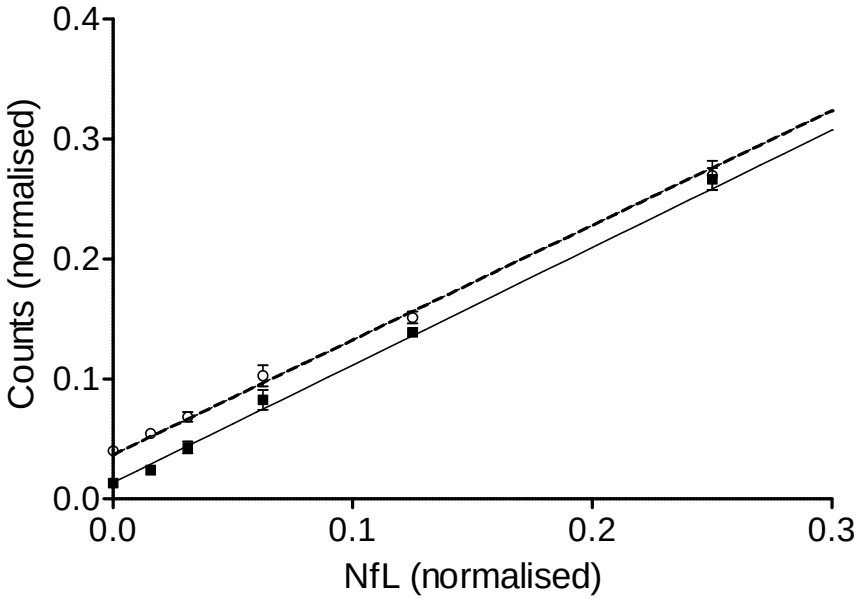


Figure 4.2. Parallelism between standards and serum dilutions.
Parallelism for NfL between standards (open line, open dots) and serum (closed line, black squares). The linear regression lines, mean (open dots or black squares) and ±SD are shown.

4.3.5. Reference populations

NfL was determined in serum of 67 HC (56.7% females, median age 35.0 years) and in serum of 68 and CSF of 66 CP (60.3% female, median age: 38.3 years) (**table 4.1.**). Serum levels between HC (3.3 pg/ml, 2.0-5.3) and CP (4.4 pg/ml, 2.4-8.1) did not differ (p=1.0) and did not correlate with either age or gender. Conversely, CSF levels in CP correlated with age (r=0.68, p<0.0001).

4.3.6. Neurological disease population

A. Serum

AD (30.8 pg/ml, 22.6-41.9), GBS (79.4 pg/ml, 24.3-259.6) and ALS (95.4 pg/ml, 57.9-157.0) had higher serum NfL levels compared with HC and CP (AD versus CP: p=0.002 all other comparisons versus HC and CP: p<0.0001). NfL levels correlated with age in GBS (r=0.48, *p*=0.038) and ALS (r=0.30, *p*=0.04). In the age-corrected group comparisons versus HC and CP, differences remained significant for all diseases except AD (**figure 4.3.**).

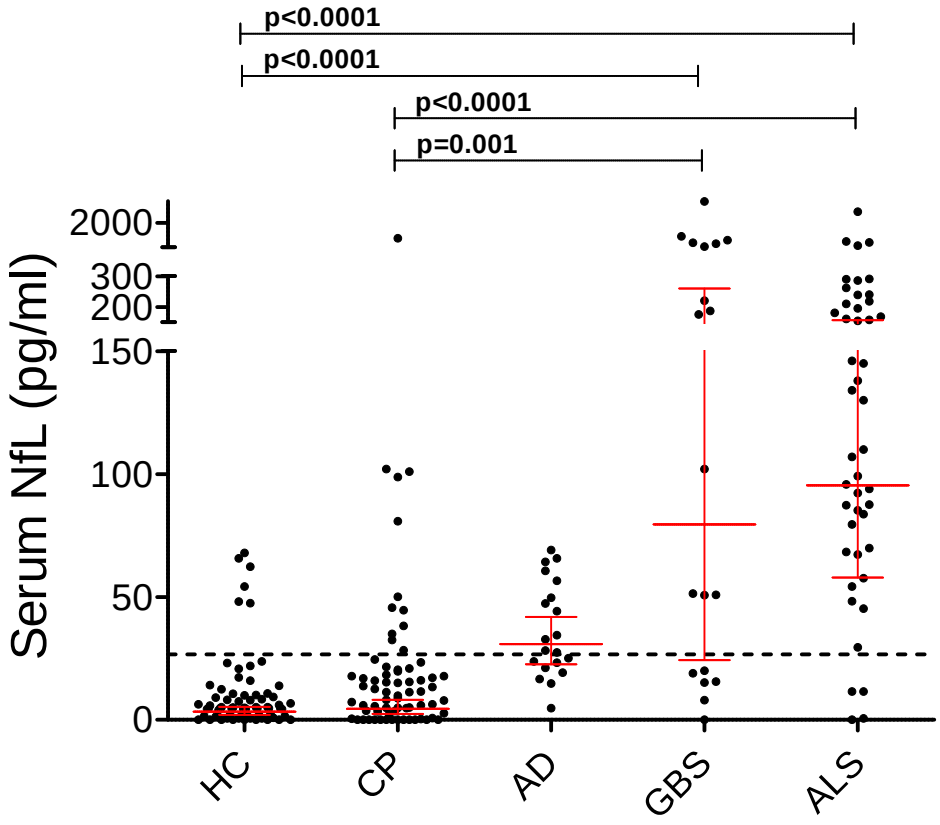


Figure 4.3. Serum NfL levels in the two reference groups (HC and CP) and neurological disease groups.

Patients with a GBS (79.4 pg/ml) or ALS (95.4 pg/ml) had higher values compared with HC (3.3 pg/ml; p<0.0001, respectively) and CP (4.4 pg/ml, p<0.0001 and p=0.001). Significances for comparisons between patients with AD and HC (p<0.0001) and AD and CP (p=0.002) were lost after age corrections. The horizontal dotted line represents the upper reference range (cut-off value) of 26.6 pg/ml. Geometric mean and 95% CI are displayed. Dots represent individual samples. P-values are adjusted for age and corrected by Bonferroni method.

A cut-off level of 26.6 pg/ml (**figure 4.3.**) for serum NfL resulted in a sensitivity of 91.3 % and a specificity of 91.0 % for differentiating ALS versus HC. A higher proportion (p<0.0001 for all comparisons) of patients had serum NfL values above this cut-off: 16/20 (80.0 %) in AD, 13/19 (68.4 %) in GBS, 42/46 (91.3 %) in ALS, compared to HC (6/67, 9.0%).

B. CSF

NfL levels in AD (1396 pg/ml, 1139-1711), GBS (1361 pg/ml, 726-2554) and ALS (5513 pg/ml, 4151-7323) were higher than in CP (324 pg/ml, 282-372, p<0.0001 for all), and CSF NfL concentrations in ALS were higher than in AD and GBS (p<0.0001, respectively).

Similar to the serum results, CSF levels of NfL correlated with age in GBS (r=0.65, p=0.002) and ALS (r=0.30, p=0.048). After correction for age, a significant difference remained between GBS (p=0.001) and ALS (p<0.0001), but not AD (p=1.0) versus CP. Similarly, I confirmed the higher levels in ALS as compared to AD and GBS (p<0.0001, for both comparisons) (**figure 4.4.**).

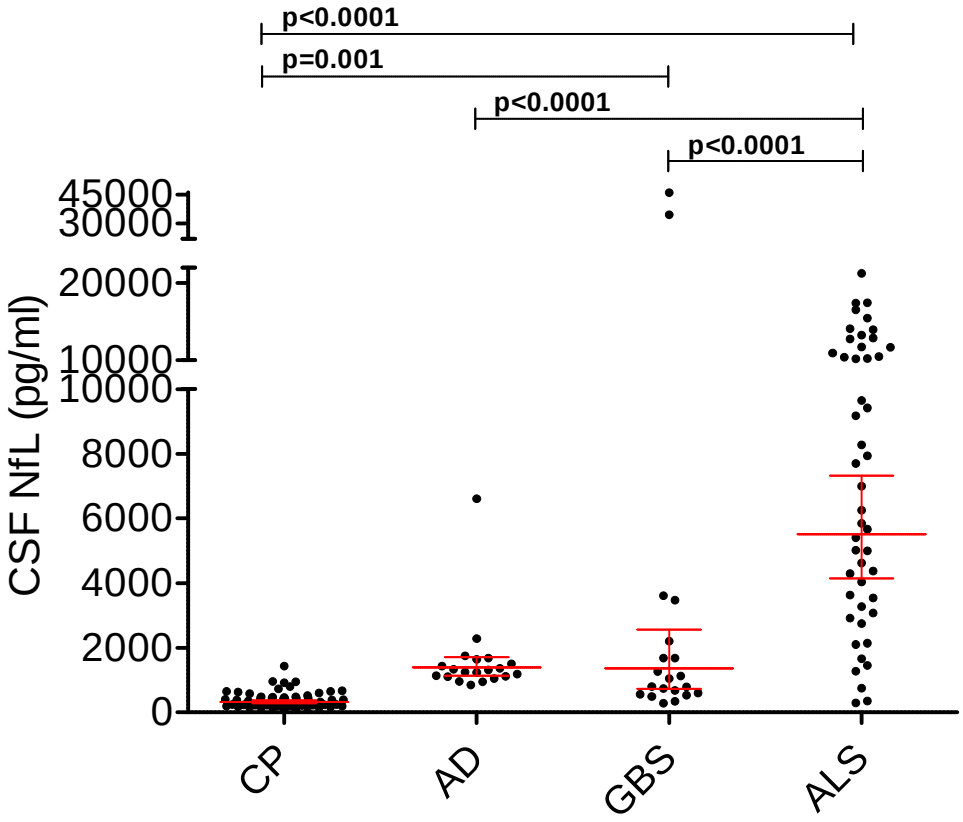
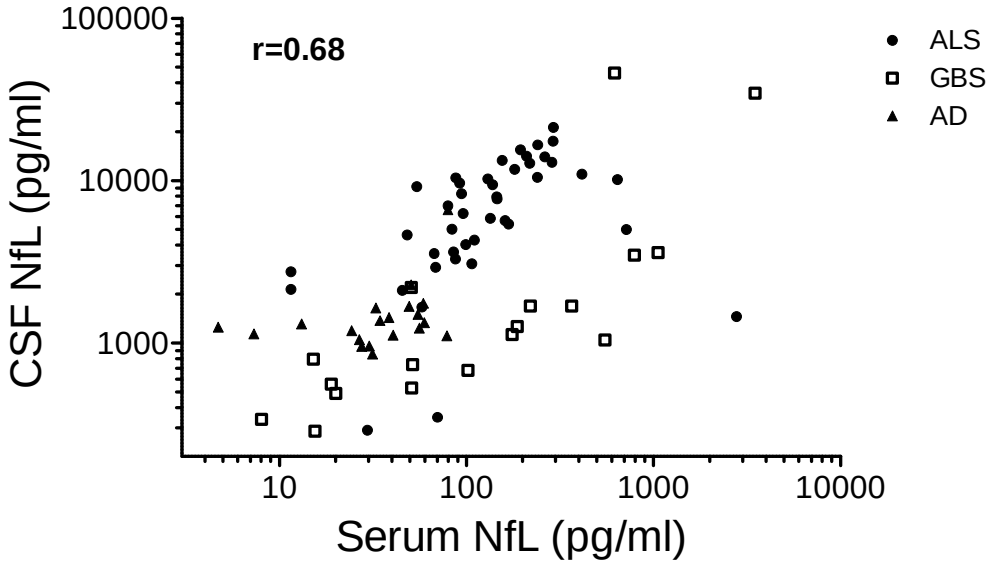


Figure 4.4. CSF NfL levels in the reference group (control patients, CP) and neurological disease cohorts.
Patients with ALS (5513 pg/ml) or a GBS (1361 pg/ml) had higher levels than CP (324 pg/ml, p<0.0001 and p=0.001). In addition ALS had higher levels than patients with AD (1361 pg/ml) and GBS (p<0.0001, respectively). Geometric mean and 95% CI are displayed. Dots represent individual samples. P-values are adjusted for age and corrected by Bonferroni method.

C. CSF – serum relationship

Overall geometric mean levels in CSF (1,142 pg/ml, 906-1,439) were 96.8-fold higher than in serum (11.8 pg/ml, 8.5-16.5, p<0.0001; fold-increase in CSF versus serum: CP: 73.6, AD: 45.3, GBS: 17.1, ALS: 57.8, p<0.0001, respectively).
Serum and CSF measurements of NfL correlated in the disease groups (**figure 4.5.A**): AD (r=0.48, p=0.033), GBS (r=0.79, p<0.0001) and ALS (r=0.70, p<0.0001), conversely this was not seen in CP (r=0.11, p=0.3739) (**figure 4.5.B**).

A. Disease groups



B. Control patients

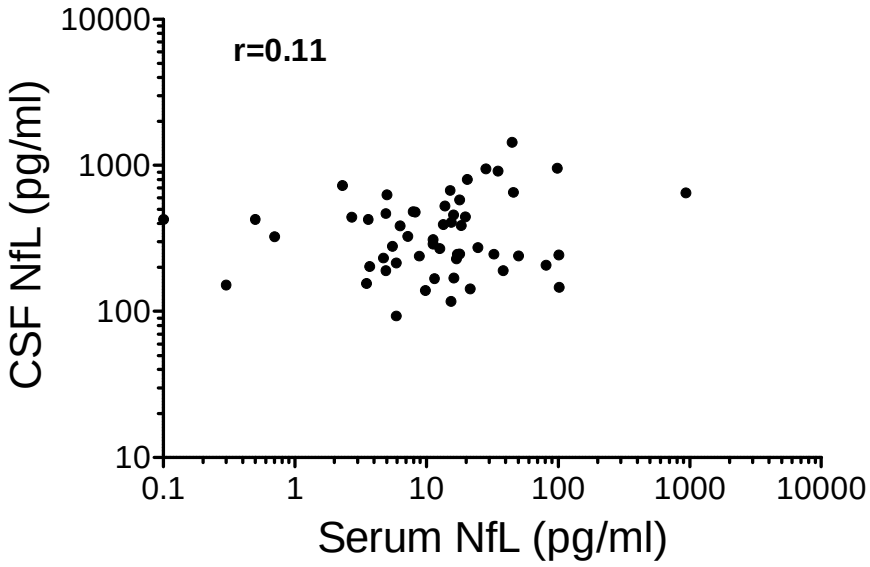


Figure 4.5. Correlation of serum and CSF NfL measurements.
Serum and CSF measurements of NfL correlated in the disease groups (A): AD (r=0.48, p=0.033), GBS (r=0.79, p<0.0001) and ALS (r=0.70, p<0.0001): overall: r=0.68, p<0.001. Conversely this was not seen in the control patients (CP) (r=0.11, p=0.3739) (B).

4.4. Discussion

A highly sensitive method for the detection of a clinically relevant biomarker of neurodegeneration has been developed. Importantly, this method allows us to make use of readily available longitudinal patient blood samples, instead of being restricted to CSF samples which are difficult to obtain because of ethical reasons. One potential clinical application for serum NfL levels is demonstrated by the diagnostic sensitivity of 91.3% for ALS, a rapidly progressive neurodegenerative disease (189, 190).
Notably, this is the first ECL based solid phase immunoassay for the NfL protein in blood based on two non-competitive, monoclonal antibodies. These antibodies have been widely used and validated in a commercial ELISA for CSF measurements of NfL (NF-light® assay) (95, 101, 191) (see chapter 1). NfL is considered to represent the most abundant and also most soluble Nf subunit (192).

The optimised ECL-NfL assay protocol proved to be highly accurate (intra-assay CV < 6%, inter-assay CV < 24%), sensitive (sensitivity 15.6 pg/ml) and demonstrated linearity and parallelism (**figures 4.1.** and **4.2.**) over a wide analytical range (15.6-10,000 pg/ml). In addition I found $NfL^{Umea47:3}$ to be stable in serum. This is relevant for a potential value to monitor drug effects by serum NfL in ALS where Nf aggregate formation is a key pathological finding (159). In contrast to NfH^{SMI34} and NfH^{SMI35}, no such aggregates were found for $NfL^{Umea47:3}$, essentially overcoming the limitations of the Nf “hook effect” (matrix effect) (159). In this context, a more than 20-fold elevation of serum $NfL^{Umea47:3}$ levels in ALS compared to HC cannot be overestimated.
Interestingly, the fold-differences between disease groups and CP for serum $NfL^{Umea47:3}$ was higher compared to the respective CSF levels (serum/CSF: ALS: 21.7/17.0; AD: 7.0/4.3 and GBS: 18.0/4,2).
CSF Nf measurements are increasingly accepted as measures of axonal injury (179, 193). Importantly in this context we found strong correlations between CSF and serum NfL measurements in all disease groups (overall: $r=0.68$, $p<0.001$). The reason behind the lack of such correlation in controls is unclear. However, the presence of very low blood NfL levels and the fact that these may approach or fall below the analytical detection limit represent potential explanations.
An important and unresolved question is whether or not there is a relevant correlation between Nf levels and age. If present, such a relationship would require age dependent cut-off values (152). A major limitation to all studies in this field to date (91, 100, 102, 151, 152, 194) is that they have not been powered to investigate this potential correlation in the CSF, due to lack of samples from a sufficiently large healthy control group across all age categories. Again, the availability of the present method to investigate this in readily available serum samples is highly relevant. Importantly, I did not find a correlation between serum $NfL^{Umea47:3}$ levels and age in either HC or CP.

Whether or not a possible relationship with age exists for ALS, GBS or AD is questionable, as older patients are often more severely affected and higher age is the most important prognostic factor in these conditions and therefore not independent of the neurodegeneration related release of $NfL^{Umea47:3}$.

The absence of the Nf hook-effect is an important analytical advantage for quantification of the ECL based serum $NfL^{Umea47:3}$ assay compared to the serum NfH^{SMI34} and NfH^{SMI35} ELISA, as there is no necessity for a time-consuming pre-incubation step with urea (159). Given the important prognostic information that NfH levels provide on a number of clinical conditions, I believe that $NfL^{Umea47:3}$ will be relevant for future studies. Serum $NfL^{Umea47:3}$ bears the potential for predicting disease progression in ALS (186, 195, 196) and MS (155), detecting particularly disabling acute episodes of optic neuritis or relapses in MS (154), identifying primary and secondary brain damage in stroke (182, 183), SAH (184), TBI (197) and in the emerging concept of chronic traumatic encephalopathy (CTE) (197, 198). Like serum NfH^{SMI35}, serum $NfL^{Umea47:3}$ may also be exploited as a safety biomarker for recognising neurotoxicity (169). There is already data that serum NfL levels are of comparable prognostic value to NfH^{SMI35} levels following cardiac arrest (170, 185). Of note there were no controls and no analytical validation data from the NfL assay that was performed in one study (170).
Similar to our previous findings for NfH^{SMI35} in CSF, a bimodal distribution of serum NfL levels was seen in patients with GBS (151). There are no previous studies on Nf in blood from patients with GBS. Earlier studies have shown that CSF levels of NfH are higher in patients with evidence of axonal damage compared to those with purely demyelinating GBS, with CSF NfH levels predictive of outcome (180, 199). Future prospective studies incorporating detailed longitudinal clinical and electrophysiological assessments, and sampling are clearly warranted. These studies will also shed light on the role of proximal versus more distal axonotmesis and secondary axonal peripheral degeneration and the relationship of increased blood NfL levels (200).

Blood levels of Nf have similarly not been investigated in patients with dementia. In this study the differences in serum and CSF NfL levels in AD compared to HC and CP ($p<0.0001$ and $p=0.002$) lost significance after age and Bonferroni correction. This is in line with previous investigations where CSF NfH^{SMI35} levels were increased, but diagnostic sensitivity, and hence potential for clinical use of NfH^{SMI35} was not superior to that of the benchmark biomarkers total tau, phospho tau, or amyloid beta 1-42 (120, 201). To explore these questions further I am currently trying to obtain a larger and well characterised cohort of AD and control patients.

Unfortunately, cross sectional or longitudinal data on disease activity or disease severity were not available in this study. Also, due to lack of follow-up clinical information I could not investigate the potential prognostic role of NfL. An important limitation is also the fact that age between controls and all three patient groups was not balanced and this needed statistical correction. In summary, I developed and validated a sensitive and reliable assay for measurements of NfL in human blood samples. For the first time, I was able to demonstrate increased blood NfL levels in patients with ALS and GBS. These differences were more pronounced for the ECL-NfL$^{\text{Umea 47:3}}$ assay than those reported in ALS for NfH in previous reports (168, 196). These data support further studies of serum NfL in well-defined longitudinal cohorts of neurodegenerative diseases. These studies will show if serum NfL measurements can be used as a biomarker for disease progression and as an outcome measure in clinical trials.

5. Serum NfL is a biomarker of human spinal cord injury severity and outcome (202)

5.1. Introduction

Acute spinal cord injuries (SCI) are one of the most devastating accidents affecting a young and active population. Mechanical injury of the spinal cord results in damage to neurons, axons, and glia at the area of impact (203). Over days to weeks several secondary injury cascades lead to further progressive tissue damage within and adjacent to the primary lesion, on top of the sequelae of exogenous trauma. Other than the trauma itself, secondary injury (204, 205) represents a window for therapeutic interventions to preserve axons and their support structures (206-208). A recent placebo-controlled trial suggested clinical improvement across several outcome measures in patients receiving the drug minocycline (161), using the American Spinal Injury Association (ASIA) exam (209). The ASIA exam can be used to determine the ASIA grade of injury severity. While this is a universally accepted classification, each grade represents a broad category of patients and the scale lacks sensitivity for longitudinal change making it insensitive as a drug response marker (210). A number of biomarkers have been evaluated for their capacity to be more sensitive and accurate tools to measure neuronal injury, but in part because these tests are restricted to cerebrospinal fluid (CSF) they have as yet provided limited clinical utility (211, 212).
In this chapter I analysed levels of NfL longitudinally in serum samples derived from subjects enrolled in a phase II clinical trial investigating the utility of minocycline to attenuate neurological deficits after spinal injury (161). I investigated the correlation of serum NfL with acute and long-term clinical outcome. Further, I analyzed the potential of serum NfL as drug response marker of the therapeutic effect of minocycline in SCI. Among several activities, minocycline downregulates microglial activation, neuroinflammation and apoptosis, effects that may reduce neuronal injury reflected in lower serum levels of NfL in SCI (206).

5.2. Methods

The research protocol was approved by the University of Calgary Conjoint Health Research Ethics Board. All patients and healthy controls provided written informed consent. Serum samples from 67 healthy controls (HC) and 27 SCI patients (with sufficient amount of serum symples available (161)) were included: 13 patients with a motor-complete SCI (cSCI; ASIA A or B; due to lack of material no 96 and 108 hours (h) samples for two patients and no 84 and 120 h samples for one patient each), 10 patients with a motor-incomplete SCI (iSCI; ASIA C or D; no 36 h sample for one

patient) and 4 patients with a central cord syndrome (CCS; ASIA C or D with more selective injury to the centrally located motor tracts and disproportionately greater motor impairment in the upper compared to lower extremities (mean lower extremity motor scores>upper extremity) (213)) (**Table 5.1.**) (161).

Table 5.1. *Baseline characteristics of healthy controls (HC) and spinal cord injury (SCI) patients.*

	HC	CCS	iSCI	cSCI	p-value
n	67	4	10	13	
Gender (n females, %)	38 (57)	1 (25)	3 (30)	5 (38)	0.254
Age (years)	35 (28-42)	49 (39-62)	33 (22-43)	32 (22-45)	0.117
Level of injury					0.999
Cervical	na	4	9	11	
Thoracic	na	0	1	2	
Injury mechanism (n, %)[a]	na				0.034
Motor vehicle accident		1 (25)	7 (70)	10 (77)	
Work accident		1 (25)	1 (10)	-	
Sport injury		-	2 (20)	3 (23)	
Fall		2 (50)	-	-	
ASIA Score (n,%)[b]	na				<0.0001
A		-	-	12 (92)	
B		-	-	1 (8)	
C		2 (50)	7 (70)	-	
D		2 (50)	3 (30)	-	
Motor score	na	59 (18-83)	45 (23-63)	16 (9-29)	0.053
Pinprick score[c]	na	65 (22-94)	81 (51-94)	24 (19-32)	0.020
Light-touch[d]	na	73 (25-106)	76 (37-97)	24 (19-32)	0.028
Delay to surgery (hours)	na	15 (10-200)	12 (8-16)	18 (12-22)*	0.285
Treatment (n,%)	na				0.499
Placebo					
Low dose**		1 (25)	5 (50)	7 (53.8)	
High dose		1 (25)	3 (30)	1 (7.7)	
		2 (50)	2 (20)	5 (38.5)	

CCS: central cord syndrome; iSCI: Motor incomplete spinal cord injury; cSCI: Motor complete spinal cord injury; ASIA: American Spinal Injury Association standardized neurological examination; * one patient without surgical decompression; ** Low dose:

200 mg twice daily; high dose: 800 mg loading dose tapered to 200 mg twice daily. Na: not applicable. Medians and IQR are shown, if not specified otherwise.
[a] More motor vehicle accidents in cSCI versus CCS ($p<0.05$).
[b] higher ASIA score in cSCI versus iSCI ($p<0.001$) and CCS ($p<0.01$).
[c] lower pinprick score in cSCI versus iSCI ($p<0.05$).
[d] lower light-touch score in cSCI versus CCS ($p<0.05$).

Surgical decompression and stabilization was performed within 24 h of injury and subjects were not treated with corticosteroids. Patients were randomized (1:1) to receive intravenous minocycline (Wyeth Pharmaceuticals either 200 mg of minocycline twice-daily (low dose) or a loading dose of 800 mg tapered by 100 mg every 12 h until 400 mg was reached (high dose), or placebo. More detailed procedures and inclusion and exclusion criteria have been described previously (161).

5.2.1. Clinical assessment and analytical procedure

Neurological function was assessed using the ASIA standardized neurological examination, including the motor (score ranging from 0-100, with higher score representing better motor function) and sensory (pinprick and light-touch ranging from 0-112 each, with higher score representing better sensory function) composites (209). These examinations were performed at days 1 (time of enrolment), 4, 5 and 7; weeks 3, 6 and 12; and months 6 and 12. Motor function observed in the study population plateaued after 3 months (161). Motor and sensory outcome was defined as the mean of the motor or sensory scores at time points 3, 6 and 12 months (161).
We examined blood samples drawn within 12 h of the SCI prior to treatment randomization and every 12 h thereafter for 7 days (15 time points, including the baseline sample). Samples were spun at 2,000 g for 10 minutes, aliquoted in polypropylene tubes and stored at -80°C within two hours (161).

The immunoassay developed in-house for NfL ($NfL^{Umea47:3}$) was used for quantification of NfL in serum (see chapter 4, (94)). The mean intra- and inter-assay coefficients of variation were 4.3% and 9.7% in these measurements respectively.

5.2.2. Statistics

Baseline characteristics were compared between groups using Kruskal Wallis test for continuous variables, or Fisher's exact test for categorical variables. Continuous variables were described by their median and interquartile range (IQR), and categorical variables by numbers and percentages. The NfL area under the curve (AUC) for the 7

days sampling period was calculated using the trapezoidal rule. NfL values were log-transformed to achieve a Gaussian distribution. The longitudinal data of the groups (twice daily NfL levels) were analysed using mixed effects linear regression, which allows for repeated measurements and missing values. One-way ANOVA with Bonferroni adjustment if significance was reached was used to compare log NfL between groups at individual time points. In addition, mixed effects linear regression was used to test for the effect of minocycline on log NfL, motor score, pin prick or light touch (baseline values were subtracted from all values at individual time points for this analysis). Since the number of patients per group was small, a pooled analysis (placebo versus both minocycline dosing schemes) was performed. Motor outcome was classified as “good” or “poor” considering the median value as cut-off. All correlation analyses were performed with Spearman’s R. A two-sided p-value < 0.05 was considered as significant. All statistical analyses were performed using Stata version 12 (StataCorp, College Station, Texas) and Graph Pad Prism 5.02 for Windows (GraphPad Software, San Diego, CA).

5.3. Results

5.3.1. Disease characteristics

Table 5.1. shows baseline characteristics of the SCI subgroups and healthy controls. The vast majority (24/27) of patients had a cervical level of injury. Across all subgroups, motor vehicle accidents represented two thirds of causes of injury. cSCI, iSCI and CCS patients differed significantly for overall injury severity (ASIA score, $p<0.0001$), pinprick ($p=0.020$) and light-touch scores ($p=0.028$), while differences in motor scores were borderline significant ($p=0.053$), at baseline. Otherwise there were no significant differences with regard to gender, age, or delay to surgery.
Fourteen patients (52%) were treated with either low dose (n=5) or high dose (n=9) minocycline, and 13 patients received placebo.

5.3.2. Levels of NfL at baseline and during follow-up

Baseline NfL levels were different between groups ($F(3,90)=9.49$, $p<0.001$); iSCI (21 (15-90) pg/ml, $p=0.006$) and cSCI (70 (17-134) pg/ml, $p<0.001$) had higher levels of serum NfL than HC (5 (2-11) pg/ml). Similarly, serum NfL levels in iSCI and cSCI were higher than in CCS (6 (0.3-18) pg/ml, $p=0.025$ and $p=0.010$, respectively) (**Figure 5.1.**).

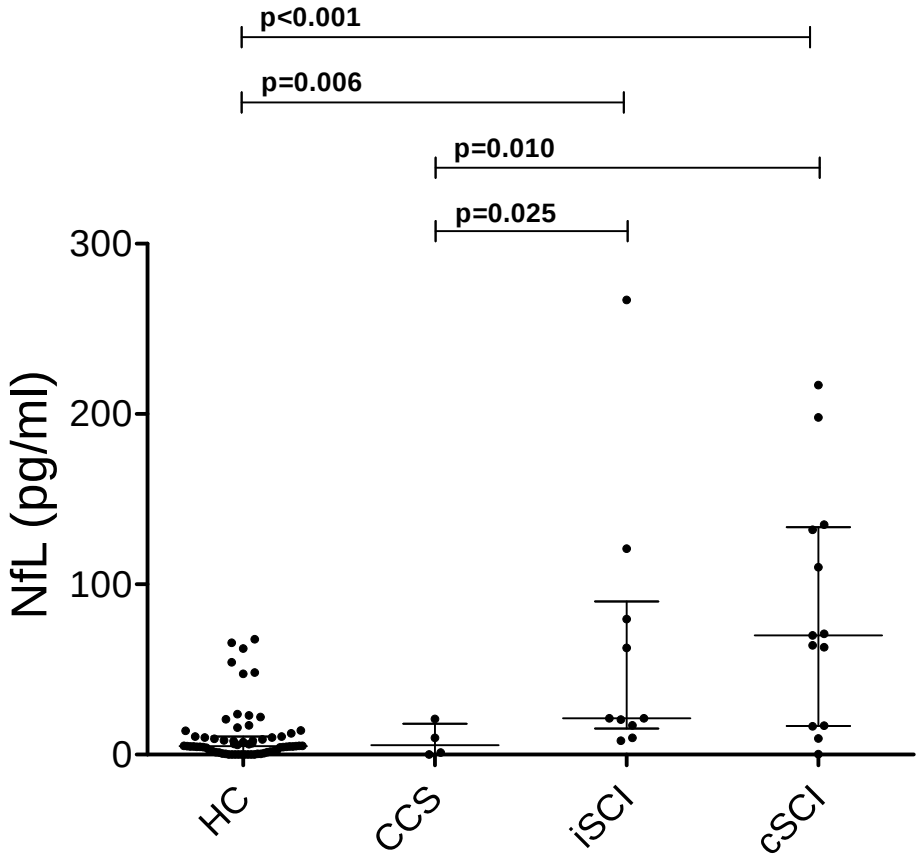


Figure 5.1. Baseline serum NfL levels in healthy controls and spinal cord injury patients.

At baseline serum NfL levels were different between the groups ($F(3,90)=9.49$, $p<0.0001$); motor incomplete SCI (iSCI, $p=0.006$) and motor complete SCI (cSCI, $p<0.001$) had higher levels of serum NfL than healthy controls (HC). Similarly, serum NfL levels in iSCI and cSCI were higher than in patients with a central cord syndrome (CCS, $p=0.025$ and $p=0.010$, respectively). Median and IQR are displayed. Dots represent individual samples.

There was an increase of serum NfL levels over time ($p<0.001$, mixed effects model). Levels increased in all three groups from baseline ($p<0.001$) and were higher in cSCI versus iSCI ($p=0.011$) and CCS ($p<0.001$) ($p=0.045$ for iSCI versus CCS). Differences for individual time points especially between cSCI and CCS were strong (**Figure 5.2.**, **Table 5.2.**). Accordingly, the NfL AUC was significantly higher in cSCI (4,614 (3,486-7,701)) versus CCS (1,260 (420-2,257), $p=0.012$; $F(2,24)=5.9$, $p=0.008$).

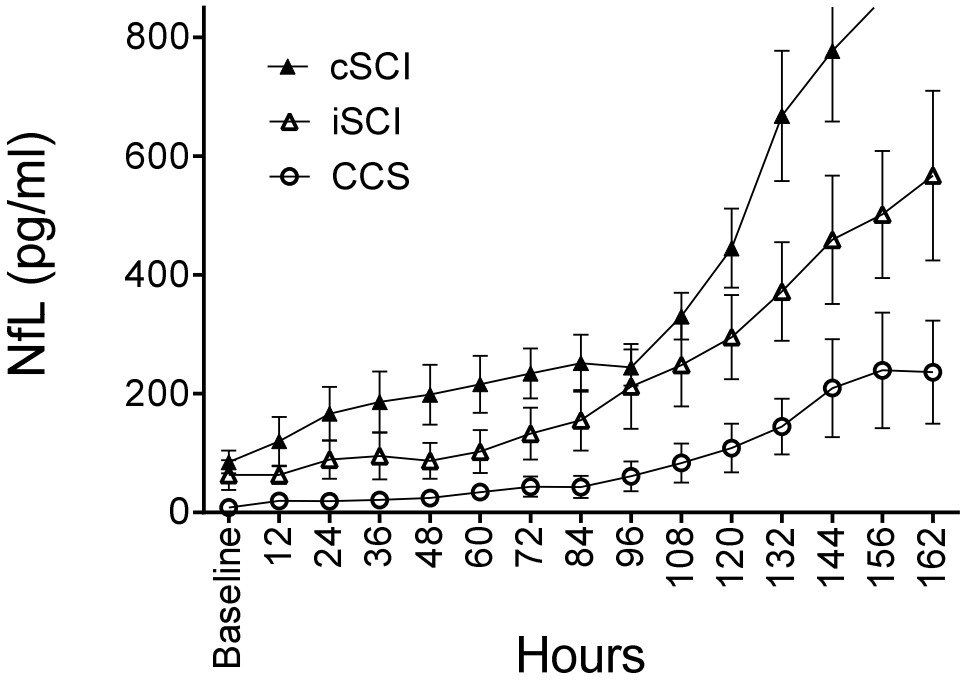


Figure 5.2. Serum NfL levels over time in different groups of spinal cord injury patients. Serum NfL levels increased over time in the overall group ($p<0.001$, n=27) and in all three groups from baseline ($p<0.001$). Serum NfL was higher in motor complete SCI (cSCI, n=13) versus motor incomplete SCI (iSCI, n=10, $p=0.011$) and central cord syndrome patients (CCS, n=4, $p<0.001$). ISCI had higher serum NfL levels as compared to CCS ($p=0.045$). Differences for individual time points especially between cSCI and CCS were strong (see table 5.2.). Mean and standard error of the mean are displayed.

Table 5.2. *Serum NfL levels in spinal cord injury patients over time.*

	CCS (NfL, pg/ml)	iSCI (NfL, pg/ml)	cSCI (NfL, pg/ml)	p-values		
				CCS vs. iSCI	CCS vs. cSCI	iSCI vs. cSCI
Baseline	6	21	70	**0.019**	**0.009**	0.999
12 h	18	48	85	0.174	**0.048**	0.999
24 h	21	47	113	0.218	**0.012**	0.191
36 h	21	58	114	**0.023**	**0.001**	0.267
48 h	24	51	129	0.065	**0.001**	0.053
60 h	34	70	163	0.136	**0.002**	0.062
72 h	38	100	199	0.080	**<0.001**	0.084
84 h	41	127	210	**0.021**	**<0.001**	0.166
96 h	56	155	237	0.053	**0.005**	0.558
108 h	88	184	303	**0.027**	**0.002**	0.434
120 h	114	220	338	**0.046**	**0.002**	0.341
132 h	153	278	558	0.087	**0.001**	0.095
144 h	193	333	617	0.127	**0.003**	0.133
156 h	194	384	710	0.138	**0.003**	0.120
162 h	230	379	796	0.153	**0.003**	0.130

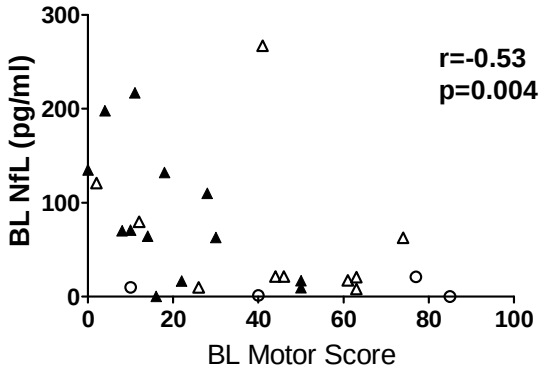
Median NfL concentrations for the individual time points in pg/ml and p-values are displayed. CCS: Central cord syndrome; iSCI: motor incomplete spinal cord injury; cSCI: motor complete spinal cord injury; vs.: versus; h: hours post baseline.

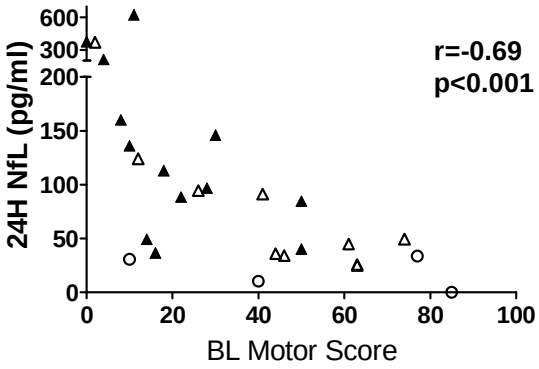
5.3.3. Baseline NfL and motor, pinprick and light-touch scores

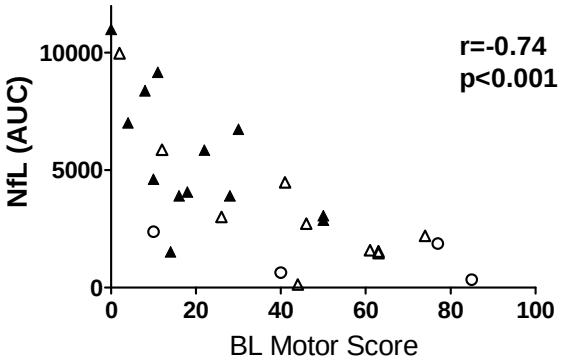
NfL levels were negatively correlated with the motor score at baseline (r=-0.53, p=0.004; **Figure 5.3.a** and **Table 5.3.**), but not with the pinprick or light-touch scores (**Table 5.3.**). Levels also inversely correlated with several of the post baseline motor scores, less so with the sensory scales (**Table 5.3.**).

Interestingly, serum NfL levels determined after 24h showed a stronger association with the baseline motor score (r=-0.69, p<0.001) and throughout all other sampling time points, including also the sensory scores (**Figure 5.3.a** and **Table 5.3.**). These correlations were of similar frequency and strength for the NfL AUC (ASIA score: r=0.60, p=0.001, **Figure 5.3.a** and **Table 5.3.**).

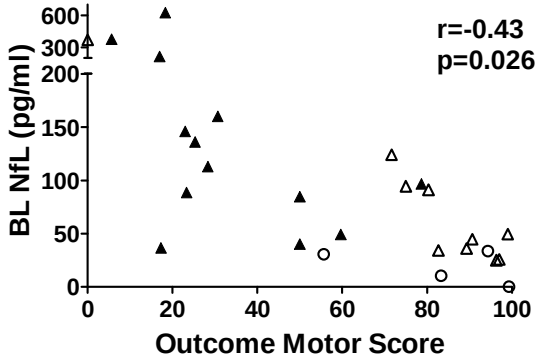
A.

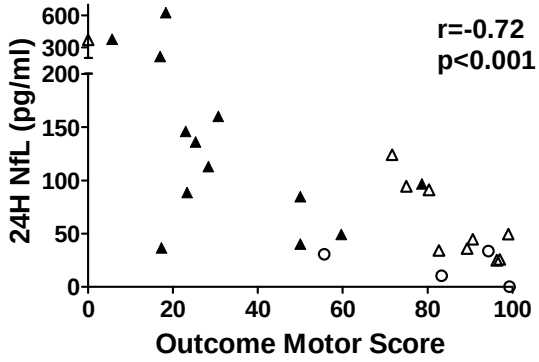

BL NfL (pg/ml)
300
200
100
0
0
20
40
60
80
100
BL Motor Score
r=-0.53
p=0.004


24H NfL (pg/ml)
600
300
200
150
100
50
0
0
20
40
60
80
100
BL Motor Score
r=-0.69
p<0.001


NfL (AUC)
10000
5000
0
0
20
40
60
80
100
BL Motor Score
r=-0.74
p<0.001

B.


BL NfL (pg/ml)
600
300
200
150
100
50
0
0
20
40
60
80
100
Outcome Motor Score
r=-0.43
p=0.026


24H NfL (pg/ml)
600
300
200
150
100
50
0
0
20
40
60
80
100
Outcome Motor Score
r=-0.72
p<0.001

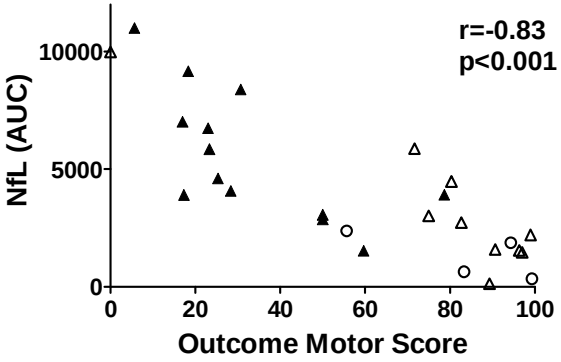

NfL (AUC)
10000
5000
0
0
20
40
60
80
100
Outcome Motor Score
r=-0.83
p<0.001

Figure 5.3. Correlation of NfL and NfL area under the curve (AUC) and motor score at baseline (A.) and motor outcome (B.).

A.

Left: Baseline serum NfL concentration and baseline motor score. Baseline NfL levels correlated with the motor score at baseline (r=-0.53, p=0.004; central cord syndrome patients, CCS: r=-0.40, p=0.6; motor incomplete SCI, iSCI: r=-0.46, p=0.184; motor complete SCI, cSCI: r=-0.63, p=0.022).

Middle: 24H NfL concentration and baseline motor score. 24H NfL levels correlated with the motor score at baseline (r=-0.69, p<0.001; CCS: r=0.5, p=0.667; iSCI: r=-0.78, p=0.008; cSCI: r=-0.64, p=0.017).

Right: Serum NfL area under the curve and baseline motor score.

The NfL AUC correlated with the baseline motor score (r=-0.74, p<0.0001, CCS: r=-0.8, p=0.2; iSCI: r=-0.73, p=0.017; cSCI: r=-0.65, p=0.017).

B.

Left: NfL levels at baseline correlated with the motor outcome (r=-0.43, p=0.026, CCS: r=-0.40, p=0.600; iSCI: r=-0.44, p=0.206; cSCI: r=-0.29, p=0.334).

Middle: 24H NfL concentration and motor outcome. 24H NfL levels correlated with the motor outcome (r=-0.75, p<0.0001; CCS: r=0.50, p=0.667; iSCI: r=-0.76, p=0.011; cSCI: r=-0.52, p=0.07).

Right: Serum NfL area under the curve and motor outcome.

The NfL AUC correlated with the motor outcome (r=-0.83, p<0.0001, CCS: r=-0.80, p=0.200; iSCI: r=-0.79, p=0.006; cSCI: r=-0.69, p=0.009).

Filled triangles: cSCI; empty triangles: iSCI, open circles: CCS. AUC: Area under the curve; BL: baseline. The Spearman’s correlation coefficients of the ranks and p-values are indicated.

Table 5.3. *Correlations between baseline NfL concentration and NfL area under the curve and motor, pinprick and light touch scores at different time points.*

Day	**BL**	**4**	**7**	**21**	**42**	**90**	**182**	**365**
Motor Score								
n	27	22	23	23	23	25	24	24
BL NfL	-0.53 0.004	ns	-0.49 0.019	ns	-0.41 0.049	-0.44 0.028	-0.44 0.031	-0.42 0.039
24H NfL	-0.69 *	-0.56 0.007	-0.64 0.001	-0.69 *	-0.68 *	-0.72 *	-0.69 *	-0.73 *
NfL AUC	-0.74 *	-0.73 *	-0.68 *	-0.72 *	-0.80 *	-0.82 *	-0.79 *	-0.82 *
Pinprick								
n	27	20	23	22	22	25	24	24
BL NfL	ns	ns	-0.55 0.007	-0.43 0.046	ns	ns	ns	Ns
24H NfL	-0.52 0.001	-0.57 0.008	-0.70 *	-0.71 *	-0.50 0.022	ns	-0.47 0.024	Ns
NfL AUC	-0.65 *	-0.56 0.011	-0.69 *	-0.79 *	-0.60 0.003	-0.43 0.033	0.58 0.003	0.44 0.032
Light-touch								
n	27	21	22	22	22	25	24	24
BL NfL	ns	ns	-0.55 0.008	-0.55 0.008	ns	ns	ns	Ns
24H NfL	-0.57 0.002	-0.46 0.037	-0.70 *	-0.80 *	-0.61 0.003	-0.46 0.025	-0.48 0.022	Ns
NfL AUC	-0.62 0.001	-0.51 0.017	-0.62 0.002	-0.85 *	-0.74 *	-0.58 0.002	-0.63 0.001	-0.49 0.016

BL: baseline examination; n: number of patients at each time point (in days post-baseline); BL NfL: NfL measurement at baseline; 24H NfL: NfL measurement after 24 hours; NfL AUC: NfL area under the curve over the 7 day sampling period; Spearman r and p values are displayed; *: $p<0.001$.

5.3.4. Correlation of NfL with clinical outcomes

Motor outcome plateaued at three months after SCI (median ASIA score: 60 points), regardless of whether the injury was motor-complete, motor-incomplete or of central

cord type. As expected, the motor outcome was better in CCS (89 points, p<0.05) and iSCI (86.0 points, p<0.01) versus cSCI (25 points), and correlated strongly with respective baseline motor score values (r=0.83, p<0.0001) (161). Similar correlations between baseline and outcome scores were seen for the pinprick and light-touch (r=0.63, p<0.001 and r=0.58, p=0.001).

During the period of 24 to 168 hours after injury, NfL levels were increasingly higher (**Figure 5.4.**) in patients with a poor outcome (as defined by below median motor score, n=13) compared to those with a better outcome (median motor score above median, n=14, p=0.001).

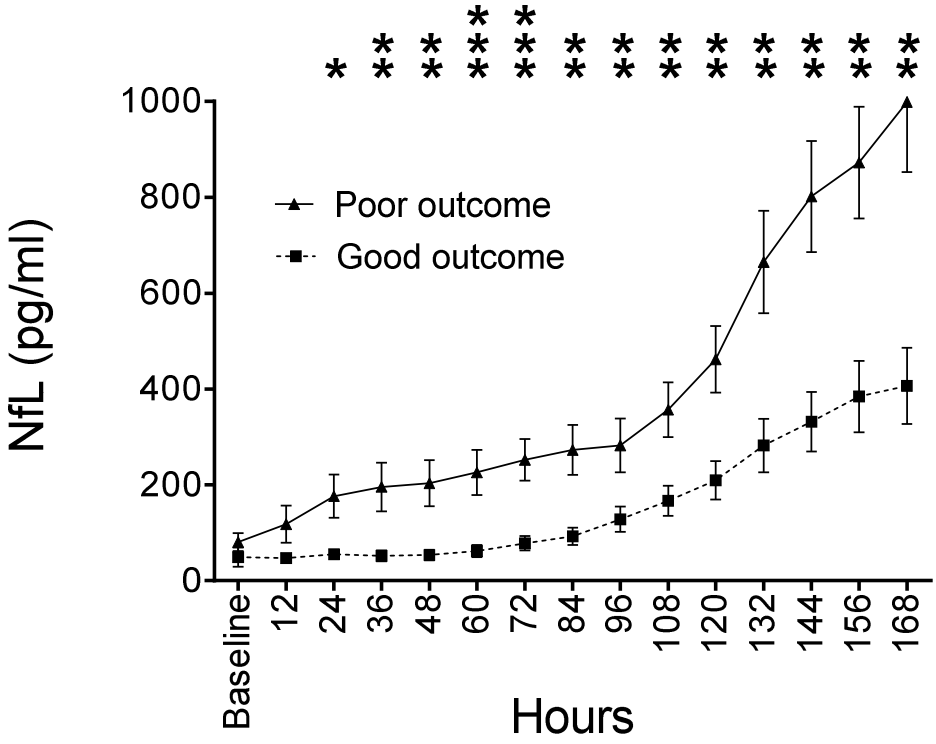


Figure 5.4. NfL over time depending on motor outcome.

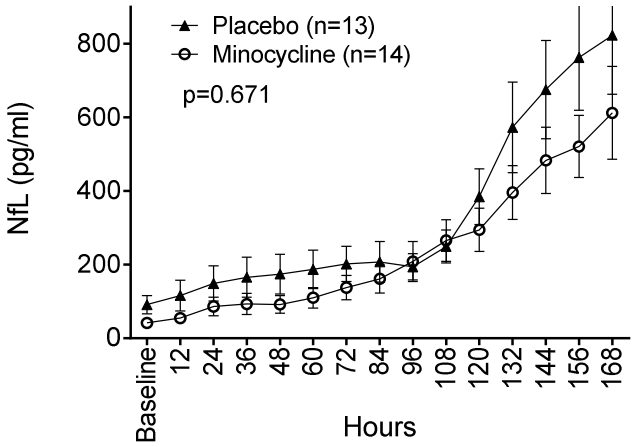
Patients with a better motor outcome (as defined by median, n=13) had lower NfL levels than patients with a poor outcome (n=14, p=0.001). For individual time points, patients with a poor outcome had higher serum NfL levels between serum sampling after 24 hours and throughout all samplings up to 168 hours. Mean, standard error of the mean and corresponding p-values (*: p<0.05; **: p<0.01; ***: p<0.001) for poor outcome versus good outcome group are displayed.

This was corroborated by an AUC analysis of NfL (better outcome: 1,878 (1,058-3,464) versus worse outcome: 5,234 (3,017-8,584), p=0.002). In line with the correlation of clinical scores at baseline with those of outcome, NfL levels at baseline correlated with motor outcome (r=-0.43, p=0.026, **Figure 5.3.b**). Again, this correlation became stronger over time for NfL measurements after 24h (12 h: r=-0.56, p=0.003; 24 h: r=-0.72 (**Figure 5.3.b**); 48 h: r=-0.82; 72 h: r=-0.81; 120 h: r=-0.79; 144 h: r=-0.83; 168 h: r=-0.82, p<0.0001 for all) and for the AUC analysis (**Figure 5.3.b**). Similar correlations were noted for the outcome of pinprick and light-touch (data not shown).

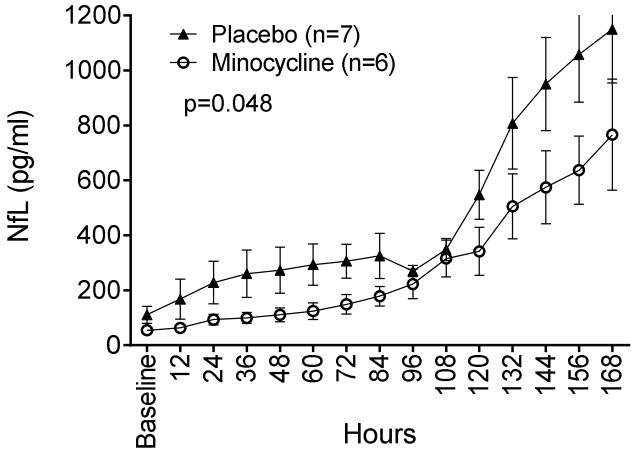
5.3.5 Correlation of NfL with Minocycline treatment

Treatment was not evenly allocated across CCS, iSCI and cSCI (**Table 5.1.**); the median motor score at baseline was 30 for patients receiving placebo (n=13, 54% cSCI patients), 46 for those receiving low dose (n=5, 20% cSCI patients), and 26 for those receiving high dose minocycline (n=9, 56% cSCI patients) (p=0.9050). Compared to placebo, treatment with low or high dose of minocycline (median baseline motor score: 27 points) versus placebo did not have a significant effect on the longitudinal profile of serum NfL levels in this group of patients (p=0.67, mixed effects model, **Figure 5.5.a**). Likewise, minocycline treatment had no effect on the longitudinal profile of motor (p=0.495), pinprick (p=0.324) or light-touch scores (p=0.264) in an all patients analysis. A more comprehensive analysis of the effect of minocycline treatment on neurological outcome including these patients was previously published (161).

A.



B.



C.

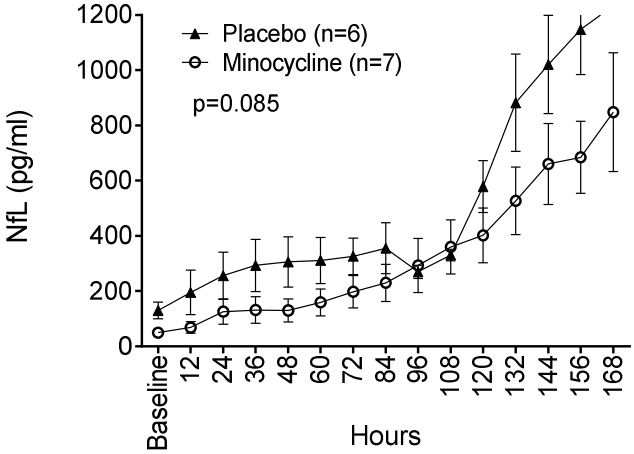


Figure 5.5. Comparison of longitudinal serum NfL between minocycline and placebo treated patients.

A. In all patients (n=27) treatment with minocycline (n=14) versus placebo (n=13) did not have an effect on the longitudinal profile of serum NfL levels (p=0.671).
B. In patients with a motor complete SCI (cSCI, n=13) patients on minocycline (n=6, 5 high dose, 1 low dose) had lower longitudinal NfL levels than placebo (n=7) treated patients (p=0.048).
C. In patients with a baseline motor score below the median of the baseline motor score of all patients (28 points, n=13) minocycline treated patients (n=7) showed a trend for lower NfL levels over time in comparison to the placebo treated group (n=6, p=0.85).
Mean and standard error of the mean are displayed.

In contrast, in cSCI (6 patients receiving minocycline, and 7 patients receiving placebo) minocycline treated patients showed lower longitudinal NfL levels than placebo (p=0.048) (**Figure 5.5.b**). The effect of minocycline was more pronounced after removing the one patient on the low dose regimen (p=0.006), or the two patients with a thoracic level of the injury (p=0.030).
This difference of NfL levels was not paralleled by clinical findings, as scores between treatment groups were not different (motor: p=0.234; after removing one patient on low dose minocycline: p=0.132, or the two patients with the thoracic injury level: p=0.686; pinprick: p=0.681, and p=0.827, p=0.417 or light-touch: p=0.185, and p=0.186, p=0.312) and failed to indicate a treatment effect of minocycline.
Similarly, in the 13 patients who had a motor score below the median motor score (28) at baseline (1 patient with CCS, 3 with iSCI and 9 cSCI), minocycline treated patients (n=7) showed a trend for lower NfL levels over time in comparison to the placebo treated group (n=6, p=0.085, **Figure 5.5.c**, after removing 2 patients on low dose minocycline: p<0.001). Again, clinical scores were not different (motor: p=0.567, and p=0.972; pinprick: p=0.551, and p=0.656; light-touch: p=0.636, and p=0.320) between minocycline and placebo treated patients.

5.4. Discussion

This is the first study exploring the utility of NfL in serum as a marker of injury severity and outcome in patients with SCI, using a high-sensitivity ECL-immunoassay (94). NfL is the most abundant and also most soluble Nf subunit, factors that likely contribute to the superior sensitivity of NfL over neurofilament heavy chain (NfH) assays (192). The assay system is validated for CSF and serum, the latter allowing the acquisition of longitudinal measures in routine clinical settings.

In the course of acute and chronic neuronal damage, disruption to the axonal cell membrane releases Nf into the interstitial fluid, that eventually reaches the CSF and blood compartments (214, 215). In SCI, trauma causes direct acute neuronal necrosis, followed by secondary injury mechanisms that increase neuronal loss by apoptosis or necrosis, a process that may last from days to weeks (216, 217).
At baseline, NfL levels in iSCI and cSCI were increased 4.2-fold and 14-fold compared to healthy controls, and 3.5-fold and 11.7-fold compared to CCS, respectively. Over the 7 day follow-up NfL levels steadily increased in all three disease groups, with maximum levels (CCS: 230 pg/ml, iSCI: 384 pg/ml, cSCI: 796 pg/ml) being markedly higher than in more chronic diseases like Alzheimer's disease (37 pg/ml), Guillain-Barré syndrome (102 pg/ml) or amyotrophic lateral sclerosis (120 pg/ml) (94). Only limited human data is available beyond this time point: three of four ASIA A and one of two ASIA C patients reached their highest NfH plasma levels after 10 days in a recent pilot study (218), leaving the question of peak and duration of release open.
Only a few studies have investigated Nf in CSF or blood in SCI so far. First evidence for the usefulness of NfL as a marker of neuronal damage in SCI arose from a study investigating CSF in acute spinal cord disease: all 6 patients with SCI and 3 of the 17 with whiplash injury showed increased concentrations of NfL (219). In a rat model of SCI, NfH levels in blood (195) were correlated with the extent of damage and were reduced by treatment with minocycline: this difference did not reach significance however (220). The same group of investigators performed the first study in humans with acute cervical SCI: NfH was detectable in plasma of 11 of the 14 included patients and ASIA A showed higher levels than ASIA C patients; however, correlation with clinical subscores, or outcome was not presented (218).
The prognostic value of the ASIA grading system is limited by its lack of dynamic change over time on individual grounds, and its susceptibility to interference due to other injuries such as head or multi-system traumas, and drug effects (210). The consequence of these constraints for clinical studies are large patient numbers to achieve adequate statistical power, to observe differences in treatment regimens (221). The inclusion of motor and sensory subscores may increase the accuracy of the clinical grading (218). Our results show a high correlation between NfL and motor scores, both at baseline, during follow-up, and for long-term outcome, indicating that serum NfL may be a reliable quantitative biomarker of the degree of SCI. This may be of specific value in the context of clinical trials where inter-rater variability of clinical assessment may increase the threshold to detect treatment effects. Furthermore, serum NfL may also allow for better evaluation of injury severity than

the ASIA grade. In particular, it may allow further stratification and prognostication of the large population of ASIA A injuries.

So far, no drug has been shown to ameliorate the course of SCI, despite several candidate compounds showing promising results in animal models (222-224). Apart from the larger heterogeneity of human disease when compared to experimental SCI (225), this failure may also be attributed in part to the lack of sensitive biomarkers with a broad dynamic measuring range.

Minocycline is a tetracycline antibiotic that has shown neuroprotective properties in a variety of models of degenerative and acute neurological diseases, including SCI (226-230), by pathways unrelated to its antimicrobial activity (206). In the minocycline trial an intravenous loading and maintenance dose to achieve serum levels similar to those efficacious in animal models of SCI was used (161, 231). In the subgroups of cSCI, despite the small sample size, treated patients showed lower NfL levels at every time point beyond 24 hours post injury, whereas the clinical scales were insensitive to detect a difference between the treatment groups. This reduction of NfL levels was more pronounced after excluding the patients with a thoracic SCI or those on low dose minocycline. These findings are in line with the clinical scores of the core study (161) in which thoracic SCI patients did not benefit from minocycline treatment, and the comparison of the low- and high-dose minocycline groups suggested a greater effect with higher doses.

The study is limited by the retrospective design and the fact that only a subfraction of participants in the original trial was available for serial serum NfL measurements (161). This may also have caused large heterogeneity in injury severity and mechanisms between minocycline treated and non-treated patients and, at least in part, precluded a potential minocycline treatment effect in the overall SCI cohort.

In summary, this data provides new evidence that serum NfL may represent a useful indicator of acute severity and long-term outcome of neuronal injury, especially in cases where accurate clinical assessment is not possible. Further studies are warranted to increase the evidence for NfL as drug response marker in SCI.

6. Predicting multiple sclerosis: a large international multicentre study (232, 233)

6.1. Introduction

The cause of MS is unknown, but the disease appears to develop in genetically susceptible populations as a result of environmental exposures. Leading candidates for environmental factors associated with MS risk are vitamin D deficiency, Epstein-Barr virus (EBV) infection and smoking (234).

In approximately 85% of MS patients the disease starts as a single demyelinating episode known as CIS (42, 57). Prospective studies have shown that approximately 60%-70% of CIS patients develop a second clinically evident demyelinating event separated in time and space within 20 years and will, therefore, be diagnosed with clinically definite MS (CDMS) (57, 235). The identification of factors influencing the risk of conversion to CDMS is relevant for prognosis, early intervention strategies and for the understanding of the biological mechanisms driving MS. Several studies have investigated which clinical and experimental variables can predict the onset of CDMS. The presence and number of MRI lesions in the CNS as well as that of oligoclonal bands (OCB) in the CSF of CIS patients have been independently associated with an increased risk of conversion (57, 235, 236). Other studies have focused on candidate environmental factors in MS and demonstrated that CIS patients with low vitamin D levels and high anti-EBV antibody titers are more susceptible to conversion to CDMS (237, 238).

CSF Nf levels are abnormally high in CIS and MS patients and correlate with disease activity (100, 104, 239, 240). Unfortunately, obtaining CSF is a relatively invasive procedure and this has limited the potential use of Nf as biomarkers in MS, especially in longitudinal study designs with repetitive samplings. As pointed out in chapter 4, I recently developed a sensitive ECL based immunoassay for quantification of NfL in serum (94).

Given the potential interdependence between risk factors, it is vital to assess all of them collectively and test their association with risk of conversion using a large population of CIS cases. To our knowledge, only one study has comprehensively analyzed a number of suggested factors in a cohort of patients experiencing a first demyelinating event. However, the cohort was relatively small (n=302) and only cases with pediatric onset were included (241). In this multicentre study we aimed to assess which clinical and environmental variables predict the risk of conversion from

adult CIS to CDMS using the largest cohort of adult CIS cases ever studied to date (n=1,047).

For the serum NfL analysis I decided to follow a two staged process: In the initial step I selected 100 CIS with the shortest time to conversion to CDMS (fast converters (FC)) and 100 CIS with the longest follow-up time in the absence of conversion (non-converters (NC)) and serum samples from 92 HC. It was decided that only if I saw a difference in serum NfL levels between FC and NC serum NfL would be measured in the entire cohort of patients.

6.2. Methods

6.2.1. Participants and inclusion criteria

This was an international collaborative study across 33 centres located in 17 different countries (**table 6.1.**).

Table 6.1. Participating centres and number of provided cases.

Centre	Country	Number of samples
Amsterdam	Netherlands	48
Barcelona*	Spain	26
Barcelona**	Spain	45
Bari	Italy	55
Basel	Switzerland	20
Belgrade	Serbia	11
Bergen	Norway	9
Buenos Aires	Argentina	4
Copenhagen	Denmark	15
Düsseldorf	Germany	25
Genoa	Italy	6
Gothenburg	Sweden	14
Graz	Austria	21
Innsbruck	Austria	46
Istanbul	Turkey	23
Lublin	Poland	14
Lyon	France	17
Madrid	Spain	32
Marseille	France	20
Milan[#]	Italy	8
Milan[##]	Italy	144
Montpellier	France	54
Novara	Italy	34
Pavia	Italy	22
Prague	Czech Republic	31
Rome	Italy	7
Rotterdam	Netherlands	81
St Petersburg	Russia	81
Stockholm	Sweden	44
Szeged	Hungary	25
Toulouse	France	20
Ulm	Germany	24
Valencia	Spain	21

Total	17	1047

* Hospital Clinic of Barcelona; ** Vall d'Hebron Research Institute; # Ospedale Policlinico; ## San Raffaele Hospital.

Each centre was asked to provide baseline clinical data and stored serum samples from CIS patients, on whom a minimum of 2 years of follow-up data were available. Inclusion criteria were: 1) the presence of a monophasic clinical episode suggestive of MS (CIS), not attributable to other diseases (for example infectious, neoplastic, congenital, metabolic or vascular disease) (242); 2) clinical follow-up of at least 2 years; 3) available serum sample collected at time of CIS; 4) available basic demographic and clinical data (age at serum sampling or month and year of birth, gender, dates of CIS onset, serum sampling, CSF examination, MRI, conversion to CDMS (if present) and last follow-up visit); 5) information on presence or absence of OCB in CSF at time of CIS; 6) available data on T2 hyperintense lesions on cranial MRI at time of CIS.

Patients with neuromyelitis optica (NMO), opticospinal MS in Asian populations or a history of a progressive course from onset were excluded. CDMS was diagnosed according to Poser criteria when new symptoms occurred after an interval of at least one month, and only when other diagnoses had been excluded (243). The study was approved by the corresponding local ethics committees and participants gave written informed consent.

6.2.2. Data collection

MRI, CSF and clinical assessments were performed in each participating centre as part of the diagnostic workup. The number of T2 hyperintense lesions on cranial MRI at time of CIS was used to group patients into three separate categories (0-1 lesions, 2-9 lesions and >9 lesions). Grouping of lesion load was performed in order to control for variations in imaging protocol and individual analysis between centres. The presence of IgG OCB was determined by isoelectric focusing combined with immunoblotting of matched serum and CSF sample pairs in all patients (244). Additional clinical and CSF data were provided for a more limited number of patients (topography of CIS, CSF IgG index and CSF cell count) (245).

Serum samples were aliquoted and stored at -80° C according international consensus guidelines (246). Liquid chromatography-tandem mass spectrometry (LCMS/MS) was used to measure 25-hydroxy-vitamin D3 levels (25-OH-D) (Royal London Hospital, Barts Health NHS Trust, London, UK). Daily internal quality

samples are measured by LCMS/MS and the institution follows an external quality assurance scheme (The International Vitamin D Quality Assessment Scheme, DEQAS). IgG titres against the EBV nuclear antigen 1 (EBNA1) and cytomegalovirus (CMV) were evaluated using commercially available ELISA (ETI-EBNA-G and ETI-CYTOK-G PLUS Diasorin, Saluggia, Italy) following the manufacturers' recommendations. Internal run validation criteria specified by the manufacturer and based on the calibrators were met for all ELISA plates measured. According to the manufacturer's instruction the cut-off for positivity was 20 arbitrary units (AU) for EBNA1 IgG and 0.4 international units (IU) for CMV IgG. Results were calculated by dividing the optical density (OD) of each sample by the OD of the 20 AU/0.4 IU calibrator on each ELISA plate. Serum cotinine levels were assessed using a commercially available ELISA (Calbiotech Inc., Spring Valley, USA) according to the manufacturer's instructions and were used as a marker for smoking behaviour with levels > 14 ng/ml indicating a positive smoking status (247). Intra- and inter-assay variability for all ELISA and the NfL immunoassay measurements (see chapter 4, (94)) was below 15%. All the ELISA assays were performed a single center (Blizard Institute London, UK), with the analyst blinded to clinical data.

6.2.3. Statistical analysis

A. NfL analysis

We selected 100 FC patients and 100 NC (two patients with insufficient sample volume). EDSS, number of T2 hyperintense and Gd+ lesions on cranial MRI and presence of OCB in the CSF at the time of CIS were assessed in each participating center as part of their diagnostic workup. EDSS and Gd+ lesion data were available on 170 and 146 CIS patients respectively. Patients were grouped in categories based on EDSS scores (0.0-1.0 vs 1.5-2.0 vs >2.0), number of T2 lesions (0-1 vs 2-9 vs >9) and presence or absence of Gd+ lesions and OCB.

Variables were described by median with IQR and counts with percentages. Normalized (log10) NfL levels were treated as a continuous variable and used for all analyses. Logistic regression models were used to assess the ability of NfL levels to predict disease status (FC vs HC, NC vs HC and FC vs NC). Similarly, we tested the association between NfL levels and markers of disease activity using logistic regression (NfL predicting OCB and Gd+ status) and ordinal regression models (NfL predicting increase in T2 and EDSS categories). In all models, results were corrected for both age and sex. All analyses were performed using R (http://www.r-project.org/).

B. All other examined risk factors

Variables were described by their median and IQR or by counts and percentages. Serum 25-OH-D varies according to season. In order to correct for this, raw 25-OH-D values were converted into deseasonalized 25-OH-D levels using the methods described by Mowry et al. (248). Briefly, sine and cosine terms were generated to model the influence of the date of blood draw on vitamin D status; these were then included in a linear regression model providing the adjusted 25-OH-D levels (248). The impact of each variable on the cumulative risk of conversion to CDMS was assessed in both univariate (Kaplan-Meier survival curves and univariate Cox regression) and multivariate analyses (multivariate Cox regression with backward stepwise selection of variables). The assumption of proportional hazards was tested by including time-dependent covariates (interactions) in the model. Centre information was included in all Cox regression models by using a generalized estimating equation sandwich estimate of variance approach. Differences between groups of CIS patients according to their OCB and MRI status were also assessed using logistic and ordinal regression models. All statistical analyses were performed using STATA (http://www.stata.com/) and R (http://www.r-project.org/).

6.3. Results

6.3.1. Overall characteristics of CIS patients

A total of 1,047 CIS patients were included in the study. These patients had presented to neurology services between November 1986 and December 2011; 1,010 (96.5%) after 2000 and 794 (75.8%) after 2005. The median time between onset of neurological symptoms and serum sampling, CSF and MRI examination was 33 (12-81), 28 (10-67) and 19 (4-59) days respectively. In 729 patients (69.6%) all evaluations were performed within three months since onset of symptoms. Patients were longitudinally followed up for a median time of 1,574 days (4.31 years). During this time 623 patients (59.5%) converted to CDMS (median survival time before conversion=1,096 days, 95% CI=973-1,267).

The demographic and clinical features of CIS patients are shown in **table 6.2.**. CSF IgG index was available in 696 patients (66.5%) and CSF cell count in 513 patients (49.0%).13 CIS patients were more likely to be female than male (female/male ratio=2.1). The majority of patients had OCB in their CSF (74.3%) and had more than one CNS T2 lesion on MRI (2-9 T2 lesions=41.8%; >9 T2 lesions=43.7%).

Clinical CIS information was available on 911 patients (87%). Of these patients, 288 (31.6%) presented with optic neuritis (ON), 188 (20.6%) with a brainstem attack (BS),

257 (28.2%) with a spinal cord syndrome and the remaining 178 (19.5%) with other symptoms.

Table 6.2. Demographic and clinical characteristics of the total CIS cohort, patients who converted and not converted to CDMS during follow-up

	All CIS patients (n=1,047)	**Missing values**	**Converted to CDMS (n=623, 59.5%)**	**Missing values**	**Not converted to CDMS (n=424, 40.5%)**	**Missing values**
Age (years)	32.0 (26.0 - 39.0)	0 (0)	31.0 (25.2 - 38.1)	0 (0)	33.2 (27.1 - 39.7)	0 (0)
Females	714 (68.2)	0 (0)	440 (70.6)	0 (0)	274 (64.7)	0 (0)
Follow-up (days)	1,574 (1,042 - 2,330)	0 (0)	1,768 (1,110 - 2,558)	0 (0)	1,332 (965 - 1,971)	0 (0)
Type of presentation	ON=288 (31.6) BS=188 (20.6) Spinal=257 (28.2) Other=178 (19.5)	136 (13)	ON=160 (30.7) BS=103 (19.8) Spinal=151 (29.0) Other=107 (20.5)	102 (16.4)	ON=128 (32.8) BS=85 (21.8) Spinal=106 (27.2) Other=71 (18.2)	34 (8.0)
Days to conversion to CDMS	NA	NA	421 (212 - 853)	0 (0)	NA	NA
Cotinine (> 14 ng/ml)	350 (33.7)	10 (1.0)	205 (33.2)	5 (0.8)	145 (34.6)	5 (1.2)
OCB positive	778 (74.3)	0 (0)	525 (84.3)	0 (0)	253 (59.7)	0 (0)
MRI T2 lesions	0-1=151 (14.4) 2-9=438 (41.8) >9=458 (43.7)	0 (0)	0-1=48 (7.7) 2-9=249 (40.0) >9=326 (52.3)	0 (0)	0-1=103 (24.3) 2-9=189 (44.6) >9=132 (31.1)	0 (0)
25-OH-D (nmol/l)	49.3 (32.2 - 72.5)	6 (0.6)	47.9 (31.3 - 71.9)	4 (0.6)	50.5 (34.7 - 73.5)	2 (0.5)
EBNA1 IgG	11.3 (6.7 - 14.7)	3 (0.3)	11.6 (6.7 - 15.0)	1 (0.2)	10.9 (6.7 - 14.2)	2 (0.5)
CMV IgG	1.7 (0.2 - 3.3)	12 (1.1)	1.9 (0.2 - 3.3)	8 (1.3)	1.2 (0.2 - 3.4)	4 (0.9)
CSF IgG index	0.7 (0.5 - 1.1)	351 (34)	0.8 (0.6 - 1.1)	231 (37.1)	0.6 (0.5 - 0.9)	120 (28.3)
CSF cell count (n/µl)	5.0 (2.8 - 12.0)	534 (51)	5.8 (2.0 - 12.0)	315 (50.6)	5.0 (3.0 - 11.0)	219 (51.7)

Median and interquartile range (IQR) or n (%).

ON: optic neuritis; BS: brainstem syndrome; Spinal: spinal cord syndrome; NA: not applicable.

6.3.2. Serum NfL analysis in FC versus NC and HC (n=290)

The median time to conversion to CDMS in FC was 110 days (79-139), while the median follow-up time in NC was 6.5 years (5.3-7.9). The general characteristics of FC, NC and HC are provided in table **6.3.**.

Table 6.3. *General characteristics of CIS patients and healthy individuals included in the study.*

Variable	**Category**	**FC (n=100)**	**NC (n=98)**	**HC (n=92)**
Age (years)	-	30.6 (25.1-37.9)	31.6 (27.4-37.8)	35.0 (29.0-45.0)
Sex	F	67 (67%)	61 (62.2%)	58 (63.0%)
OCB	Positive	87 (87%)	60 (61.2%)	-
T2 lesion count	0 to 1	5 (5%)	21 (21.4%)	-
	2 to 9	38 (38%)	52 (53.1%)	-
	> 9	57 (57%)	25 (25.5%)	-
Gd+ lesions	Positive	46 (58.2%)	25 (37.3%)	-
EDSS	-	2.0 (1.5-3.0)	1.5 (1.0-2.0)	-
NfL (pg/ml)	-	24.1 (13.5-51.8)	19.3 (13.6-35.2)	7.9 (5.6-17.2)

FC: fast converters to CDMS; NC: non-converters to CDMS; HC: healthy controls; OCB: oligoclonal bands in CSF; T2: T2 hyperintense MRI lesions; Gd+: gadolinium enhancing MRI lesions; EDSS: Expanded Disability Status Scale; NfL: neurofilament light chain in serum.

The number of patients with OCB, Gd+ and >9 T2 lesions was higher in FC than NC (87% vs 61.2%, 58.2% vs 37.3% and 57% vs 25.5% respectively). EDSS scores were also overall higher in FC than NC (median EDSS=2.0 (1.5-3.0) vs 1.5 (1.0-2.0) respectively).

There was no correlation between age and NfL in either CIS (Pearson's r=-0.11, p=0.11) or HC (Pearson's r=-0.02, p=0.83).

NfL levels were higher in FC (24.1 pg/ml (13.5-51.8) and NC (19.3 pg/ml (13.6-35.2) than in HC (7.9 pg/ml (5.6-17.2)) (**figure 6.1.**). Increasing NfL levels were significantly associated with an increased risk of being either FC (OR=5.85, 95%CI=2.63-13.02, p=1.5x10-5) or NC (OR=7.03, 95%CI=2.85-17.34, p=2.3x10-5) compared to HC. When comparing FC vs NC, NfL levels were not associated with fast conversion.

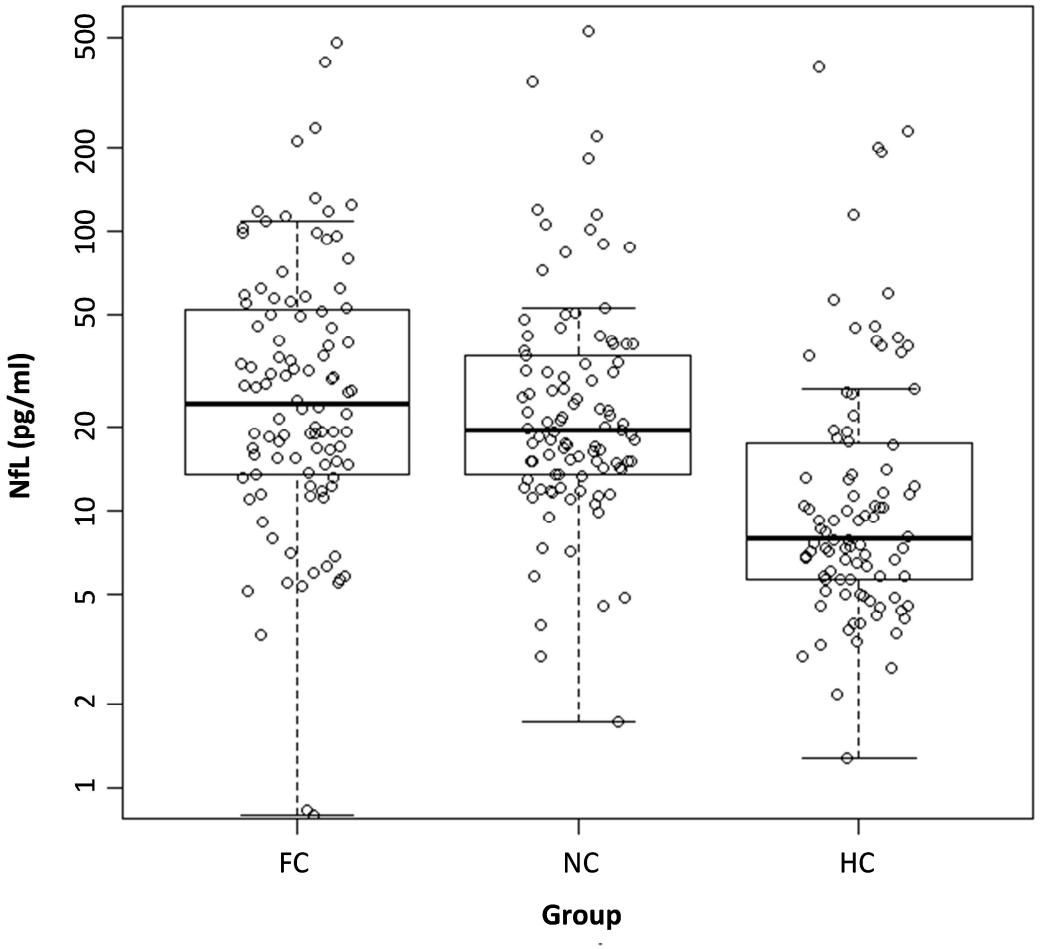


Figure 6.1. Serum NfL concentrations across the three investigated groups (FC=fast converters to clinically definite MS (CDMS); NC=non-converters to CDMS;

HC=healthy controls). Each dot represents NfL concentration in a single individual. Boxplots indicate median and IQR with whiskers extending 1.5 times the IQR.

However, presence of OCB, increasing number of T2 lesions, presence of Gd+ lesions and higher EDSS scores were all positively associated with FC status (**table 6.4**. upper panel).

Table 6.4. *Upper panel: results of logistic regression models investigating serum NfL, OCB, T2, Gd+ and EDSS as predictors of disease status (adjusted by age and sex).*
Lower panel: results of logistic and ordinal regression models investigating NfL concentration as predictor of OCB, EDSS, T2 and Gd+ lesions (adjusted by age and sex).

Comparison	**Predictor**	**OR**	**95%CI**	***p***
NC vs HC	NfL	7.03	2.85 - 17.34	2.3×10^{-5}
FC vs HC	NfL	5.85	2.63 - 13.02	1.5×10^{-5}
FC vs NC	NfL	1.36	0.69 - 2.69	0.376
FC vs NC	OCB	4.13	2.02-8.45	9.9×10^{-5}
FC vs NC	T2 (2-9 lesions)	3.11	1.07-9.06	0.037
	T2 (>9 lesions)	9.63	3.25-28.58	4.4×10^{-5}
FC vs NC	Gd+	2.45	1.23-4.86	0.011
FC vs NC	EDSS (1.5-2.0)	1.40	0.67-2.94	0.374
	EDSS (>2.0)	5.29	2.18-12.84	0.0002
Predicted variable	**Predictor**	**OR**	**95%CO**	***p***
OCB	NfL	1.66	0.75-3.70	0.214
T2	NfL	2.36	1.21-4.59	0.011
Gd+	NfL	2.69	1.13-6.41	0.026
EDSS	NfL	2.54	1.21-5.31	0.013

OR: odds ratio; 95%CI: 95% confidence interval; SE: standard error; NC: non-converters to CDMS; HC: healthy controls; FC: fast converters to CDMS; NfL: neurofilament light chain in serum; OCB: oligoclonal bands in CSF; T2: T2 hyperintense lesions; Gd+: gadolinium enhancing lesions.

Finally, NfL concentration was positively associated with presence of Gd+ lesions (OR=2.69; 95%CI=1.13-6.41; p=0.026), increasing T2 lesion load (OR=2.36; 95%CI=1.21-4.59; p=0.011), increasing EDSS category (OR=2.54; 95%CI=1.21-5.31; p=0.013), but not OCB status (OR=1.66; 95%CI=0.75-3.70; p=0.214) (**table 6.4.** lower panel, **figure 6.2.**).

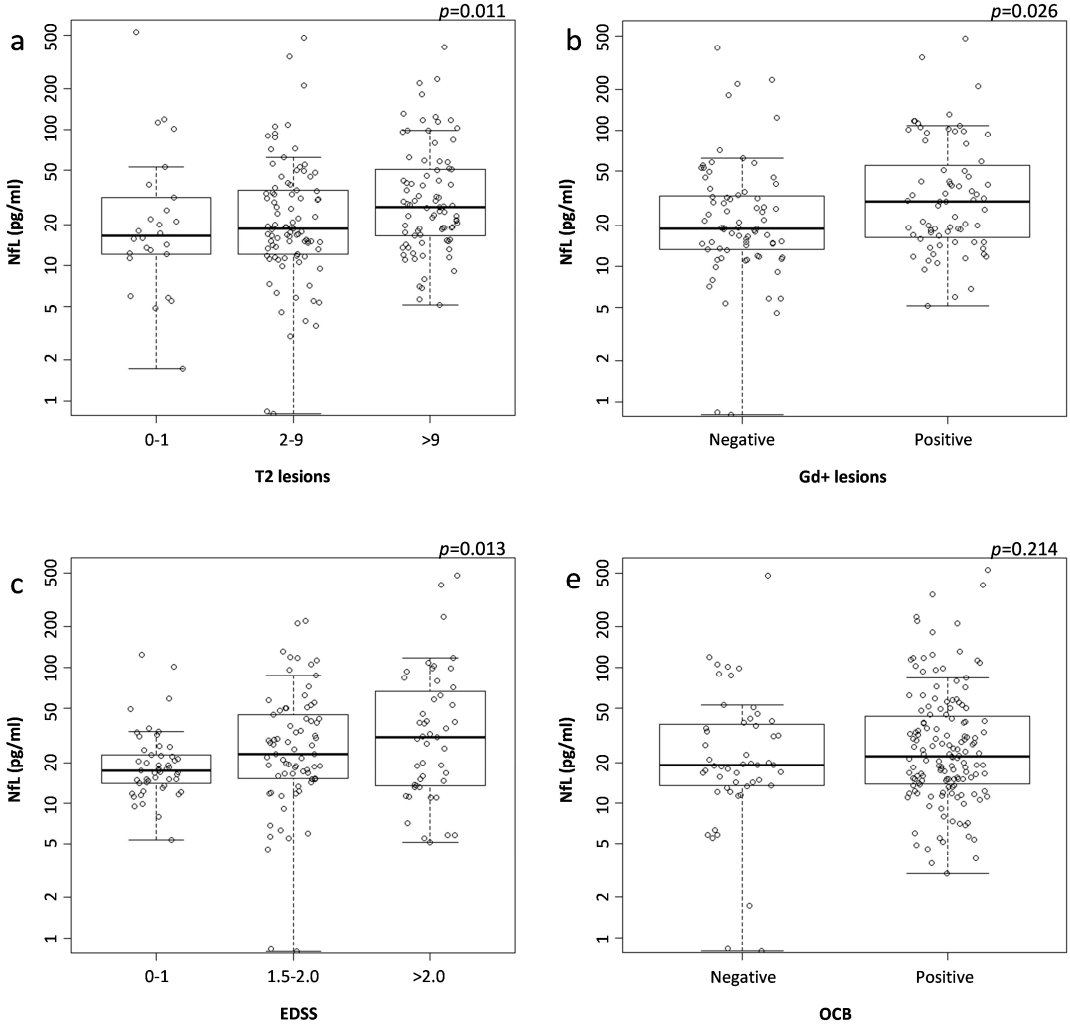


Figure 6.2. a) serum NfL levels across T2 lesion categories; b) serum NfL levels in patients with and without gadolinium enhancing MRI lesions (Gd+); c) serum NfL levels across EDSS categories; d) serum NfL levels in patients with and without oligoclonal bands (OCB). Each dot represents NfL concentration in a single individual. Boxplots indicate median and IQR with whiskers extending 1.5 times the IQR.

Since there was no significant difference in serum NfL concentrations between FC and NC, the a priori decision criterion to not measure serum NfL in the entire study cohort was fulfilled.

6.3.3. Predictors of conversion to CDMS in the entire CIS cohort (n=1,047)

Each variable was tested as a predictor of conversion from CIS to CDMS using a univariate Cox regression model (**table 6.5.**). OCB positive CIS patients were more than twice as likely to convert to CDMS as OCB negative individuals (hazard ratio (HR)=2.49, 95%CI=1.91-3.23, p<0.001; **figure 6.3.a**). Similarly, a higher IgG index was associated with conversion to CDMS (HR=1.22, 95%CI=1.10-1.36, p<0.001), whilst CSF cell count was not. A gradient of risk of conversion was observed with increasing numbers of T2 lesions (2-9 vs 0/1 lesions: HR=2.28, 95%CI=1.70-3.06, p<0.001; >9 vs 0/1 lesions: HR=3.26, 95%CI=2.28-4.65, p<0.001; >9 vs 2-9 lesions: HR=1.43, 95%CI=1.21-1.68, p<0.001; **figure 6.3.b**). Females were at slightly higher risk of CDMS but this did not reach statistical significance (HR=1.17, 95%CI=0.94-1.46, p=0.160; **figure 6.4.a**). Age at CIS onset was inversely associated with risk of conversion (HR per year increase=0.98, 95%CI=0.976-0.99, p<0.001; **figure 6.4.b**).

Table 6.5. *Univariate and multivariate stepwise Cox regression results for all variables investigated as predictors of conversion to CDMS.*

		Univariate			**Multivariate**		
Variable	**Category**	**HR**	**95%CI**	***p***	**HR**	**95%CI**	***p***
Age	-	0.98	0.976 - 0.99	<0.001	0.98	0.98 - 0.99	<0.001
Sex	F vs M	1.17	0.94 - 1.46	0.160	-	-	-
OCB	Positive vs Negative	2.49	1.91 - 3.23	<0.001	2.18	1.71 - 2.77	<0.001
CSF IgG index	-	1.22	1.10 - 1.36	<0.001	-	-	-
MRI T2 lesions	2-9 vs 0/1	2.28	1.70 - 3.06	<0.001	1.97	1.52 - 2.55	<0.001
	> 9 vs 0/1	3.26	2.28 - 4.65	<0.001	2.74	2.04 - 3.68	<0.001
25-OH-D (quartiles)	2^{nd} vs 1^{st}	0.76	0.60 - 0.95	0.016	0.82	0.70 - 0.97	0.019
	3^{rd} vs 1^{st}	0.69	0.58 - 0.83	<0.001	0.76	0.63 - 0.91	0.003
	4^{th} vs 1^{st}	0.76	0.62 - 0.94	0.010	0.85	0.68 - 1.07	0.167
EBNA1 IgG (quartiles)	2^{nd} vs 1^{st}	0.85	0.69 - 1.05	0.127	0.75	0.60 - 0.94	0.014
	3^{rd} vs 1^{st}	0.92	0.70 - 1.23	0.586	0.81	0.59 - 1.13	0.220
	4^{th} vs 1^{st}	1.11	0.83 - 1.49	0.464	1.00	0.75 - 1.34	0.986
lgG (quartiles)	2^{nd} vs 1^{st}	1.13	0.92 - 1.38	0.238	1.22	0.98 - 1.52	0.075
	3^{rd} vs 1^{st}	1.24	0.94 - 1.62	0.126	1.36	1.10 - 1.67	0.004
	4^{th} vs 1^{st}	1.12	0.89 - 1.41	0.346	1.25	0.97 - 1.60	0.07
Cotinine	>14 vs <14 ng/ml	0.95	0.79 - 1.14	0.549	-	-	-
CSF cell count	-	1.00	0.99 - 1.01	0.988	-	-	-
Type of CIS	BS vs ON	1.04	0.74 - 1.46	0.831	-	-	-
	Spinal vs ON	1.12	0.89 - 1.41	0.318	-	-	-
	Other vs ON	1.22	0.93 - 1.59	0.155	-	-	-

HR: hazard ratio; CI: confidence interval; F: female; M: male; vs: versus; CSF: cerebrospinal fluid; OCB: oligoclonal bands in CSF; 25-OH-D: 25-hydroxyvitamin D3; EBNA1: Epstein-Barr nuclear antigen 1; CMV: cytomegalovirus; CIS: clinically isolated syndrome; BS: brainstem syndrome; ON: optic neuritis; Spinal: spinal cord syndrome.

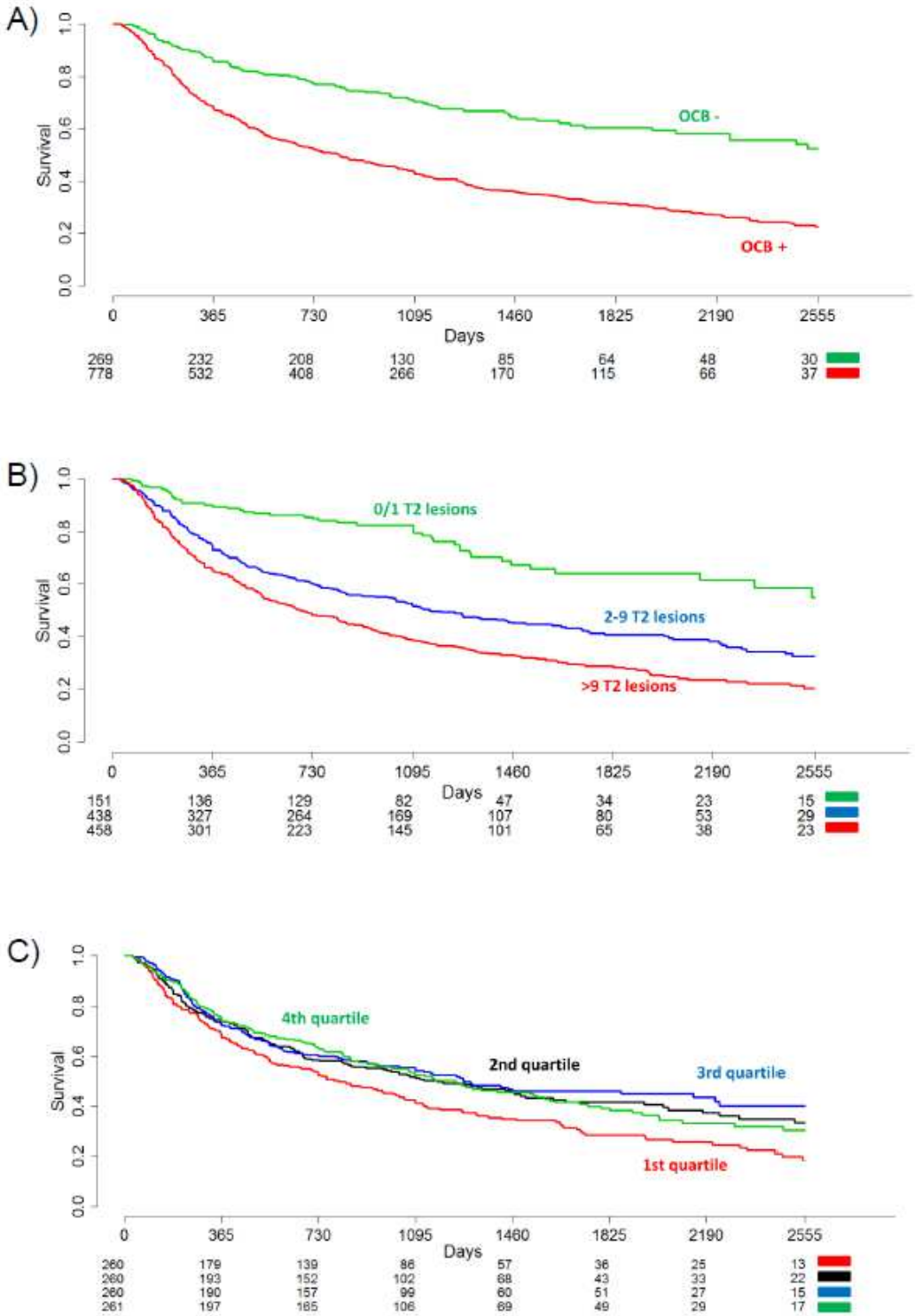


Figure 6.3. Survival curves of conversion to CDMS and numbers of individuals at risk of conversion (below the x axis) stratified according to: a) OCB status; b) number of T2 lesions on MRI; c) quartiles of 25-OH-D levels.

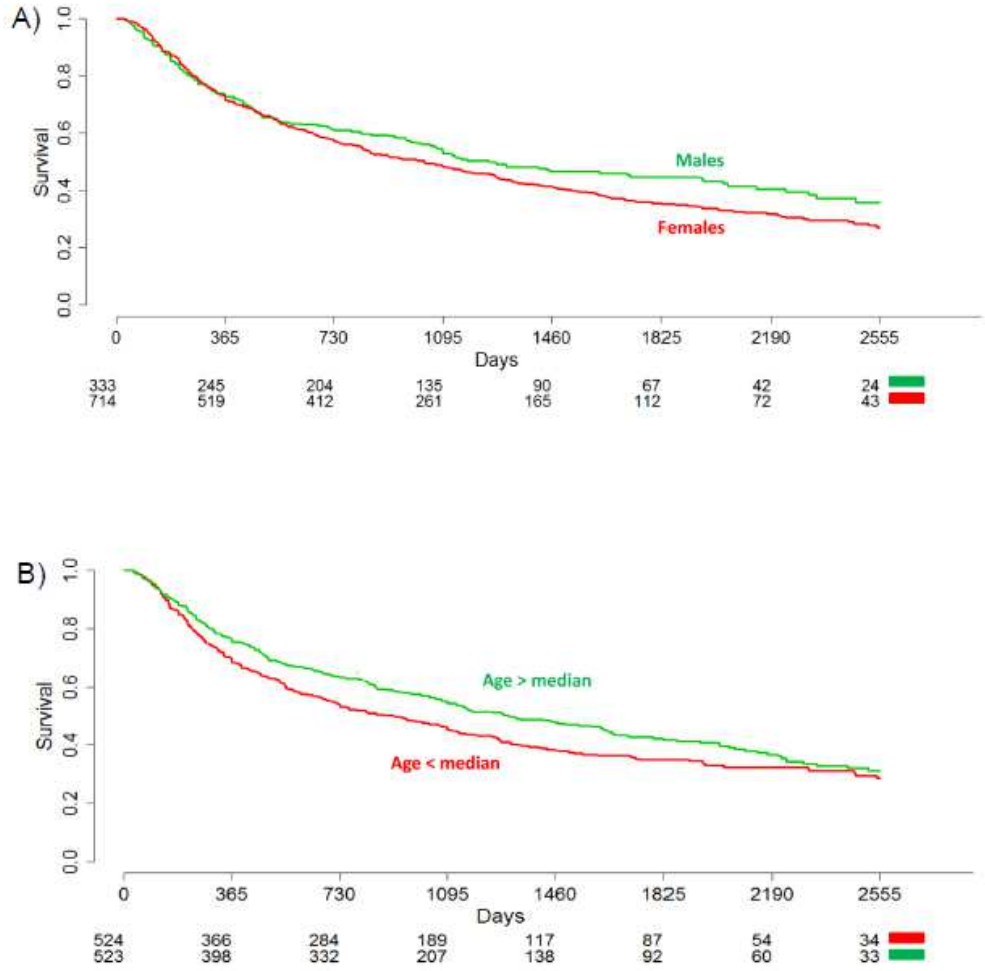


Figure 6.4. Survival curves of conversion to CDMS and numbers of individuals at risk of conversion (below the x axis) stratified according to: a) gender; b) age.

We attempted to make these statistical measures of risk more clinically relevant by estimating the risk of conversion to CDMS in different categories of CIS patients at 2 and 5 years of follow-up. The risk of conversion varied widely across different exposure categories (**table 6.6.**) and was high among individuals with evidence of OCB and more than 9 T2 lesions on MRI (57.0% and 85.5% at 2 and 5 years of follow-up respectively). Including 25-OH-D information did not considerably change these estimates. Notably, the risk of conversion among OCB negative CIS cases with either 0 or 1 T2 lesions and higher vitamin D status was low (6.8% and 21.4% at 2 and 5 years respectively). These estimates should however be interpreted with caution given the small number of patients lacking all risk factors for conversion and the correspondingly wide confidence intervals.

Table 6.6. *Risk of conversion to CDMS in different categories of CIS patients after 2 and 5 years of follow-up.*

Category	At 2 years of follow-up		At 5 years of follow-up	
	n	% risk of conversion (95%CI)	n	% risk of conversion (95%CI)
OCB positive	778	47.9 (44.4 - 51.5)	607	81.1 (77.6 - 84.0)
OCB negative	269	22.7 (17.9 - 28.2)	154	58.4 (50.2 - 66.2)
MRI > 9 T2 lesions	458	51.7 (47.1 - 56.4)	372	82.5 (78.2 - 86.2)
MRI 2-9 T2 lesions	438	39.9 (35.4 - 44.7)	314	74.5 (69.3 - 79.2)
MRI 0-1 T2 lesions	151	14.6 (9.5 - 21.4)	75	54.7 (42.8 - 66.0)
25-OH-D 1st quartile	260	47.3 (41.1 - 53.6)	205	82.4 (76.4 - 87.2)
25-OH-D 2nd quartile	260	41.5 (35.5 - 47.8)	182	76.4 (69.4 - 82.2)
25-OH-D 3rd quartile	260	40.0 (34.0 - 46.3)	179	71.5 (64.2 - 77.9)
25-OH-D 4th quartile	261	36.8 (31.0 - 43.0)	191	74.3 (67.4 - 80.2)
OCB positive / > 9 T2	377	57.0 (51.8 - 62.1)	310	85.5 (80.9 - 89.1)
OCB negative / > 9 T2	81	27.2 (18.1 - 38.4)	62	67.7 (54.5 - 78.7)
OCB positive / 2-9 T2	328	43.9 (38.5 - 49.5)	251	77.7 (71.9 - 82.6)
OCB negative / 2-9 T2	110	28.2 (20.2 - 37.7)	63	61.9 (48.8 - 73.6)
OCB positive / 0-1 T2	73	19.2 (11.2 - 30.4)	46	69.6 (54.1 - 81.8)
OCB negative / 0-1 T2	78	10.3 (4.8 - 19.7)	29	31.0 (16.0 - 51.0)
OCB positive / >9 T2 / 25-OH-D < median	192	56.2 (48.9 - 63.3)	154	87.0 (80.4 - 91.7)
OCB negative / 0-1 T2 / 25-OH-D > median	44	6.8 (1.8 - 19.7)	14	21.4 (5.7 - 51.2)

CI: confidence interval; OCB: oligoclonal bands in CSF; 25-OH-D: 25-hydroxyvitamin D3.

6.3.4. Predictors of OCB and MRI lesions

The clinical and biological parameters associated with the presence of OCB and T2 hyperintense lesions at disease onset were assessed. The median age, sex ratio, vitamin D status, EBNA1 IgG and CMV IgG levels of OCB positive and OCB negative CIS patients are described in **table 6.7.**. OCB positive individuals were younger, more likely to be female, had more T2 lesions and lower 25-OH-D levels than those who were OCB negative. OCB positive patients also had higher EBNA1 but not CMV IgG levels compared to OCB negative patients. In a multivariate logistic regression model predicting OCB status, the number of T2 lesions (>9 vs 0/1 lesions: odds ratio (OR)=5.03, 95%CI=3.35-7.58, p<0.001), lower age at CIS onset (OR per year increase=0.98, 95%CI=0.97-0.997, p=0.019) and higher EBNA1 IgG levels (OR=1.08, 95%CI=1.04-1.11, p<0.001) were significantly associated with OCB (**table 6.7.**).

Table 6.7. *Association between demographic and clinical features of CIS patients with presence or absence of OCB.*

				Univariate			Multivariate		
Variable		**OCB positive**	**OCB negative**	**OR**	**95%CI**	***p***	**OR**	**95%CI**	***p***
Age	Median (IQR)	31.3 (25.4 - 39.0)	33.6 (27.7 - 38.8)	0.98	0.97 - 0.998	0.025	0.98	0.97 - 0.997	0.019
T2 MRI	2 – 9	328 (42.2%)	110 (40.9%)	3.19	2.17 - 4.68	<0.001	3.16	2.13 - 4.70	<0.001
	> 9	377 (48.5%)	81 (30.1%)	4.97	3.34 - 7.41	<0.001	5.03	3.35 - 7.58	<0.001
EBNA1 IgG	Median (IQR)	11.8 (7.6 - 14.9)	9.3 (4.2 - 14.1)	1.08	1.05 - 1.11	<0.001	1.08	1.04 - 1.11	<0.001
25-OH-D	Median (IQR)	49.0 (31.2 - 71.3)	50.1 (36.1 - 76.8)	0.995	0.99 - 0.999	0.035	-	-	-
Sex	Females	544 (69.9%)	170 (63.2%)	1.35	1.01 - 1.81	0.042	-	-	-
CMV IgG	Median (IQR)	1.5 (0.2 to 3.4)	2.2 (0.2 to 3.3)	1.03	0.98 - 1.09	0.225	-	-	-

OCB: oligoclonal bands in CSF; OR: odds ratio; CI: confidence interval; IQR: interquartile range; EBNA1: Epstein-Barr nuclear antigen 1; 25-OH-D: 25-hydroxyvitamin D3; CMV: cytomegalovirus.

The general features of CIS patients in the three different categories based on number of T2 lesions are shown in **table 6.8.**. We used a multivariate ordinal regression model to investigate which variables were independently associated with an increased number of T2 lesions. Only presence of OCB was significantly associated with an increased lesion load (OR=2.73, 95%CI=2.08-3.58, p<0.001) (**table 6.8.**).

Table 6.8. *Demographic and clinical features of CIS patients associated with MRI status.*

Variable		0-1 T2	2-9 T2	> 9 T2	Univariate			Multivariate		
					OR	**95%CI**	***p***	**OR**	**95%CI**	***p***
OCB	Positive	73 (48.3%)	328 (74.9%)	377 (82.3%)	2.73	2.08 - 3.58	<0.001	2.73	2.08 - 3.58	<0.001
Age	Median	31.2 (25.5-36.9)	32.3 (26.6 -39.1)	32.2 (26.0 - 39.3)	1.00	0.99 - 1.02	0.458	-	-	-
Sex	Females	104 (68.9%)	301 (68.7%)	309 (67.5%)	0.95	0.74 - 1.21	0.667	-	-	-
EBNA1 IgG	Median	10.1 (5.3 -13.7)	11.8 (7.0 – 15.0)	11.3 (6.9 - 14.7)	1.02	0.99 - 1.04	0.206	-	-	-
CMV IgG	Median (IQR)	2.1 (0.2 - 3.5)	1.7 (0.2 - 3.6)	1.7 (0.2 - 3.2)	0.97	0.94 - 1.01	0.119	-	-	-
25-OH-D	Median (IQR)	50.2 (35 - 77)	50.0 (33 - 73)	48.0 (30 - 70)	1.00	0.99 - 1.00	0.132	-	-	-

OR: odds ratio; CI: confidence interval; OCB: oligoclonal bands in CSF; IQR: interquartile range; EBNA1: Epstein-Barr nuclear antigen 1; CMV: cytomegalovirus; 25-OH-D: 25-hydroxyvitamin D3.

6.4. Discussion

I report the largest study ever performed on CIS patients and aimed to elucidate the factors driving the onset of CDMS. This is also the first study investigating serum NfL in CIS patients. These were significantly higher in both FC and NC than in HC. This supports the presence of accumulating axonal injury starting in the very early phases of the disease and ongoing Wallerian degeneration from focal lesion(s) presenting as CIS. We noted some HC had higher NfL levels than CIS patients. To what extent these high NfL levels in HC represent minor neurological insults occuring in day-to-day life (e.g. minor head injuries or compression of peripheral nerves) rather than normal synaptic and neuronal turnover is unclear and needs further study.

Interestingly, the difference in serum NfL between FC and NC was not statistically significant (and hence the decision to restrict this analyses to extreme outcome groups in this study). Therefore, while increased NfL levels appear a good indicator of axonal damage, this does not appear to be specific to CIS with a relatively rapid conversion to CDMS. Increased serum NfL and NfH levels have been found in neurological conditions other than MS such as ALS and GBS, and have been used as a marker of neurotoxicity after chemotherapy (94, 168, 169). Results from previous studies in CIS have been controversial with one study showing slightly higher CSF NfL levels in CIS patients converting to CDMS than in non-converters (100), while others have not (102, 178). It should be noted that, even in this cohort, NfL levels were to some extent higher in FC than NC and it is plausible that by increasing the sample size the difference may become statistically significant. As compared to CSF, serum samples are more easily accessible but also more distant from the pathological process taking place in the CNS. This may affect the sensitivity of serum NfL to predict conversion to CDMS.

Importantly, higher serum NfL concentration was associated with the presence of both T2 and Gd+ lesions; the former a burden of disease marker and the latter a marker of acute focal inflammation. Furthermore, NfL levels increased with increasing EDSS scores at the time of CIS. These results are in agreement with previous studies showing that Nf levels in the CSF are correlated with both MRI and clinical activity of MS (98, 100, 152, 240). It will be important to determine if raised serum NfL levels at presentation predict long-term disability outcomes similarly to CSF NfL levels (99).

I confirmed a strong association between an increasing number of T2 hyperintense lesions on baseline MRI and risk of conversion in keeping with several previous studies (57, 235, 249). Similarly, CSF markers of B cell activity (OCB and a higher IgG index) were strongly and independently associated with conversion to CDMS (250-252). Notably, the risk of conversion appeared particularly high in those patients carrying both OCB and a high number of T2 lesions (57% and 86% risk of conversion after 2

and 5 years of follow-up respectively). As previously suggested, age at disease onset was also inversely associated with risk of conversion (253).

Two recent studies have demonstrated a significant inverse relation between vitamin D status and risk of conversion (237, 254). Our study appears to confirm this finding, and indeed extends this in a heterogeneous sample of CIS patients from a variety of latitudes. It is noteworthy that our study included both a considerably larger number of patients (237) and also incorporated information on OCB (254). In the univariate model predicting conversion to CDMS the 2nd, 3rd and 4th quartiles versus the 1st (lowest) quartile of vitamin D levels were all significantly associated with a lower risk of converting to CDMS. Nevertheless, already in this analysis and more so in the multivariate approach no apparent dose-response relationship was visible. In addition, significance was lost in the multivariate model for comparing the 4th with the 1st quartile. Even if the effect of 25-OH-D levels on risk of conversion appears partially attenuated in the multivariate analysis, our overall results confirm that CIS patients with lower 25-OH-D levels tend to convert to CDMS more rapidly. Studies outside the field of MS have reported that vitamin D levels may fall in the presence of systemic inflammation and this may happen to a greater extent in those CIS patients, who go on to convert to CDMS (255). Although reverse causation cannot be excluded, the increasing evidence for regulatory effects of vitamin D on the immune system support a potential causal link between vitamin D deficiency and conversion to CDMS (256, 257).

Previous studies have suggested that individuals with higher antibody levels against EBV are at increased risk of conversion (238, 241). However, we did not see any association between IgG production against EBNA1 and CMV and conversion to CDMS in univariate analyses. Some quartiles appeared significantly associated in the multivariate model, but this was not consistent across quartiles. Given the lack of significance in univariate analyses and the known risk of false positive associations in stepwise models, we believe these data should be interpreted with caution. The difference in the methods used by previous studies could at least partly contribute to the discrepancy in these results. We only tested a single EBV antigen using an ELISA based kit, which is less accurate than immunofluorescence based methods (258).

We were particularly interested in the relation between OCB and IgG against EBNA1, as previous studies have suggested a potential link between these markers of B cell activation in MS. A similarly large proportion of patients with MS have OCB in their CSF and antibodies against EBV in their serum (244, 259). In addition, both the presence of OCB and high antibody titres against EBV proteins are positively associated with the HLA-DRB1*1501 allele, the main genetic risk factor in MS (260-263). Our finding that EBNA1 but not CMV IgG levels are significantly associated with the presence of OCB

further strengthens the link between EBV and intrathecal B cell activation. This highlights a potential connection between exposure to this virus and the most consistent immunological finding in MS patients.

OCB status was also positively associated with MRI T2 lesion load and lower age at onset. Notably, these variables were also associated with risk of conversion and this confirms the importance of OCB and B cell activation within the CNS in the pathology of this disease.

Our multicentre approach enabled the retrospective collection of more than 1,000 CIS patients who have been followed up for several years, but this real-life clinical setting also inevitably leads to some limitations. Potential bias can arise from centre specific effects, despite adjusting for them in the regression models. This includes the potential consequences of various different clinical care protocols employed across the participating sites, and possible recall bias depending on the frequency of follow-up visits. Furthermore, we should remember that patients who participate in studies are those who fulfill the inclusion criteria, and differences may be present between these and the overall patient population. We did not include additional variables that could potentially act as confounders or independently influence the risk of conversion to CDMS such as the potential effect of disease modifying treatment, genetic factors, history of infectious mononucleosis, latitude, time spent outdoors, vitamin D intake and previous history of smoking in our analysis. Vitamin D levels were only measured at a single time-point, which may not appropriately reflect vitamin D status over the long term. Finally, MRI protocols and T2 lesion counting methods could not be pre-specified and hence not uniform across the different centres. We therefore chose not to use MRI as evidence of dissemination in time and space (as in the current diagnostic criteria (264)). We instead applied Poser criteria to define CDMS, which allow a more reliable evaluation of disease activity in multicentre studies such as this one. Nonetheless, the occurrence of new neurological symptoms represents an important and unequivocal clinical endpoint which is relevant for both patients and neurologists. Similarly, in order to reduce inter-rater variability in T2 lesion counts, we grouped patients in categories (0-1, 2-9 and >9 lesions) and used these rather than the original lesion counts for all analyses.

In conclusion, despite not discriminating between fast and slow converting CIS patients, serum NfL levels are abnormally high in the earliest stages of MS, correlate with MRI activity and disability scores and represent a promising and easily accessible biomarker in this debilitating disease. These results strongly support future studies with longitudinal sampling and well conducted clinical and imaging follow-up to investigate serum NfL's potential prognostic potential in CIS and also later disease stages.

Conversely, OCB status, number of T2 lesions and lower age at CIS onset are associated with an increased risk of conversion from CIS to CDMS. A role for lower vitamin D levels is also suggested. We confirm that patients with both OCB and several T2 lesions are highly likely to convert, with their risk of developing CDMS in 5 years at almost 90%. Given that MRI activity and OCB status are inextricably linked with immune activation, these data support targeting the immune system early in the disease in order to slow or prevent MS disease activity.

The effect of vitamin D is intriguing and supports the need for large-scale clinical trials of vitamin D supplementation in order to conclusively answer the question as to whether this environmental factor is causal or consequential for disease activity. The future integration of additional parameters including genetic variants associated with the risk of MS will allow a more accurate assessment of risk of conversion and more targeted intervention strategies.

7. Summary and Conclusions

The incidence of neurodegenerative diseases increases with age. At present about 16% of the European population is older than 65 years with an estimated 30% by 2060. In contrast, neurodegeneration in children and young adults is more related to accidents such as traumatic brain or spinal cord injury. In addition, there are neurodegenerative diseases which affect adults at the prime of their active working life. Notably, neurodegeneration is now recognised as the key pathological feature driving disease progression in MS and other demyelinating syndromes, stroke and ALS. There is a lack of analytically and clinically validated in vitro diagnostic tests for assessment of neurodegeneration in this context. Until now, MRI findings have attracted most interest as measures and surrogates of axonal loss (265). While classical MRI measures (focal T2 hyperintense lesions, Gadolinium enhancement) have been disappointing in this regard, more elaborated quantitative outcomes including whole brain or regional brain atrophy were found to better depict the destructive process (266). Unfortunately, brain volume is a "mixed baggage" resulting from many different factors, pathological and physiological, not directly related to axonal loss such as fluid shifts, ageing, remyelination and astrogliosis, all of them affecting measures of brain atrophy in sometimes opposite directions (e.g. inflammation may increase water content and volume and its suppression – although beneficial in the long run – may result in volume reduction via reduction of edema (a phenomenon apostrophized as "treatment-induced pseudo-atrophy" (267))). Hence whole brain atrophy is a rather unspecific surrogate of neurodegeneration. Another significant problem of measuring brain atrophy relates to the delayed responsiveness of this outcome measure (268). To date, the best validated universal body fluid biomarkers for axonal damage are the Nf proteins.

My findings support this view: NfL proved to be a stable analyte with respect to repeated freeze thawing cycles and prolonged exposure to room temperature. This does not support previous concerns about its susceptibility to proteases, especially in the protease-rich CSF or blood (160). It is important to conclude that there is no basis to prefer NfH over NfL as biomarker of axonal damage due to concerns of sample stability.

My results demonstrated that CSF Nf levels are increased in the earliest stages of MS and correlate with signs of inflammation in CSF and relapses. Differences in CIS and MS were stronger for NfL than for NfH^{SMI35}. The higher abundance of NfL and/or better performance of the UmanDiagnostics NF-light® assay may outweigh the known high sensitivity and higher dynamic range of the ECL technology used in our previously developed NfH^{SMI35} assay (95).

This is also supported by more pronounced reductions of CSF NfL levels as compared to NfH^{SMI35} after 12 months of natalizumab treatment in MS patients. However, it would be premature to generalize this to the assumption that NfL measurements would be universally superior to NfH. Rather these findings could reflect specific assay characteristics, and although both proteins originate from the same source, it is foreseeable that increased levels of one or the other bear differential information.
I performed the first double-blind, placebo controlled evaluation of the effects of an approved MS therapy on CSF NfL levels. The data demonstrated that CSF NfL levels decrease substantially in fingolimod-treated patients, whereas a similar magnitude of change is not observed in patients on placebo. Importantly, the treatment effect on NfL levels was associated with improved clinical and MRI outcomes of disease activity. At the individual patient level, extreme changes in CSF NfL levels, irrespective of treatment, were reflected in the clinical and paraclinical findings. Consistent with these results, a recent uncontrolled study in RRMS patients with high disease activity also suggested an association of natalizumab treatment with a decrease in CSF NfL levels and corresponding improvements in clinical, MRI and other laboratory measures (101).

A notable limitation of cross-sectional studies on biomarkers of neurodegeneration is that data are obtained almost exclusively from CSF samples. This invasive, potentially harmful procedure has to be performed under sterile conditions and is not well tolerated by all patients which explains the lack of large multi-centre longitudinal studies. Longitudinal data is essential for monitoring of progression of neurodegeneration and efficacy of neuroprotective treatment strategies. However, obtaining longitudinal CSF samples is considered too invasive outside the clinical trial arena, precluding the broader clinical use of Nf.
Several reports have suggested peripheral blood levels of NfH as a potential marker of neurodegeneration (154, 155, 168, 182, 184, 185, 198, 269). In contrast to studies on NfH, there is only one recent report investigating NfL in serum; this work examined the relationship between serum NfL and neurological outcome following cardiac arrest (170).
A commercially available ELISA (UmanDiagnostics NF-light® assay) uses two highly specific, non-competing monoclonal antibodies (47:3 and 2:1) to quantify soluble NfL in CSF samples. However, this cannot be used in its present form for analysis of blood samples (95). In a collaborative effort with UmanDiagnostics I developed a highly sensitive ECL-based NfL assay ($NfL^{Umea47:3}$) suitable for the quantification of NfL in serum at concentrations relevant to clinical settings (94). We applied this immunoassay to cohorts of patients with AD, GBS, ALS. All patient groups had higher serum NfL values than a CP and HC. Further strengthening these results, CSF and serum levels

correlated significantly in all disease groups and sensitivity and specificity of serum NfL for separating ALS from healthy controls was over 90%.

To further my initial findings, I had access to a unique collection of longitudinally collected serum samples from well characterized patients with SCI. In SCI, the severity of disease is quantified exclusively by clinical measures that have limited sensitivity and reliability, and no blood-based biomarker has been established to further stratify the degree of injury. I investigated the role of serum NfL determined by $NfL^{Umea47:3}$ as predictor of the clinical outcome in 27 SCI patients. I found that baseline NfL levels (in samples collected within 12 hours after the trauma) were significantly higher in iSCI and cSCI than in HC and patients with CCS, and levels increased over time. NfL levels correlated with ASIA motor score at baseline and after 24 hours and the long term motor outcome. Minocycline treatment showed decreased NfL levels in the subgroup of cSCI patients.
These findings are important since they show for the first time that serum NfL concentrations in SCI patients are closely correlated with injury severity and functional outcome and provide evidence that serum NfL is of prognostic value in SCI patients. Further, blood NfL levels may qualify as drug response markers in SCI.
Finally, I collected and studied the largest cohort of CIS patients to date in order to investigate serum NfL levels in CIS patients and which factors predict conversion from CIS to CDMS, defined by the occurrence of a second clinical relapse.
NfL levels were higher in FC (24.1 pg/ml) and NC (19.3 pg/ml) than in HC (7.9 pg/ml, $p=1.5x10^{-5}$ and $p=2.3x10^{-5}$ respectively). In addition, increased serum NfL concentration was associated with increasing numbers of T2 hyperintense MRI lesions (p=0.011), Gd+ lesions (p=0.026) and higher EDSS scores (p=0.013) at CIS diagnosis. However, NfL levels were not associated with fast conversion to CDMS (p=0.37). Based on this I decided not to investigate serum NfL further in the entire cohort of CIS patients (n=1047). Predictors of conversion in multivariate analyses were OCB (p<0.001), number of T2 lesions (p<0.001) and age at CIS (p<0.001). Lower 25-OH-D levels were associated with CDMS in univariate analysis, but this was attenuated in the multivariate model. Strikingly, OCB positivity was associated with higher EBNA-1 IgG titres. In conclusion, I validated MRI lesion load, OCB and age at CIS as the strongest independent predictors of conversion to CDMS in this multicentre setting. A role for vitamin D is also suggested but requires further investigation.

Neurodegeneration is now recognised as the key pathological feature driving disease progression in MS and other demyelinating syndromes, but also stroke and ALS. There is a significant unmet need for analytically and clinically validated in vitro diagnostic

tests for assessing of axonal injury; and in this context, assessing the efficacy of neuroprotective agents in the setting of delayed axonal loss is proving extremely problematic. Taken together, the results of my thesis confirm and expand on previous findings of Nf as quantitative markers of neurodegeneration in CSF. In particular, I showed that NfL is a stable molecule, can be measured in both CSF and serum, is increased in a wide range of neurodegenerative conditions, correlates with clinical features of disease activity and progression and is potentially responsive to disease treatment. Nf is a universal measure of axonal injury, therefore specificity is limited. According to the definitions set up by the biomarker expert working group (137), the greatest potential for Nf can be anticipated in their use as a tool for quantifying disease severity, prognosis and monitoring therapy response. Future studies will also have to show if NfL may qualify as surrogate endpoint in clinical trials. It will be important to have independent research groups being able to technically reproduce serum NfL measurements. The near past has shown several examples of relevant scientific findings that could not be independently validated (270-272). Longitudinal samples are essential for monitoring of the progression of neurodegeneration. Medium-term longitudinal studies investigating serum NfL in MS will have to include large patient numbers with well conducted clinical and paraclinical follow-up examinations. Clinical follow-up will have to be long enough (ie 5-10 years) to draw conclusions on the relationship between NfL measurements and disease severity and prognosis. This should also include conventional and more advanced imaging measures. I propose samples that have already been collected in the framework of observational cohort studies or randomized-controlled trials could be well suited to fulfill these requirements. Depending on outcome, these retrospective projects will have to be followed by prospective trials investigating the potential of serum NfL measurements as a surrogate endpoint and to measure disease severity, treatment response and prognosis in individual patients further.

8. Publications in peer reviewed journals associated with this thesis

Chapter 2:

1. Kuhle J, Plattner K, Bestwick JP, Lindberg RL, Ramagopalan S, Norgren N, Nissim A, Malspina A, Leppert D, Giovannoni G*, Kappos L*. *equal contribution.
   A comparative study of CSF Neurofilament light and heavy chain protein in MS.
   Mult Scler, 19(12):1597-1603, 2013.

Chapter 3A:

2. Kuhle J, Malmeström C, Axelsson M, Plattner K, Yaldizli O, Derfuss T, Giovannoni G, Kappos L, Lycke J.
   Neurofilament light and heavy subunits compared as therapeutic biomarkers in multiple sclerosis.
   Acta Neurol Scand, 128(6):e33-62013, 2013.

Chapter 3B:

3. Kuhle J, Disanto G, Lorscheider J, Stites T, Chen Y, Dahlke F, Francis G, Shrinivasan A, Radue E-W, Giovannoni G, Kappos L.
   Fingolimod and CSF neurofilament light chain levels in relapsing-remitting multiple sclerosis.
   Neurology, 84(16): 1639-1643, 2015.

Chapter 4:

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