Neurofilaments as biomarkers of neuronal damage

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Qui cerca, troba

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ABSTRACT

Different neurodegenerative diseases have overlapping symptomatology and pathology and have thus become a challenge to modern medicine to achieve a correct diagnosis. The aim of the thesis was to evaluate the use of neurofilaments as biomarkers of neuronal damage by testing their ability to discriminate between different neurodegenerative diseases as well as assessing whether higher neurofilments predict a poorer clinical outcome in ischemic stroke.

For these purposes, we developed two new Enzyme-Linked ImmunoSorbent Assays (ELISAs) for the quantification of neurofilament light (NFL) and phosphorylated neurofilament heavy (pNFH) in cerebrospinal fluid (CSF).

The new NFL and pNFH ELISAs presented good analytical performance and both NFL and pNFH concentrations were valid across different analytical approaches. CSF-NFL concentrations were significantly higher in inflammatory demyelinating diseases and Alzheimer's disease when compared to Parkinson's disease or controls. In ischemic stroke, both CSF and blood NFL and pNFH reflected the temporal dynamics of post ischemic damage of axons. Finally, both CSF-NFL and CSF-pNFH were increased in amyotrophic lateral sclerosis (ALS) compared to other neurological conditions mimicking ALS and controls.

Both NFL and pNFH proved to be sensitive and reliable biomarkers of neuronal damage. These findings support the use of neurofilaments as disease intensity markers and suggest that both NFL and pNFH can be useful

laboratory tests in the diagnostic work-up of patients with suspected neurodegenerative diseases.

Keywords: neurofilaments, biomarker, neurodegenerative diseases, stroke, cerebrospinal fluid, blood.

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SAMMANFATTNING PÅ SVENSKA

Neurodegenerativa sjukdomar har blivit en stor utmaning inom modern medicinsk forskning och diagnostik på grund av överlappande patofysiologiska mekanismer. Målet för denna avhandling var att utvärdera neurofilamentproteiner som potentiella biomarkörer för nervcellsskador. Detta genom att kartlägga deras förmåga att skilja olika sjukdomar åt samt identifiera patienter med större skador och allvarligare sjukdomsförlopp.

För att utforska detta utvecklades två nya analysmetoder för att kvantifiera neurofilamentproteiner i ryggvätska, baserade på Enzymkopplad Immunoadsorberande Analys (ELISA). En för att kvantifiera neurofilament light (NFL) och en för att kvantifiera fosforylerat neurofilament heavy (pNFH).

Dessa nya metoder uppvisade god analytisk styrka och mätresultaten var konsistenta samt reproducerbara med andra analysmetoder. Koncentrationen av NFL i ryggvätska var signifikant högre i de-myeliniserande sjukdomar och Alzheimers sjukdom i jämförelse med Parkinsons sjukdom och friska kontroller. Både NFL och pNFH återspeglade tidsförloppet av post-ischemisk celldöd efter stroke. Båda uppvisade också högre nivåer vid amyotrofisk lateralskleros (ALS) i jämförelse med ALS-liknande tillstånd och friska kontroller.

Både NFL och pNFH visade sig vara både känsliga och pålitliga biomarkörer för nervcellsskador, vilket stödjer deras användning som biomarkörer både inom diagnostik av neurodegenerativa sjukdomar och för värdering av sjukdomsaktivitet.

Nyckelord: neurofilament, biomarkör, neurodegenerativ sjukdom, stroke, ryggvätska, blod.

RESUMEN EN CASTELLANO

El hecho que distintas enfermedades neurodegenerativas tienen una sintomatología y patología similar y/o común hace que su correcto diagnóstico se haya convertido en un gran reto para la medicina moderna. El objetivo de esta tesis doctoral fue evaluar el potencial de los neurofilamentos como biomarcadores de daño neuronal, valorar su capacidad para discriminar entre distintas enfermedades neurodegenerativas, así como examinar si unos mayores niveles de neurofilamento predicen un peor pronóstico en el ictus isquémico.

Con este objetivo, se desarrollaron dos nuevos ensayos "Enzyme-Linked ImmunoSorbent Assays" (ELISAs) para la cuantificación del neurofilamento ligero (NFL) y del neurofilamento pesado fosforilado (pNFH).

Los nuevos ELISAs para NFL y pNFH presentaron un buen perfil analítico y las concentraciones de NFL y pNFH fueron parecidas a las halladas usando otras metodologías analíticas. Las concentraciones de NFL en el líquido cefalorraquídeo fueron significantemente más elevadas en pacientes con enfermedades inflamatorias desmielinizantes y enfermedad de Alzheimer que en pacientes con enfermedad de Parkinson o controles. En el ictus isquémico, tanto NFL como pNFH en el líquido cefalorraquídeo y en sangre reflejaban la dinámica temporal de degeneración axonal post-isquémica. Finalmente, NFL y pNFH en el líquido cefalorraquídeo estaban más elevados en pacientes con esclerosis lateral amiotrófica (ELA) que en controles o pacientes con síntomas similares a los de la ELA.

Tanto NFL como pNFH han demostrado ser biomarcadores sensibles y fiables de daño neuronal. Estos resultados apoyan la utilización de los neurofilamentos como marcadores de la intensidad de la enfermedad y concluyen que tanto NFL cómo pNFH pueden ser herramientas útiles en el diagnóstico de las enfermedades neurodegenerativas.

RESUM EN CATALÀ

El fet que diferents malalties neurodegeneratives tinguin una simptomatologia i patologia similar i/o comuna fa que la seva correcte diagnosi hagi esdevingut tot un repte per a la medicina moderna. L'objectiu d'aquesta tesi doctoral ha sigut avaluar el potencial dels neurofilaments com a biomarcadors de dany neuronal, valorar la seva capacitat per discriminar entre diferents malalties neurodegeneratives, així com examinar si uns majors nivells de neurofilament prediuen una pitjor prognosi en un ictus isquèmic.

Per aquest motiu, vàrem desenvolupar dos nous assajos "Enzyme-Linked ImmunoSorbent Assays" (ELISAs) per a la quantificació del neurofilament lleuger (NFL) i del neurofilament pesat fosforilat (pNFH).

Els nous ELISAs per a NFL i pNFH presentaren un bon perfil analític i les concentracions de NFL and pNFH foren similar a aquelles trobades usant diferents mètodes analítics. La concentració de NFL al líquid cefaloraquidi va ser significadament més elevada en pacients amb malalties inflamatòries desmielinitzants i amb malaltia d'Alzheimer que en pacients amb malaltia de Parkinson o controls. En els ictus isquèmics, tant NFL com pNFH mesurats en el líquid cefaloraquidi o a la sang reflectiren la dinàmica temporal de la degeneració axonal post-isquèmica . Finalment, NFL i pNFH en el líquid cefaloraquidi estaven més elevats en pacients amb esclerosis lateral amiotròfica (ELA) que en controls o pacients que tenen símptomes similars a la ELA.

Tant NFL com pNFH han demostrat ser biomarcadors sensibles i fiables de dany neuronal. Aquests resultats recolzen la utilització dels neurofilaments com a marcadors de la intensitat de la malaltia i conclouen que ambdós NFL i pNFH poden ser eines útils en la diagnosi de malalties neurodegeneratives.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Gaetani L, Höglund K, Parnetti L, **Pujol-Calderón F**, Becker B, Eusebi P, Sarchielli P, Calabresi P, Di Filippo M, Zetterberg H, Blennow K. *A new enzyme-linked immunosorbent assay for neurofilament light in cerebrospinal fluid: analytical validation and clinical evaluation*. Alzheimer's Research & Therapy. 2018;10(1):8.

II. **Pujol-Calderón F**, Portelius E, Zetterberg H, Blennow K, Rosengren LE, Höglund K. *Neurofilament changes in serum and cerebrospinal fluid after acute ischemic stroke.* Neuroscience Letters. 2019; 698:58-63.

III. Wilke C, **Pujol-Calderón F**, Barro C, Stransky E, Blennow K, Michalak Z, Deuschle C, Jeromin A, Zetterberg H, Schüle R, Höglund K, Kuhle J, Synofzik M. *Correlations between serum and CSF pNfH levels in ALS, FTD and controls: a comparison of three analytical approaches.* Clinical Chemistry and Laboratory Medicine. 2019 [Epub ahead of print].

IV. **Pujol-Calderón F**, Zetterberg H, Portelius E, Löwhagen Hendén P, Rentzos A, Karlsson JE, Höglund K, Blennow K, Rosengren LE. *Prediction of outcome after endovascular embolectomy in anterior circulation stroke using biomarkers.* (Manuscript).

V. Behzadi A*, **Pujol-Calderón F***, Tjust AE, Wuolikainen A, Höglund K, Forsberg K, Portelius E, Blennow K, Zetterberg H, Andersen PM. *Neurofilament light and heavy can differentiate ALS patients from commonly encountered diagnostic mimics.* (Manuscript).

CONTENT

ABBREVIATIONS v
1 INTRODUCTION 1
1.1 Biomarkers 2
1.2 Cerebrospinal fluid 2
1.3 Neurofilaments 3
1.4 Neurodegenerative diseases 7
1.4.1 Alzheimer's disease 7
1.4.2 Parkinson's disease 8
1.4.3 Frontotemporal dementia 9
1.4.4 Amyotrophic lateral sclerosis 10
1.4.5 Multiple sclerosis 11
1.4.6 Neurofilaments in neurodegenerative diseases 12
1.5 Acute brain injuries 14
1.5.1 Ischemic stroke 14
1.5.2 Neurofilaments in acute brain injuries 15
1.6 Other biomarkers studied in this thesis 17
1.6.1 Tau 17
1.6.2 Glial fibrillary acidic protein 17
1.6.3 Neuron-specific enolase 17
1.6.4 S100B 18
2 AIM 19
2.1 General aim 19
2.2 Specific aims of each paper 19
3 MATERIAL AND METHODS 21
3.1 Participants samples 21
3.2 Immunoassays 21
3.2.1 Enzyme-linked immunosorbent assay 21
3.2.2 Single molecule array 23

3.3 Immunoprecipitation 23
3.4 Western blot 24
3.5 Statistics 24
4 RESULTS AND DISCUSSION 27
4.1 Paper I 27
4.2 Paper II 31
4.3 Paper III 35
4.4 Paper IV 39
4.5 Paper V 43
5 CONCLUSIONS AND FUTURE PERSPECTIVES 47
ACKNOWLEDGEMENTS 49
REFERENCES 52

ABBREVIATIONS

AD	Alzheimer's disease
AD-dem	Alzheimer's disease dementia
ALS	Amyotrophic lateral sclerosis
ALS-FTD	Amyotrophic lateral sclerosis and Frontotemporal Dementia
APS	Atypical parkinsonian syndrome
ASPECTS	Alberta stroke program early computed tomography score. ASPECTS ranges 0-10, the higher the score the better the prognosis
AUC	Area under the curve
BI	Barthel index. BI ranges 0-100, the higher the score the better the prognosis
CBD	Corticobasal degeneration
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computed tomography
ECF	Extracellular fluid
EDSS	Expanded disability status scale. EDSS ranges 0-10 and the lower the score the better the patient status
ELISA	Enzyme-Linked ImmunoSorbent Assay
FET	The FET protein family includes Fused in sarcoma (FUS), Ewing’s sarcoma (EWS), and TATA-binding protein-associated factor 15 (TAF15).

FTD Frontotemporal dementia

FTLD Frontotemporal lobar degeneration

GFAp Glial fibrillary acidic protein

H&Y Hoehn & Yahr scale. H&Y ranges 0-5, the lower the score the less symptoms

HRE Hexanucleotide repeat expansion

HS Haemorrhagic stroke

IDD Inflammatory demyelinating disease

INA Alpha-internexin

IQR Interquartile range

IS Ischemic stroke

KSP Lysine-serine-proline

LLOQ Lower limit of quantification

LOD Limit of detection

LP Lumbar puncture

LVO Large vessel occlusion

MCI Mild cognitive impairment

MCI-AD Mild cognitive impairment with impairment in episodic memory and with evidence of a progressive decline in cognitive performance over time

MMSE Mini-Mental State Examination. MMSE ranges 0-30, the higher the score the better the cognitive function

MND Motor neuron disease

MRI	Magnetic resonance imaging
mRS	Modified ranking scale. mRS ranges 0-6, the higher the score the worse outcome. 6 = death
MS	Multiple sclerosis
MSA	Multiple system atrophy
mTICI	Modified thrombolysis in cerebral ischemia. mTICI ranges 0-3, the higher the score the better the recanalization
NFH	Neurofilament heavy
NFL	Neurofilament light
NFs	Neurofilament proteins
NIHSS	National Institute of Health Stroke Scale. NIHSS ranges 0-42, the higher the score the worse the stroke severity
NSE	Neuron-specific enolase
OND	Other neurodegenerative disease
PD	Parkinson's disease
PET	Positron-emitted topography
PLPH	Post-lumbar puncture headache
pNFH	Phosphorylated neurofilament heavy
PPMS	Primary progressive multiple sclerosis
PSP	Progressive supranuclear palsy
ROC	Receiver operating characteristic
RRMS	Relapsing-remitting multiple sclerosis
Simoa	Single molecule array

SOD1 Superoxide dismutase 1

SPMS Secondary progressive multiple sclerosis

SSI Scandinavian Stroke Scale Index. SSI ranges 2-56, the higher the score the better the prognosis

TBI Traumatic brain injury

TDP Transactive response DNA-binding protein

tPA Tissue plasminogen activator

t-tau Total tau

ULF Unit-length-filament

UPDRS III Unified Parkinson's Disease Rating Scale, part III. UPDRS III ranges 0-120, the lower the score the less motor symptoms

VAPB Vesicle-associated membrane protein B

VaD Vascular dementia

WB Western blot

WML White matter lesion

1 INTRODUCTION

The common denominator for all neurodegenerative diseases and acute brain injuries is the damage and loss of neurons; it is this phenomenon that gives rise to symptoms and loss of cognitive and motor functions [1]. Damaged neurons release their cytoplasm contents such as different proteins and molecules into the extracellular fluid, from where they can diffuse into adjacent body fluids such as cerebrospinal fluid (CSF) and blood. Due to this phenomenon, samples of serum, plasma or CSF can be used to measure changes in the levels of these proteins [2]. This allows the usage of protein concentrations in biofluids as biomarkers that reflect the current state of the brain and estimate the degree of neuronal damage [3, 4] (figure 1).

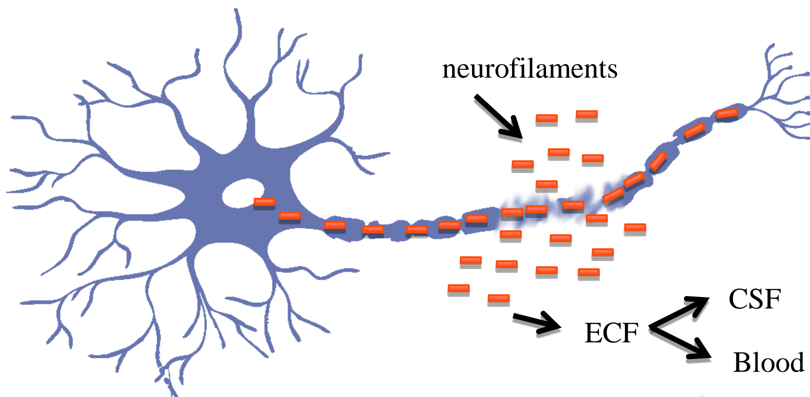

Figure 1. Protein release into extracellular fluid after neuronal damage. *Schematic description of the release of the neurofilaments into the body fluids after the axon has been damaged. ECF= extracellular fluid, CSF=cerebrospinal fluid.*

1.1 BIOMARKERS

According to the definition provided by The Biomarkers Definitions Working Group (2001) a biological marker (biomarker) is described as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [5]. The desired properties of a biomarker are that:

- The biomarker is strongly associated with the disease and absent in healthy individuals and other diseases.
- It captures a biological important aspect of the disease.
- The concentration of the biomarker reflects the severity of the disease and can predict the prognosis.
- The effect of a therapy is reflected in the change of the biomarker concentration [6].

A biomarker can have one or more applications including:

- Use as a diagnostic tool for the identification of patients with a disease or abnormal condition.
- Use as a tool for staging or classifying the extent of the disease or condition.
- Use as an indicator of disease progression and prognosis.
- Use as a monitor of the response to a treatment [5].

In humans, biomarkers can be measured in tissue or body fluids such as blood, cerebrospinal fluid, saliva or urine.

For a biomarker to be used in the clinic, it needs to first be validated and qualified. The validation process assesses the biomarker’s characteristics, such as sensitivity, specificity and determines the conditions under which the results are reproducible. Qualification is a process where evidence linking the biomarker with a biological process and/or a clinical end point is acquired through clinical studies [7].

1.2 CEREBROSPINAL FLUID

Cerebrospinal fluid (CSF) is a clear, colourless body fluid produced in the choroid plexus of the ventricles situated in the centre of the brain, as well as from the brain interstitial fluid. It occupies the ventricular system as well as

surrounds the brain and the spinal cord. CSF has two main functions, first, by submerging the brain it provides a cushion against trauma preventing mechanical injuries to the brain, and second, it provides a medium to transport nutrients and waste products to and from the brain tissue [8]. In normal healthy adults, the total volume of CSF is around 150mL. CSF is produced at a rate of around 500mL/day, which ensures a constant flow into and around the brain and the spinal cord; old CSF is eventually cleared into the blood, ensuring a stable environment and assisting in the removal of waste products [9]. Thus, by being in close contact with the central nervous system (CNS), CSF provides a good reflection of the status of the brain.

The most common procedure to obtain CSF is through a lumbar puncture (LP) between L3/L4 or L4/L5 vertebrae. It is a safe procedure and the only potential complication is post-lumbar puncture headache (PLPH). However, the incidence of PLPH is very low [10]. As a standardized protocol, 12mL of CSF are collected, then centrifuged at 2000g for 10 minutes and finally the supernatant is aliquoted and stored at -80°C until further use [11].

1.3 NEUROFILAMENTS

Visually, the most distinct characteristic of neurons is their extreme morphology with long extensions and protrusions, which makes their cytoskeleton key for their stability and consequently good cellular function.

The principal elements of the cytoskeleton are actin filaments (~7nm in diameter), intermediate filaments (~10nm in diameter) and microtubules (~25nm in diameter). Intermediate filaments have a basic role in cells providing mechanical strength and stability, whereas actin filaments and microtubules are responsible for cell movement [12].

Intermediate filaments can be classified into six types based on similarities in their amino acid sequences (Table 1) [12].

From all the cytoskeletal proteins, the ones belonging to the Type IV intermediate filaments are called neurofilament proteins (NFs) and are the only ones expressed specifically in neurons, at the exception of peripherin, a type III intermediate filament expressed in the peripheral neurons. Due to their long axons, NFs are the key in the extreme neuron morphology maintenance for a good cellular function. They share a conserved α-helical rod domain flanked by an N-terminus head domain and a variable C-terminus tail domain [13].

Table 1. Intermediate filaments classification.

Type	Protein	Size (kDa)	Site of expression
I	Acidic keratins	40–60	Epithelial cells
II	Neutral or basic keratins	50–70	Epithelial cells
III	Vimentin	54	White blood cells, fibroblasts, other cell types
	Desmin	53	Muscle cells
	Glial fibrillary acidic protein	51	Glial cells
	Peripherin	57	Peripheral neurons
IV	NFL	68	Neurons
	NFM	150	Neurons
	NFH	200	Neurons
	α-Internexin (INA)	66	Neurons (CNS-specific)
V	Nuclear lamins	60–75	Nuclear lamina of all cell types
VI	Nestin	200	CNS stem cells

The structural neurofilaments are composed of four NFs, including α-internexin (INA) (66kDa), which is CNS-specific, and the neurofilament triplet consisting of NF light (NFL) (68kDa), NF medium (NFM) (150kDa) and NF heavy (NFH) (200kDa), identified by their molecular weights (Table 1) [14, 15].

The diversity in NFs is primarily due to the length and sequence of the C-terminal tail, where NFH exhibits the longest. Its most distinctive feature is the presence of numerous lysine-serine-proline (KSP) repeat motifs, varying between 8 and 58 repeats, depending on species (figure 2a) [14].

NFs are synthetized in the cell body and then transported to the axon, where they assemble to form the filaments that give structure and stability to the neuron [16].

The mechanism behind the assembly of the NFs to form filaments follows several steps. First INA or NFL aligns with any of the other NFs, through association of the conserved rod domains, to form parallel and coiled-coil dimers. Then, two dimers line up side by side in an antiparallel manner (head to tail) to form tetramers. Thereafter, about eight tetramers will aggregate laterally to form a unit-length-filament (ULF) of approximately 55nm in length. A longitudinal aggregation of ULFs lead to the formation of immature filaments of about 16 nm of diameter. The last step is a radial compaction resulting in a close packing of the molecular filaments to form the final 10nm neurofilament (figure 2b-c) [17]. This structure is a so called "bottlebrush" because NFM and NFH tails form side arms that protrude from the central filament core formed by the compaction of all NFs rod domains.

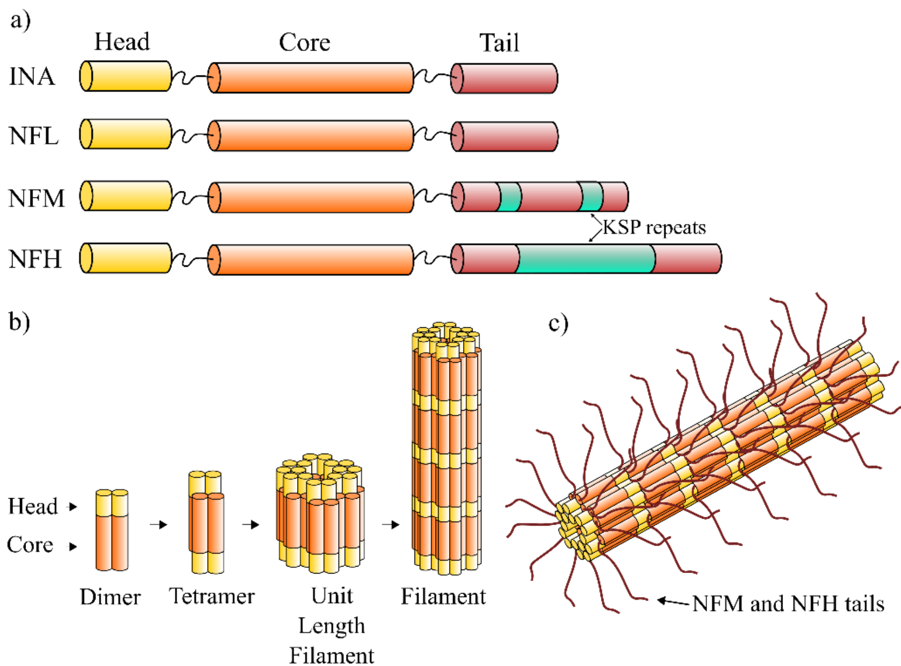

Figure 2. Type IV intermediate filaments. *a) Neurofilament protein structures. b) Neurofilament protein assemblies. c) "Bottlebrush" structure representation.*

In vitro experiments have shown that NFL is essential for the formation of neurofilaments, since it is the only neurofilament that can homopolymerize, meaning that NFM and NFH have to bind to NFL to be able to form filaments

[18, 19]. In support, *in vivo* studies have observed that NFs are compulsory heteropolymers [20]. Furthermore, it has been reported that the central α-helical coiled-coil rod domain is essential for NF assembly into dimers [21], whereas the head domain directs the lateral association of the tetramers into ULFs and the tail domain guides the axial association of ULFs into immature neurofilaments [17, 22].

Phosphorylation plays an important role in the assembly of the neurofilaments and is involved in various other functions. The phosphorylation of the NFL head domain controls the heteropolymer formation [20], inducing assembly when not phosphorylated, or disassembly when phosphorylated [18, 23]. The head domain phosphorylation may occur shortly after NFL synthesis in the cell body, suggesting that a premature assembly of NFs is avoided before their transport to the axon [24].

Many roles have been associated to the phosphorylation of the KSP motifs in the tails of NFM and NFH. Some examples are; the slowing of the NFs axonal transport, the formation of cross-bridges between neurofilaments or microtubules and the expansion of the axonal calibre [18].

NFM and NFH are found to be heavily phosphorylated after they have been transported through the axon suggesting that NFM and NFH tail phosphorylation occurs in a gradient manner along the axon, beginning when the NFs enter the axon and continuing along it until they reach their final destination [25, 26]. Another suggested function of the tail phosphorylation of NFM and NFH is to protect them from protease degradation. Dephosphorylated NFs are easily degraded by calpain, a protease found in the axons [27].

Tail phosphorylation has also been shown to modulate NFs interactions with other cytoskeletal proteins such as microtubules [28]. When NFH tail is dephosphorylated it has a high binding affinity to the microtubules, but when phosphorylated it causes their dissociation [29].

Some studies using different NF mouse models (knockout and transgenic mice) suggest that the phosphorylation of the NFM and NFH tails only contributes in part to the radial growth of big calibre axons [30] and that instead it is the subunit composition and the ratio of the NFs that determines axon calibre by controlling their number [31]. It has been shown that in the adult mouse CNS the stoichiometry of the NFs is 4:2:2:1 being NFL, INA, NFM and NFH respectively [32].

1.4 NEURODEGENERATIVE DISEASES

Neurodegenerative diseases can be defined as diseases that result from a progressive impairment in neuronal function and structure, which eventually leads to neuronal death. Neurodegenerative diseases include both dementias and movement disorders and their presentation depends on the affected area of the brain and the employed degenerative mechanism [33]. Common aspects of neurodegenerative diseases are the deposition of protein aggregates in the nucleus, cytosol and/or extracellular space [34, 35].

Some neurodegenerative diseases have a clear genetic component; if inherited, the familial form of the neurodegenerative disease in question develops [36].

In most neurodegenerative diseases, the onset of symptoms does not equate with the onset of disease pathology. The symptoms begin after enough neurons have been damaged and/or died, and the functions of the affected area cannot be maintained, meaning that the onset of the disease pathology occurs earlier in time. The time needed for the appearance of symptoms depends on the neurodegenerative process and can range from a few months to several years, depending on the neurodegenerative disease. This means that the disease may be relatively advanced by the time the symptoms are observed [33, 36].

Giving an accurate diagnose is quite complicated due to that many neurodegenerative diseases share a progressive clinical course of the disease. As such, postmortem neuropathologic evaluation is still the gold standard for the diagnosis of neurodegenerative diseases [37]. Hence finding diagnostic tools to facilitate accurate and earlier diagnosis is much needed.

1.4.1 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) was first described during the early 20th century by Alois Alzheimer in a patient who presented with memory disturbances and later develop dementia [38]. Today, AD is classified as a neurodegenerative disease clinically characterized by progressive cognitive decline, usually starting with an impairment in the ability to form recent memories, and progressing into a disruption of executive function, affecting the ability to perform daily basic activities [39]. AD is the most common cause of dementia, accounting for about 60-80% of all cases [40]. The disease affects over 46 million people worldwide (2015) with a prevalence of 5 per 100 individuals in Europe [41, 42].

Neuropathologically, AD is characterised by extracellular depositions of amyloid-beta (Aβ) forming large aggregates called plaques, and intraneuronal accumulation of hyperphosphorylated microtubule-associated protein tau (p-tau) forming fibrillary tangles [39, 43, 44]. It has been demonstrated that AD has a long pre-clinical phase that is suggested to start decades before the earliest clinical symptoms arise [45].

The diagnosis of AD is traditionally based on clinical history and cognitive testing by the clinician, and according to the latest reviewed diagnostic criteria, the clinical onset of AD can be divided into three different phases: preclinical, mild cognitive impairment (MCI), and AD dementia (AD-dem) [46-49].

The CSF biomarkers Aβ, total tau (t-tau), and phosphorylated-tau (p-tau), which are primarily used in research settings, combined with volumetric magnetic resonance imaging (MRI) and positron-emitted topography (PET) can help evaluate and monitor the progression of AD pathology as well as significantly improve the differentiation of AD from other diseases [50]. These biomarkers are used in the revised definitions of all three AD phases for different purposes. In the preclinical phase, the biomarkers are used only in research for the establishment of AD pathology in study subjects with no or very subtle clinical symptoms. In MCI and dementia stages of the disease, the biomarkers are used as a complement to the clinical diagnosis to establish the underlying pathology [47, 51].

To date, only symptomatic treatments exist for this disease [43], and treatments capable of stopping or at least effectively modifying the course of AD are still not available but under extensive research [52, 53].

1.4.2 PARKINSON'S DISEASE

Parkinson's disease (PD) is the second most common neurodegenerative disorder after AD with prevalence estimates ranging from 66 to 12500 per 100000 individuals in Europe [54]..

PD symptomatology is characterized by the classical parkinsonian motor symptoms, such as bradykinesia, resting tremor, rigid musculature and postural imbalance, as well as non-motor features including cognitive impairment, sleep disorders, olfactory dysfunction, psychiatric symptoms, autonomic dysfunction, pain, and fatigue [55, 56].

Neuropathologically, PD is characterised by the loss of dopaminergic neurons in the substantia nigra, as well as aggregation of misfolded alpha-synuclein protein in intracellular inclusions within the cell body and processes of neurons (Lewy bodies and Lewy neurites, respectively) [55, 57].

The diagnosis of PD is based on clinical features and it can only be confirmed after autopsy. However, a DaTscan, a dopamine transporter single photon emission computerized tomography imaging technique has the potential to provide support in diagnosis, especially for those patients who have an unclear presentation of parkinsonian motor symptoms [58]. Currently, no biomarkers are used in the diagnosis of PD; however, CSF alpha-synuclein has been suggested as a potential candidate [59, 60].

There are no treatments that slow the neurodegenerative process of PD. However, therapies to treat motor symptoms of PD, mainly by increasing dopamine concentrations or stimulating dopamine receptors, are available and should be administered when the symptoms cause disability or discomfort to the patient with the aim of improving their quality of life [55, 61].

1.4.3 FRONTOTEMPORAL DEMENTIA

Frontotemporal dementia (FTD) is an umbrella term that encompasses a group of neurodegenerative diseases characterized by a selective degeneration of the frontal and temporal lobes, causing progressive deficits in behaviour, executive function and/or language [62].

The estimated prevalence of FTD is 15 to 22 per 100000 people and it is the second most common dementia in persons under 65 years of age [63] and the survival time from diagnosis is around 3 to 4 years [64].

FTD is clinically diagnosed [65] and can be sub divided into three broad molecular subgroups depending on the major constituent of the intracellular protein aggregates. These groups are frontotemporal lobar degeneration (FTLD) with tau, FTLD with transactive response DNA-binding protein 43 (TDP-43) and FTLD with FET [66]. The FET protein family includes Fused in sarcoma (FUS), Ewing's sarcoma (EWS), and TATA-binding protein-associated factor 15 (TAF15). As of today, there are no specific biomarkers for FTD, however, the core biomarkers for AD (Aβ, t-tau and p-tau) can be used to differentiate AD patients from FTD patients [67, 68].

There are currently no approved disease-modifying treatments for FTD. However, there are medication strategies for the management of behavioural symptoms [69].

1.4.4 AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative motor neuron disease (MND) characterized by progressive loss of upper and lower motor neurons [70]. ALS remains a relatively rare disorder with a prevalence of five patients per 100000 people in Europe [71].

In most patients, ALS starts with a spinal onset with asymmetric, painless weakness in a limb. Weakness, muscle atrophy and fasciculations are signs of lower motor neuron damage, whereas hyperreflexia and hypertonia indicate upper motor neuron involvement [72]. In the other cases, the weakness starts in the bulbar muscles (bulbar onset), which results in symptoms such as dysarthria, dysphagia and tongue fasciculations [72]. In the majority of cases, the cause of ALS is unknown, but in about 15% a genetic cause can be found [72]. More than half of cases of familial ALS present mutations in super oxide dismutase 1 (*SOD1*), *TDP-43*, *FUS* and *C9orf72* genes [72].

Patients typically survive 2 to 5 years after symptom onset and only 5–10% survive beyond 10 years [73, 74]. There is no cure for the disease and the cause of death is most commonly due to respiratory failure [73]. However, two different treatments to slow the disease progression and increase the survival of patients are available [75].

An ALS diagnosis is made on the basis of clinical evaluation of motor symptoms and the ruling out of differential diagnoses occasionally masquerading as ALS. Due to the lack of definitive diagnostic tests for ALS and the occasionally lengthy investigations, most patients will have to wait up to a year for a diagnosis [76].

A biochemical diagnostic biomarker could be of assistance to physicians to increase the diagnostic certainty, as well as to accelerate the diagnostic work-up, especially during the early stages of the disease when it may be difficult to differentiate ALS from common mimics, such as Kennedy disease, motor neuropathies, myopathies and myelopathies [77, 78].

Approximately 15% of ALS patients show cognitive and/or behavioral dysfunction and TDP-43 positive inclusions in cortical neurons as in FTD [79].

While 15% of FTD patients present motor neuron symptoms as in ALS. Patients with clinical evidence for both disorders have ALS-FTD [80].

1.4.5 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an immune-mediated demyelinating disease that damages the CNS. The concept that the immune system plays a critical role in the pathogenesis of MS is indisputable [81, 82]. However, axonal demyelination and consequent neuronal degeneration is accepted as the major cause of permanent disability in MS patients [83, 84]. Therefore, MS is described as a primary inflammatory demyelinating disease with secondary axonal and neuronal degeneration, hence, the inclusion of MS under neurodegenerative diseases in this thesis.

MS affects approximately 2.3 million people worldwide with a prevalence of 108 cases per 100000 in Europe [85]. It causes a heterogeneous array of symptoms and signs, such as tremors, clumsiness and poor balance, vertigo, impaired swallowing, stiffness, painful spasms, temperature sensitivity and pain [86].

MS often starts with a course of recurrent and reversible neurological deficits. This phase is termed relapsing-remitting MS (RRMS). With time, the majority of RRMS patients enter a second disease phase, termed secondary progressive MS (SPMS), and characterised by continuous, irreversible neurological decline unrelated to relapses. The transition from RRMS to SPMS can only be delayed by treatment but not prevented [87]. In some patients, the course of the disease is progressive from the very first symptoms, which is called primary progressive MS (PPMS) [88]. The clinically isolated syndrome (CIS) is characterised by an episode comparable to an MS relapse but the patient does not fulfil the criteria to be classified as MS. A patient with CIS may convert to RRMS if, for example, new relapses occur [89].

The diagnosis of MS is based on typical clinical symptoms with support of MRI and laboratory tests such as oligoclonal bands in CSF and not in blood [89].

The treatment of MS can be divided into three categories: symptomatic treatment, relapse treatment and disease-modifying treatment. Symptomatic treatments are not specifically approved for MS only and are used to improve several of the patient symptoms such as pain, balance impairment, spasticity, depression and weakness. While relapse treatments improve symptoms and

short-term disability after an acute relapse, disease-modifying treatments can mitigate the disease course and improve prognosis by inhibiting inflammation [90-93].

1.4.6 NEUROFILAMENTS IN NEURODEGENERATIVE DISEASES

Since the first developed NFL assay with a high enough sensitivity to analyse CSF [94], high CSF-NFL concentrations have consistently been found in a variety of neuroinflammatory and neurodegenerative diseases.

CSF-NFL concentration has been shown to be increased in AD compared with healthy controls [95], and NFL levels correlate with disease progression [96]. NFL concentration has also been reported to be increased in serum and plasma from AD patients compared with controls, and these levels correlate with those in CSF [97, 98].

CSF-NFL has also been shown to be a possible useful biomarker for the differentiation of PD from atypical parkinsonian syndromes (APS) such as multiple system atrophy (MSA), progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD). NFL concentration is reported to be higher in APS than PD and healthy controls [99-102]. Elevated NFL concentrations in CSF have been reported also for other types of dementias, such as frontotemporal dementia and vascular dementia (VaD), where NFL levels are found to be even higher than those found in AD patients [95, 103].

Some neurodegenerative diseases share part of their symptomatology and neuropathology making it difficult to differentiate between them, as in the overlap between FTD and ALS [80]. NFL could become a useful tool since it has been shown that both serum and CSF-NFL concentrations are higher in ALS than in FTD as well as than in AD and healthy controls [98, 104-108]. Furthermore, CSF-NFL has been found to be significantly higher in patients with FTD-ALS than in patients with FTD without ALS [109].

Interestingly, phosphorylated neurofilament heavy (pNFH) also showed capability to differentiate ALS patients from controls [110, 111]. In addition, concentrations of NFL and pNFH in CSF have been shown to correlate with survival length in ALS [106, 108, 112], and CSF-NFL concentration predicted the conversion from bulbar/spinal to generalised ALS [113]. ALS mutation carriers with ALS symptoms had higher NFL in CSF and serum than those

without ALS symptoms [114], showing that elevated NFL levels are linked to the symptomatic disease phase, and that it probably is an indicator of an ongoing neurodegenerative process, suggesting the use of NFL as a progression marker. However, it is suggested that pNFH is a better diagnostic marker because it can better differentiate ALS from other diseases mimicking ALS symptoms [115].

NFL is one of the most studied biomarkers in MS. It is found to be increased in both CSF and blood in CIS, RRMS, PRMS and SPMS, especially after relapse [116-118]. CSF-NFL at disease onset may be able to predict the disease severity and the conversion from CIS to clinically diagnosed MS [119-121]. NFL concentration in both CSF and blood is reduced after treatment [122-126]. CSF-NFH correlated to relapses and disability in MS patients [127]. However, NFL has been suggested to be a better biomarker than NFH to monitor treatment effects [128].

In addition, NFL in CSF and serum is also a biomarker reflecting induced neuronal damage in a mouse model, where NFL is increased after induction of neurodegeneration but does not increase after the induction is stopped, suggesting that NFL mirrors the ongoing neurodegeneration and neuronal loss [129]. The levels of NFL also correlated with the extent of neuronal damage (assessed through immunostaining), suggesting that NFL could be used as a dynamic marker of neurodegeneration [129]. This is a confirmation that NFL is also increased in a pre-clinical model where neurodegeneration can be induced, concluding that NFL is a translational biomarker that can be used when developing therapeutics to monitor treatment effects.

1.5 ACUTE BRAIN INJURIES

Acute brain injuries are medical emergencies that differ from neurodegenerative diseases in that the death of neurons is acute rather than progressive. In these events, the neuronal damage is caused by a sudden insult to the brain. Stroke, traumatic brain injury (TBI), subarachnoid haemorrhage and hypoxic brain injury following cardiac arrest are examples of acute events.

1.5.1 ISCHEMIC STROKE

A stroke can cause long-term disability, lasting brain damage or death, making it a major public health problem [130]. Stroke occurs when the blood supply to a part of the brain is blocked (ischemic stroke; IS) or when a blood vessel in the brain bursts (haemorrhagic stroke; HS) (figure 3.). In western countries, IS accounts for 87% of total stroke type while the rest 13% is subjected to HS [131].

Common signs of stroke are face dropping, arm weakness and speech difficulty [132]. Currently, non-contrast computed tomography (CT) imaging of the brain is most routinely used for confirming the diagnosis of stroke and distinguishing IS from HS. No blood biomarkers have been validated for diagnosis and differentiation purposes [131].

During IS, the brain tissue of the affected area is deprived of oxygen, usually resulting in a fatally injured core and a salvageable surrounding area called the penumbra. The core, where the blood clot occurs, can receive about 10-25% of the normal blood flow, leading to infarction and tissue necrosis. The penumbra, which is the ischemic tissue surrounding the core, can receive blood from collateral circulation, delaying completion of the infarct and therefore the neurons in the penumbra are salvageable if the area is re-perfused in time [133].

Treatment of IS relies on the possibility to administer thrombolytic agents, such as tissue plasminogen activator (tPA), within a narrow time window of 3-4.5 hours after symptom onset [134]. After the publication of five crucial clinical trials [135-139], endovascular thrombectomy has been accepted as the standard care for patients with large vessel occlusion (LVO) in the anterior circulation [140].

The affected tissue releases neuronal and glial proteins into the CSF and blood. Potentially, these proteins can be used as biomarkers to determine the degree

of damage, as indicators of disease prognosis, as well as to predict and monitor the response to an intervention [5, 141, 142].

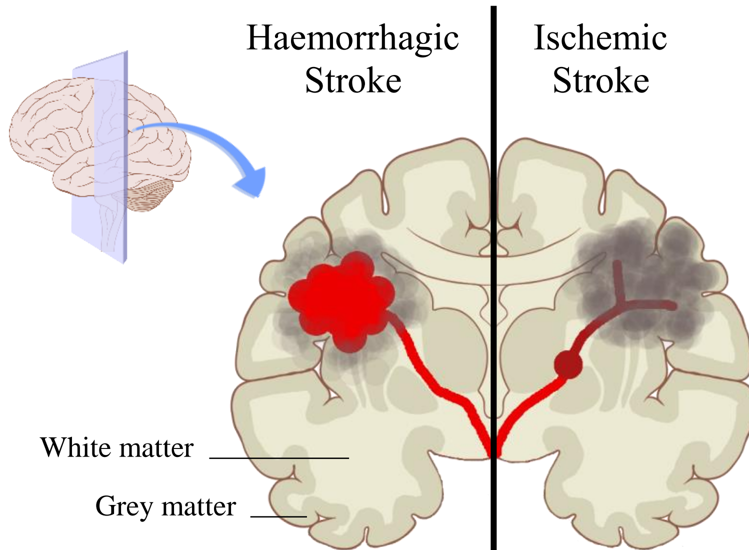

Figure 3. Schematic representation of haemorrhagic and ischemic stroke. *During haemorrhagic stroke a vessel in the brain bursts causing bleeding (red area) inside the brain, then the brain tissue is deprived of oxygen and nutrients (grey area) and begins to die; if the bleeding is severe pressure can build up inside the skull and cause tissue damage in other areas. During ischemic stroke, a vessel in the brain is clogged, and then the brain tissue is deprived of oxygen and nutrients (grey area) and begins to die.*

1.5.2 NEUROFILAMENTS IN ACUTE BRAIN INJURIES

NFL has been assessed in mild traumatic brain injuries in contact sports such as boxing, where serum-NFL was able to differentiate boxers with severe concussion from boxers with milder impacts as well as identify those boxers who would recover faster from post-concussion symptoms [143]. Similarly, NFL was increased in CSF after acute ischemic stroke

compared to controls and it was significantly associated to white matter lesions (WML) [141]. In severe brain trauma, serum-NFL concentration after time of admission in the hospital was able to differentiate patients with favourable outcome from those with poor outcome and survivors from non-survivors [144].

To date, NFM has only been reported in one clinical study, where its concentration was measured in the CSF of patients with HS, IS and controls and serum of patients with TBI and controls. The results showed that NFM was elevated in the HS group compared with IS and controls, and that there was no difference between IS and controls [145]. In addition, NFM was significantly higher in TBI than in controls [145].

However, NFH levels in CSF and serum of stroke patients have shown conflicting results. Petzold *et al.* showed no differences in CSF and serum NFH levels comparing IS and controls [142], while Sellner *et al.* reported higher levels of serum-NFH in stroke patients than in controls [146]. The contrasting results between the two studies can possibly be explained by the fact that the Sellner stroke cohort included both IS and HS and that there may be differences in how NFH behaves in these two conditions, resembling NFM [145].

Serum-pNFH has also been reported in TBI and IS studies. In IS, increasing concentrations of pNFH from day 1 to day 8 and 3-6 weeks after arrival in the hospital were reported [147]. Serum levels of pNFH at 3 weeks after IS correlated with infarct volume and the final outcome evaluated 6 months after hospital discharge [147]. In TBI, serum-pNFH levels at 72h after hospital arrival were significantly higher than at 24h after arrival. Serum-pNFH at 24 hours after injury was shown to be a good predictor of fatal outcome in patients with TBI [148].

1.6 OTHER BIOMARKERS STUDIED IN THIS THESIS

1.6.1 TAU

Tau is a microtubule-associated protein, mainly located in unmyelinated axons [149]. Its main function is to stabilize microtubules. By regulating the microtubule assembly, it allows the reorganisation of the cytoskeleton [150]. It has also been reported that tau regulates axonal transport by different mechanisms [151]. Tau has also been detected in dendrites; however, its function there is still unclear [152]. Tau is an established biomarker in neurodegenerative diseases, such as Creutzfeldt-Jakob disease and AD [153, 154], and is believed to reflect ongoing axonal degeneration. Levels of tau have also been found to be increased in both blood and CSF after stroke [141, 154]. Immunohistochemistry staining for tau has been shown to be decreased in the infarcted region of a rodent brain 24h after experimental large vessel occlusion when compared to controls, suggesting that tau is released or degraded during ischemia [155].

1.6.2 GLIAL FIBRILLARY ACIDIC PROTEIN

Glial fibrillary acidic protein (GFAp) is the main intermediate filament protein in astrocytes [156]. GFAp is a vital component of the astroglial cytoskeleton providing mechanical strength to the cell [157, 158]. It also has a number of other functions, such as playing a role in suppressing neuronal proliferation in the mature brain [159], forming a physical barrier to isolate damaged tissue [160, 161], as well as regulating the blood flow following ischemia [162]. GFAp immunoreactivity has been shown to be decreased in infarcted regions of post-mortem human brain [163]. CSF levels of GFAp are increased in neurodegenerative diseases, such as AD and multiple sclerosis [164, 165], as well as after stroke [166, 167] and TBI [168].

1.6.3 NEURON-SPECIFIC ENOLASE

Neuron-specific enolase (NSE) is an isozyme of the glycolytic enzyme enolase [169]. Human NSE is a major brain protein that constitutes between 0.4% and 2.2% of the total soluble protein of brain, depending on the region [170] making it a plausible marker of neurons [171], but NSE is also expressed in neuroendocrine tissue, erythrocytes and platelets [172, 173]. NSE has been

proposed as a biomarker for neuronal damage in TBI and stroke, and as a tool in cancer diagnostics [174-176]. Blood NSE dynamics after stroke are controversial; while some studies show an increase of NSE [177], others report no significant changes over time [178, 179].

1.6.4 S100B

S100B is one of the 20 proteins that belong to the S100 protein family; they represent the largest subgroup of Ca^{2+}-binding proteins characterized by the EF-hand structural motif [180]. In the nervous system, S100B is mainly found in astrocytes but also in other cell types and its presence is not restricted to neuronal tissue, as it is expressed, *e.g.*, in adipose tissue [181, 182]. S100B has been reported to increase in CSF and blood after stroke [167], but also in other acute disorders, such as traumatic brain injury [183].

2 AIM

2.1 GENERAL AIM

The overall aim of this thesis is to investigate and evaluate the use of neurofilaments as biomarkers in different situations involving neuronal damage.

2.2 SPECIFIC AIMS OF EACH PAPER

Paper I: To confirm the diagnostic utility of NFL using a newly developed NFL ELISA based on in-house-produced antibodies. Its performance was evaluated in different neurological disorders.

Paper II: To examine the temporal pattern of NFL and pNFH concentrations in serum and CSF after acute ischemic stroke. To test this aim, a new pNFH ELISA was developed.

Paper III: To compare the analytical sensitivity and reliability of three novel analytical approaches for the quantification pNFH in both CSF and serum in samples of ALS, FTD and control subjects.

Paper IV: To investigate the progression of nervous tissue damage and their relationship to outcome after endovascular treatment of acute ischemic stroke by the parallel analyses of tau, NFL, NSE, GFAp and S100B in blood, as well as to determine their possible use as prognostic biomarkers.

Paper V: To test the hypothesis that CSF NFL and pNFH can differentiate ALS patients from patients with ALS-like symptoms who eventually received a different diagnosis. Examine if the biomarkers are correlated to survival and if the mutation type of the mutation carriers has an influence on the biomarker levels.

3 MATERIAL AND METHODS

3.1 PARTICIPANTS SAMPLES

All participants in the studies gave their informed consent and sample collection was performed according to the ethical permissions approved by the corresponding ethical committees. More detailed information about the participants can be found in the respective papers.

For method development, de-identified CSF and serum samples from the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital were used.

3.2 IMMUNOASSAYS

There are different types of immunoassays but what all of them have in common is the use of antibodies to detect and quantify the analyte of interest.

3.2.1 ENZYME-LINKED IMMUNOSORBENT ASSAY

An enzyme-linked immunosorbent assay (ELISA) is a type of immunoassay that uses an enzymatic reaction to detect and quantify an analyte of interest. In general, the analyte (antigen) is immobilized on a surface and then detected with an antibody that is linked to an enzyme. To detect the immunocomplex, the conjugated enzyme activity is assessed by the incubation with a substrate to produce a product that can be measured through a change in colour or through light emission. The amount of the product is directly proportional to the concentration of the analyte of interest in the sample and can be quantified when compared to the assay signal generated from a set of standard samples with known concentrations of the target analyte (a standard curve).

There are different types of ELISAs depending on how the antigen is immobilized to the assay plate and detected. The analyte can be either directly bound to the assay plate or bound through a capture antibody and then, directly detected with another antibody (primary antibody) linked to the detection enzyme or indirectly detected when the enzyme is linked to a secondary antibody that binds the primary detection antibody. Commonly, the term direct ELISA is used when the analyte is directly bound to the plate, irrespectively if

it is directly or indirectly detected, and the term sandwich ELISA is used when the analyte is immobilized to the plate with a capture antibody, irrespective of a direct or indirect detection (figure 4). The most common ELISA assay format is the sandwich assay since it is more sensitive, specific and robust.

In paper I, II, IV and V a sandwich ELISA was used to detect NFL with a colorimetric substrate whereas the sandwich ELISA used to measure pNFH used a chemiluminiscent substrate.

In paper I, a direct ELISA was used to screen the cell media for NFL antibodies.

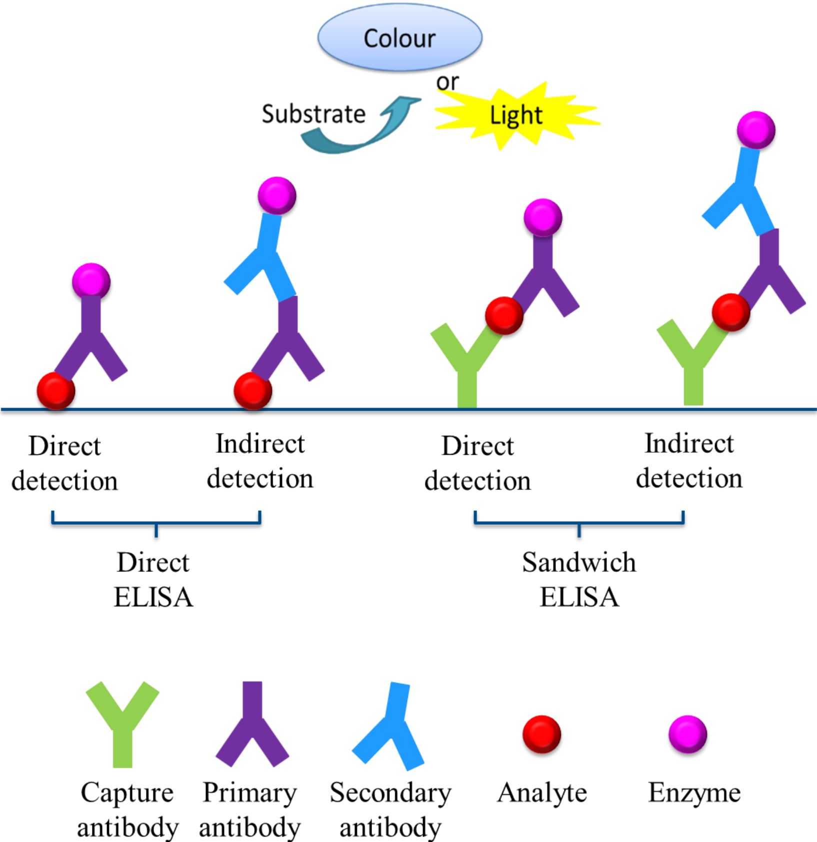

Figure 4. Schematic representation of different types of ELISA.

3.2.2 SINGLE MOLECULE ARRAY

Single molecule array (Simoa) is the newest type of immunoassays and is somewhat similar to a sandwich ELISA with the difference that it uses magnetic beads coated with the capture antibody and a fluorescent detection enzyme with a compartmentalized detection reaction in microwells into which only one magnetic bead per well can fit.

The general procedure for a Simoa is as follows: after the binding of the analyte to the antibody-coated beads, nonspecific proteins are washed away and the beads are then incubated with a biotinylated detector antibody. The incubation with detector antibodies is followed by another incubation with β-galactosidase-labelled streptavidin (SBG). Beads are then individually placed in wells containing substrate, sealed in with oil and finally imaged. The concentration of the analyte is determined by counting the wells that contain a bead compared to the total amount of wells containing a bead that emits a fluorescent signal (digital signal). If the concentration of the analyte is too strong or takes too long, the samples will be detected using an analogue technique wherein the total amount of fluorescence emitted by all wells is detected [184].

The Simoa is capable of improving the sensitivity of a normal ELISA ~100-1000 times depending on the analyte [184].

In paper II, Simoa was used to measure NFL concentration in blood.

In paper III, two different Simoa methods were used to measure pNFH concentrations in CSF and blood.

In paper IV, Simoa was used to measure NFL, tau and GFAp concentrations in blood.

3.3 IMMUNOPRECIPITATION

Immunoprecipitation (IP) is an antibody-based enrichment technique, where the principle is to couple a specific antibody capable to capture the protein of interest onto magnetic beads. The coupled beads are then incubated in the sample where the antibodies will bind the protein of interest and form a bead-antibody-protein complex that can be separated from the rest of the sample

proteins by using a magnet. In the end, there is an elution step isolating your protein of interest.

In paper I, IP was used to characterize the in-house produced NFL antibodies.

3.4 WESTERN BLOT

Western blot (WB) is a technique that can be used to both characterize antibodies and to detect specific proteins in a sample. Its principal consists of three steps. First, the sample proteins are separated based on size in an electrophoresis gel. Second, the proteins are transferred onto a membrane, where they are fixed. Third, the protein of interest is detected with an antibody. If you would stain the gel after the electrophoresis, using a general protein stain, such as silver or Coomassie, you would see all the protein bands that your sample contained, whereas using WB you only see the bands corresponding to the protein your antibody has bound to. To characterize antibodies, you check for cross-reactivity by including other similar proteins (in our case the other NFs) and evaluate if the antibody can bind to them. Fragments of the target protein can also be included to define where on the protein the antibody binds.

In paper I, WB was used to characterize the in-house produced NFL antibodies.

3.5 STATISTICS

In paper I, a likelihood ratio test based on generalized linear models was used to test if the distribution of demographics and clinical features differed between each diagnostic group subcategories. NFL was log-transformed to reach normality and an analysis of covariance (ANCOVA) was used to test if CSF NFL log values would differ between the diagnostic groups. The diagnostic accuracy of NFL was assessed by the receiver operating characteristic (ROC) curve at the point that maximized the Youden index (sensitivity + specificity-1). The statistical analyses were performed using R software, version 3.3.1. All tests were two-sided and significance was set at $p \leq 0.05$.

In paper II, a Mann-Whitney test was used for comparisons between controls and the other time points. Mixed effect model analyses were performed on each biomarker repeated measurements where subjects were included as random

factors and age and gender as covariates. Correlations between groups were analysed with Spearman's non-parametric test. All tests were two-sided and significance was set at $p \leq 0.05$. Statistical analyses were performed using SPSS software, version 23.0. Samples below the limit of detection (LOD) were included in the statistical analysis as half the LOD (0.033ng/mL).

In paper III, Spearman's non-parametric test was used to analyse correlations of pNfH concentrations between and within analytical approaches. Mann-Whitney tests were used to determine if there are differences in pNFH concentrations between the different diagnosis groups. All tests were two-sided and significance was set at $p \leq 0.05$. Statistical analyses were performed using SPSS software, version 23.0.

In paper IV, the change of the biomarker concentration over time was analysed with mixed effect models. Subjects were included as random factors and age, gender, NIHSS at admission, type of anaesthesia, the use of intravenous thrombolysis and the use or no use of vasoactive drug during the embolectomy procedure as covariates. The correlations between groups was calculated with Spearman's non-parametric tests. To predict the unfavourable outcome of ischemic stroke patients based on the biomarker level and the clinical income parameters ROC analysis was performed and Youden index was used to describe their performance. Statistical significance was set at $p \leq 0.05$. Statistical analyses were performed using SPSS software, version 23.0 and Microsoft excel (2016) was used to calculate Youden index.

In paper V, to compare the neurofilament concentrations between ALS patients and the other groups, a one-way ANOVA with pre-determined contrast tests and a 1000 run, bias-corrected accelerated bootstrap was used. The relationship between neurofilament concentrations and patient survival was investigated with a Spearman's non-parametric test. Patients were grouped into three groups based on a survival of <2 years, $2<10$ years and $10<$ years and analysed the differences in biomarker levels between the groups using a one-way ANOVA with a polynomial linear, weighted progression test. Statistical significance was set at $p \leq 0.05$. Statistical analyses were performed using SPSS software, version 24.

4 RESULTS AND DISCUSSION

4.1 PAPER I

To date, most previous studies have used the same commercial ELISA to measure NFL in CSF. To confirm the extensive evidence for NFL as a reliable biomarker for axonal damage, we developed a new ELISA method and in this paper we present its performance for the measurement of CSF-NFL in different neurological disorders.

Two antibodies were produced for this ELISA, NFL-21 and NFL-23. Both antibodies showed high affinity binding full length bovine NFL and human recombinant NFL, as well as to the core of recombinant NFL in western blot and direct ELISA.

The newly developed ELISA had a range between 5000pg/mL and 39pg/mL with a lower limit of quantification (LLOQ) of 78pg/mL and an upper limit of quantification (ULOQ) of 10000pg/mL. Its within-plate and inter-plate variations were 8% and 13%, respectively. The CSF samples diluted linearly, meaning that a sample with a concentration above the ULOQ can be diluted to a concentration within the working range and still give a reliable result, and the spike recovery was between 80 and 109% concluding that the concentration–signal relationship is similar in the calibration curve and the samples. The new ELISA uses bovine NFL as standard protein, it did not cross-react with NFM or NFH and correlated strongly with the commercial one ($r=0.9984$, $p<0.001$).

For the entire cohort, we found a weak correlation between CSF-NFL and age ($r=0.19$, $p<0.01$) as well as that males had slightly increased NFL than females (889pg/mL vs 808pg/mL respectively, $p<0.05$).

CSF-NFL was significantly higher in both the inflammatory demyelinating diseases (IDD) and MCI-AD/AD-dem groups when compared to the other neurological diseases (OND) group ($p<0.001$ for both comparisons) as well as when compared to the Parkinson's disease group (IDD vs PD $p<0.05$ and MCI-AD/AD-dem vs PD $p<0.01$). There were no statistical difference between the IDD and MCI-AD/AD-dem groups or between the PD and OND groups. When adjusted for age, NFL could differentiate IDD patients from OND patients with a sensitivity and specificity of 86% and 83%, respectively (AUC=0.87); MCI-AD/AD-dem versus OND with a sensitivity and specificity of 94% and 75%, respectively (AUC=0.84) and PD was differentiated from OND with a

sensitivity and specificity of 97% and 42%, respectively (AUC=0.69) (figure 5).

Within the IDD group, CSF-NFL was not significantly different between the MS sub-groups (CIS, RRMS, PPMS and SPMS). However, it was significantly higher in patients who had a recent relapse (30 days before CSF sampling) than in patients with no evidence of recent disease activity (p<0.001). Moreover, the CSF-NFL levels correlated with the degree of neurological impairment, scored with the Expanded Disability Status Scale (EDSS), at the time of the lumbar puncture (r=0.23, p<0.05). Several associations were found between different magnetic resonance imaging (MRI) features and CSF-NFL concentrations. Finally, CSF-NFL did not correlate with the time between the first clinical symptom manifestation and the CSF sampling.

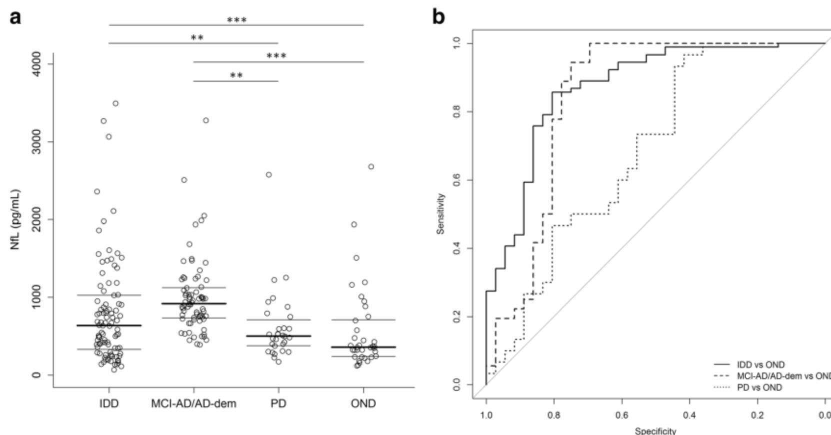

***Figure 5. CSF NfL values (pg/ml) in the different diagnostic groups**. a) CSF neurofilament light (NfL) values in IDD, MCI-AD/AD-dem, PD, and OND groups; p values are from the ANCOVA adjusted for age and sex; *p < 0.05; **p < 0.01; ***p < 0.001. b) Diagnostic value of CSF NfL. IDD vs OND comparison (age-adjusted AUC = 0.87, 95% CI 0.80–0.95) is reported as the solid line. MCI-AD/AD-dem vs OND comparison (age-adjusted AUC=0.84, 95% CI 0.74–0.95) is reported as the dashed line. PD vs OND comparison (age-adjusted AUC = 0.69, 95% CI 0.56–0.81) is reported as the dotted line. AD-dem Alzheimer's disease dementia, IDD inflammatory diseases of the central nervous system, MCI-AD mild cognitive impairment due to Alzheimer's disease, OND other neurological diseases, PD Parkinson's disease. Reprinted from Alzheimer's Research & Therapy volume 10, Article number: 8. © 2018 The Author(s).*

Within the MCI-AD/AD-dem group, no statistical difference was found between the concentrations of CSF-NFL from MCI-AD patients and AD-dem patients. Furthermore, no correlation was seen between CSF-NFL and cognitive function at baseline, follow-up nor the change over time. The cognitive function was scored with the Mini-Mental State Examination (MMSE) test. In addition, CSF-NFL concentration did not correlate with disease duration.

Within the PD group, we observed a positive correlation between CSF-NFL levels and motor symptoms scored with the Unified Parkinson's Disease Rating Scale, part III (UPDRS III) ($r=0.48$, $p<0.05$). However, when adjusting for age it became non-significant. No statistical significant correlations were found between CSF-NFL and symptom progression, baseline and follow-up cognitive function, or disease duration. The symptom progression was scored with the Hoehn & Yahr scale (H&Y).

In this paper, we reported the performance of a new established ELISA to analyse NFL in CSF. The assay displayed robust and accurate measurements of NFL in CSF with no cross-reactivity with NFM or NFH. There was a strong correlation with the previously established commercial ELISA.

Our observation of increased NFL in the CSF of IDD patients is in line with the results from other studies showing that CIS, RRMS,PPMS and SPMS patients have higher CSF-NFL than controls (OND) [121, 185] and that the diagnostic accuracy of our ELISA is similar to the one reported with the commercial ELISA [185]. In our IDD cohort, CSF-NFL concentrations were not significantly different between the different sub-groups. A reason for this result could be the low number of patients with progressive MS ($n = 8$). However, we found a non-significant increasing trend in CSF-NFL values from the isolated syndromes to RRMS, and to the progressive MS patients, thus suggesting that axonal damage increases during the disease course. Furthermore, we showed that CSF-NFL correlated to several clinical and MRI measures of disease severity.

Similarly, the observation that the MCI-AD/AD-dem had higher CSF-NFL than PD or controls (OND) is concordant with other studies [96, 97]. The results suggest that measurement of CSF-NFL with the newly developed ELISA has a similar diagnostic accuracy compared with the commercially available ELISA. Compared to the classical AD biomarkers, NFL lacks in pathophysiological specificity. Therefore, it is desirable to use it in combination to Aβ42, t-tau, and p-tau in the diagnostic work-up of neurodegenerative dementias.

The non-significant difference between CSF-NFL concentrations in PD and controls (OND) that we observed was also consistent with the results of a previous study [186].

The key findings of this study are: 1) good analytical performance of the new ELISA for CSF-NFL, and 2) the confirmation of significantly higher CSF-NFL concentrations in IDD and MCI-AD/AD-dem groups when compared to PD or controls (OND).

4.2 PAPER II

Neurofilament light is a well-known biomarker for neuronal injury and neurodegeneration. However, NFH has been less extensively studied and the potential of phosphorylated NFH (pNFH) as a stroke biomarker and for the prediction of clinical outcome is unknown. In this study, we aimed to examine the temporal pattern of NFL and pNFH concentrations in serum and CSF from patients who have been followed over time after acute ischemic stroke. For this purpose, we developed a new ELISA to detect pNFH.

The new pNFH ELISA used two commercial available antibodies (NF-01 and NF-05) and a NFH recombinant protein as standard. It had a measurement range between 0.077 and 50ng/mL, a limit of detection (LOD) of 0.066ng/mL and the inter-plate precision was 8.8%. The samples did not dilute in a linear way, hence all samples were analysed neat. When the calibrator was incubated together with alkaline phosphatase, the ELISA signals were reduced. Therefore, we conclude that our assay is specific for the phosphorylated form of NFH.

The levels of NFL in CSF and serum showed a similar distribution pattern over time. The levels increased over time from Day 0-1 reaching the highest value on the 3rd week and decreased almost back to normal after 3-5 months after stroke. Similarly, serum and CSF pNFH levels increased over time reaching their peak at 3 weeks and returning to baseline values after 3-5 months of stroke onset (figure 6).

CSF-NFL and serum-NFL correlated with each other at several time points with the best correlation at 3-5 months ($r=0.782$; $p<0.0001$). However, serum-pNFH and CSF-pNFH did not correlate to each other at any time point. A low correlation between CSF-NFL and CSF-pNFH was observed on Day 2-3 ($r=0.513$; $p<0.05$). No other correlations were significant between CSF-pNFH and CSF-NFL or serum-NFL and serum-pNFH.

The size (cm^2) of the infarcted area was calculated 3-5 months after stroke trough neuroimaging techniques such as computed tomography (CT) or magnetic resonance imaging (MRI). CSF-NFL and serum-NFL correlated with the size of infarct on Day 7-9 ($r=0.44$; $p<0.05$ and $r=0.91$; $p<0.0001$ respectively), 3 weeks ($r=0.72$; $p<0.0001$ and $r=0.81$; $p<0.001$ respectively) and 3-5 months ($r=0.63$; $p<0.001$ and $r=0.69$; $p<0.001$ respectively). CSF-pNFH and serum-pNFH did not correlate with the infarct volume at any time point.

The volume (mL) of the infarct was calculated for those patients who received an MRI at 3-5 months. CSF-NFL correlated significantly with the volume at 3 weeks and 3-5 months (r=0.76; p<0.001; r=0.65; p<0.01 respectively), whereas serum-NFL only correlated on Day 7-9 (r=0.72; p<0.05). CSF and serum pNFH did not correlate with the volume of the infarction at any time point.

The patient's status was evaluated with the modified Scandinavian Stroke Scale index (SSI) at the same time points as when the samples were taken. A correlation was found between SSI and CSF-NFL at three weeks (r=0.60; p<0.01) and 3-5 months (r=0.43; p<0.05). However, none of the other biomarkers correlated with SSI at any time point. SSI was found to correlate with the size of infarction on Day 7-9 (r=0.43; p<0.05) and at three weeks (r=0.45; p<0.05), but did not correlate with infarction volume at any time point.

Barthel Index (BI) was used to score the performance in activity of daily living 3-5 months after stroke. We found that CSF-NFL correlated negatively with BI at three weeks (r=-0.72; p<0.0001) and 3-5 months (r=-0.67; p<0.0001), whereas serum-NFL and BI correlated negatively on Day 2-3 (r=-0.79; p<0.001), Day 7-9 (r=-0.77; p<0.01), three weeks (r=-0.77; p<0.001) and 3-5 months (r=-0.72; p<0.0001). BI also correlated with patient status (SSI) on Day 2-3 (r=-0.45; p<0.05), Day 7-9 (r=-0.59; p<0.01), at three weeks (r=-0.59; p<0.01) and 3-5 months (r=-0.67; p<0.0001).

In this paper, we show the progression of CSF and serum NFL and pNFH over the course of 3-5 months after stroke, showing that both biomarkers had their peaks at 3 weeks after stroke. It has been previously reported that from day 0 to 3 weeks serum-pNFH progressively increases after stroke, here we corroborate that finding as well as show that both pNFH and NFL in serum and CSF decrease back to baseline values after 3-5 months of stroke onset, showing that axonal breakdown is no longer prevailing.

NFL showed a better correlation with size than with volume, this could be attributed to the lower number of volume determinations consequent to MRI contraindication in several patients (eg cardiac pacemaker).

Outcome measured as BI correlated well with the levels of NFL in serum from all time points, except Day 0-1, whereas CSF-NFL levels only correlated at three weeks and 3-5 months. The reasons why pNFH from both serum and CSF or NFL in CSF at the early time points did not correlate with outcome is obscure. However, little is known about how these biomarkers are transported from the damaged tissue to serum. Different pathways would probably

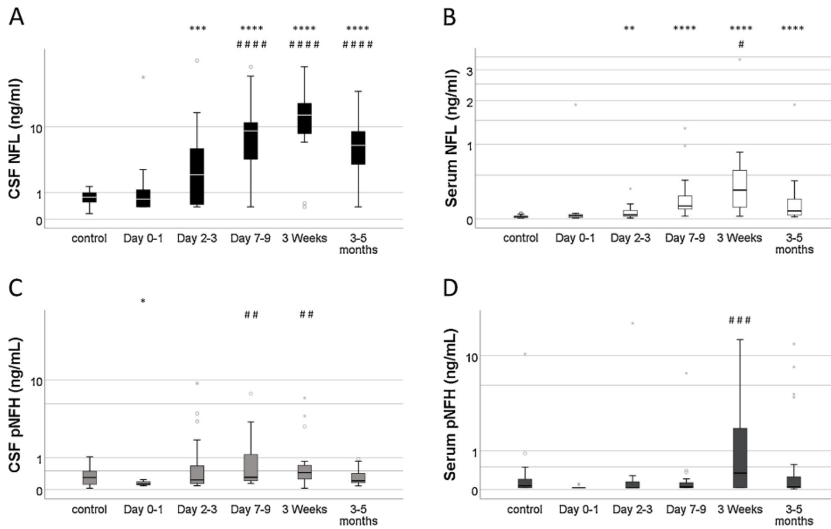

Figure 6. Biomarkers evolution over time after acute ischemic stroke. *Represented as median with inter quartile range, °=outlier, x=extreme value. Statistical significance comparing to group control *=p≤0.05; **=p≤0.01; ***=p≤0.001; ****=p≤0.0001. Statistical significance comparing to Day 0-1 #=p≤0.05; # #=p≤0,01; # # #=p≤0.001; # # # #=p≤0.0001.*
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implicate different kinetics. Also, regarding pNFH, the antibody-antigen interaction is dependent on the phosphorylation state of the protein. pNFH released from degraded axons in anoxic tissue might also get dephosphorylated, at least partly. This could be one reason for the overall lower values for pNFH compared with NFL observed in this study. The NFL protein, on the contrary, is only lightly phosphorylated and dephosphorylation probably would not change the antigen-antibody interaction of the antibodies used in the assay system in this study.

Patient status, periodically evaluated with the SSI, correlated with the size of infarction on Day 7-9 and at three weeks. On Day 2-3, Day 7-9, at three weeks and after 3-5 months, SSI also correlated with outcome (BI). Interestingly, both CSF and serum NFL correlated with outcome and the size of the infarction at the same time points as SSI but with better correlation values, suggesting that the extension of the damage in the brain is better reflected by the biomarkers and that they can predict outcome more precisely than the clinical scoring. However, when we intended to calculate at which time point the biomarkers

would have a better prediction of outcome, it became evident that our data set did not have enough patients classified as “bad outcome” to be able to compute receiver operating characteristic (ROC) curves. Therefore, the potential use of NFL and pNFH as predictors of outcome could not be evaluated.

The key finding of this study is that both CSF and serum NFL and pNFH show the dynamics of neuronal injury after stroke with the highest levels on week 3 and a decrease to almost baseline concentrations at 3-5 months after stroke.

4.3 PAPER III

Analytical methods for the measurement of pNFH by ELISA differ largely between laboratories [187-191] and promising novel Simoa approaches have not yet been compared in a standardised manner. Furthermore, the associations of CSF-pNFH with disease progression and survival have not yet been consistently reproduced in blood [3, 187, 191]. In view of these challenges, we aimed to compare three novel analytical approaches (one ELISA, a homebrew Simoa and a commercial Simoa) for the measurement of pNFH concentrations by analysing matched pairs of CSF and serum samples of ALS, FTD and control subjects.

All CSF samples were above the analytical sensitivity of each method whereas for serum, 97.1% of the samples were over the analytical sensitivity in the homebrew Simoa, 100% in the commercial Simoa and 50% in the ELISA. Overall, the coefficients of variation (CVs) were lower in CSF than in serum.

CSF-pNFH concentrations were highly correlated between the different methods: homebrew Simoa vs commercial Simoa: $r=0.91$, $p<0.001$; homebrew Simoa vs ELISA: $r=0.99$, $p<0.001$ and commercial Simoa vs ELISA: $r=0.94$ $p<0.001$ (figure 7 A-C).

Serum-pNFH measurements were also highly correlated between both Simoa methodologies ($r=0.95$, $p<0.001$). However, correlations between ELISA and both Simoa approaches were weak (homebrew Simoa: $r=0.55$, $p<0.001$; commercial Simoa: $r=0.37$, $p<0.05$) (figure 7 D-F).

CSF and serum sample measurements strongly correlated in both Simoa approaches (homebrew Simoa: $r=0.62$, $p<0.001$, and commercial Simoa: $r=0.62$, $p<0.001$) but not in the ELISA ($r=0.19$, $p>0.05$) (figure 7 G-I).

All three methods showed significantly higher CSF-pNFH concentrations in ALS than in FTD and controls (ALS vs FTD $p<0.001$ and ALS vs controls $p<0.001$ for all assays). Furthermore, if measured with the ELISA method CSF-pNFH was higher in FTD vs controls ($p<0.05$) but not when measured with any of the Simoa assays (figure 7 J-L).

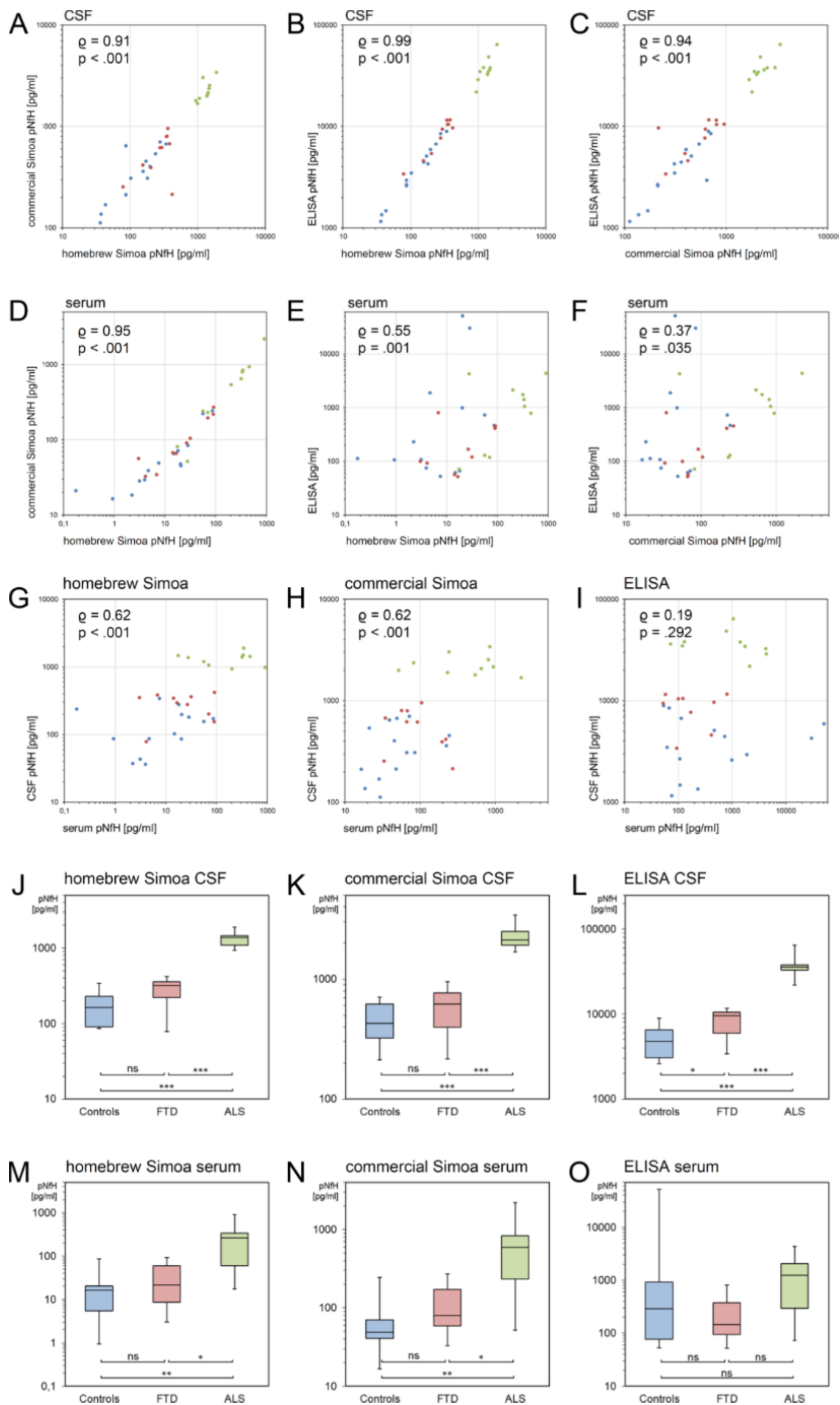
A
CSF
ρ = 0.91
p < .001
commercial Simoa pNfH [pg/ml]
homebrew Simoa pNfH [pg/ml]
B
CSF
ρ = 0.99
p < .001
ELISA pNfH [pg/ml]
homebrew Simoa pNfH [pg/ml]
C
CSF
ρ = 0.94
p < .001
ELISA pNfH [pg/ml]
commercial Simoa pNfH [pg/ml]
D
serum
ρ = 0.95
p < .001
commercial Simoa pNfH [pg/ml]
homebrew Simoa pNfH [pg/ml]
E
serum
ρ = 0.55
p = .001
ELISA [pg/ml]
homebrew Simoa pNfH [pg/ml]
F
serum
ρ = 0.37
p = .035
ELISA [pg/ml]
commercial Simoa pNfH [pg/ml]
G
homebrew Simoa
ρ = 0.62
p < .001
CSF pNfH [pg/ml]
serum pNfH [pg/ml]
H
commercial Simoa
ρ = 0.62
p < .001
CSF pNfH [pg/ml]
serum pNfH [pg/ml]
I
ELISA
ρ = 0.19
p = .292
CSF pNfH [pg/ml]
serum pNfH [pg/ml]
J
homebrew Simoa CSF
pNfH [pg/ml]
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Figure 7. Associations between pNfH measurements across analytical approaches in CSF (A–C) and serum (D–F), correlations between CSF and serum pNfH measurements within analytical approaches (G–I), pNfH concentrations in FTD, ALS and control subjects measured with different approaches in CSF (J–L) and serum (M–O). *CSF pNfH measurements were highly correlated across all three analytical approaches (A–C). Dot colour indicates diagnosis (blue: controls, red: FTD, green: ALS). For serum measurements, pNfH levels were highly correlated between both Simoa approaches, while the correlations between ELISA and both Simoa approaches were considerably weaker (E–F). Correlation between paired CSF and serum samples was strong for both the homebrew Simoa and the commercial Simoa approach, but not significant for the ELISA measurements (G–I). In CSF, all three approaches yielded significantly higher pNfH levels in ALS than in both FTD and age-matched control subjects (J–L), CSF pNfH levels of FTD subjects were significantly higher than those of controls if measured by ELISA (L) (p-values Bonferroni-corrected for multiple comparisons, *** p < .001, ** p < .01, * p < .05, ns: not significant). In serum, significantly higher pNfH levels in ALS than in FTD and control subjects were only found by the Simoa approaches (M–O). Central horizontal lines indicate median values, boxes illustrate the ranges between lower and upper quartiles, and error bars represent the full ranges of data. Please note the logarithmic scale of the x- and y-axis.*
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When analysed with both Simoa methodologies serum-pNFH was significantly higher in ALS vs FTD (p<0.01 for both) and in ALS vs controls (p<0.001 for both) but not when measured with the ELISA. Though cohorts were not sufficiently powered to yield statistical significance, serum-pNfH levels were also quantitatively higher in FTD than in control subjects across all three approaches (figure 7 M-O).

In this paper, we report that pNfH concentrations could be sensitively and reliably quantified by all three novel analytical approaches in CSF and that the Simoa assays also provided reliable and sensitive measurements of pNFH in serum.

In CSF, pNFH concentrations were highly correlated between the three analytical assays, an important characteristic for the comparison across centres and future multicentre trials. However, the serum pNFH measurements only

showed a strong correlation between the two Simoa assays. A weaker correlation between the ELISA and both Simoa methods might be due to the lower analytical sensitivity and the high number of serum samples that fell outside the analytical range of the ELISA.

Serum-pNFH measurements correlated strongly with CSF measurements, when measured by either of the two Simoa approaches but not when analysed with ELISA. The correlation between CSF and serum pNFH levels measured by ELISA might result from its limitations in sensitivity, affecting the serum measurements.

All three pNFH assays seemed suited to unveil relevant findings in the neurodegenerative disease cohorts. All three methods distinguished ALS subjects from control and FTD subjects by CSF-pNFH concentrations, with the Simoa assays also detecting this distinction in serum. These findings support the validity of the assays and extend previous findings of increased pNFH levels in ALS by demonstrating that this increase is consistent and reliable across different analytical approaches.

The key finding of this paper is that CSF-pNFH concentrations are correlated between the different assays and consistently increased in ALS patients compared to controls, independently of which assay was used.

4.4 PAPER IV

We have previously reported how NFL concentrations in CSF and serum progress after acute ischemic stroke (paper II). However, we could not determine its prognostic capabilities due to the characteristics of our cohort. In this paper, we aimed to investigate several biomarkers (NFL, tau, GFAp, NSE and S100B) in blood to further understand the progression of nervous tissue damage and their relationship to outcome after endovascular treatment of acute ischemic stroke, as well as to determine their possible use as prognostic biomarkers and their relationship with clinical biomarkers.

Blood samples were taken before endovascular treatment (pre) and 2h, 24h, 48h, 72h and 3 months after treatment. Each biomarker progressed slightly differently over time. Tau was lowest before endovascular treatment, progressively increased until 72h after treatment and decreased back to baseline at 3 months. NFL had a slower increase over time when compared to tau, with its lowest concentration found at pre-embolectomy to slow and constantly increase until 3 months after treatment. GFAp and S100B both reached their peak at 48h and decreased back to baseline values after 3 months, whereas NSE remained constant over time (figure 8).

The volume of the infarcted area was measured on day 1 using a CT and on day 3 using an MRI. Both volumes correlated to all blood biomarkers at least at two time points. However, GFAp was the only marker that already at 2h correlated significantly with volumes day 1 and 3 ($r=0.51$, $p<0.0001$ and $r=0.47$, $p<0.0001$, respectively).

Clinical stroke severity was estimated using the National Institute of Health Stroke Scale (NIHSS) at admission and at 24h after endovascular treatment. Tau, NFL and GFAp correlated to admission NIHSS at least at one time point while NSE and S100B did not correlate at any. All blood biomarkers correlated to NIHSS at 24h at least at one time point.

The Alberta Stroke Program Early CT score (ASPECTS) was used to assess stroke severity using available CT data. On day 3, all blood biomarkers correlated with ASPECTS, at least at one time point, whereas only NFL, tau and GFAp correlated with ASPECTS at admission.

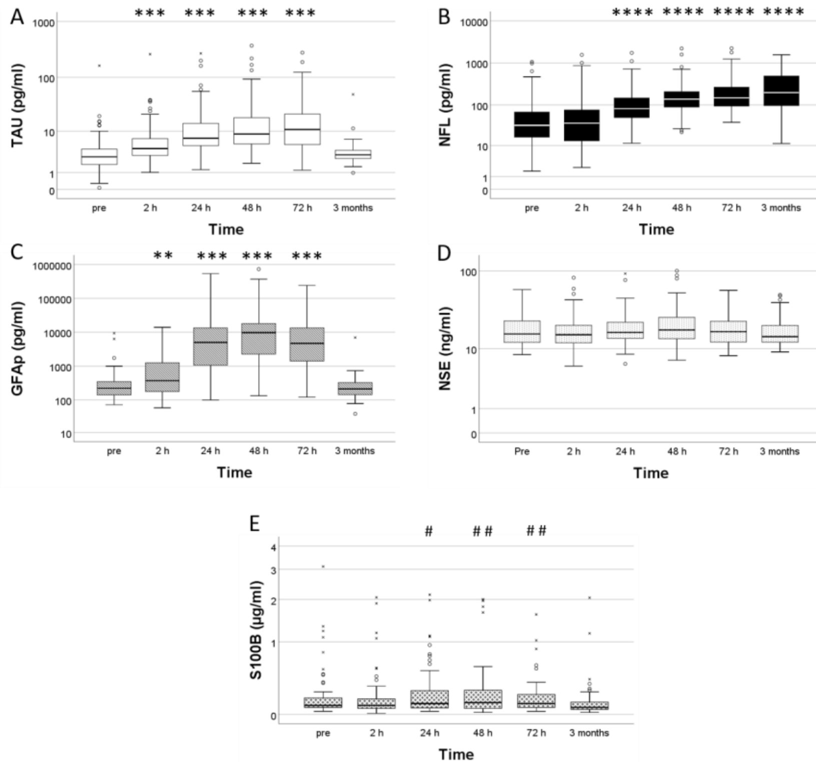


Figure 8. Biomarker progression. Concentration of biomarkers per time point (pre, 2h, 24h, 48h, 72h and 3 months). *A) TAU pg/ml. B) NFL pg/ml. C) GFAP pg/ml. D) NSE ng/ml. E) S100B µg/ml. °=outlier, x=extreme value. Statistical significance comparing to pre* ✱*=p≤0.05;* ✱✱*=p≤0.01;* ✱✱✱*=p≤0.001;* ✱✱✱✱*=p≤0.0001. (S100B: statistical significance comparing to 3 months #=p≤0.05; # #=p≤0.01; # # #=p≤0.001; # # # #=p≤0.0001).*

The degree of recanalization after the embolectomy, evaluated by angiography, was defined according to the modified thrombolysis in cerebral ischemia (mTICI). Only GFAp and NSE correlated with mTICI (two time points and one time point respectively). Tau, NFL and S100B did not correlate to mTICI at any time point.

The modified ranking scale (mRS) was used to assess the degree of disability or dependence in the daily activities 3 months after stroke. All blood biomarkers correlated with mRS at least at one time point, with both tau and GFAp already correlating to mRS at 2h after stroke. All clinical biomarkers

(admission NIHSS, NIHSS at 24h, ASPECTS at 72h, infarct volume day 1, infarct volume day 3 and mTICI) at the exception of admission ASPECTS, also correlated with the severity of outcome 3 months after stroke.

The best time to predict poor outcome, defined as mRS≥3, was for tau at 2h or 48h (AUC=0.76 both) and for GFAp at 72h (AUC=0.81). NFL had the highest predictive capacity at 3 months (AUC=0.88). However, since 3 months is the time as the outcome was evaluated, we therefore assigned 72h (AUC=0.79) as the best time for NFL to predict for outcome. Both NSE and S100B predicted best at 24h (AUC=0.67 and 0.70 respectively). Most clinical biomarkers had a good prediction of poor outcome. Admission NIHSS (AUC=0.65), NIHSS at 24h (AUC=0.86), admission ASPECTS (AUC=0.60), ASPECTS at 3 days (AUC=0.77), infarct volume on day 1 (AUC=0.73), infarct volume on day 3 (AUC=0.78) and mTICI (AUC=0.61).

To determine if the prediction for poor outcome could be improved, we combined clinical parameters and blood biomarkers. The combination of the clinically accessible parameter NIHSS at 24h, and biomarkers with AUCs>0.7 at 48h, *i.e.*, tau, NFL and GFAp were chosen. All combinations had similar predictive capacity (AUC=0.891, 0.894 and 0.890, respectively) and improved the prediction of poor outcome when compared to the prediction with a single factor.

In this paper, we reported the different progression patterns of tau, NFL, GFAp, NSE and S100B in blood after acute ischemic stroke and their potential use together with clinical biomarkers to predict for poor outcome after 3 months.

The different kinetics of each blood biomarker possibly reflect the different cellular and subcellular locations, considering that GFAp and S100B are located in astroglial cells and tau, NFL and NSE are located in neurons. Furthermore, tau is more prevalent in thin unmyelinated axons whereas NFL is mainly expressed in large myelinated axons. However, S100B and NSE are also present outside the nervous system, *e.g.*, in adipose and neuroendocrine tissue, as well as in blood cells.

Tau, NFL and GFAp were moderately to highly correlated with infarct volume, NIHSS at 24h and mRS at 3 months. Regarding NSE and S100B, however, correlations were very low and mainly insignificant. In the scientific community, there is controversy regarding the usefulness of NSE and S100B as biomarkers of CNS injury since there are opposite results in different studies [192-194]. Our results suggest that tau, NFL and GFAp in blood reflect the

extent of nervous tissue damage after ischemic stroke, whereas NSE and S100B are of little value.

Regarding the prediction of outcome, the best performing biomarker was NFL at 72h followed by GFAp at 72h and tau at 2 hours. NIHSS at 24h predicted outcome slightly better than the blood biomarkers, whereas ASPECTS at day 3 and infarct volume at both days 1 and 3 had similar prediction levels. Admission NIHSS and ASPECTS, as well as mTICI, had a poor prediction of outcome compared with the biomarkers. The combination of NIHSS at 24h and NFL, tau or GFAp at 48h improved the performance in predicting poor outcome compared with a single biomarker, suggesting that these blood biomarkers could be used as complementary tests to confirm the severity of the stroke.

The key findings of this study are: 1) the different blood biomarkers show different kinetics over time after ischemic stroke, and 2) their performance in predicting for poor outcome, also in combination with clinical parameters such as NIHSS at 24h, was high.

4.5 PAPER V

ALS is a fatal neurodegenerative MND characterized by progressive loss of upper and lower motor neurons. CSF concentrations of NFL and pNFH have been found to be increased in ALS [77, 94, 115]. In this study, we aimed to test the hypothesis that CSF NFL and pNFH can differentiate ALS patients from patients with ALS-like symptoms who eventually received other diagnoses. In addition, we examined if the biomarkers were correlated with disease survival and if the mutation type in genetic cases had an influence on the biomarker levels.

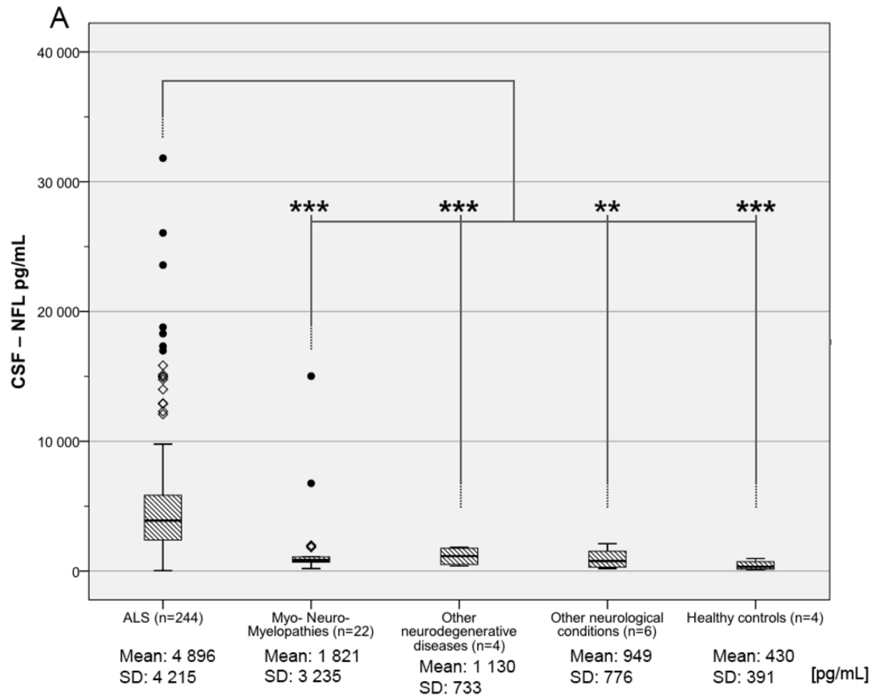
In the whole cohort, CSF-NFL and CSF-pNFH were significantly correlated to each other (r=0.817, p<0.0001).

ALS patients had higher CSF NFL and pNFH compared with referral patients with myo-/neuro-/myelopathies (p<0.001 for both NFL and pNFH), patients with other neurological conditions (NFL: p<0.01, pNFH: p<0.001)), patients with other neurodegenerative diseases (p<0.001 for both) and healthy controls (p<0.001 for both) (figure 9).

Neither NFL nor pNFH was significantly different between patients with bulbar or spinal onset.

Both NFL and pNFH correlated with survival time from symptom onset, however; NFL had a stronger correlation than pNFH (NFL: r=-0.307, p<0.0001; pNFH: r=-0.158, p<0.05).

By grouping the patients in intervals of short (<2 years), intermediate (2-10 years) and long (>10 years) survival time, we observed that for NFL there was a significant difference (p<0.001) between the 3 groups in a linear negative trend, where patients with longer survival had lower concentration of NFL. The same trend, but not significant, was seen for the pNFH concentrations.


A
CSF – NFL pg/mL
40 000
30 000
20 000
10 000
0
***
***
**
***
ALS (n=244)
Myo- Neuro-Myelopathies (n=22)
Other neurodegenerative diseases (n=4)
Other neurological conditions (n=6)
Healthy controls (n=4)
Mean: 4 896
SD: 4 215
Mean: 1 821
SD: 3 235
Mean: 1 130
SD: 733
Mean: 949
SD: 776
Mean: 430
SD: 391
[pg/mL]

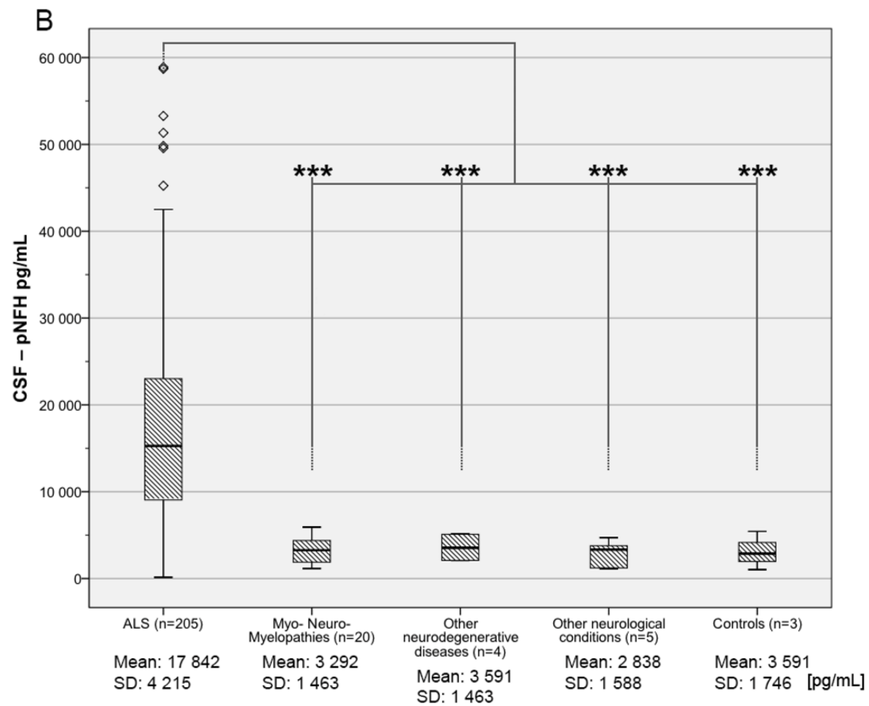

B
CSF – pNFH pg/mL
60 000
50 000
40 000
30 000
20 000
10 000
0
***
***
***
***
ALS (n=205)
Myo- Neuro-Myelopathies (n=20)
Other neurodegenerative diseases (n=4)
Other neurological conditions (n=5)
Controls (n=3)
Mean: 17 842
SD: 4 215
Mean: 3 292
SD: 1 463
Mean: 3 591
SD: 1 463
Mean: 2 838
SD: 1 588
Mean: 3 591
SD: 1 746
[pg/mL]

*Figure 9. **Biomarker concentrations in the different diagnostic groups.** A) CSF-NFL (pg/mL) B) CSF-pNFH (pg/mL). Center of box defines median values, upper and lower box limits defines first (Q1) and third (Q3) quartiles. Whiskers defines +/-1.5 times interquartiles range (IQR). Diamonds indicate cases outside 1.5 times IQR. Filled circles indicate cases outside 3 times IQR. When comparing all groups, the overall One-Way ANOVA was significant at p<0.0001. Statistical significance comparing other groups to the ALS group is denominated with asterisk *=p≤0.05; **=p≤0.01; ***=p≤0.001.*

Within our ALS group, there were 26 *C9orf72* hexanucleotide repeat expansion (HRE) carriers, 24 superoxide dismutase 1 (*SOD1*) mutation carriers and 4 vesicle-associated membrane protein B (*VAPB*) mutation carriers. However, the great majority (n=192) did not have a detectable mutation. Neither NFL nor pNFH differed between the different mutation carrier groups or between the carrier and non-carrier groups. However, the *C9orf72* HRE patients had noticeably higher NFL concentrations (median; IQR: 5419; 4258-6621pg/mL) compared with *SOD1* mutation carriers (median; IQR: 3248; 2333-4472pg/mL) and non-carriers (median; IQR: 3740; 2260-5802pg/mL).

In this paper, we showed that the ALS group had higher NFL and pNFH concentrations in CSF compared with the healthy controls, referral patients with myo-/neuropathies and patients with other non-degenerative neurological conditions. Due to the different inclusion criteria for the diagnosis of ALS and differences in the terminology used across the different publications, it is complicated to make proper comparisons between studies. However, our results are in line with the general observation from other studies to show that both NFL and pNFH are elevated in the CSF of ALS patients [111].

Our results support previous findings that CSF neurofilament levels cannot differentiate ALS patients according the site of onset (spinal or bulbar) [195], suggesting that the extent of ongoing neuronal degeneration is similar in both types of onset, despite the poorer prognosis in bulbar onset patients. In some way, the worse prognosis in bulbar onset cases could be in part due to the earlier dysphagia, which in turn would affect the nutritional intake and by extension influence the course of the disease due to the increased metabolic demand present in ALS [196-198].

We found that ALS patients with lower concentrations of the biomarkers had longer survival times; we therefore assume that patients with lower biomarker concentrations had less intense neurodegeneration.

It has been previously reported that ALS patients carrying *C9orf72* HREs have higher pNFH levels compared to non-mutation carriers and that *SOD1* mutation carriers have lower NFL levels when compared with non-mutation carriers [108, 189]. Our results corroborate these results. In addition, we show that NFL is also higher in *C9orf72* HRE carriers compared with non-mutation carriers, and that pNFH is higher in *SOD1* mutation carriers when compared to non-mutation carriers, which contrasts NFL. The transcriptome of induced pluripotent stem cells (iPSC)-derived motor neurons carrying *C9orf72* HREs is different from the one of iPSC-derived motor neurons with *SOD1* mutations [199], and perhaps the differences we observed in the biomarker concentrations across the different mutation carriers could be a consequence of the pathophysiology caused by such mutation.

The key findings of this study are: 1) the increased concentrations of both NFL and pNFH in the CSF of ALS patients as compared to ALS-mimics and healthy controls, 2) the correlation of both biomarkers with survival and 3) the different biomarker profile in the different mutation carrier groups.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The work included in this thesis includes method development and clinical studies to qualify the potential use of neurofilaments as biomarkers of neuronal damage in different situations.

The newly developed ELISA to measure NFL in CSF showed a good analytical performance, correlated with the established commercial assay, and showed similar diagnostic accuracy in distinguishing IDD patients from controls. Therefore, we conclude that our assay represents a valid alternative method for the measurement of NFL in CSF and that CSF-NFL is a reliable biomarker of the severity of the axonal damage processes occurring in different neurological diseases.

The newly developed ELISA for the measurement of pNFH was specific for the phosphorylation form of NFH. On the one hand, it performed well for the measurement of pNFH in CSF. On the other hand, its analytical sensitivity was not high enough to be used for serum analyses. Therefore, one of the Simoa assays would be preferable for the analysis of pNFH in serum. The increased concentration of pNFH in several neurodegenerative diseases and the correlation between CSF and serum values suggest that pNFH, like NFL, could become a promising blood biomarker of neuronal damage, which is valid across different analytical platforms. Therefore, more studies are needed to further prove this point, as well as to further qualify the diagnostic differentiation capabilities of the neurofilaments. These future studies should be performed, preferably, including other established biomarkers such as tau GFAp and/or clinical parameters which could be used together with the NFs, or if time is of importance, perhaps instead of them, as for tau at 2h had a reasonable prediction performance compared to NFL at 72h. Furthermore, the combination of different biomarkers improved the prediction of outcome in stroke, so it may be possible that it can also improve the diagnostic capabilities of one single biomarker.

NFL and pNFH in either blood or CSF are biomarkers reflecting the temporal dynamics of post-ischemic degeneration of axons. Their use in the early setting after endovascular treatment of stroke could lead to a simplified and standardized way to estimate the nervous tissue damage and possibly complement the clinical judgment in foreseeing the need of rehabilitation measures, especially if combined with other biomarkers such as NIHSS at 24h.

However, for a better understanding of the full potential of these markers, further studies in different subsets of stroke are needed.

The observed correlations of CSF-NFL with serum-NFL and CSF-pNFH with serum-pNFH suggest the possibility of the use of future blood-based tests in the clinic due to the easier accessibility to blood samples.

An interesting point to study in the future would be the relationship between pNFH and NFH. Since the phosphorylation-dephosphorylation dynamics could be altered in different ways in different neurodegenerative diseases depending on their neurodegenerative pathway. Similarly, the study of INA, which is CNS-specific, could complement the information given by the other neurofilaments and allow for making stronger statements regarding the CNS-specificity of a blood neurofilament concentration change.

Also further studies in the protein structure are needed since it has been postulated that NFL in CSF could be degraded and/or aggregated [129]. This would give insight into the dynamics of neurofilament degradation that could also vary in different neurodegenerative diseases, similarly to the phosphorylation status of NFH.

In conclusion, the work presented in this thesis shows that both NFL and pNFH proved to be sensitive and reliable biomarkers of neuronal damage, which further qualifies their use as disease intensity markers and shows their great potential as laboratory tests in the diagnostics of neurodegenerative diseases.

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